Silvernanowires and their potential applications in 3D scaffolds for biomedical applications

The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

Arianna De Mori

October, 2018
Preface

The three years project was supervised by Dr. Marta Roldo, Dr. Gianluca Tozzi and Dr. Eugen Barbu.

Experiments were conducted at the School of Pharmacy and Biomedical Sciences, the School of Engineering, the School of Biological Sciences, the School of Geography and the School of Geology of the University of Portsmouth.

Arianna De Mori
Abstract

The aim of this work was to design, prepare and characterize novel composite materials with inherent antibacterial properties to be used in bone repair and bone regeneration applications. The project was developed in three stages, looking at novel filler materials initially and then investigating their use in two different types of scaffolds, namely soft hydrogels and hard cements.

The first part of the project dealt with the in vitro elucidation of the antibacterial activity and cytocompatibility of silver nanowires (AgNWs). These silver nanoparticles have been attracting increasing attention in comparison to other silver nanospecies due to their high aspect ratio that influences their thermal and electrical properties for use in flexible transparent conductive films (TCFs). Nevertheless, a limited number of papers have explored AgNWs antibacterial activity and eukaryotic toxicity. In this work, a polyol method, in presence or absence of carbon nanotubes (CNTs) has been used to guide the silver nanowires formation. Initially, the synthesised nanomaterials were fully characterized in terms of their physicochemical properties. Then, their toxicity, reactive oxygen species (ROS) formation and release of cytoplasmic proteins was studied against four different bacterial species and against three different mammalian cell types, to simulate different infection types and routes of exposure, respectively. Results showed that AgNWs, with similar characteristics, can be synthetized using either of the two synthetic methods, with no advantages imparted by the inclusion of CNTs in the synthetic process. Moreover, AgNWs were proven to be affective antibacterial agents acting via ROS formation and cellular membrane damage. Finally, we showed that AgNWs presented dose-dependent and time-dependent cytotoxicity following ROS formation in all the studied mammalian cell lines.
The second part of the project looked at applications of silver nanowires addressing the occurrence of infections as a complication following bone implant surgery. These infections can lead to destruction of the bone and consequently to an increased rate of treatment failure and delayed osseous-union. A chitosan-based hydrogel incorporating hydroxyapatite and AgNWs with potential bioactive, biocompatible properties and antibacterial properties was formulated. Physicochemical characterization, Ca/P deposition, silver release, antibacterial and cytocompatibility studies were carried out, and results suggested that the hydrogels herein developed present good bioactivity and biocompatible properties.

The third part focused on trying to solve some of the drawbacks related to the use of polymethyl methacrylate (PMMA) cements in vertebroplasty and total joint replacement: stiffness mismatch between the bone and the cement, high exothermic reaction temperature, leakage of toxic monomer and lack of antibacterial properties. In this part, we encapsulated AgNWs in PMMA bone cements with different concentrations of chitosan or methacryloyl-chitosan, to ensure a long lasting and increased release of Ag\(^{+}\) over time. Cytocompatibility, antibacterial and mechanical properties of the novel composite cements were investigated. This study suggested that the inclusion of CS/CSMCC (between 10 and 20%) and AgNWs (1%) in the existing commercial materials could provide bone cements with good results in terms of cytocompatibility combined with appropriate thermal, mechanical, and antibacterial properties. However, no advantages were shown by the inclusion of CSMCC over CS.
This thesis is organized as follows:

**Chapter 1:** Silver nanowires and their suitability for biomedical applications.

**Chapter 2:** Evaluation of antibacterial and cytotoxicity properties of silver nanowires and their composites with carbon nanotubes for biomedical application.

**Chapter 3:** Composite hydrogels for bone regeneration. Published review paper: Tozzi, G.; De Mori, A.; Oliveira, A.; Roldo, M. Composite hydrogels for bone regeneration. Materials. 2016. 9 (4), 267.

**Chapter 4:** Injectable composite gels containing silver nanowires as bone scaffold materials. Paper in preparation to be submitted to Journal of Materials Science: Materials in Medicine.

**Chapter 5:** Novel antibacterial PMMA composited as alternative bone cements with tunable thermal and mechanical properties. Paper in preparation to be submitted to Journal of the mechanical behavior of biomedical materials.

**Chapter 6:** Conclusions and Future Plans

**Chapter 7:** Bibliography

**Appendix I**

Carbon nanotubes play an important role in the spatial arrangement of calcium
Carbon nanotubes play an important role in the spatial arrangement of calcium deposits in hydrogels for bone regeneration. Journal of Materials Science: Materials in Medicine 2016. 27 (8). 126.

Appendix II

3D printing and electrospinning of composite hydrogels for cartilage and bone tissue engineering.

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

This is a continental thesis comprising two peer reviewed published reviews and a paper. Moreover, three further chapters have been paginated as papers and will be shortly submitted.

Word count: 44,673
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<tr>
<td>1 D</td>
<td>One-dimensional</td>
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<tr>
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<td>Two-dimensional</td>
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<tr>
<td>3 D</td>
<td>Three-dimensional</td>
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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
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<td>AAO</td>
<td>Anodic aluminium oxide</td>
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<td>AgNPs</td>
<td>Silver nanoparticles</td>
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<td>AgNWs</td>
<td>Silver nanowires</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>AuNPs</td>
<td>Gold nanoparticles</td>
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<td>A.U.</td>
<td>Arbitrary unit</td>
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<td>ASCs</td>
<td>Adipose-derived stem cells</td>
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<td>BCA</td>
<td>Bicinchoninic</td>
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<tr>
<td>BFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>BPs</td>
<td>Bisphosphonates</td>
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<tr>
<td>BTE</td>
<td>Bone tissue engineering</td>
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<td>C60</td>
<td>Fullerene</td>
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<tr>
<td>Caco-2</td>
<td>Human epithelial colorectal adenocarcinoma cells</td>
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<tr>
<td>CaP</td>
<td>Calcium phosphate</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<td>CS</td>
<td>Chitosan</td>
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<td>Carbon nanotubes</td>
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<tr>
<td>DAPI</td>
<td>(4',6-diamidino-2-phenylindole)</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Embryonic stem cells</td>
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<td>f-CNT</td>
<td>Functionalized-carbon nanotubes</td>
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<td>FGF</td>
<td>Fibroblast growth factors</td>
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<tr>
<td>FESEM</td>
<td>Field emission scanning electron microscopy</td>
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<tr>
<td>FTEs</td>
<td>Flexible transparent electrodes</td>
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<tr>
<td>FTIC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>GG</td>
<td>Gellan gum</td>
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<td>Glycerolphosphate</td>
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<td>IGF</td>
<td>Insulin like growth factor</td>
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<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma-optical emission spectroscopy</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>MBC</td>
<td>Minimal bactericidal concentration</td>
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<td>MIC</td>
<td>Minimal inhibitory concentration</td>
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<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MTPs</td>
<td>Multi twinned nanoparticles</td>
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<tr>
<td>nHap</td>
<td>Nano hydroxyapatite</td>
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<td>MMA</td>
<td>Methylmethacrylate</td>
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<td>MRSA</td>
<td>Methicillin resistant staphylococcus aureus</td>
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<td>MWNTs</td>
<td>Multi-wall nanotubes</td>
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<td>NOSC</td>
<td>N-octyl-O-sulphate chitosan</td>
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<tr>
<td>NWs</td>
<td>Nanowires</td>
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<td>O.D.</td>
<td>Optical density</td>
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<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
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<td>PMMA</td>
<td>Polymethyl methacrylate</td>
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<tr>
<td>Ppb</td>
<td>Parts per billion</td>
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<td>Ppm</td>
<td>Parts per million</td>
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<tr>
<td>PVP</td>
<td>Polyvinlypirrolidone</td>
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<td>ROS</td>
<td>Oxygen reactive species</td>
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<td>PTH</td>
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Acknowledgements

I would like to first thank my supervisor, Dr. Marta Roldo, for her optimism, kindness and guidance throughout my PhD at the University of Portsmouth. I am grateful she let me be independent during my work and she gave to me a chance to take part to many different research projects. Also, I would like to thank my second and their supervisors, Dr. Gianluca Tozzi and Eugen Barbu, for their input along the way.

I would also like to thank the Institute of Biology and Biomedical Science (IBBS) for financial support of my scholarship. Furthermore, I would like to thank the Technicians, Research Fellows, Lecturers and PhD students that shared their knowledge with me and did their best to help me with this project: Dr. Roger Draheim, Colin Lupton, Dr Alex Peter Kao, Elaine Dyer, Linley Hastewell, Dr. Anita Sanghani, Silvia Fancellio, Emanuela Di Gregorio, Dr. Stella Pastore, Lowrie Vayro, Dr Robin Rumney, Christine Hughes, Torquil Jackson, Dr. Salman Goudarzi and Marta Peña Fernández.

Most importantly, I would like to express my gratitude towards my family and friends for all of their love and constant support throughout this difficult life and work time: Giorgio, Antonieta, Valentina, Melina, Than, Yujiao (Sharon), Rahmi, Regina, Dien, Monica, Alicia and Ana.
Dissertation

Publications


Manuscripts in preparation


Conference Posters

(1) BIRAX 3rd Regenerative Medicine Conference. 11th-12th April 2016. University of Oxford. ‘Carbon nanotubes play an important role in the special arrangement of of calcium
deposits for bone regeneration’. **De Mori, A.**, Oliveira, A., Cancian, G., Hussain, A. A. B., Tozzi, G., Roldo, M.


Antibacterial resistance is currently one of the most serious health problems for medicine. Indeed, a recent review has estimated that, by 2050, 10 million people a year will die as a result of antimicrobial resistance [1]. Unfortunately, as bacteria have a short doubling time and can acquire resistance genes from other strains via horizontal gene transfer (transformation, conjugation and transduction), they can both accumulate mutations faster than humans and increase the risk of spread of antibiotic resistance from species to species [2]. Moreover, in the case of implant-surgeries, the presence of infections can cause longer length of hospital stay, pain, implant failure, amputations and ultimately death [3]. In this context, there is a huge demand for novel antibiotics in order to prevent a health disaster. Nanotechnology has an important part to play in the fight against antimicrobial resistance, in fact nanoparticles (NPs) (particles having at least one dimension in the size range 1- 100 nm [4]) seem to exert their antimicrobial activity via multiple mechanisms rendering the development of simultaneous defence mechanisms very unlikely. Therefore, attention has been focused on novel NPs with antibacterial activity, such as silver nanoparticles.

1.1 Silver

Silver is the metallic element with atomic number 47, atomic mass 107.89 and symbol Ag (from Latin argentum, derived from Greek ἄργυρος, literally "shiny" or "white"). Ag possesses unique chemical and physical properties such as the highest electrical and thermal
conductivities of all metals, catalytic and optical properties, chemical stability and malleability [5]. Moreover, silver has been used in engineering technologies (e.g. sensors) because of its superior plasmonics and surface-enhanced Raman scattering (SERS). Finally, silver is widely used in medicine for its antimicrobial activity.

1.1.1 Silver as an antimicrobial material

Silver is known for its antimicrobial properties against a wide range of microorganisms since times immemorial, even though its use was based on simple empirical observations. One of the first documented uses of silver has been reported by Herodotus (V century B.C.) to keep fresh and pure the water for long periods of time. In the XIX century isolated attempts were made to use silver, until 1880s, when Doctor Carl Siegmund Franz Crede, a German obstetrician, formulated 1 % silver nitrate eye drops to prevent ophtalmia in new-born infants and later used colloidal silver for the treatment of septic wounds. Through the 20th century, silver fell into disuse as the medical world concentrated on antibiotics for the control of infections, as they presented less toxicity to the host and they were more effective for systemic use. In the 60s, the rise of bacterial resistance brought the attention of silver back to research because of its effective antimicrobial activity against a wide spectrum of unicellular organisms [6]. Nowadays, silver is used into sanitary devices, such as catheters and plasters for preventing urinary tract and wound infections, respectively [7]. When in the 1980s the first findings on unique properties of nanoparticles (NPs) were published [8], new doors opened up to tailor the chemical and physical properties of materials for various applications.
In this perspective, several studies have focused on the synthesis and development of different shapes and sizes of silver nanoparticles (AgNPs) for a wide variety of engineering and biomedical applications.

1.2 Metallic Nanoparticles and Silver Nanoparticles

Engineered nanomaterials have unique properties that make them suitable for uses in various industrial applications, such as agriculture [9], chemistry, electronics, food [10], medicine, automotive telecommunication, energy, textiles, etc.

The main motivation to perform research on NPs is based on the so-called quantum size effect of nano-scale materials [8]. As their size reduces from macro to nano, materials not only acquire a larger surface area per unit mass, but also a higher surface energy, due to the high friction of atoms/molecules on the particle surface. Surface energy increases in magnitude with a decreasing particle size, generating unique physical, chemical and biological properties [11]. The large fractions of surface atoms and surface energy seem to influence the optical, thermal and magnetic properties of nanomaterials. Consequently, tailoring the size and shape of nanomaterials, it is possible to obtain defined properties and characteristics. Furthermore, NPs possess surface atoms which have a smaller coordination number (number of atoms, ions or molecules bonded to an atom in crystal) than bulk atoms and thus, highly polarized surfaces (a dielectric surface is said ‘polarized’ when its molecules acquire an electric dipole moment, in response to an applied external field) [12]. Consequently, NPs can adsorb and bind to biological materials non-specifically through their need for steric and electrostatic stabilization [13, 14].

In comparison to other types of nanomaterials, metal nanoparticles show the advantages
of a high heat resistance, excellent antimicrobial properties, low cost preparation and less chances to induce microbial resistance in comparison to conventional antibiotics. Metal NPs include Ag, silver oxide (AgO), titanium dioxide (TiO₂), alluminiun oxide (Al₂O), silicon, copper oxide (CuO), zinc oxide (ZnO), Au, calcium oxide (CaO) and magnesium oxide (MgO). All these nanomaterials seem to exert their antibacterial effects by the release of free ions from dissolution of metals and oxidative stress via the generation of reactive oxygen species (ROS) on the surfaces of the nanoparticles [15].

Among the other types of metallic NPs, silver nanoparticles (AgNPs) are the most studied due to their outstanding antimicrobial properties. Indeed, studies have shown that AgNPs are effective against a broad range of Gram positive and Gram negative bacteria, mycobacteria and fungi [16]. Few researches have also indicated that AgNPs act as potent antiviral agents against various virus species including hepatitis, herpes simplex virus, monkeypox virus and even human immunodeficiency virus (HIV-1). AgNPs possess therapeutic advantage over conventional and narrow targeting antibiotics, due to a rare chance of microbes to develop resistance against them [17].

Finally, AgNPs have been synthesized in many shapes and sizes, such as spherical, triangular, square, cubic, rods, rectangular disks, oval and wires [18]. Depending on their dimensions, they can also be classified into zero-dimensional (0D), one-dimensional (1D), two-dimensional (2D) and three dimensional (3D) [19].

1.3 Silver nanowires (AgNWs)

Among other types of silver NPs, AgNWs (Fig. 1.1) have been attracting more and more attention due to their high aspect ratio and electrical and thermal properties. AgNWs are
one dimensional silver nanostructures with diameters in the range 10-200 nm and length in a range of 5-100 µm with an aspect ratio higher than 10.

So far, AgNWs have been mainly used to fabricate flexible transparent electrodes (FTEs) which can be applied to many optoelectronic devices such as portable solar cells and touch screens. When applied on a substrate, they form a highly conductive network that enables an interplay between the user and the system [20]. More recently, the antibacterial properties of AgNWs have also emerged as potential alternative to traditional antimicrobial agents in a wide range of applications, such as wearable or medical devices.

Due to the economic importance of AgNWs, many researchers have focused on the investigation of synthetic methods to obtain of high-yield and low costs AgNWs with different length and diameter. In general, AgNWs can be synthetized using different methods that can be broadly classified as hard- and soft-template methods, even though other techniques can be used, such as hydrothermal method, ultraviolet irradiation technique and electrochemical processes.
1.3.1 Hard-template synthesis of AgNWs

The hard-template method (or template-directed method) is based on the use of rigid materials as a mould, whose firm structure guides the formation of crystals on the surface, determining their size and morphology [22]. In this method, water and alcohols are used as solvents, whereas sodium borohydride, citrate, ascorbic acid, etc. are used as reductant [23]. At the end of the process, the metal wires can be removed from the template.

In the template-directed method, the metal is deposited chemically or electrochemically within the mould. Different hard templates are available to build-up materials with this method: membranes, such as anodic aluminium oxide (AAO), carbon nanotubes, carbon fibers, plastic foams, nanoporous silica and alumina membranes, zeolites and track-etched
polycarbonate [24] [25].

The advantage of this technique is that the NWs produced in this way are perfectly customized and they are homogeneous. Finally, the process can be highly reproducible. However, this process presents several drawbacks, such as: high cost, difficulty to scale-up and the separation of the wires from the template can require a harsh process, causing damage to the wires [24, 26].

1.3.2 Soft-template synthesis of AgNWs

To overcome the limitations associated with the hard-template method, the soft-template (or template-free methods) were developed [24]. In these synthetic methods, no fixed rigid structures are needed, but various types of surfactants, micelles, and many other polymers are used as soft-template [23, 27, 28].

1.3.2.1 Polyol process

Among the other soft Template methods, the polyol method is the most popular [29], as it is a faster process compared to the hard-template methods, and it produces a greater yield of AgNWs, with controllable diameters and size [30]. Moreover, the use of polyols offers different relevant advantages in the synthesis of metallic NPs. Firstly, metal salts can be solubilized in hydrophilic polyols, reducing the production of toxic waste products (such as carbonyl metals or advanced organometallic compounds). Secondly, the polyols work both as stabilizing and reductive agents, allowing the formation of NWs in a one-pot reaction synthesis [28]. Finally, polyols can be highly-boiling solvents (this is advantageous when high temperature of synthesis are necessary to synthetize the desired NPs).
Typically, in the synthesis of AgNWs, ethylene glycol (EG) or glycerol, poly(vinyl pyrrolidone) (PVP), and AgNO₃ are used as polyol, capping agent (to prevent agglomeration of the colloidal particles), and salt precursor, respectively. In this synthetic process, the polyol that is the reducing agent, promotes the reduction of ionic silver (Ag⁺) into metallic silver (Ag°), forming multiply-twinned nanoparticles (MTPs). The MTPs tend to have a decahedron configuration with ten {111} facets and (111) planes, as shown in Fig. 1.2 A. The multiply-twinned NPs serve as seeds for of subsequent formation of AgNWs. In particular, bigger NPs can form, through a process called Ostwald ripening. PVP, adsorbing on (100) planes, leaves (111) planes active for anisotropic growth in the <110> direction, forming at first nanorods and then nanowires.

As also reported by Jones et al. [31], the formation of AgNWs of different sizes and diameters is controlled by several factors (e.g. small amount of salts, stirring rate, temperature and presence of oxygen) and different strategies can be adopted, making the polyol method very flexible and adaptable for the production of AgNWs for different applications.
Figure 1.2 Schematic growth mechanism of AgNWs. (A) At the beginning, multiply-twinned nanoparticles (MTPs) of silver are formed after reduction of Ag$^+$ to Ag$^\circ$. [111] facets are highlighted in red and [111] planes in violet. Then, PVP passivates on (100) planes, blocking the access of Ag$^\circ$ to Ag seeds. (B) Schematic model illustrating the diffusion of silver atoms towards the two ends of a nanorod, with the side surfaces completely passivated by PVP. This drawing shows a projection perpendicular to one of the five side facets of a nanorod, and the arrows represent the diffusion fluxes of silver atoms. Reproduced from [32].

1.4 Gram-positive vs Gram-negative bacteria

Bacteria are prokaryotic organisms, whose size ranges between 0.1 to 10 μm. These microorganisms reproduce themselves through binary fission and they exist in a variety of morphologies, such as bacilli, cocci and others (spirilla, vibrios and spirochaetes). Most of the bacteria can be primarily grouped into Gram-negative and Gram-positive (Fig. 1.3) strains based on their ability to retain or not Gram stain.
Gram-positive bacteria are those that retain crystal violet-iodine complex in their cell walls, due to their thicker and less porous peptidoglycan cell wall than Gram-negative [34, 35]. Moreover, Gram-positive bacterial cell wall is characterized by the presence of teichoic acid that is a negatively charged glycoprotein which is not present in Gram-negative bacteria. Gram-negative bacteria present a plasma membrane located outside of the cell wall, known as the outer membrane (OM) and, composed of lipids, proteins and lipopolysaccharide (LPS). This extra layer makes Gram-negative bacteria more resistant to antibiotics than Gram-positive. Furthermore, LPS plays an important role in the pathogenesis of many Gram-negative bacterial infections, inducing the release of proinflammatory cytokines and the activation of the complement, leading to endotoxin shock.

There are essentially two pathways that antibiotics can take through the outer membrane: a lipid-mediated pathway for hydrophobic antibiotics, and general diffusion through porins for hydrophilic antibiotics [36].

**Figure 1.3** Schematic representation of differences between the cell walls of Gram-positive and Gram-negative bacteria. Reproduced from [33].
Finally, on the inner side of the cell wall there is a cell membrane, known as ‘inner’ in Gram negatives or ‘cytoplasmic’ in Gram-positives, composed of a phospholipid bilayer and proteins and, it performs multiple functions, such as active transport, respiratory chain components and energy-transducing systems. The membrane also contains components of protein export systems that enable proteins to exit the cell and it is the anchoring site for DNA, too [34, 37].

1.5 Mechanisms of action of AgNPs in bacteria

To date very little is known about the exact mechanism of action of AgNPs, Ag ions or other silver nano species. Various theories on how AgNPs effect their microbicidal action have been proposed [38], with a combined action of both Ag⁺ and integral AgNPs being the most plausible explanation [39]. As little is known about AgNWs mechanism of action, here we propose the main hypotheses of AgNPs bacterial toxicity (Fig. 1.4 A-B) [40]:
Figure 1.4 A comparison of the silver ions (A) and silver nanoparticles’ (B) mode of action to Gram negative and Gram-positive bacteria. (1) Pore formation; metabolites and ions leakage (shown as plus and minus in the figure above) (2) Denaturation of structural and cytoplasmic proteins; enzymes inactivation. (3) Inactivation of respiratory chain enzymes. (4) Increase of intracellular reactive oxygen species (ROS) concentration. (5) Interaction with ribosome. (6) Interaction with nucleic acids. (7) Inhibition if signal transduction. Reproduced from [41, 42].
(1) **Adhesion of NPs to the bacterial surface, altering membrane properties.**

This phenomenon depends on the electrical interaction/repulsion between bacteria and materials’ surface charge. El Badawy et al. found that surface charge is the most important factor for nanosilver-bacteria interaction [43]. Indeed, on one hand, bacterial cells have a negative charge on their cell wall, mainly due to the presence of negatively charged teichoic acid in Gram positive and phospholipids and lipopolysaccharides in Gram negative bacteria [44]. On the other hand, AgNPs can be positively-, negatively- and neutrally- charged and their attachment and bactericidal activity is different. Abbaszadegan et al. demonstrated that positively- charged AgNPs presented the highest bactericidal activity against both Gram + and Gram – bacteria while neutral or negative charged NPs provided the intermediate and lowest bactericidal activity [45]. Interestingly, negatively charged NPs coming closer to the bacteria can overcome the repulsive forces and somehow interact with cells or they can interact with sulphur-binding proteins within the cell membrane [46]. As a consequence, it has been suggested that NPs can cause local membrane damage, with consequent cytoplasmic materials leakage.

(2) **DNA damage.**

Lee et al. demonstrated that AgNPs can induce cell death in *E.coli* as an apoptosis-like response that includes the following: accumulation of reactive oxygen species (ROS), increased intracellular calcium levels, phosphatidylserine exposure in the outer membrane, disruption of membrane potential, activation of a bacterial caspase-like protein and DNA degradation [47]. DNA fragmentation was also shown in *H. pylori*, *Proteus* sp. and *Klebsiella* sp. [48].
(3) **Ag^+** released from AgNPs can interact with sulphur and nitrogen-containing compounds.

Metallic silver present in AgNPs can be oxidized to form Ag^+. Usually, the oxidation of AgNPs can be due to either reaction with oxygen or with hydrogen peroxide in acidic medium [24]. Ag ions, once inside the cells, interact with thiol (-SH) and amine (-NH_x, x=1,2,3) groups of proteins, nucleic acids and enzymes. [42] Consequently, a cascade of damages happens to bacterial cell wall, cytoplasmic structures and genomic components [49, 50]. For instance, Jung et al. proved that the accumulation of Ag^+ in the bacterial envelope was followed by the separation of the cytoplasmic membrane from the cell wall in both Gram-positive and Gram-negative bacteria [51]. A further major target in bacterial cells are S2 proteins in ribosomes that result in denaturation of the ribosome native structure and inhibition of protein biosynthesis [52]. Finally, damage can be due to the interaction of Ag^+ with transport pumps leading to proton leakage and a decrease in proton motive force; or with inhibition of phosphate uptake causing cytoplasmic shrinkage and detachment of the cell membrane [53].

(4) **Generation of reactive oxygen species (ROS).**

Many studies provide evidence for the production of ROS by AgNPs. ROS can be generated outside the cell, in medium, or inside the cell, also as a consequence of cell damage/disruption. Exposure to a concentration of ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2•⁻) and hydroxyl radical (OH•), over the level of tolerance of antioxidant defence systems or inhibiting scavenging enzymes (e.g. superoxide dismutase), causes oxidative stress, protein damage, DNA breakage, inhibition of respiratory chain enzymes and consequently cell death [24, 54] [55].
(5) **Disruption of proton electrochemical gradient.**

Cao et al. reported that AgNPs could attach to cells creating proton depleted regions, thus interfering with ATP production in the intermembrane space: the disruption of the transmembrane proton electrochemical gradient may inactivate ATP synthesis, ion transport, and metabolite sequestration, leading to cell death [56].

1.5.1 **Ag⁺ and AgNPs uptake in bacteria**

Little is known about the mechanisms of uptake of both Ag⁺ and AgNPs in bacteria.

The mechanism of Gram-negative cells uptake of ionic Ag has been explored more than that of Gram positive bacteria. Most likely, to reach the plasma membrane of Gram-negative bacteria, Ag ions should first pass outer membrane via major outer membrane proteins (OMPs), especially OmpF and its homolog OmpC [51, 57], whose bacterial porin family is usually responsible for the efflux of specific toxic metals out of the cells [58].

On the other hand, it seems that the majority of the works suggest that bacteria are unable to uptake NPs, due to the lack of uptake mechanisms [59].

1.5.2 **Studies on antibacterial properties of AgNWs**

Similarly to the more studied spherical AgNPs, the mechanism of action of AgNWs is still to be elucidated; some researchers suggest that the reason for AgNWs toxicity is due to only to Ag⁺ release, while others claim a shape and size effect [60, 61].

Visnapuu et al. compared toxicity of AgNWs (600 x 6100 nm) and Ag nanospheres (83 nm)
against *E. coli* to test the effect of shape and size. The surface charge of both nanostructures was negative and Ag⁺ was dissolved at similar rates in the aqueous suspensions. They found that AgNWs and spheres presented inhibition of bacterial growth at concentrations of 0.42 ± 0.06 and 0.68 ± 0.01 mg/L, respectively. Moreover, they found that the toxic effects of AgNWs, Ag spheres and AgNO₃ were driven by dissolved Ag⁺ ions and that the toxicity was not dictated by nanoparticle shape [60].

Other studies seem to show that the shape and size of the metallic nanoparticles play an important role in toxicity. Hong et al., for instance, compared the antibacterial activity of Ag nanocubes (55 ± 10 nm), spheres (60 ± 15 nm) and wires (60 ± 10 nm in diameter and 2-4 μm in length) against *E. coli*. They showed that spheres were more efficient than nanocubes and nanowires in inhibiting *E. coli* growth, presenting MIC values (for cell density if 1 x 10⁴ CFU/ml) of 37.5 ± 5.3, 75 ± 2.6 and 100 ± 5.3 μg/mL, respectively. According to TEM observation, it was concluded that nanocubes and nanospheres, presenting a larger specific surface area than NWs could attach to the cell membranes more closely, causing more cell membrane damage and stronger antibacterial performance than AgNWs [62].

The most complete study on AgNWs mechanism of action against bacteria has been carried out by Cui et Liu. They found that AgNWs were able to inhibit the growth of both *E. coli* and *S. aureus* at the concentrations of 28 and 35 μg/ml, respectively. Furthermore, they found out that exposure of *E. coli* to concentrations as low as 10 μg/ml were sufficient to cause leakage of proteins and DNA/RNA from bacteria walls and generation of ROS [63].
1.6 Mechanisms of action of AgNPs in mammalian cells

As already reported for bacteria, even for mammalian cells little is known about the precise mechanisms of action of AgNWs. Thus, here we report the possible mechanism of cytotoxicity of AgNPs in mammalian cells:

1) **Generation of ROS**

High levels of ROS, in mammalian cells, can cause DNA damage, alteration of gene expression, chromosomal aberrations, lipid peroxidation and reduction of glutathione (GSH), often accompanied by proliferation arrest. Several studies have demonstrated that AgNPs can induce ROS generation within different cell lines, by 1) directly interfering with organelles, such as mitochondria, 2) binding transition metals or 3) leading to ROS formation, due to their surface chemistry [64]. For instance, Gurunathan et al. demonstrated that AgNPs (ca. 20 nm in diameter) increased ROS formation in a dose-dependent manner in both human lung (L-132) and lung carcinoma cells (A549) ([Fig. 1.5](#)) [65], whereas Kim et al. found dose-dependent ROS generation in human hepatoma cells (HepG2) by AgNPs (ca. 5-10 nm in diameter) [66].
Figure 1.5 AgNPs induce generation of ROS in AgNP-treated A549 cells. Fluorescence (given by, 2’-7’-Dichlorofluorescin, DCF) images of A549 cells without silver nanoparticles (AgNPs) (0) and cells treated with AgNPs 30 μg/mL and incubated at different time points. Reproduced and adapted from [65].

2) Interaction with and damage to cellular proteins

Both Ag⁺ and AgNPs can interact with aminoacids and proteins (such as serum albumin, haemoglobin, cytoskeletal proteins and antioxidant molecules), altering their functionality [67]. For instance, Paula et al. found that AgNPs (5-45 nm range in diameter) inhibited creatine kinase from the rat brain and skeletal muscle cells [68]. Asharani et al. found that AgNPs (6-20 nm) can bind to cytosolic proteins forming a protein-starch coating able to control cellular responses[69].
3) Mitochondrial damage

AgNP-induced apoptosis can also be achieved by the mitochondrial pathway. Mitochondrial-mediated apoptosis involves two phenomena: the alteration in the permeability of the outer mitochondrial membrane (due to the formation of mitochondrial membrane pores) and the loss of the electro chemical gradient. As a consequence, an event cascade occurs, leading to ROS production [70], reduced ATP production, increased mitochondrial calcium sequestration and release of cytochrome c [70]. For instance, Gurunathan et al. showed that after exposure of A549 cells to AgNPs (20 nm) the transmembrane potential decreased in comparison to the control [65]. Hsin et al. determined that nanosilver was able to cause mitochondrial damage, releasing cytochrome c into the cytosol in mouse fibroblasts [71]. Nair et al. also determined that AgNPs (6-20 nm) could induce a time-dependent drop in ATP synthesis in human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251), which in turn caused DNA damage [72].

4) Induction of apoptosis

AgNPs exposure can also induce cell death. It is not well known if this effect is due to the direct action of AgNPs and/or Ag\(^+\) or it is a consequence of other toxic mechanism induced by AgNPs in the cells. Kawata et al. found that non-cytotoxic doses of AgNPs (7-10 nm) induced the expression of genes associated with cell proliferation and apoptosis in human hepatoma cells (HepG2) [73].
5) **Cell membrane damage**

Studies found that AgNPs could cause cell membrane damage in different cell lines. For instance, Ghosh et al. evaluated AgNPs (20 nm)-induced morphological changes in human leukocytes of cetaceans, finding nuclear membrane rupture, after 4 hours of culture [74].

6) **Genotoxicity**

Some studies have also reported that AgNPs induce genotoxicity in both cancer and normal cell lines [65]. For instance, Foldbjerg demonstrated a dose-dependent formation of DNA adducts in human alveolar cells (A519), after exposure to AgNPs (ca. 69 nm) for 24 hours [75].

1.6.1 Uptake of Ag⁺ and AgNPs in mammalian cells

As for bacteria, very little is known about the systems involved in the uptake of Ag⁺ and AgNPs or AgNWs into mammalian cells.

So far, studies suggest that silver may be transported inside the cells through the copper transporter 1 (Ctr1), which is a transmembrane protein [76].

On the other hand, theories suggest that AgNPs uptake by mammalian cells may happen though endocytosis or diffusion (Fig. 1.6). However, as yet, the exact mechanism of the endocytic pathways (clathrin- and caveolin-mediated endocytosis, phagocytosis or micropinocytosis)[77, 78] is not known. Further, the mechanism of NP uptake seems to be influenced by several factors such as their morphology, size, concentration and surface factors [64]. Asharani et al. showed that normal human IMR-90 lung fibroblasts and U251
glioblastoma cells treated with AgNPs (6-20 nm) presented endosomes with NPs in the cytosol, suggesting that nanoparticles were entering the cells mainly through endocytosis rather than diffusion [72]. According to Sabella et al., once inside the endosomes, NPs are uptaken by lysosomes whose acidic pH triggers an enhanced release of toxic ions [79].

**Figure 1.6** Schematic illustration of AgNPs uptake, via endocytosis or diffusion, and possible mechanisms of cell toxicity. Reproduced from [79].
1.6.2 Studies on cytotoxicity of AgNWs

To date, studies on the interaction of AgNWs with mammalian cells are limited and seem to indicate that their reactivity depends on the specific tissue they come in contact with and the chemical composition of the extracellular liquid.

One of the most relevant occupational risk of exposure to AgNWs is the respiratory route. For example, AgNWs suspensions are deposited onto substrates to make flexible and lightweight touchscreens via aerosolized droplets [20]. Pleural effects of AgNWs have been explored, for example, by Schinwald et al [80]. In particular, they have defined a threshold length (≥5 μm) at which AgNWs produce pathogenicity in the pleural space. They thoroughly demonstrated that the threshold length at which frustrated phagocytosis of AgNWs occurs in vivo (≥5 μm) is lower than that in vitro (≥14 μm) due to the abnormal experimental conditions inherent in the latter [80]. Silva et al. studied the biological responses of rats after intratracheal (IT) administration of PVP-coated long and short AgNWs (S-AgNWs, 2 μm; L-AgNWs, 20 μm) [81]. Both short and long AgNWs produced significant pulmonary toxicity (inflammation), and 21 days post exposure, silver was still present in the lungs. Shu Chen et al. characterized the mechanism of action of AgNWs against human alveolar epithelial cells type 1 (TT1). They determined that AgNWs (72 ± 36 nm in diameter and 1.5 ± 1.4 μm long) could rapidly access the cells, as they were found within endosome-like vesicles after a 1 h pulse and in the cytoplasm after 24 h. Moreover, they found Ag₂S NPs formation around the AgNWs already after 1 h of exposure: Ag⁺ ions dissolved from AgNWs and rapidly precipitated as Ag₂S nanoparticles. The precipitation of highly insoluble Ag₂S became increasingly extensive over the 7 days post-exposure, finally coating all surface of AgNWs. Interestingly, in this study, they did not find evidence of ROS production after 1 h exposure and the overall cell viability was not significantly reduced by AgNWs up to 24 h. Overall,
these results suggested that AgNWs can be degraded within the cellular environment and transformed to Ag$_2$S by sulfidation, limiting cell death from ROS production [82]. Theodorou et al. investigated 1.5 and 10.5 µm long AgNWs reactivity in human monocyte-derived macrophage cells (HMMs), as they are thought to phagocyte AgNPs to clear the lungs from these toxicants. They showed that 10 µm AgNWs were completely internalized by HMMs, whereas 1 µm were less phagocytized, with numerous lysosomal vesicles observed in close vicinity to the AgNWs. Even though AgNWs were close to the nuclei, they did not enter in them. Cell viability decreased in a dose dependent manner and it was higher for short AgNWs than for long AgNWs. Even in this study, they observed the formation of precipitates of Ag$_2$S, as well as AgCl, maybe as a consequence of acidic degradation in lysosomes [83].

Another route of exposure that has been studied was the endovenous one. Kim et al. reported moderate toxicity of silver nanowires on red blood cells. In particular, they studied the toxicity of AgNWs (40 nm in diameter and 1-2 µm long) and Ag nanospheres (30 or 100 nm in diameter) on human erythrocytes (RBC) rheology. They found that the haemolytic effect depended on the particle size: the smaller the particles, the more haemolysis occurred. In addition, AgNWs were safer than Ag nanospheres. Cellular deformity depended on nanomaterials concentration and incubation time: the small AgNPs (30 nm) caused the most significant reduction in RBC deformity by time in comparison to AgNPs (100 nm) and AgNWs. Finally, AgNWs more significantly reduced RBCs aggregation at lower concentration than Ag nanospheres [84].

Verma et al. compared the cytotoxicity of AgNWs (3-6 µm long) against various types of cells (lung epithelial cells, A549; umbilical vein endothelial cells, HUVEC; gastric epithelial cells, AGS; and phagocytic cells, THP-1), finding that the cytotoxic effect was dependent on
cell type, doses and incubations times [85]. All cell lines showed accumulation of AgNWs after 24 hours of exposure and no visual signs of cellular or nuclear abnormalities. Furthermore, all cell types treated with 5 µg/ml AgNWs showed significant decrease in cell viability. Finally, THP-1 cells, after 4 hours of exposure to 5 µg/ml of AgNWs, presented an increase in vesicle size, suggesting presence of autophagy.

These studies demonstrate that a careful in vitro and in vivo study is needed in order to provide comprehensive datasets regarding the toxicity of these nanomaterials and their derivatives.

1.7 Potential biomedical applications of AgNWs

Due to the history of silver use in wound dressing there is interest in exploring ways of exploiting AgNWs in the design of textiles, wound dressing materials or surface coating for medical devices.

Giesz et al. fabricated cotton and viscose fabrics using colloidal AgNWs and titanium dioxide (TiO₂) to obtain a multifunctional material for the purification of air from nicotine. These materials were designed to have photocatalytic properties under UV and VIS light, as well as conductive and antibacterial properties. The application of AgNWs/TiO₂ on fabric showed 3-4 times faster photocatalytic decomposition of nicotine in comparison to unmodified cotton fabric. Cotton fabric modified with AgNWs/TiO₂ showed excellent bacteriostatic and bactericidal effect against S. aureus and K. pneumoniae [86]. Nateghi et al. also produced an electroconductive fabric made of AgNWs loaded into cotton by repeating iterations of submerging the cotton in alcohol suspensions of AgNWs and drying at room temperature. The results showed that the fabric caused 100% microbial death against Gram-positive and Gram-negative [31]. Another study focused on developing a film for wound dressing or skin
grafting Shahzadi et al [45]. They prepared an AgNWs-chitosan film by solution casting. The resulting film presented improved tensile strength and Young modulus compared both to chitosan and chitosan-silver nanoparticles films. Antimicrobial activity was shown by CS-AgNWs films in comparison to bare CS films that had no areas of inhibition against either *E. coli* or *S. subtilis* [45]. All these studies showed that AgNWs have antibacterial properties against both Gram + and Gram -, even when included in complex 3D scaffolds.

Other researchers have tried to combine both the electrical and antibacterial properties of AgNWs for decreasing bacterial attachment and/or improving mammalian cell growth on AgNWs-loaded scaffolds. Tan et al. developed, for instance, a membrane to efficiently purify water from bacterial infections through the release of Ag⁺ in presence or absence of an applied field. The nanofiber membrane was prepared using polyacrylonitrile (PAN) and thermoplastic polyurethane (TPU) via electrospinning, and then embedding AgNWs on the surface of the electrospun nanofibers through vacuum filtration deposition. AgNWs formed a highly conductive network that can generate an electric field for electrochemical disinfection. Indeed, electroporation causes damages to bacterial membranes, which can lead to ion leakage and escape of metabolites. With the application of an electrical field, *E. coli* and *S. aureus* (10⁵ CFU/ml) were inactivated within 20-25 min at 1.5 V, compared to 6 h without electrical field application [87]. In another study, Wickham et al., developed an electroactive nanocomposite material made by compression of self-assembled collagen fibrils and silver nanowires (AgNWs) for neural and cardiac regeneration. The scaffolds with the lowest concentrations of AgNWs (0.1 mg/ml) showed good embryonic cardiac cell proliferation within 7 days, whereas higher concentrations (0.5 and 1 mg/ml) led to a reduced cell proliferation in the same period of time. Prevention of *E. coli* and *S. epidermidis* biofilm formation was found up to 72 h for all the samples containing AgNWs [69]. Lee et al.
developed, instead, a conductive polyethylene glycol (PEG)/hydrogel enriched with AgNWs as a micropattern to direct differentiation of neuronal stem cells (NSCs) and neurite outgrowth under the application of electrical stimuli. In order to successfully seed the neurospheres and to guide the neurite growth a micropattern with 200 µm width was chosen. In the system, neural progenitor cells could differentiate into mature neuronal cells and the cells could better grow when a voltage between 5 and 10 V was used rather than lower or higher voltages [88].

1.8 Research gaps

Despite some studies on AgNWs, this brief review highlighted the large gaps in the existing knowledge of the these nanomaterials’ antibacterial/ cytotoxic behavior and mechanisms of action against different bacterial and mammalian cell lines. Nevertheless, AgNWs seem to exhibit a promising activity as antimicrobial agents, in a world where bacterial infections are still a major cause of morbidity and mortality. Indeed, current antibiotics show low success in contrasting biofilm-associated infectious diseases, which account for over 80 percent of microbial infections in human body. All such agents have difficulty in penetrating the extracellular polysaccharide layer covering the biofilm, promoting the development of antibiotic resistance [89]. More and more studies should be carried out in order to explore the potential of AgNWs in inhibiting bacterial proliferation on surfaces and medical devices.

1.9 Aims and Hypothesis

Therefore, the aim of this study has been:
• To screen the potential toxicity of AgNWs, obtained through soft-template method, in absence or presence of carbon nanotubes (CNTs) against four strains of bacteria (Escherichia coli, Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus and Staphylococcus saprophyticus) and three types of cell lines (human foreskin fibroblasts (Hs27), human colon adenocarcinoma (Caco-2) and human osteoblasts (hFOB 1.19)). Furthermore, studies on ROS production, cell morphology and release of cytosolic components have been carried out.

• To incorporate AgNWs into thermo-sensitive chitosan-based hydrogels to establish their potential in inhibiting biofilm formation, without significantly interfering with cell proliferation on the scaffold.

• To investigate how different ratios of CS/CSMCC and AgNWs in a commercial bone cement could modify the thermal, mechanical, cytotoxic and antibacterial properties of the material.

The hypothesis of the study is that the introduction of AgNWs within 3D scaffolds will provide antibacterial properties to both a soft and a hard material, without statistically affecting the cell viability on the scaffolds. Additionally, when CSMCC is included in the bone cement, lower polymerization temperature and lower cytotoxicity are expected following to crosslinking of methacrylic group of CSMCC with MMA.
Chapter 2: Evaluation of antibacterial and cytotoxicity properties of silver nanowires and their composites with carbon nanotubes for biomedical applications.

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Authors’ contributions

Marta Roldo coordinated the team and is responsible for conception and design of experiments as well as data analysis and writing. The work was carried out in majority by Arianna De Mori, with support from Richard Jones and Matteo Cretella. Guido Cerri carried out X-ray diffractometric analysis. Linley Hastewell gave support for carrying out AAS analyses. Eugen Barbu and Gianluca Tozzi contributed the supervision and guidance of the researchers who carried out the experiments.

Abstract

In this work, we prepared silver nanowires \textit{via} polyol method in presence or absence of single wall carbon nanotubes (CNTs), in order to test the physicochemical, antibacterial and cytotoxic properties of the two synthesis products. Results showed that the introduction of CNTs lead to the formation of AgNWs at lower temperature, but the final product
characteristics of AgNWs and AgNWs-CNT were not significantly different. AgNWs exhibited antibacterial properties against all the studied bacterial species via the formation of oxygen reactive species (ROS) and membrane damage. Furthermore, AgNWs exhibited a dose-dependent and time-dependent toxicity at concentrations ≥ 10 µg/ml. Fibroblasts appeared to be more resistant than Caco-2 and osteoblasts to the toxicity of AgNWs. The cytotoxicity of AgNWs was found to be related to the formation of ROS, but not to membrane damage. Overall, these results suggest that AgNWs are potential antibacterial agents against *E. coli*, *S. aureus*, MRSA and *S. saprophyticus*, but their dosage needs to be adjusted according to the route of administration.

2.1 Introduction

Silver has been known for its antimicrobial properties against a wide range of microorganism for centuries. When in the 1980s the first findings on the unique properties of nanoparticles (NPs) were published, researchers started to investigate novel applications for silver nanomaterials in electronic, optical and biomedical fields. In order to satisfy specific needs, several morphologies have been developed, including silver nanospheres, nanocubes, nanorods, nanotriangles and nanowires.

Silver nanowires (AgNWs) are one-dimensional silver structures and are, for example, currently being used as alternative materials to indium tin oxide (ITO) for the production of conductive films in displays and touch panels, due to their excellent conductive properties [90]. Despite the fact that the antibacterial and cytotoxic properties of other silver nanoparticles have been widely explored and medical devices loaded with AgNPs have been developed, research on AgNWs antibacterial properties, mechanisms of action and possible
biomedical applications is still scarce [91]. So far we know that AgNWs are potential antibacterial agents against *E. coli* and *S. aureus*, but we know little about how they exert their action. Visnapuu et al., for instance, stated that the toxicity of AgNWs against *E. coli* was due to dissolved Ag+ ions rather than to a direct effect of the silver nanostructures [60]. Cui and Liu, instead, highlighted that *E. coli* is more sensitive than *S. aureus* to the action of AgNWs, determining their toxicity was a consequence of both AgNWs induced bacteria disruption and induction of ROS generation [63].

Moreover, AgNWs from commercial products can be released into the environment during manufacturing, use, or disposal, thus there is an impelling need to understand the short- and long-term toxicity of these materials in humans [20]. High-aspect ratio materials, such as asbestos or carbon nanotubes, have indeed been shown to be actively absorbed into eukaryotic cells, causing different types of damages [92]. As the respiratory route in one of the major possible risk of occupational exposure to AgNWs, the majority of studies focused on the possible damages through this route. Schinwald et al. found that AgNWs longer than ≥14 μm or ≥ 5 μm were able to induce pleural inflammation, *in vivo* and *in vitro* respectively [80]. Further studies have also highlighted that AgNWs can enter and accumulate in epithelial cells, interstitial sites, airway smooth muscle cells, the vascular endothelium, the pleural membrane and macrophages. Stoehr et al. compared the cytotoxicity of AgNWs (length: 1.5-25 μm; diameter 100-160 nm) and Ag nanospheres (30 nm) on human alveolar epithelial cells (A549), finding that whereas no effects were observed for the spherical particles, significantly reduced cell viability and increased LDH release were induced by AgNWs [93].

In light of this diverse evidence and *in vitro* studies on different routeS of administration, this study aims to investigate the *in vitro* antibacterial properties of AgNWs, obtained
through two synthetic methods (a soft template and a heterogeneous method) tested against *E. coli*, *S. aureus*, MRSA and *S. saprophyticus*. Moreover, the internalization, cytotoxicity and possible mechanisms of action of AgNWs, on human osteoblasts (hFOB 1.19), human skin fibroblasts (Hs27) and human colon adenocarcinoma (Caco-2) were studied to investigate future potential applications such as inclusion in bone implants, and wound dressing for skin and mucosa.

2.2 Materials and methods

2.2.1 Materials

2′,7′-Dichlorofluorescin diacetate (≥97%), acetone, agar, chitosan from shrimp shells low viscosity (degree of deacetylation ~85%, calculated by H\(^1\)-NMR), dimethyl sulfoxide anhydrous, (≥99.9%), glutaraldehyde solution, hexamethyldisilazane (≥99%), sodium chloride, octaldehyde (99%), poly(vinylpyrrolidone) powder (55 kDa), phosphate buffered saline tablets, silver standard for AAS, sodium cacodylate trihydrate, trypsin-EDTA 0.25% solution, trypitone enzymatic digest from casein, Triton™ X-100 and yeast extract for microbiology were purchased from Sigma-Aldrich (Irvine, UK). Ethanol, glycerol (99%), hydrochloric acid (37%), isopropanol, L-(+)-lactic acid (90 %) and sodium borohydride were purchased from Acros Organics (Geel, Belgium). 3-(4, 5-dimethylthiazol-2)-2, 5 diphenyl tetrazolium bromide (MTT), 4′,6-Diamidino-2-Phenylindole dihydrochloride (DAPI), dimethylformamide, DMEM (high glucose, with GlutaMAX™ and pyruvate), foetal bovine serum (FBS), Hank's Balanced Salt Solution (HBSS), HPLC-grade water, methanol, nitric acid (70%), penicillin/streptomycin solution, phalloidin Dylight 550, Pierce ™ BCA Protein Assay and silver nitrate were purchased from Fisher (Loughborough, UK). Single-wall carbon
nanotubes-COOH OD 1-4 nm (SW-CNTs) were purchased from Cheaptubes.com (Grafton, VT). Corning DMEM/F12 (with L-glutamine and 15mM HEPES) was purchased from Scientific Laboratory Supplies (UK).

2.2.2 Synthesis of AgNWs by soft template method

AgNWs were synthesised via the polyol method, using silver nitrate (AgNO₃) as the Ag source. PVP (3 g) was fully dissolved in glycerol (95 ml) by heating to 80 °C. The solution was cooled down and AgNO₃ (0.79 g) was added under vigorous stirring (800 rpm) until the powder was fully dissolved. Subsequently, NaCl (5 mM in final solution) was mixed into 5 ml of glycerol and added to the PVP/glycerol solution. The reaction temperature of the mixture was raised to 210 °C. Once the temperature was reached, it was maintained for further 10 min. Samples (1 ml) were taken at different temperatures, diluted with water and analysed by UV (300-900 nm, Thermo Scientific Nicolet Evolution 100 UV-Visible Spectrophotometer) to follow the formation of the AgNWs. Finally the reaction was cooled down to room temperature, diluted 1:1 with deionized water and centrifuged (2880 x g, 1 hour), the pellet was then washed twice with isopropanol (2880 x g, 30 min) and twice with deionized water (2880 x g, 10 min) (Thermo Electro Corporation, B4i Jouan Multifunction Centrifuge, UK). The product was stored in deoxygenated purified water at room temperature, protected from light.

2.2.3 Synthesis of AgNWs in presence of CNTs (AgNWs-CNT)

AgNWs were synthetized in presence of CNTs with a slightly modified method. After
complete dissolution of PVP in glycerol, 5.8 mg of carbon nanotubes were added and
sonicated for 2 hours to favour dispersion. After sonication, silver nanowires were
synthesized and washed as described above.

2.2.4 Preparation of physical mixture of CNT and AgNWs (AgNWs-CNTmix)

N-octyl-O-sulphate chitosan (NOSC) was synthetised as previously described [94]. NOSC (10
mg) was dissolved in 10 ml of purified water, before the addition of 12.5 mg of CNTs. The
sample was sonicated for 4 hours. Then, 40 ml of deoxygenated water and 2 g of PVP were
added to the mixture. The mixture was then heated and maintained at 80°C for 30 min.
AgNWs in deoxygenated water (10 ml, 1.5mg/ml) were then added to the reaction mixture,
this was stirred at 800 rpm for 6 hours in the dark. The sample was then centrifuged at 2880
g for twenty min and the supernatant removed. This step was repeated twice. The solid pellet
obtained was then washed and stored as described above.

2.2.5 TEM characterization of AgNWs, AgNWs-CNT, AgNWs-CNT-mix and swCNTs

Freeze dried products were suspended in chitosan solutions (1%, in lactic acid 0.1 M), to
achieve different concentrations (0.5-1.5 mg/ml), by vortexing and sonicating for several
min. Samples were then dropped onto TEM grids (Agar scientific square mesh TEM support
grids-copper) and allowed to dry at room temperature and pressure. Dry grids were stored
in sealed petri dishes in a desiccator until observation using a Jeol JEM 2100 Transmission
Electron Microscope.
2.2.6 XRD characterization of AgNWs, AgNWs-CNT, AgNWs-CNT-mix and CNTs

The crystal structure of nanoparticles was determined with a Bruker D2-Phaser diffractometer. Instrumental parameters were: CuKα radiation, 30kV, 10 mA, LynxEye PSD detector with an angular opening of 5°, 2θ range 6-84°, step size 0.020°, time per step 2 s, spinner 15 rpm. The alignment of the instrument was calibrated using an international standard (NIST 1976b). A low-background silicon crystal specimen holder (Bruker) was used. The analysis was performed at room temperature (25°C). The XRD pattern was evaluated using the software Bruker EVA 14.2 (DIFFRACplus Package) coupled with the database PDF-2 (ICDD).

2.2.7 Total silver content and silver release by AAS

For total silver content, freeze-dried NPs were digested in an equal volume of MilliQ water and 70% nitric acid. The volume of digested samples was brought up to 20 ml with MilliQ water. Finally the solutions were filtered 5 times by a glass microfiber filter (GF/D) to get rid of the CNTs.

For silver cation release studies, freeze-dried NPs were transferred to a water bath maintained at 37°C. At scheduled time points, the AgNWs suspensions were centrifuged at 1440 x g for 3 min and 1 ml of supernatant was taken and substituted with 1 ml of fresh water. The determinations of Ag content of both experiments were carried out by a furnace absorption spectro-photometer (Varian SpectrAA 220FS) at a wavelength and spectral bandwidth of 328.1 and 0.2 nm, respectively. The experiments were carried out in triplicate.
2.2.8 Autofluorescence detection

In order to determine whether either AgNWs or CNTs possessed any autofluorescence, freeze-dried NPs were suspended in deionized water and sonicated to provide a homogeneous suspension. Samples were excited at different wavelengths (390, 488, 550, 570, 633 and 670 nm) and the respective emission spectra were recorded. Images of the suspensions were then acquired by fluorescence microscopy (Zeiss Axio Imager Z1).

2.2.9 Zeta potential determination

Surface zeta potentials were measured using Malvern Zetasizer (Nano ZS, UK). Nanoparticles were suspended by sonication in deionized water. An average of twelve measurements per samples were carried out. The selected refractive index for silver nanoparticles was 1.333.

2.2.10 Bacterial cultures preparation

The antibacterial activity of AgNWs was examined by a suspension assay against gram negative \textit{E.coli} (ATCC 25922) and gram positive \textit{S. aureus} (ATCC 25923), Methicillin-resistant \textit{S. aureus} (ATCC 12403) and \textit{S. saprophyticus} (ATCC 15305). The bacteria were transferred from -80°C (30 % glycerol) into 5 ml of fresh sterile LB by a sterile toothpick and incubated (at 37°C and 200 rpm) until the bacterial suspension was cloudy (1 day for \textit{E.coli} and \textit{S. aureus}, 2 days for MRSA and 3 days for \textit{S. saprophyticus}) (MaxQ™ 8000, Thermo Scientific). Then, 50 µl of the bacterial suspension were transferred into 5 ml of fresh sterile LB and the bacteria were further incubated at 37°C until the suspension was newly cloudy (1 day for \textit{E.}}
coli, S. aureus and MRSA and 2 days for S. saprophyticus).

2.2.11 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Bacterial suspensions were prepared as described above. Serial dilutions of NPs and silver nitrate were tested (10, 100, 200, 500, 750, 1000, 1500, 2000 and 3000 µg/ml) against bacterial suspensions (1 x 10^7 CFU/ml) to a final volume of 150 µl in 96 well plates. Batch suspensions (10 mg/ml) of NPs were prepared in deionized water and then, diluted in LB. Instead, AgNO₃ was dissolved in water in order to have an initial concentration of 10 mg/ml of cationic silver. Medium with just bacteria served as negative control. The plates were incubated at 37°C for 24 hours with gentle shaking (60 rpm). The absorbance was read at 600 nm using nanoparticles suspensions as blanks. MIC was determined as the lowest concentration visibly inhibiting growth. MBC was determined by transferring 25 µl samples from clear wells into nutrient agar plates. After overnight incubation, the MBC was determined as the concentration at which there was no microbial growth. All experiments were performed in duplicate and results were reported as mean ± SD.

2.2.12 SEM imaging of bacteria with nanoparticles

Bacterial suspensions were prepared as described above. Then, bacteria were diluted to an OD₆₀₀ of 0.01 and they were treated with different concentrations of nanoparticles (50 and 800 µg/ml) and incubated for 8 hours. The experiment was carried out in 96 well plates (150 µl). The samples were then aseptically removed and centrifuged at 5000 x g for 10 min, the medium was removed and 800 µl of 0.2 M cacodylate was added to suspend the pellet. Then,
50 µl of cell suspension was added to a sterile silicon wafer (shiny side up) (Agar Scientific) and it was allowed to settle for 40 min. Glutaraldehyde (0.5 ml, 2%) in 0.2 M cacodylate was gently added to each silicon wafer, making sure the specimens were completely immersed. The samples were fixed for 1 hour, at 4°C. Then, the samples were washed once with buffer and were dehydrated through a series of ethanol solutions (30, 50, 70, 90, 95 and 100%) for 5 min each at room temperature. Finally, samples were covered with acetone (until evaporation) and in 100% hexamethyldisilazane (HMSD) before being allowed to air-dry overnight. Images were taken by a scanning electron microscope (Zeiss EVO MA10), after having sputter coated the samples with gold and palladium (Polaron e500, Quoram Technologies, UK).

2.2.13 Protein leakage from bacteria

Bacteria were grown as described above. Then, bacteria suspensions were centrifuged (13000 x g for 5 min) and the pellets were suspended in sterile PBS to a final concentration of 0.6 CFU/ml in presence of two concentrations of NPs: 12.5 and 100 µg/ml. Bacteria were incubated at 37°C and 200 rpm. After 1 hour and 24 hours of incubation, 1 ml of sample was taken from each test tube and centrifuged at 13000 g for 5 min. The supernatant was then stored at -20°C until further analysis. The bicinchoninic assay (Pierce ™ BCA Protein Assay) was carried out according to the manufacturer’s instructions. Briefly, 25 µl of samples were mixed with 200 µl of working solution. Samples absorbance was read at 562 nm after two hours of incubation at 37°C. The experiment was carried out in duplicate.
To determine levels of ROS generated, a DCFH-DA probe was used. 2, 7- dichlorofluorescein diacetate (DCFH-DA) is converted to highly fluorescent 2, 7- dichlorofluorescein (DCF) in the presence of reactive oxygen species (H$_2$O$_2$, HO$^*$ and ROO$^*$) [95]. Initially, the DCFH-DA probe was suspended in DMSO at a 10mM concentration, before being diluted with LB medium to create a 100 μM working concentration. At the 24-hour time point of the bacterial suspension studies, 1 ml samples were removed and centrifuged at 1300 rpm for 1 minute. The supernatant (40 μl) was removed and incubated with 60 μl of the fluorescent probe for thirty min in the dark at 37°C. Using an excitation wavelength of 485 nm and an emission wavelength of 520 nm, the fluorescence of the samples was read on a bench top, top read fluorimeter (Agilent Cary Eclipse).

Human foetal osteoblasts (hFOB 1.19), human foreskin fibroblasts (Hs27) and human colorectal adenocarcinoma cells (Caco-2) were purchased from ATCC®. hFOB were maintained at 37°C in 5% CO$_2$ in flask in Dulbecco’s Modified Eagle’s Medium Ham/F12 medium (with L-glutamine and 12 mM HEPES) containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Hs27 were maintained in the same physical conditions, but using DMEM (high glucose, with GlutaMAX™ and pyruvate), 10% FBS and 1% P/S. Caco-2 cells were maintained at 37°C in 5% CO$_2$ in MEM containing 10% FBS, 1% P/S, 2mM L-glutamine and 1% non-essential amino acids. Cell lines were used between passage 4 and 12.
Cell viability study

Cell viability was evaluated by the MTT [3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide] colorimetric technique. Briefly, 5000 cells/well were plated in a 96 well plate with 100 µl of complete medium and incubated overnight, to permit cells attachment. Meanwhile, stock suspensions of nanoparticles in complete medium were sonicated for 4 h at 40 Hz. Aliquots of initial AgNPs solution were added to cell medium to final NPs concentrations of 0.1, 1, 10, 25, 50 and 100 µg/ml. After 24 hours of incubation, 100 µl of treatments were added to each well containing cells and incubated for 2 and 24 h. At scheduled time points, the treatments were removed and cells were treated with 100 µl of complete medium containing MTT (0.5 mg/ml). The cells were incubated for 4 h in 5% CO₂ incubator for reduction of MTT by metabolically active cells. The reagent was removed and the purple formazan crystal inside the cells were solubilized with 100 µl of DMSO. The formazan content was quantified by spectrophotometry, at a wavelength of 570 nm (SpectraMax® i3x, Molecular Devices). DMSO served as a blank. The experiments were carried out at least in triplicate.

Determination of membrane integrity by LDH assay

To evaluate cell membrane integrity, the lactate dehydrogenase (LDH) leakage assay was performed. Cells were plated at 10,000 cells/well. Then, cells were treated with nanoparticles in complete medium containing 2% FBS (as the serum may interfere with the assay). Spontaneous activity and maximum LDH activity were used as controls. The assay was carried out according to the manufacturer’s instructions (Pierce ™ LDH cytotoxicity assay).
The absorbance was read at 490 and 680 nm. The absorbance read at 680 nm (background) were subtracted from the 490 nm absorbance before calculation of LDH release.

2.2.18 Intracellular production of ROS

Cells were plated in a sterile black 96 well plate at 25,000 cells/well in 100 µl of medium. Cells were incubated for 24 h and then, the medium was removed and the cells were washed once with sterile HBSS containing Ca and Mg. The cells were incubated with a solution 28 µM of DCFH-DA in HBSS for 45 min. After washing with HBSS, the cells were treated with complete media (2% FBS), containing nanoparticles, for desired periods of time. Fluorescence was measured with a fluorescence reader, with an excitation of 485 nm and emission of 525 nm.

2.2.19 Cell uptake of silver

Human osteoblasts were plated at 30,000 cells/well (1 ml medium) in a 24 well plate and incubated for 24 h, as described above. Cells were exposed to nanoparticles at different concentrations (1 ml). After 24 hours of exposure, the medium was removed and the cells were gently washed twice with cold PBS to remove loosely attached Ag ions and/or NPs from the cell membrane. To each well, HPLC grade water (600 µl) was added, and then 70% HNO₃ (600 µl). The nanoparticles were then digested for 20 min and the solutions were brought to 21 ml with HPLC grade water. Controls were made with just cells. Silver content was determined by an inductively coupled plasma optical emission (ICP-OES) spectrometer (Spectroblue OEP-TI, Ametek, Germany) equipped with an ASX-520 autosampler. External
calibration was performed by analysis of a blank and five solutions of dissolved Ag in 2% HNO3 ranging from 0 to 100 μg L\(^{-1}\). The charged Ag ions were measured at two wavelengths (328.068 and 328.289 nm) and the results were averaged. The ICP-OES was equipped with a SPECTRO SMART ANALYZER software (vs. 6.01.0943). The set parameters for the analysis were: 1450 W for plasma power, 30 rpm for pump speed, 13.00 l/min for coolant flow and 0.75 l/min for nebulizer flow.

2.2.20 Fluorescence imaging of AgNWs uptake

Osteoblasts were seeded on coverslips at a cell density of 10000 cells/well in 24 well plates (300 ul of medium). After overnight incubation at 37°C and 5% CO\(_2\), cells were treated with AgNWs. After 24, cells on coverslips were fixed directly with paraformaldehyde 4% in PBS for 15 min, washed with PBS, permeabilized for 10 min in 0.1% Triton X-100/PBS, washed twice with PBS, blocked with 2% bovine serum albumin (BSA) in PBS for 1 hour, washed twice with PBS, stained with Phalloidin Dylight 550 in PBS (2 units/ml, stock solution 300 units/ml in methanol) for 1 hour (300 µl, at room temperature), washed twice with PBS, stained with DAPI 2 µg/ml in PBS for 10 min and finally rinsed again with PBS. Coverslips were mounted on glass slides using PermaFluor Aqueous Mounting Medium. Samples were kept protected from light until imaging. Photos were taken with a fluorescence microscope (Zeiss Axio Imager Z1) equipped with a Hamamatsu HR camera and also a color AxioCam MRc camera. Images were processed by Volocity 6.3 software.
2.3 Results

2.3.1 AgNWs physicochemical characterisation

2.3.1.1 UV-vis and TEM characterization

Figure 2.1 Physicochemical characterization of synthesised products. UV-spectra of (A) AgNWs and (B) of AgNWs-CNT at different temperatures during the synthesis reaction. TEM images of (C) AgNWs (scale bar 500 nm), (D) AgNWs-CNT (scale bar 500 nm), (E) AgNWs-CNT-mix (scale bar 500 nm), (F) CNTs (scale bar 500 nm), (G) AgNWs-CNT, presenting particles aligned along the CNTs (scale bar 200 nm) and (H) AgNWs-CNT-mix with NPs aligned along CNTs (scale bar 500 nm).

AgNWs were synthesised via the polyol method using PVP as soft template, whereas
AgNWs-CNT were synthesised with the same method, in presence of CNTs, in order to evaluate whether the addition of nanotubes influenced the morphological, physical and biological properties of the AgNWs. AgNWs-CNT-mix was used as control to investigate whether any potential difference between AgNWs in AgNWs-CNT was due to the mere presence of CNTs or if they had an effect when introduced in the synthetic procedure.

The UV-Vis spectra of AgNWs and AgNWs-CNT, at low temperatures, showed a peak at 410 nm, indicative of the formation of nucleation sites with initial presence of silver nanoparticles and nanorods (Figure 2.2 A-B). A shift towards lower wavenumbers and the formation of a double peak (350 and 380 nm) was observed as the temperature increased, indicating the formation of longer structures such as nanowires [96]. Interestingly, the shift was observed at lower temperatures when the synthesis of AgNWs was performed in the presence of CNTs; this could be due to the excellent heat conductivity of CNTs that can affect the reaction kinetics. This would allow for silver nanoparticles in close proximity to carbon nanotubes to benefit from a localised increase in temperature and thus would allow for AgNWs formation at lower temperatures of the overall reaction mixture.

Nanoparticles were also observed by TEM (Fig 2.1 C-H). TEM images confirmed the successful formation of AgNWs in both synthesis conditions. However, in all the cases, other types of nanoparticles, such as nanospheres and nanorod, were visualized. Moreover, the amount of CNTs found in AgNWs-CNT appeared significantly inferior to AgNWs-CNT-mix. Interestingly, in both AgNWs-CNT and AgNWs-CNT-mix samples, the smaller particles tended to align along the surface of CNTs (Fig. 2.1 G-H). In the synthesis of AgNWs, a coordination complex is formed between silver ions and PVP through donation of loan pair electrons of oxygen and nitrogen atoms of PVP to sp orbitals of the metallic silver reduced by glycerol. We hypothesized that –COOH groups of CNTs can interact with Ag+ forming the first nucleation
centres for the growth of silvernanowires. This supports the theory that nucleation centres form on the surface of carbon nanotubes and lead to formation of nanowires along the axis of CNTs. Average lengths were 5.23 μm (± 1.5), 5.21 μm (± 2.7) and 5.04 μm (± 1.7), for AgNWs, AgNWs-CNT and AgNWs-CNT-mix, respectively. Average diameters 73.70 nm (± 25.79), 67.04 nm (± 25.23) and 68.54 nm (±17.47) for AgNWs, AgNWs-CNT and AgNWs-CNT-mix, respectively. One-way ANOVA showed no significant difference in lengths and diameters across all three materials (p>0.05).

2.3.1.2 Total silver content and Ag⁺ release

Total Ag content in each of the products and the cumulative Ag⁺ released over time were determined by AAS. The total Ag (Fig. 2.2 A) found in AgNWs (97.33± 1.14 wt/wt %) was higher than in AgNWs-CNT (89.11±1.25 wt/wt %), but not significantly different (One-way ANOVA, p>0.05), meaning that both the synthesis and purification processes lead to a high yield of silver products. Furthermore, these values suggest that whereas CNTs might have played a role in the nucleation and reduction of heat required in the synthesis, they are not chemically bound to AgNWs, and as a consequence, they are efficiently removed by the purification process (Ag content: 89.11±1.25 wt/wt %). On the other hand, the amount of Ag in AgNWs-CNT-mix was significantly inferior to the other two batches (ca. 35.27±5.33 wt/wt %) (p<0.001). This was expected as CNTs and AgNWs, in this case, were added in a 1:1 ratio.
Figure 2.2 Silver content and release. (A) Percentage silver content of AgNWs, AgNWs-CNT and AgNWs-CNT-mix; One-way ANOVA returned p<0.05; results of Post-hoc Tukey’s multicomparison test, when comparing all the formulations to AgNWs, are reported in the graph (** p=0.001); results of Post-doc Tukey’s test, when comparing AgNWs-CNT to AgNWs-CNT-mix ($$$ p=0.003). (B) Percentage cumulative release of silver from AgNWs (red), AgNWs-CNT (black) and AgNWs-CNT-mix (green).

The normalized cumulative release of Ag⁺ from AgNWs, AgNWs-CNT and AgNWs-CNT-mix, in water, is reported in Fig. 2.2 B. Ag⁺ release started immediately in the aqueous solution and its concentration increased over time. After 2 days in water, the percentage amount of Ag⁺ released was of ca. 0.32 wt/wt% from AgNWs, 0.23 wt/wt% from AgNWs-CNT and 0.71 wt/wt% from AgNWs-CNT-mix. One-way ANOVA revealed no statistical significance among the samples (p>0.05). After 30 min, the concentration of Ag⁺ in suspension was ca. 0.31±0.04 ppm from AgNWs, 0.22±0.17 ppm from AgNWs-CNT and 0.09±0.02 ppm from AgNWs-CNT-mix. Kumar et al. reported that a minimum concentration level of 0.1 part per billion (ppb) could provide effective antimicrobial activity [97]. As in our samples, the silver ion concentration was higher than 0.1 ppb already after 30 min, the nanomaterials may be seen as potential platforms for Ag⁺ delivery for antibacterial purposes.
The X-ray diffraction (XRD) performed on samples was used to reveal information about the chemical composition of nanoparticles, as well as their detailed crystallographic structure. **Fig. 2.3 A** shows the XRD spectrum of AgNWs sample and of AgNWs-CNT. The diffraction peaks at 2θ values of 38.1°, 44.3°, 64.4° and 77.3° were assigned to the crystal planes (111), (200), (220) and (311), which are typical of AgNWs [36]. Furthermore, the (111)/(200) peak ratio was 3.7, indicating the formation of well-elongated AgNWs. Minimal trace impurity of AgCl can be seen at 2θ value of 32.1° and 46.2°.

**Figure 2.3** XRD and fluorescence spectra. (A) XRD spectra of AgNWs (pink) and AgNWs-CNT (blue). (B) XRD spectra of AgNWs (violet), AgNWs-CNT-mix (green) and CNTs (black). (C) The emission fluorescence peaks of AgNWs (red), AgNWs-CNT-mix (black), CNTs (grey) and water (blue). (D) Fluorescence microscope image of AgNWs-CNT in water: white arrow indicates AgNWs and yellow arrow indicates CNTs.
Superimposed spectra of AgNWs and AgNWs-CNT confirmed that the two synthetic processes lead to similar products. The (111)/(200) intensity ratio for AgNWs-CNT was 3.2. In Fig. 2.3 B the XRD pattern spectra of AgNWs (violet), AgNWs-CNT-mix (black) and swCNTs (green) are reported. According to the AAS results, this product was mainly composed of swCNTs and a smaller portion of the powder was made of AgNWs, thus the expected AgNWs-CNT-mix spectrum should have been a combination of both swCNTs and AgNWs XRD profiles. The peaks at 2θ angles of 25.7°, 43.4° and 57.3° of swCNTs profile in Fig. 2.3 B, correspond to the graphite d-spacing (distance between atomic layers of a crystal) of the swCNTs and the (111) and (200) reflections of carbon, respectively [24]. The origin of peaks at 18.2° and 35.6° is not explained. The (111)/(200) intensity ratio of AgNWs-CNT-mix was found to be 3.2, as for AgNWs-CNT. The fluorescence emission of AgNWs, AgNWs-CNT and CNTs aqueous solutions was also investigated. Excitation at wavelengths between 390 nm and 400 nm lead to fluorescence emission between 450 and 600 nm and lambda maxima at 465 nm, corresponding to the blue-green emitting region. In literature, the fluorescence emission properties of silver clusters and silver nanoparticles of different dimensions has already been described [98-100], but, as far as we know, it is the first time this effect has been reported for AgNWs. The autofluorescence of AgNWs can be exploited for different applications, such as fluorescence imaging of nanowires in cells.

Surface zeta potential (ζ) was also measured. The zeta potentials values were found to be negative for all the preparations containing AgNWs, as already reported by Visnapuu et al. [60]. In particular, the zeta potential of AgNWs was -12.43±1.23, of AgNWs-CNT was -18.24±5.92 and of AgNWs-CNT-mix was -19.8±7.66. These results suggested that no one of the preparations was stable in suspension, having ζ < -30 mV.
2.3.2 Antibacterial properties

2.3.2.1 MIC and MBC

Table 2.1 MIC and MBC values (mg/ml) for AgNWs, AgNWs-CNT, AgNWs-CNT-mix, AgNO3 and CNTs.

MIC and MBC values are reported as a mean ± SD (n≥3). The One-way ANOVA returned p<0.05. Results of the post Tukey’s multiple comparisons test was carried out to compare the experimental values among each other. For E. coli MIC values: a p<0.05 (compared to AgNO3); for MRSA MIC values: a p<0.01 (compared to AgNO3); for S. saprophyticus MIC values: a p<0.05 (compared to AgNO3). For E. coli MBC values: a p<0.01; b p<0.001; c p<0.05 (compared to AgNO3); for S. aureus MBC values: a p<0.001 (compared to AgNO3); for MRSA MBC values: a p<0.0001 (compared to AgNO3).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Material</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AgNWs</td>
<td>0.42±0.26</td>
<td>1.16±0.27a</td>
</tr>
<tr>
<td></td>
<td>AgNWs-CNT</td>
<td>0.53±0.54a</td>
<td>1.38±0.55b</td>
</tr>
<tr>
<td></td>
<td>AgNWs-CNT-mix</td>
<td>0.49±0.07a</td>
<td>0.99±0.16c</td>
</tr>
<tr>
<td></td>
<td>AgNO3</td>
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<td>0.17±0.05</td>
</tr>
<tr>
<td></td>
<td>CNTs</td>
<td>&gt;3.00</td>
<td>&gt;3.00</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AgNWs</td>
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<td>1.55±0.63a</td>
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</tr>
<tr>
<td></td>
<td>AgNO3</td>
<td>0.01±0.00</td>
<td>0.1±0.00</td>
</tr>
<tr>
<td></td>
<td>CNTs</td>
<td>&gt;3.00</td>
<td>&gt;3.00</td>
</tr>
<tr>
<td><strong>MRSA</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>AgNO3</td>
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<td>0.07±0.05</td>
</tr>
<tr>
<td></td>
<td>CNTs</td>
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<td>&gt;3.00</td>
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<td><strong>S. saprophyticus</strong></td>
<td></td>
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<td></td>
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<tr>
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<td>AgNWs</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>AgNO3</td>
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<td>0.01±0.00</td>
</tr>
<tr>
<td></td>
<td>CNTs</td>
<td>&gt;3.00</td>
<td>&gt;3.00</td>
</tr>
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</table>

The potential bacteriostatic and bactericidal activity of the synthesized materials was tested against four different bacterial species: *E. coli*, *S. aureus*, MRSA and *S. saprophyticus*. 

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In order to compare the activity of AgNWs, AgNWs-CNT, AgNWs-CNT-mix and AgNO₃, the MIC and MBC values obtained were normalized to the amount of Ag present in each material.

All the studied materials inhibited cell growth at the concentrations tested, except for CNTs which didn’t show any antibacterial activity (Table 2.1). In particular, the normalized MIC values of AgNWs, AgNWs-CNT and AgNWs-CNT-mix were not statistically different from each other (p>0.05) for all bacterial species. Furthermore, the order of efficacy in inhibiting bacterial growth was: \textit{S. saprophyticus} > \textit{S. aureus} > \textit{E. coli} > \textit{MRSA}. All the preparations were less efficient than AgNO₃ in inhibiting cell growth. The normalized MBC values for AgNWs, AgNWs-CNT and AgNWs-CNT-mix were also not statistically different from each other (p>0.05) against \textit{E. coli}, \textit{S. aureus} and MRSA. In the case of MRSA, it was not possible to compare the results, as the MBC of AgNWs-CNT-mix was higher than the maximum tested concentration. The order of efficiency was: \textit{S. saprophyticus} > \textit{E. coli} = \textit{S. aureus} > \textit{MRSA}. All the preparations were less efficient than AgNO₃ in killing the different bacterial strains. Once again, CNTs did not show any bactericidal activity against the studied bacterial species.

Even though the MIC values found for \textit{E. coli} and \textit{S. aureus} were higher than what reported by Cui and Liu for AgNWs (28 and 35 µg/ml against \textit{E. coli} and \textit{S. aureus}, respectively)[63], Hong et al. found values > 100 µg/ml against \textit{E. coli}. The results could be explained considering that the initial bacterial density of our experiment was higher (1 x 10^7 CFU/ml) than the ones used in the other two studies. For instance, Hong et al. tested the AgNWs against an initial cell density equal to 1 x 10^5 CFU/ml. Indeed, it is well known that the efficacy of antibiotics, as well as silver nanoparticles, declines with the increase of the bacterial density [61].
In this work, AgNWs-CNT-mix was used as a control to check whether the silver nanoparticles in AgNWs-CNT presented different characteristics from AgNWs in the first synthesis method. As the MIC and MBC values tend to be similar among AgNWs, AgNWs-CNT and AgNWs-CNT-mix, we could assume the three preparations had similar antibacterial properties with no effect due to the presence of CNTs.

2.3.2.2 Bacterial growth kinetics

In order to study how the bacterial growth kinetics were influenced by different concentrations of studied materials, growth was followed by measuring O.D.\textsubscript{600nm} in LB medium. The kinetics of the bacterial growth curves followed a typical pattern (Fig. 2.4): a lag phase, an exponential phase and a stationary phase. The lag phase is the time in which no bacteria replication happens as the bacteria adapt into the medium after being inoculated. During this phase, cells express transient genes that promote transcriptional processes in order to synthetize transporters and enzymes for the uptake of fundamental carbon nutrients \cite{101}. Then, bacteria start to divide asexually in an exponential rate, originating the so called exponential phase. When bacteria exhaust nutrient sources, cells enter into stationary phase in which no increase in cell number is observed and an equilibrium between death and life is reached.

The activity of AgNWs, AgNWs-CNT against the different bacterial strains is reported in Fig. 2.4. AgNWs showed an interference in the duration of both lag phase (100 \(\mu g/ml\), \(p=0.0359\)) and time to reach the stationary phase (50 \(\mu g/ml\), \(p=0.0252\)) against \textit{E. coli} (Fig. 2.4 A). MRSA showed a significantly longer lag phase than the control only in presence of AgNWs 100 \(\mu g/ml\) (\(p=0.0031\)) and no effect on the time to reach the stationary phase (Fig. 2.4 E).
**E. coli** (Fig. 2.4 A) was more sensitive to AgNWs-CNT than AgNWs: its lag phase (12.5 µg/ml, \(p=0.0003\), whereas 50 µg/ml and 100 µg/ml had a \(p<0.0001\)). The time to reach the stationary phase was statistically longer for concentrations equal or higher than 50 µg/ml (\(p=0.0382\)). Finally, **S. saprophyticus** (Fig. 2.4 G) lag phase duration was increased for the two lowest concentrations (12.5 µg/ml had \(p=0.0071\) and 25 µg/ml had \(p=0.0141\)), whereas no growth was found for the higher concentrations of nanoparticles and the time for reaching the stationary phase was significantly longer even for the lowest tested concentration.

**E. coli** was more sensitive to AgNWs-CNT (Fig. 2.4 A) than AgNWs: its lag phase (12.5 µg/ml, \(p=0.04447\), whereas 25, 50 and 100 µg/ml had a \(p<0.0001\)) and the time to reach stationary phase were significantly longer for the lowest concentration, too. Seemingly, **S. aureus** (Fig. 2.4 D) was more sensitive to the action of AgNWs-CNT than AgNWs: no growth was found for the highest concentration, whereas 12.5 µg/ml had a \(p=0.0019\) and the higher concentrations had a \(p<0.0001\). No difference in the time to reach the stationary phase was found for concentrations 12.5 and 25 µg/ml in comparison to the control. **MRSA** (Fig. 2.4 F) growth was affected by AgNWs-CNT: 12.5 µg/ml (\(p=0.0051\)), 25 µg/ml (\(p<0.0001\)), 50 µg/ml (\(p<0.0001\)) and 100 µg/ml (\(p=0.0003\)). No effect on the time to reach the stationary phase was observed. **S. saprophyticus** lag phase (Fig. 2.4 H) was affected for the two lowest concentrations (\(p=0.0210\)) and no growth was observed for the higher concentrations. The time to reach the stationary phase of **S. saprophyticus** was statistically longer for the concentration 25 µg/ml than the control.
<table>
<thead>
<tr>
<th>Figure 2.4</th>
<th>Growth curves of bacteria with different concentrations of AgNWs (A, C, E, G) and AgNWs-CNT (B, D, F, H). Results are reported as a mean ± SD (n=3).</th>
</tr>
</thead>
</table>

For all the studied nanoparticles and bacterial strains whenever the stationary phase was reached, no statistical difference in OD values was found among treated and untreated
Overall, results suggest that for all the bacterial strains, introduction of AgNWs affected the growth kinetics as compared to the negative control. Similar results were observed by Cui et Liu which described the influence of increasing concentrations of AgNWs on *E. coli* growth [63]. However, whereas Cui and Liu showed growth inhibition at concentrations higher than 25 µg/ml, in this study, none of the concentrations used could inhibit cell growth in the same time frame. Variations in the results can be due to microbial strains, but also to physicochemical characteristics of AgNWs suspension [102]. Finally, as already found in MIC and MBC study, MRSA seemed to be the least sensitive strain among the species studied, whereas *S. saprophyticus* was the most susceptible. On the other hand, similar trends were reported for *E. coli* and *S. aureus*. These results seemed to show that it is not possible to explain the species sensitivity to silver nanoparticles in terms of bacterial classification (Gram + and -), but in terms of specific differences among species, as reported for instance by Agnihotri et al. [102]

2.3.2.3 *Leakage of proteins from bacteria*

Cui and Liu reported that AgNWs could cause the leakage of cytoplasmic content, such as DNA and proteins, from *E. coli* [63]. This phenomenon may be due to several alterations in bacterial cells, such as the inhibition of the activity of membranous enzymes or DNA damages.

In this work, a bicinchoninic acid (BCA) assay was used to determine whether or not, AgNWs obtained by the two methods induced release of proteins in the extracellular liquid. All bacterial species tested showed an increased release of proteins in comparison to the control
(Fig. 2.6 A-H). Moreover, this effect is dose-dependent, but no difference was found between 1 and 24 h, except for *S. saprophyticus*. Interestingly, higher amount of proteins leaked out of *E. coli* compared to *S. aureus* and MRSA, suggesting that Gram-negative bacteria could be more sensitive than some of the Gram-positive bacteria to the action of AgNWs. A higher protein release in gram negative than in gram positive bacteria was already highlighted by Soo-Hwan et al. who tested AgNPs (no information about the size) against *E. coli* and *S. aureus* up to 6 h [103]. In this case, even in the control group an increase in protein release was found, probably due to physiological bacterial cell death and the release of extracellular proteins. No statistical difference was found between AgNWs and AgNWs-CNT, when comparing the same concentrations.
Figure 2.6 Absorbance relative to protein release after 1 h and 24 h of treatment for *E. coli* (A and B), *S. aureus* (C and D), MRSA (E and F) and *S. saprophyticus* (G and H) treated with 12.5 µg/ml or 100 µg/ml of AgNWs or AgNWs-CNT. Data are represented as a mean ± SD (n=4). The One-way ANOVA performed on all the samples showed significantly different release for both AgNWs and AgNWs-CNT (p<0.05). Results of the post-hoc Tukey’s multicomparison test are shown in the graphs (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001 and **** indicates p < 0.0001).
Previous studies on silver nanoparticles suggest that the oxidative stress plays a crucial role in the origin of the bacterial toxicity of nanoparticles. In this work, the ROS production in the medium outside cells was quantified 24 h after treatments with nanoparticles (Fig. 2.7 A-H). Fluorescence production was measured through 2’,7’-dichlorofluorescin diacetate (DCF-DA) probe. DCF-DA in the presence of ROS is oxidised to form a highly fluorescent product, called 2’, 7’ –dichlorofluorescein (DCF). Results showed that ROS were produced when bacteria were treated with all the types of metallic nanoparticles. Moreover, generally, we could observe that the amount of DCF produced increased with the increase of the concentration of nanoparticles, as reported in literature for other types of AgNPs, such as nanospheres against *S. aureus*, *E. coli* and *P. aeruginosa* [104]. Interestingly, MRSA did not show any significantly increased ROS generation in comparison with the no treated cells. For *S. saprophyticus* treated with AgNWs, no significant difference was detected (*p*>0.05), but when cells were treated with AgNWs-CNT, there was statistical difference for concentrations equal or higher to 25 µg/ml.

AgNWs were found to produce statistically more ROS than AgNWs-CNT in *E. coli* and *S.aureus*. In particular, the unpaired t-test for *E. coli* showed the following values: 100 µg/ml presented *p*=0.0188, 50 µg/ml presented *p*=0.0346, 25 µg/ml presented *p*=0.0007 and 12.5 µg/ml presented *p*=0.0064. On the other hand, the unpaired t-test against *S. aureus* showed the following results: 100 µg/ml presented *p*=0.0243 and 50 µg/ml presented *p*=0.0154.

All together these results suggest that bacterial toxicity of AgNWs was probably mediated through oxidative stress, in case of *E. coli* and *S. aureus*, whereas the ROS levels in MRSA were not significant, at the tested concentrations, suggesting that MRSA could present more efficient antioxidant mechanisms than *E. coli* and *S. aureus*. As regards *S. saprophyticus* more
studies should be carried out to understand the difference between the two treatments.

<table>
<thead>
<tr>
<th></th>
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<th>AgNWs-CNT</th>
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<td><img src="image2" alt="" /></td>
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<tr>
<td><strong>S. aureus</strong></td>
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<td><img src="image6" alt="" /></td>
</tr>
<tr>
<td><strong>S. saprophyticus</strong></td>
<td><img src="image7" alt="" /></td>
<td><img src="image8" alt="" /></td>
</tr>
</tbody>
</table>

**Figure 2.7** ROS production (% of the control) from bacterial cells, after 24 hours from treatment. *E. coli* (A and B), *S. aureus* (C and D), MRSA (E and F) and *S. saprophyticus* (G and H) treated with 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml of AgNWs or AgNWs-CNT. Data are represented as a mean ± SD (n=3). The One-way ANOVA performed on all the samples showed significantly different ROS production for both AgNWs and AgNWs-CNT (*p*<0.05) for some of the bacterial strains. Results of the post-hoc Tukey's multicomparison test are shown in the graphs (* indicates *p* < 0.05, ** indicates *p* < 0.01, *** indicates *p* < 0.001 and **** indicates *p* < 0.0001)
SEM images of the 4 bacterial strains were acquired by SEM (Fig. 2.7) to detect potential morphological changes, after 8 h treatment with AgNWs and AgNWs-CNT. In Fig. 2.7, it is possible to notice that AgNPs and AgNWs tend to deposit on the surface of bacteria and that some of the cell surfaces were damaged, in particular E. coli showed some fragmentary bacteria, as indicated by arrows. The ability of AgNPs to cause big gaps formation in E. coli membrane has already be shown by Das et al.[105].
Figure 2.8 SEM photos of bacteria morphology after treatment either with AgNWs or AgNWs-CNT. (A), (B) and (C): *E. coli*; (D), (E) and (F): *S. aureus*, (G), (H) and (I): MRSA; (J), (K) and (L): *S. saprophyticus*.
2.3.3 \textit{In vitro} cell toxicity studies

The toxicity of the two types of AgNWs- was tested against three cell lines: osteoblasts (hFOB 1.19), human foreskin fibroblasts (Hs27) and Caco-2 cells. These cell lines were chosen to mimic three possible route of exposures: such as application of treatments on bone, skin and digestive system, respectively. In particular, Caco-2 are an \textit{in vitro} model for prediction of the toxicity and adsorption in the intestine of humans \cite{106}. Cell viability was determined by the MTT assay, based on the cleavage of the tetrazolium salt by metabolically active cells from a water insoluble formazan dye (\textbf{Fig. 2.9 A-F}). During the MTT assay protocol, the medium containing AgNWs was removed prior to the addition of the MTT solution, in order to reduce the interference of the NWs with the MTT or formazan.

The viability results of osteoblasts showed that both the AgNWs treatments decreased the metabolism of cells in a dose- and time-dependent-manners (\textbf{Fig. 2.9 A and B}). After the 2 h treatment with the highest concentration of AgNWs (100 µg/ml), the viability decreased to 92.86 ± 17.10 % and to 95.52 ± 20.07 % for AgNWs-CNT. Whereas, after 24 hours of exposure, the cell viability was around 62.28 ± 16.68 % for AgNWs and 63.48 ± 18.27 % for AgNWs-CNT. One-way ANOVA performed on the same concentration of the two treatments showed no statistical difference ($p$>0.05).

Fibroblasts viability results showed that the highest concentration of AgNWs and AgNWs-CNT (\textbf{Fig. 2.9 C and D}) reduced cell metabolic activity up to 91.40±10.99% and 85.96±3.77%, after 2 h. While, after 24 h, cell toxicity reduced to 70.33 ± 20.01% and 66.61 ± 18.16%, after 24 h. One-way ANOVA performed on the same concentration of the two treatments showed no statistical difference. Overall fibroblasts were less sensitive to the effect of AgNWs.
<table>
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<td><strong>Fibroblasts</strong></td>
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<tr>
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<td><img src="image5" alt="Graph E" /></td>
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</tbody>
</table>

**Figure 2.9** Cytotoxicity for different types of cell lines, after 2 and 24 h of treatment. Osteoblasts (A and B), fibroblasts (C and D), Caco-2 (E and F). Data are represented as a mean ± SD (n>3). The One-way ANOVA performed on all the samples showed significantly different viability for both AgNWs and AgNWs-CNT (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$).

Caco-2 cells (Fig. 2.9 E and F) were the most sensitive to the action of AgNWs. The treatment with the highest concentration of AgNWs, showed a cell viability of 96.59 ± 13.00
% after 2 h, whereas for AgNWs-CNT viability was 87.26 ± 10.52 %. After 24 h, cell viability decreased dramatically to 49.62 ± 17.99 % and 45.56 ± 12.90 % for AgNWs and AgNWs-CNT, respectively. One-way ANOVA performed on the same concentration of the two treatments showed no statistical difference.

Overall, these results show that AgNWs present a dose-dependent toxicity and the extent of the cell toxicity depends on the cell type, as already reported by Verma et al. [85]

2.3.3.1 Oxidative stress in cells

Several studies have shown evidence of a link between AgNPs toxicity and production of ROS in mammalian cells [107] [108]. In order to measure the AgNWs-induced ROS generation, DCF-DA probe was used. The results in Fig.2.10 A-F highlight that ROS induced by AgNWs are a significant factor in toxicity for all the cell lines. Indeed, all the cell lines presented a significant increase of fluorescence values that then decreased over time. Fibroblasts seemed to be less affected than Caco-2 and osteoblast cells by AgNWs and AgNWs-CNT ROS generation effect. Also Sweeney et al. showed that AgNWs (72 nm x 1.5 µm) could induce a significant increase of ROS production; they showed that AgNWs promoted a significant ROS generation, in human type-I epithelial-like cells (TT1), both at 4 (≥25 µg/ml) and at 24 h treatment (≥ 10 µg/ml).
Figure 2.10 Time course of ROS production in different cell lines: osteoblasts (A and B), fibroblasts (C and D) and Caco-2 (E and F) treated with AgNWs or AgNWs-CNT for 15 min. One-way ANOVA returned $p<0.05$. Data are reported as mean±SD (n=3). Results of the post-hoc Tukey’s multicomparison test are shown in the graph. Statistical differences are reported in the graph: * was used to compare 100 with 0 µg/ml (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$); $ was used to compare 50 with 0 µg/ml ($ indicates $p<0.05$, $$$$$ indicates $p<0.0001$); # was used to compare 50 with 10 µg/ml (# indicates $p<0.05$, ### indicates $p<0.001$, #### indicates $p<0.0001$); £ was used to compare 100 with 10 µg/ml (£££ indicates $p<0.001$, ££££ indicates $p<0.0001$).
However, differently from Sweeney et al., in our experiment, we observed a reduction of ROS production, over time, in comparison to the percentage of the control. This is in agreement with what reported by Onodera et al.: treating BALB/3T3 A31-1-1 cells with 1-nm AgNPs, they observed a decrease in fluorescence between 5 min and 60 min [109]. Moreover, ROS intensity tend to increase with the increase of AgNWs concentrations, as shown for silver nanospheres (26.2 ± 7.6 nm) by Lee et al. in mouse embryonic fibroblasts (NIH 3T3) [108], for peptide-coated silver nanospheres (20 nm) by Böhmert et al. in Caco-2 cells [110] and for Ag nanospheres by Castiglioni et al. in Saos-2 osteoblast-like cells [111].

2.3.3.2 Membrane damage evaluation

Membrane integrity was assessed by measuring extracellular lactate dehydrogenase (LDH). Cells were treated with AgNWs (0, 10, 50 and 100 µg/ml) for 24 h and results are reported in Fig. 2.11 A-F. No significant toxicity was observed after 24 h for all the concentrations tested in comparison to the spontaneous LDH release (0 µg/ml) (p>0.05). These results are in disagreement with what found, for instance, by Gurunathan et al. which found that silver nanoparticles (20 ± 3.5 nm) caused an increased and significant LDH leakage from lung carcinoma cells (A549), after 24 h treatment, with concentrations ≥ 6.125 µg/ml [112]. However, Gliga et al. have previously observed that LDH release from human lung cells (BEAS-2) depended on the dimensions of AgNPs. Testing particles of 10, 40 and 75 nm, they found no significant LDH release for the NPs ≥ 40 nm of diameter [113]. Thus, we hypothesized that AgNWs may cause less membrane damage than other AgNPs due, for instance, to their lower specific surface area.
Figure 2.11 Percentage LDH release from osteoblasts, fibroblasts and Caco-2 after 24 hours of exposure to different concentrations of AgNWs or AgNWs-CNTs. The one-way ANOVA calculated among the different concentrations of each test did not show any statistical difference ($p>0.05$). Data are reported as a mean $\pm$ SD ($n=3$).
Several studies have reported that AgNWs tend to be uptaken and accumulate in different types of lung cells, such as alveolar type-I and type-II epithelial cells[82].

In our work, we wanted to find out whether or not AgNWs could be uptaken into osteoblast cells and at which concentration. In our study, osteoblast cells were treated with Ag⁺, AgNWs or AgNWs-CNT for 24 h. Concentrations up to 10 µg/ml of NPs and AgNO₃ were tested as they caused no relevant differences in toxicity to osteoblasts after 24 h of exposure (Fig. 2.12).

![Figure 2.12](image)

**Figure 2.12** Amount (µg) of Ag found in osteoblasts after 24 h of exposure to AgNWs, AgNWs-CNT and AgNO₃. The one-way ANOVA calculated among the different concentrations of AgNWs and AgNWs-CNT returned \( p<0.05 \). Results of the post-hoc Tukey’s multicomparison test are shown in the graph: * indicates \( p<0.05 \), ** indicates \( p<0.01 \) and **** indicates \( p<0.0001 \). Values are reported as a mean ± SD (n=3).

We examined the accumulation of both AgNPs and Ag⁺ at various concentrations applied, after digestion of cells in nitric acid. Results showed that all the NWs studied and Ag⁺ have been efficiently taken up in a concentration-dependent manner by osteoblasts, but the Ag
concentration was significantly lower for AgNO₃ than for AgNWs and AgNWS, for all the concentrations (Tukey’s multicomparison test showed p<0.0001): for instances, detected Ag contents in cells exposed to 10 µg/ml of AgNWs, AgNWs-CNT and Ag⁺ were 0.46 ± 0.07, 0.33 ± 0.08 µg and 0.0007 ± 0.0004 µg, respectively. A similar behavior was found with mammalian kidney cells (PkJ15) for silver nanoparticles of different sizes (ca. 13.8-61.2 µm) by Milic et al [114]. We have hypothesized that as there is not a specific transporter for Ag⁺, but it has to compete for transporters with other ionic species, the amount of Ag that entered in the cells was much inferior in comparison to AgNWs that could enter endocytosis.

<table>
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Figure 2.13 Phalloidin Dylight 550 and DAPI stained osteoblast cells at 24 hours of incubation with AgNWs (10 and 100 µg/ml). (A) (D) and (G) Merged images of Phalloidin (staining F-actin) and DAPI (staining nuclei) of
osteoblasts. (B), (E) and (H) AgNWs excited at wavelength of 495 nm and emitting at a wavelength of 519 nm (FTIC). (C), (F) and (I) Merged images of Phalloidin/DAPI/AgNWs. Scale bar 270.00 µm.

Results of ICP-OES were confirmed by fluorescence microscopy (Fig. 2.13). AgNWs accumulated in osteoblasts in a dose-dependent manner. Moreover, through fluorescence microscopy it was possible to observe that cells presented AgNWs either close to the membrane (Fig. 2.13 F) or within the cytoplasm (Fig. 2.13 F and I).

2.4 Conclusions

In summary, we successfully synthetized AgNWs through two routes. The materials were characterized by TEM, XRD, UV-vis and fluorescence spectroscopy and found to be similar. Then, we studied their antibacterial properties, confirming their similarity. The toxicity of the materials was found to be related both to the generation of ROS and membrane damage. Moreover, the cytotoxicity studies indicated that AgNWs had a dose- and time-dependent toxicity profile. The cells presented high levels of ROS after exposure to AgNWs and cells did not present any significant membrane damage. Furthermore, AAS results on osteoblasts revealed that AgNWs could be efficiently uptaken by this cell type. Future work will be carried out in order to determine whether AgNWs could determine DNA damage in both bacteria and cells.
Chapter 3: Review - Composite hydrogels for bone regeneration

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This review has been published in:


Authors’ contributions

Marta Roldo conceived, designed and coordinated the work. Marta Roldo and Gianluca Tozzi contributed to the writing and were responsible for the reviewing of the manuscript. Arianna De Mori and Antero Oliveira equally contributed to literature review and writing of the manuscript.

Abstract

Over the past few decades bone related disorders have constantly increased. Among all pathological conditions, osteoporosis is one of the most common and often leads to bone fractures. This is a massive burden and it affects an estimated 3 million people only in the UK furthermore, as the population ages, numbers are due to increase. Therefore, novel
Biomaterials for bone fracture regeneration are constantly under development. Typically, these materials aim at favoring optimal bone integration in the scaffold, up to complete bone regeneration. This approach to regenerative medicine is also known as tissue engineering (TE). Hydrogels are among the most promising biomaterials in TE applications. They are very flexible materials that allow a number of different properties to be targeted for different applications, through appropriate chemical modifications. The present review will focus on the strategies that have been developed for formulating hydrogels with ideal properties for bone regeneration applications. In particular, aspects related to the improvement of hydrogels mechanical competence, controlled delivery of drugs and growth factors are treated in detail. It is hoped that this review can provide an exhaustive compendium of the main aspects in hydrogel related research and therefore stimulate future biomaterial development and applications.

3.1 The bone

The bone is a solid composite living material that represents the main constituent of the vertebral skeleton[115]. This dynamic tissue plays many important roles in the human body: first of all, by a joint action with the digestive and renal systems, it contributes to the regulation of the concentration of electrolytes, such as Ca and P [116, 117]. Secondly, its hard and moderately elastic nature allows it to form a framework for the support and attachment of softer tissues such as muscles, and for the protection of vital organs and the bone marrow. Finally, it provides body support for locomotion and muscular contraction [118].
3.1.1 Bone anatomy

Bones are typically formed by an outer layer of compact bone (also called cortical bone) and an inner portion of spongy bone (also called trabecular or cancellous bone). Trabecular bone is formed by various trabeculae arranged in a honeycomb structure. The relative proportion between the cortical and trabecular bone varies with the skeletal segments and their function. The major difference between compact and trabecular bone lies in their porosity that ranges from 5 to 30% in the compact bone and from 30 to 90% in the trabecular bone.

Long bones (i.e. humerus, radius and ulna) are constituted by three regions: diaphysis, the central shaft; epiphysis, the bulbous extremities; and metaphysis, located between the two. In the middle of the shaft a medullary (or marrow cavity) is present. This contains red bone marrow for hematopoiesis during infancy and yellow marrow for energy storage during adulthood. Short bones include the tarsal and carpal bones; flat bones include the frontal and parietal bones of the cranium, ribs, scapula and pelvis; finally, irregular bones include the bones of the spine (vertebrae, sacrum and coccyx) and some bones of the skull such as sphenoid and ethmoid [119, 120]. Bone shape is genetically determined in order to satisfy particular requirements according to the anatomic position and function. Furthermore, during life, bone shape is altered by the process known as remodeling, based on a combination of periosteal and endosteal apposition and resorption[121-123].

3.1.2 Chemical composition
At a microscopic level, bone can be divided into three main components: matrix, cells and bioactive factors. The calcified bone matrix is formed by an organic protein-rich matrix (20 % of dry weight), a mineral substance (65 %) and water (ca. 10 %). The organic phase has a role in determining the form of the bone and affords resistance to tension. The major component of the organic matrix is collagen type I (90 %); the remaining 10 % is constituted of noncollagenous proteins, proteoglycans and phospholipids. Moreover, the matrix is constituted by growth factors and enzymes such as phosphatase and metalloproteinase. Collagen fibrils are formed by three filamentous polypeptide chains in a helical configuration, stabilised by intra- and intermolecular cross-links. These bonds allow both mineralisation and reinforcement of the tensile strength of the fibrils. The non-collagenous proteins have different functions in the bone structure: phospholipids and proteoglycans have a regulatory effect in the calcification process [124]. The inorganic phase serves as an ion reservoir (predominately Ca, Mg, Na and P) and increases the strength of the bone due to the presence of apatite, carbonate, acid phosphate and brushite. The main inorganic component of the bone is phosphate hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2] \) [125, 126].

3.1.3 Histology of the bone

The bone consists of cells from two cell lines: mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC).

1. MSCs are multipotent stromal cells that can differentiate into diverse cell types such as myocytes and adipocytes but also osteoblast progenitors, osteoblasts, bone-lining cells and osteocytes. Osteoblasts are the most prevalent cell type in the bone and their function
is to secrete matrix components, such as collagen I, in response to mechanical stimuli and to promote the mineralisation of the bone matrix. Osteocytes, derived from osteoblasts, have mechanosensor and modulator (promotion of nerve growth) activities. Bone-lining cells are instead able to release enzymes to remove the layer of osteoids that covers mineralised matrix allowing osteoclasts to attach and begin resorption.

2. HSCs are stem cells that give rise to blood cells such as monocyte, macrophages or platelets, but also preosteoclasts and osteoclasts. Osteoclasts are responsible of bone resorption; by the secretion of protons, they can lower the pH and so solubilize the mineral phase. These cells are responsible for an intricate balance between formation, maintenance and destruction of bone tissue. This equilibrium is maintained by the action of hormones, cytokines and mechanical factors such as calcitonin and parathyroid hormone (PTH) that can control the levels of calcium and phosphate in the blood. Calcitonin is a thyroid hormone that reduces blood calcium levels by inhibiting osteoclasts and decreasing Ca resorption in the kidneys. Conversely, PTH is a hormone that increases blood calcium levels, acting upon the PTH1 receptor in bone and kidney, and the PTH2 receptor in the central nervous system, pancreas, testis, and placenta [120]. It is evident from the above brief description that the bone is an extremely complex tissue and that many factors can play a role in its physiology and function.

3.1.4 Bone healing
Bone tissue possesses the intrinsic capacity of healing itself; the bone repairing process is an interplay of biomechanical, cellular and molecular factors [128]. If successful, bone regenerates itself with newly formed bone in children and in a mechanically stable lamellar structures in adults [129]. The full understanding of this process may help researchers to develop new strategies to treat slowly healing or non-healing fractures [130]. First of all, when a fracture occurs, the following local tissue damages are observed: interruption of skeletal integrity, disruption of vascular structures and of nutrient flow at the fracture site leading to oxygen tension and disruption of marrow architecture [131]. These are the prelude to the real bone regeneration process [132]. The bone regeneration healing is divided into three phases: inflammation phase, reparative phase and remodelling phase (Fig. 3.2) [129]. During the inflammation phase, blood vessel disruption causes a blood clot, called hematoma, while fibroblasts fill the defect site with a fibrin-rich extracellular matrix [132]. Later, the immigrating platelets, neutrophils and macrophages release signalling molecules, such as fibroblast growth factors (FGF), tumour necrosis factor-α (TNF-α), platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF), transforming growth factor (TGF-β) and cytokines, such as IL-1 and IL-6 [131]. Hence, IL-1 and IL-6 chemotactically attract mesenchymal cells precursors [132]. Finally, mesenchymal precursors proliferate and differentiate into the chondrogenic and osteogenic lineages [131]. During the second phase, called repairing phase, it is possible to see that chondroblasts form hyaline cartilage while osteoblasts form woven bone. The latter brings on a cartilaginous callus that bridges and stabilises the bone wound. Then, the so-called endochondral ossification happens: woven and hyaline cartilage are substituted by lamellar tissue due to mineralisation of the tissue. The calcified tissue, by the activity of matrix metalloproteinase expressing cells, is penetrated by blood vessels and the cartilaginous
septum is removed. The last phase is the remodelling phase in which there is the conversion of fracture callus into real bone. Firstly, osteoclasts adsorb trabecular bone, creating a shallow resorption pit, called Howship’s lacunae. Then, an enzymatic destruction of the bone matrix, promoted by the locally present cells, leads to the releasing of growth factors and cytokines. The latter induce the conversion of osteoprogenitors to osteoblasts that enter the resorption pits created by osteoclasts and create new bone matrix of either the woven and lamellar cells. Finally, the entrapped cells within the bone matrix generate osteocytes [131].

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<tr>
<th>Inflammation</th>
<th>Repair</th>
<th>Remodelling</th>
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<tr>
<td>Formation of hematoma</td>
<td>MSCs and endothelial cells secrete growth factors such as BMP-2</td>
<td>Osteoclasts absorb trabecular bone</td>
</tr>
<tr>
<td>Fibroblasts infiltration</td>
<td>MSCs differentiate into chondrogenic and osteogenic lineages</td>
<td>- Enzymatic destruction of bone matrix</td>
</tr>
<tr>
<td>Infiltration of platelets, neutrophils and macrophages</td>
<td>- Formation of cartilaginous callus</td>
<td>- Release of growth factors and cytokines</td>
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<tr>
<td>Release of FGF, TNF-α, PDGF, VEGF, BFGF, TGF-β, IL-1 and IL-6</td>
<td>- Endochondral ossification and blood vessels infiltration</td>
<td>- Differentiation of osteoprogenitor cells to osteoblasts</td>
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<td>- Formation of new bone</td>
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**Figure 3.2** Bone healing process

3.2 Bone tissue engineering

3.2.1 The need for effective bone repair strategies: economic, social and clinical aspects

Over the past few decades the prevalence of bone related disorders has steadily been on the rise [133]. The most notable cause for this is our increasingly aging population,
however other factors such as disease (i.e. Paget’s disease or osteoporosis) and recurrent sports injuries are also relevant [134]. While these issues may have taken a back seat in comparison to the attention that heart disease and cancer have attracted; bone diseases are common and significant healthcare issues, and this has prompted a steady stream of research in the field of bone regenerative medicine [133].

Osteoporosis is one of the most common diseases leading to bone fractures; it affects an estimated 3 million people in the UK and, as the population ages, numbers are due to increase [135]. The National Institute for Health and Care Excellence has published a guidance indicating that the direct cost from fragility related fractures in the year 2000 was estimated to £1.8 billion, with the potential to increase to £2.2 billion by 2025 and to reach £6 billion by 2036, in the UK only [136].

Bone fractures also have a great impact on the patients’ quality of life, for example they can infringe on the ability of a person to live independently where the fracture site is along a load bearing bone or it can affect self-esteem and social interaction when it affects the spine and the posture of the patient [135], leading to high mortality in the elderly [135, 136]. Moreover, co-morbidities can reduce the bone regeneration capacity with recurrence of delayed- or non-union fractures; these significantly prolong the recovery period and constitute a considerable societal burden [137].

3.2.2 Limitations of the current treatments

Current treatment protocols for complex bone fractures involve the use of auto-/allo-grafts or inert metallic/ceramic implants. Autograft implantation represents the golden standard for treatment of small bone defects [138]. This technique transplants the donor’s bone from
a non-load-bearing site in the patient (i.e. iliac crest) into the defect site of the same patient [139]. Bone autografting presents all the essential elements to heal a bone fracture since the transplanted bone tissue has osteoconductive, osteoinductive and osteogenic properties. However, autografting shows some disadvantages such as high costs and limited available tissue from donor sites. Allograft is the second most common bone-grafting technique. This method involves the transplantation of bone from a different donor (i.e. cadavers) and the main shortcomings are related to infections and demand of donor bone tissue [140]. When both auto- and allografts are not successful or adequate, different biomaterials are considered. Metallic devices such as plates/screws, rods and fixators are widely used, but despite their excellent mechanical properties, they are not bioactive and bioresorbable. Thus, limiting their performance and involving additional surgical procedures in case of revision [141]. Moreover, due to stress shielding effects they can cause bone resorption and consequent implant loosening [141]. Ceramics represent a valuable alternative due to their availability and adaptation to various applications. Calcium phosphate (CaP) cements, for instance, well resembles bone tissue chemical/functional properties, being both biocompatible and bioactive. However, low tensile strength and high brittleness are main drawbacks of such materials [142, 143]. CaP showed ability in promoting bone repair, although they typically provide poor revascularisation/mineralisation, limited life time, and inability to adapt to skeletal changes [144]. Furthermore, a single-phased material cannot efficiently guarantee bone growth and tissue engineering approaches are needed [145].

3.2.3 Requirements for successful development of bone tissue engineering scaffolds

As reported by the pioneers Langer and Vacanti, the term tissue engineering (TE) defines
“an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue or organ function”. In general, TE aims at developing temporary 3D multicomponent scaffolds, also called composites, to induce the physiological regeneration of functional tissues (i.e. bone), overcoming the downfalls of classic biomaterials systems [141, 145, 146]. Scaffolds are firstly intended to work as fillers, occupying the available space in the damaged organ/tissue and slowly their programmed bioerosion/resoption allows them to provide a framework for growth of new tissue that finally partially or completely replaces the scaffold.

An ideal scaffold for bone tissue engineering (BTE) must present the following three main properties [147]:

1. **Osteoconductivity** refers to the growth of new tissue on the external and internal (pores) surfaces of the implant. This is greatly dependent on the physical form and chemical composition of the material. Factors such as hydrophilicity, porosity, biocompatibility and biodegradability of the material will affect its osteocductive properties. For example, porosity (optimal pore size 200-350 µm) is crucial for allowing neovascularization and diffusion of nutrients and gases required for the formation of the new bone [148]. Mechanical competence of the material is also important to provide an osteoconductive scaffold. This review will look at different approaches that have been studied in an attempt to improve the mechanical performance of hydrogel scaffolds.

2. **Osteogenicity** is the property of those scaffolds that contain osteoprogenitor cells and favour their adhesion and proliferation [149]. This review will look at the different types
of stem cells that can be used in bone regeneration and strategies for their inclusion in scaffolds hydrogel scaffolds.

3 **Osteoinduction** is the capacity of attracting immature cells to a healing site and stimulating these cells to develop into bone-forming cells. Materials that are osteoinductive are able to induce bone formation in ectopic sites [147]. This review will consider the complexity of controlled drugs and growth factor delivery from bone regeneration scaffolds.

4 **Osteointegration**, defined at a histological level as the direct contact between the implant and the new bone without the formation of fibrous tissue and the ability to maintain a strong biomaterial/bone interaction over time, should be the overall outcome of the application of a scaffold that present all above described properties. Importantly, osteointegrated implants should re-establish mechanical function of the repaired bone.

3.3 Hydrogels as scaffolds and delivery platforms

In their letter to Nature in 1960, Wichterle and Lim investigated for the first time the role of hydrogels for biological use [150]; the following decades have seen a wealth of research aimed at exploiting the particular properties of hydrogels that allow their use for many purposes such as: matrices for tissue engineering and regenerative medicine, diagnostics, cellular immobilization, separation of biomolecules or cells and barrier materials to regulate biological adhesion [125, 151].

Hydrogels are three dimensional, hydrophilic, polymeric cross-linked networks[151, 152]. They are able to swell without disintegrating and can absorb up to several times their dry
weight in water [153]. Their high content in water makes them resemble the living tissue; therefore they are ideal for a wide range of biomedical and pharmaceutical applications [154]. Hydrogels can be produced by different techniques: one-step methods like polymerization and parallel cross-linking of multifunctional monomers, or multiple step methods by the synthesis of polymer molecules characterised by the presence of reactive groups that originate cross-links in presence of a low molecular weight crosslinking agents [155]. The crosslinking points are essential to avoid the dissolution of the polymer chains and the type and degree of crosslinking determine many of the properties of the hydrogel [155]; for example crosslinking establishes the mesh size of the polymeric network and the water content that the gel can reach [156]. While the hydrophilic groups of the network chains attract and hold the fluid, the cross-links inside the structure impart an elastic force responsible for the solidity and stress resistance of the hydrogel [155, 156].

Hydrogels are very flexible materials, amenable to different chemical modifications that allow obtaining the desired properties according to the intended application; the present review will focus on the strategies that have been developed for formulating hydrogels with ideal properties for bone regeneration applications: osteoconduction, osteoinduction and osteogenicity.

3.4 Osteoconductive composite hydrogels: Strategies to improve hydrogels mechanical competence

It is widely accepted that scaffolds used for bone tissue engineering should be able to provide temporary mechanical integrity at the defect site immediately upon implantation. Scaffolds mechanical properties should be tailored to match the demands of the implant site
to decrease or avoid complications such as stress shielding, implant-related osteopenia, and subsequent re-fracture [157, 158]. Consequently, the design of the scaffold will vary with the site of implantation and with the type of bone. However, specific mechanical requirements for scaffolds are still to be defined by the research community and clinicians.

The soft nature of hydrogels makes them unsuitable for applications where a certain mechanical competence is required. Even though the mechanical properties of hydrogels can be increased by manipulating a number of parameters such as type and density of crosslinking [159], polymeric molecular weight, chemistry and concentration of the hydrogel precursors, it is not always possible to achieve the desired properties [160]. It must be also considered that some changes to the hydrogel formulation such as the use of high crosslinking density may lead to toxicity [161]. Therefore, designing hydrogel composites that are capable of synergising the biocompatibility and flexibility of the polymeric network and the structural support provided by the filler materials, can enhance the mechanical performance of hydrogels without affecting their beneficial properties. This results in a biomimetic approach where the physicochemical properties of nanostructured hybrid materials can stimulate cell growth and guide bone healing [162]. Different inorganic/organic composites for BTE have been studied; the most common fillers used are bioceramics (hydroxyapatite, tricalcium phosphate), bioglass particles, and carbon nanotubes. The mechanical properties of these nanocomposites can be controlled either by altering the properties of the matrix or of the nanofiller (i.e. concentration and size); interfacial bonding also has great importance as it may affect the effectiveness of load transfer.

3.4.1 Hydroxyapatite
Biomedical applications of nanohydroxyapatite (nHap) bioceramics have gained increasing interest due to their superior biological and biomechanical properties. Synthetic hydroxyapatite exhibits a strong affinity to host hard tissues due to the chemical similarity between hydroxyapatite and mineralised human bone tissue[163]. Several studies have combined polymers and nHap to create composite hydrogels, merging the desirable properties of the different organic and inorganic phases in order to achieve a synergistic effect in the resultant composite properties including the enhancement of the mechanical properties. Li et al. developed nHap/polyacrylamide composite hydrogels that presented higher fracture tensile stress, higher extensibility, and higher compressive strength (35.8 MPa with 15% nHap vs. 22 MPa for pure gel) in comparison to the parent hydrogels; furthermore, these composites showed excellent shape recovery[164]. The authors justified the enhanced mechanical properties as the result of the chelating effect and the hydrogen bonding between the polymer chains and nHap particles. Natural polymers are also interesting biodegradable materials used in the formulation of scaffolds for bone tissue engineering, mainly due to their similarities with extracellular matrix, chemical versatility and overall good biological performance [165]. For example, silk fibroin displays slow biodegradation, adjustable mechanical properties, high permeability to oxygen and water vapour, resistance to enzymatic degradation, favourable processability and biocompatibility, that have produced wide interest in this material for a number of applications [166]. In addition, the mechanical integrity and low inflammatory response of silk fibroin ensure its role as a promising material for osteogenic applications. A nHap/silk fibroin composite hydrogel was developed by Ribeiro et al. [167]. In this composite an increase in nHap concentration corresponded to an increase in mechanical properties of the composite with values of 90 and 100 kPa obtained. Another natural polymer, agarose, extracted from marine
red algae, can form thermoreversible gels via physical crosslinking. It is also a biocompatible and relatively bioinert material, promising for the formulation of gel matrices for biomedical applications [166]. Hu *et al.* studied nHAp/agarose composites and reported that with 70/30 organic/inorganic weight ratio the highest compressive strength value (~400 MPa) was obtained, while a 65/35 weight ratio afforded the highest elastic modulus (~1100 MPa, twofold that of pure agarose gel). This improvement in mechanical properties was justified by the formation of intermolecular hydrogen bonds between the two components, with a role also played by the average size (50 nm) of the well-dispersed spherical nHA particles [168]. Spherical hydroxyapatite granules prepared by liquid nitrogen method and then encapsulated into oxidised alginate–gelatin–biphasic calcium phosphate hydrogel networks were produced by Sarker *et al.* Also in this case, an increase of granules amount led to an increase in compressive strength due to mechanical interlocking of granules into the hydrogel matrix [169]. Chitin is another biocompatible, biodegradable and bio-resorbable biopolymer with also antibacterial and wound-healing abilities and low immunogenicity [170]. Chang *et al.* fabricated hybrid nHap/chitin hydrogels that exhibited porous structure, high mechanical strength and excellent biocompatibility [171]. Addition of nHap increased 10 fold (274 kPa) the reported compressive strength of chitin only hydrogel. The Young’s modulus of the hybrid hydrogel was ~320 kPa, compared to ~23 kPa of the chitin only hydrogel.

3.4.2 Bioactive glass

Bioactive glasses or bioglasses are inorganic amorphous materials that present variations of composition based on the original bioglass (45S5): 45% silica (SiO$_2$), 24.5% calcium oxide
(CaO), 24.5% sodium oxide (Na$_2$O), and 6% phosphorous pentoxide (P$_2$O$_5$) in weight percentage [172]. They display the ability to degrade at a controllable rate, releasing ions during this process and develop a carbonated phosphate surface layer that allows them to chemically bond to the native bone [173]. Bioactive glasses induce hydroxyapatite precipitation in the presence of a biological fluid, resulting in the enhanced mineralisation of bone tissue [173, 174]. They also possess the ability to induce differentiation of mesenchymal cells into osteoblasts, to stimulate vascularisation and enhance osteoblast proliferation [173, 175]. These properties are dependent primarily on the glass composition and microstructure [176]. Nevertheless, these materials are not suitable for load-bearing applications because of their intrinsic brittleness. Alone, they exhibit poor flexibility and fatigue strength, so their use in the formulation of composite hydrogels has been evaluated [177].

Gellan-gum represents a good candidate for the formulation of biocompatible scaffolds due to its non-cytotoxicity, biodegradability, hydrophilic and affordable nature [178]. However, similarly to other natural polymers, it presents relatively poor mechanical properties that narrow its applications in bone tissue engineering. Although a combination of physical (i.e. temperature) and chemical crosslinking approaches (i.e. photocrosslinking) can produce gellan-gum hydrogels with tunable physical and mechanical properties without affecting their biocompatibility, the mechanical properties of the hydrogel alone are not suitable for bone tissue engineering [179]. Gantar et al. reinforced gellan-gum hydrogels with a nanoparticulate glass to improve the microstructure and the mechanical properties of the material [180]. The composite hydrogel scaffold containing 50 % bioactive-glass exhibited a Young’s modulus of ~ 1.2 MPa. Although this value is not sufficient to accommodate biomechanical loading, when compared with the gellan-gum alone, the
incorporation of the bioglass significantly increased the Young’s modulus from 0.4 to ~1.2 MPa and the failure stress increased from 0.02 to ~0.11 MPa. Photopolymerised poly(ethylene glycol) dimethacrylate hydrogels combined with bioactive glass particles were synthetised and characterised by Killion et al. who demonstrated that the incorporation of bioactive glass increases the mechanical strength of the composite material, with a synergic action by the bioactive glass absorbing the initial compressive load and the polymeric matrix distributing the load between the reinforcement [181]. Lacroix et al. reported the synthesis of a bioactive glass/gelatin composite scaffold with well-controlled porosity [182]. Compressive stress–strain tests were performed on pure bioactive glass and composite scaffolds: the glass foams presented a step-by-step cracking characteristic of brittle materials, while the deformation of the gelatin-bioactive glass composite remained in the linear elastic regime. Besides these improved mechanical properties of the composite, its in vitro bioactivity was found to be as high as pure bioactive glasses.

3.4.3 Carbon nanotubes (CNTs) and other carbon materials

CNTs are allotropes of carbon composed of rolled up graphene sheets. According to the number of sheets concentrically aligned to form the one dimensional nanostructures, single-wall nanotubes (SWNTs) or multi-wall nanotubes (MWNTs) can be obtained [183]. CNTs present interesting and unique properties such as extreme toughness, high electrical conductivity and high surface area [184]; and are of interest in tissue engineering as they can support the building of flexible and porous structures similar to the extracellular matrix (ECM), environment in which cells physiologically migrate and proliferate to form tissues and organs [185]. Seo et al. developed a degradable membrane composed by chitosan/silica
incorporating functionalized-carbon nanotubes (f-CNT) for guided bone regeneration [186].

In their study, the incorporation of 2 % f-CNT substantially enhanced the mechanical properties (tensile strength and elastic modulus) of CNT/chitosan/silica membranes in comparison with chitosan/silica or bare chitosan membranes, while it did not have an effect on elongation rates. In our lab, thermosensitive chitosan gels were reinforced with chitosan grafted CNTs. It was found that CNTs not only increased the resistance of the gels to compression but, due to their thermal properties, they also had a major role in determining the time of gelation of the composite gels reducing it from around 1h for chitosan only gels to about 7-8 min [111]. Nanocomposites of the biodegradable and biocompatible polymer poly(propylene fumarate) (PPF) reinforced with three carbon nanostructures (SWNTs, ultra-short SWNTs (US-tubes) and fullerenes (C60)) were fabricated by Sitharaman et al. and the nanostructure size and surface area effects on the rheological properties of un-cross-linked PPF dispersions, as well as the mechanical properties of cross-linked nanocomposites were investigated as a function of the nanostructure concentration [187]. The US-tube nanocomposites showed the best mechanical enhancement effects. The mechanical properties for US-tube nanocomposites peaked at concentrations of 0.5 % and significantly enhanced flexural and compressive mechanical properties (up to 200%), when compared to the pure PPF. The study concluded that US-tubes and SWNTs contributed to better mechanical reinforcement than C60, due to the higher aspect ratios and larger surface areas suggesting that the surface area of carbon nanostructures may be a more important parameter than size for mechanical reinforcement. Furthermore, it was suggested that the fibril-like morphology of the SWNTs and US-tubes may also contribute to the improved mechanical properties of the nanocomposites.

Other carbon structures have also been evaluated; Lalwani et al. compared the efficacy of
two dimensional (2D) carbon and inorganic nanostructures as reinforcing agents of crosslinked PPF composites: single-walled graphene oxide nanoribbons, multi-walled graphene oxide nanoribbons, graphene oxide nanoplatelets, and molybdenum di-sulfite nanoplatelets (MSNPs) [188]. The mechanical properties (compressive modulus, compressive yield strength, flexural modulus and flexural yield strength) of all the 2D nanostructure-reinforced nanocomposites as a function of 2D nanostructure concentration (between 0.01 and 0.2 %) were significantly higher in comparison to PPF. It was found that the mechanical reinforcement is closely dependent on the nanostructure morphology and follows the order nanoplatelets > nanoribbons > nanotubes. The inorganic 2D nanostructure MSNPs showed better mechanical reinforcement than 1D or other 2D carbon nanostructures. The 2D nanostructures increased the crosslinking density of PPF nanocomposites compared to 1D nanostructures. Accordingly the authors suggested that harnessing the reinforcing potential of 2D nanostructures could lead to an entire new class of ultra-strong, lightweight biomaterials for tissue engineering applications [188].

The inorganic components considered above enhance the mechanical properties leading to reinforcement of the scaffold structure. Although the composite strategy is very promising, so far the scaffolds obtained present mechanical properties far from those of the human cortical bone and those that would be clinically relevant. Limited literature reports properties such as fracture toughness, reliability, or energy to failure of the composites. There are studies that provide some mechanical analysis of the composites produced; however only few provide all the characteristics that aid interpretation and comparison of the data. [189]. It is necessary to develop standard procedures regarding the mechanical analysis of the materials, in order to facilitate the comparison between different studies and obtain a complete characterisation of the materials. One of the biggest remaining challenges
for composites it is to define and achieve the appropriate mechanical requirements of scaffolds for load-bearing defects.

3.5 Osteoinductive composite hydrogels: controlled delivery of drugs and growth factors

While a well-designed scaffold can favour new bone formation in healthy patients by simply providing a support for the growth of the new tissue, the compromised bone homeostasis in osteoporosis and other bone conditions hinders the healing process and requires the use of drugs and growth factors to stimulate bone regeneration activity. In many cases controlled and sustained release of these pharmaceutical entities is required and hydrogels present an ideal platform for their release. Several classes of drugs and different growth factors have been evaluated; these are discussed in detail below.

3.5.1.1 Bisphosphonates

Bisphosphonates (BPs) are one of the most important drug classes for treating bone tissue diseases such as metastatic bone disease and osteoporosis [190]. They are carbon-substituted pyrophosphate analogues and they reduce bone resorption, causing loss of osteoclastic activity and accelerating osteoclast apoptosis by inhibiting farnesyl pyrophosphate synthase (an enzyme in the 3-hydroxy-2-methylglutaryl-coenzyme A (HMG-CoA) reductase pathway) [191]. The bisphosphonates currently used in clinical practice are all biologically stable due to presence of a carbon atom connecting the two phosphates (P-C-P) [190]: alendronate, clodronate, ibandronate, risedronate and zoledronate [192, 193].
Each bisphosphonate presents a certain bone binding affinity: the higher the affinity, the stronger the binding and slower the release and vice versa. These molecules exhibit high affinity for the mineralized bone matrix where they can be retained for several years, leading to potent pharmacological effects on the bone. The order of efficacy in inhibiting farnesyl pyrophosphate synthase is zoledronate > risedronate > ibandronate > alendronate. The order in the kinetic binding affinity to hydroxyapatite (Hap) is clodronate < etidronate < risedronate < ibandronate < alendronate < pamidronate < zoledronate. Controlled trials have highlighted that BPs reduce the incidence of vertebral fractures by 40 to 50 % and non-vertebral fractures by 20 to 40 % [194]. Unfortunately, these drugs present some drawbacks when orally administered, in particular low adsorption (0.6-1.5 %) and high toxicity (esophageal disease, atrial arrhythmias, osteonecrosis of the jaw and atypical femur fractures) [195].

Hydrogel systems have therefore been developed to allow controlled local delivery with minimal invasiveness and time-persistence at the targeted area of the affected tissue. Despite the promising effects of these drugs, BPs local release is still poorly explored. A common strategy investigated for BPs controlled local delivery is to combine the controlled release from a biodegradable particulate system with the diffusion barrier provided by a hydrogel scaffold. For example, Posadowska et al. encapsulated alendronate in PLGA (poly(lactide-co-glycolide)) nanoparticles by a solid/oil/water emulsification method (final drug loading 5 %) [196]. The obtained nanoparticles (average diameter 230 nm) were then suspended in a gellan gum (GG) hydrogel and the hydrogel matrix was cross-linked with calcium ions to improve stiffness. PLGA was chosen because it is characterized by a tunable degradation rate and FDA-approved, while GG is a low cost anionic natural polysaccharide widely applied for pharmaceutical purposes. This formulation allowed obtaining a release of
17% of the drug after 1 day, while the entire encapsulated drug was released within 25 days. Furthermore, *in vitro* studies confirmed cytocompatibility of the formulation with MG-63 osteoblast-like cells and its capacity to inhibit osteoclastic differentiation.

Furthermore, the high affinity of BPs for HAp can be exploited to facilitate controlled release of these drugs from hydrogel scaffolds. Verron *et al.* [197] formulated a suspension of zoledronate (Zol) granules loaded onto calcium deficient apatite (CDA-Zol) in a cellulosic-derived hydrogel. CDA was chosen to reinforce the osteoporotic site, while Zol to inhibit osteoclast resorption activity. The implantation of CDA-Zol in distal femurs of osteoporotic female rats showed a significant increase in bone volume fraction (BT/TV) and improved trabecular architecture compared to apatite alone. Further histological examination did not show the presence of abnormal tissue (e.g. fibrosis, necrosis, and granuloma) in the new-mineralised area.

### 3.5.1.2 Statins

Statins are competitive inhibitors of HMG-CoA reductase. They are generally used for lowering serum cholesterol, blocking the conversion of HMG-CoA to mevalonate. Hence, they are commonly used for treating diseases such as hyperlipidemia and arteriosclerosis. More recently, some studies have highlighted statins ability to increase new bone formation in cell cultures and in animal models [198, 199]. This anabolic effect has been firstly elucidated by an increased mineral density in bones of type 2 diabetes patients when treated with statins [200]. Bradley *et al.* demonstrated that these effects are associated with an increased expression of bone morphogenetic protein-2 (BMP-2) and nitric oxide synthase.
However, clinical use of statins is limited by the really low systemic availability (~ 2 %) and serious side effects such as liver toxicity, acute hepatic failure and episodes of myalgia [201-204]. Hence, local delivery (via bypassing hepatic metabolism) could lead to higher concentrations at the bone and a reduction of the side effects. In this context, the formulation of an appropriate scaffold is really important to achieve appropriate release kinetics and a local action. Simvastatin (SIM) is recognised as the most potent statin in stimulating bone growth, with a biological half-life of 1-3 hours and susceptibility to cytochrome P450 metabolic activity [203]. This fungal metabolite induces osteoblastic differentiation by increasing the expression levels of osteogenic markers such as alkaline phosphatase, osteocalcin and osteopontin. Many studies have been carried out for testing different materials and developing a suitable hydrogel scaffold [205].

Tanigo et al. successfully obtained a controlled release of SIM from gelatin/micelles composite hydrogels [206]. In particular, SIM was firstly water-solubilized into lactic acid oligomer grafted gelatin micelles. These micelles were then loaded into gelatin hydrogels, with tunable degradation rate based on degree of crosslinking. The system allowed controlled release of SIM over 20 days and enhanced the effect of the drug on bone regeneration in a rabbit tooth defect model. Sukul et al. prepared a three components hydrogel scaffold for the controlled release of SIM composed of: β tricalcium phosphate (β-TCP), an osteoconductive material; nanofibrillar cellulose, a slow degrading polymer that can improve control over the release rate of the gel; and crosslinked gelatin as the main hydrogel matrix [207]. The presence of nanofibers and β-TCP produced a scaffold with osteoconductive properties and able to better control the drug release over a period of time exceeding 30 days. Furthermore, a concentration of 0.5 µM SIM was identified as the most effective both in vitro (highest proliferation and differentiation of rat MSCs) and in vivo in a
8-mm rat calvarial defect with 33% new bone formation after 8 weeks treatment (compared to 25 and 17% obtained with lower and higher concentrations, respectively). Another well studied statin for bone regeneration applications is fluvastatin. Benoit et al. explored the covalent binding of this drug to the gel forming polymer, as a strategy for controlled release [201]. They used a biodegradable poly(lactic acid) spacer to load fluvastatin to a poly(ethylene glycol) dimethacrylate hydrogel. The release rate of the drug was controlled by the length of the spacer; the longer the spacer the quicker the release due to the presence of a higher number of hydrolysable bonds and an increased probability of release. This system showed potential for controlled release over 40 days, in vitro the drug was able to induce hMSCs differentiation, increase BMP-2 production and facilitate calcium deposition.

3.5.1.3 Growth factors

Growth factors such as Bone Morphogenic Protein (BMP) are clinically relevant therapeutics for tissue regeneration in musculoskeletal conditions. However, their current use has shown very limited success because even though collagen scaffolds have been developed for the controlled release of BMP, the protein short half-life and diffusion to other tissues requires the use of very high doses (10 µg) rendering the treatment costly and unsafe. Complications such as ectopic bone formation, compromised airways function, swelling at surgery site and neurological side effects have all been reported in spinal fusion application of BMP [208, 209]. Thus developing new strategies for the formulation and delivery of these growth factors could address unmet clinical need for a safe use of bone inducing biomaterials. Furthermore, research has demonstrated that physiological bone healing involves several different factors released at different times (Figure 1), indicating that release
of a single growth factor would be an oversimplification in an attempt to mimic the
physiological response [150].

As seen in the case of controlled release of smaller drugs, also for the delivery of growth
factors, composite gels are used. The strategies for sustained release are very similar; in
general a biodegradable or bioerodible multiparticulate system is introduced in the gel to
further delay the release of the entrapped macromolecule. Strategies such as core-shell and
layer-by-layer deposition are commonly used for the encapsulation of one or more growth
factors [150].

The limitations of the current clinically available BMP-2 formulations have induced a lot of
research into improving the mechanism of controlled release of this protein, to provide
better release and address its short half-life and rapid local clearance. A commonly used
strategy is that of exploiting the known affinity of BMP-2 for heparin therefore including
heparin into hydrogels; a study conducted by Bhakta et al. demonstrated that both the initial
burst, from the plain hyaluronic acid gel, and the prolonged sustained release of BMP-2,
obtained with the addition of heparin to hyaluronic acid gels, were essential for efficient
bone formation and suggested that a compromise between diffusion and affinity release
must be found for best performance of the gel [210]. Chung et al. exploited the same strategy
in their fibrin gel loaded with heparin-functionalised nanoparticles in which the presence of
heparin was instrumental in reducing the rate of release of BMP-2 and enhance quantity and
mineralisation of the newly formed bone in vivo [211]. Recent advances have looked at
introducing multiple control systems, this is for example the case of a dual interaction
nanoparticles developed by Seo et al. [212]. They proposed a hydrogel that forms in situ after
injection of polymeric nanoparticles that bind to BMP-2 by both ionic and hydrophobic
interactions; and demonstrated that dual interactions were essential in generating a more
effective formulation in vivo.

As recently reviewed by Bayer et al., growth factor delivery is moving towards the sequential release of 2 factors from individual scaffolds with the release of a factor involved in angiogenesis in the first release phase, followed by a growth factors such as BMP-2 in a second release phase [150]. Table 3.1 summarises the growth factors involved in bone healing that have been most commonly studied for bone tissue engineering applications. Dyondi et al. carried out a study to investigate the effect of dual growth factor release [213]. They developed a gellan-xanthan gum gel loaded with chitosan nanoparticles. BMP-2 was adsorbed on the surface of the nanoparticles while FGF was loaded in the gel. A comparison between single and dual growth factor release was carried out in vivo and showed that the combined release of the two growth factors resulted in a higher ability to promote osteoblast proliferation and differentiation. Interestingly, the gels also presented antimicrobial properties. Doubtlessly, as demonstrated by the examples reported above, due to their physical and chemical flexibility, hydrogels formulations and even more hydrogels composite formulations, represent an essential tool in the development of controlled release systems for the local delivery of therapeutic molecules to the bone.

Table 3.1. Growth factors employed in bone tissue engineering applications [150].

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Mechanism of action</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2 (Bone maphogenic protein)</td>
<td>Induces osteoblasts proliferation and MSCs differentiation.</td>
<td>Needs to be delivered in a controlled manner</td>
</tr>
<tr>
<td></td>
<td>Induces VEGF-A secretion therefore has a role in angiogenesis¹</td>
<td>Variable outcomes have been seen in humans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limited capacity to initiate vascular proliferation</td>
</tr>
<tr>
<td>VEGF (Vascular endothelial growth factor)</td>
<td>Induces endothelial cells mitogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attracts MSCs and induces their differentiation</td>
<td></td>
</tr>
</tbody>
</table>
| **PDGF**  
| (Platelet derived growth factor) | Attracts cells that stabilise growing vasculature  
|  | Recruits MSCs  
|  | Upregulates VEGF production  
|  | Delivered alone they lead to the inability to produce organized bone regeneration |

| **FGF**  
| (Fibroblast growth factor) | Involved in the formation of new capillaries |

| **IGF**  
| (Insulin like growth factor) | Involved in adult neo angiogenesis |

1 pro- or anti- osteogenic effect can be observed depending on the type of BMP used and the type of cells targeted.

3.6 Osteogenic composite hydrogels

Osteogenesis is characterised by a high number of cells compared to the mature bone tissue. These are osteoprogenitor cells that produce the extracellular matrix, which will support bone formation and will be later resorbed [214]. A good strategy to be employed in bone regeneration is that of recreating the environment in which osteogenesis takes place; this can be mimicked with a 3D hydrogel network that allows osteoprogenitor cells to proliferate and differentiate into osteoblasts. In fact hydrogels are able to create a complex and dynamic network that replicates the characteristics of the extracellular matrix providing physical structure, mechanical integrity and biocompatibility with the host tissue [215]. Studies have investigated the use of different cell types that will be considered in detail below [126].

3.6.1 Mesenchymal stem cells

Stem cells are undifferentiated cells capable of self-renewal and differentiation into specialised cells [202]. Bone mesenchymal stem cells (MSCs), otherwise known as bone
marrow stromal cells, are multipotent adult stem cells which can be found in the bone marrow [155, 200]. MSCs can proliferate in vitro and differentiate into diverse mesenchymal lineages (adipocytes, chondrocytes, myocytes, osteoblasts and tenocytes) in response to appropriate signaling by chemicals, growth factors and hormones (i.e. dexamethasone, β-glycerophosphate and ascorbic acid) [200, 216]. Interestingly, MSCs possess trophic factors that suppress the local immune system, decreasing the risk of autoimmune rejection and promote local vascularization [126, 217]. Moreover, they are easy to isolate from bone marrow and to manipulate, in fact they can be enriched to obtain a relatively pure population of cells and differentiate into osteoblasts that are capable of secreting extracellular matrix [202]. Many researches are focusing on the identification of a suitable three-dimensional scaffold for cell transplantation to promote the localised healing of the desired tissue. The aim is to develop a scaffold that is both biodegradable and injectable in order to allow the growth of the new tissue as degradation of the polymer scaffold occurs and to perform a single minimally invasive procedure [218]. However, the shortcomings of current methods underline the need of combining our understanding of what cell types can form bone and what scaffold best facilitates the differentiation of these cells [219]. Several hydrogels including alginate, collagen, fibrin glue, hyaluronic acid, oligo[poly(ethylene glycol) fumarate], pluronic F127 and silk fibroin have been studied to encapsulate MSCs and to promote their osteogenic differentiation [202, 220].

Nuttleman et al. successfully proposed the encapsulation of hMSCs into a photocrosslinkable, injectable scaffolding system based on a dimethacrylated PEG (MW 4.6 kDa) as the hydrogel precursor [221]. These gels presented an excellent cytocompatibility (relative cell survival: 99 %) and osteogenic-specific differentiation was confirmed by the expression of osteonectin, osteopontin and alkaline phosphatase genes. Finally, a staining
procedure revealed extensive mineralisation of the PEG hydrogels. Dimethacrylated PEG is also suitable to prepare scaffolds via the novel approach of biorprinting. Gao et al. [222] loaded hMSCs on photocrosslinked PEG-hydrogel scaffolds enriched with natural cell binding motifs (Arg-Gly-Asp or RGD peptide and matrix metalloproteinase-sensitive peptide). The bioprinting process allowed them to condense multiple steps of scaffold fabrication into one single step and to obtain a matrix that leads to a homogeneous development of bone and cartilage. Moreover, the bioprinted PEG-peptide scaffold showed excellent mineral and cartilage matrix deposition and the addition of the peptides showed a sustained effect over time on cell differentiation compared to the polymer only gel.

Another well studied polymer for BTE is hyaluronic acid (HA), a naturally derived, linear, high molecular weight polymer and one of the major components of ECM [223]. Many studies have been carried out in order to modify its structure, allowing the attachment of therapeutic drugs, functional groups, crosslinkers, and other bioactive moieties to HA [217, 224]. Kim et al., following a similar approach to the one by Nuttleman et al. described above, prepared an acrylated HA hydrogel for loading BMP-2 and hMSCs [223]. The formulation had a gelation time of 10 min at physiological conditions; however mechanical testing (complex modulus 1.8 kPa, elastic modulus 1.8 kPa) revealed this hydrogel was not strong enough for load bearing applications. Even though the formulation presented a relatively low in vitro cell viability (72 % within 2 days, increased to 81 % with the addition of BMP-2), the in vivo results showed that it was able to induce angiogenesis and osteogenesis. The HA hydrogel alone demonstrated good compatibility but no activity, while the addition of BMP-2 and hMSCs revealed a synergistic effect with formation of thicker and denser new bone, compared to the hydrogels containing only one of the two components.

Collage type I matrices are currently used in clinical practice for example for the delivery of
BMP-2 (InductOs); collagen is one of the most abundant fibrous proteins in the human body, found in tendons, ligaments, bone, teeth, skin, arteries and, in general, in extracellular matrix [225]. Collagen is a common tissue culture matrix due to its ability to facilitate cell attachment and its cell-based degradation. The ability of collagen type I hydrogels to favour attachment, migration and proliferation of rat bone marrow stromal cells was demonstrated by Hesse et al. [226].

Fibrin glue is another polymeric gel already exploited in the clinic, which has attracted interest in bone regeneration applications for the implantation of stem cells [227]. Seebach et al. implanted fibrin glue hydrogels loaded with rat derived MSCs into rat femoral bone defects to test host cell-recruitment, immunomodulation and tissue regeneration [228]. Results highlighted fibrin-MSC composite promoted host macrophage invasion, in comparison to cell-free fibrin hydrogels, due to the MSCs expression of trophic factors (e.g. IL-6, VEGF and MIP-2). Then, MSCs seemed to stimulate femoral bone healing, despite a light induction of a pro-inflammation process (TNF-α and IL-1β).

MSCs harvesting from the bone marrow requires painful procedures of aspiration from the iliac crest or from bone marrow biopsies that can however give low cells yields [219]. Hence, due to these practical constrains, researches have focused on the use of multi-lineage mesenchymal progenitor cells from other sources.

3.6.2 Adipose derived stem cells

Human adipose-derived stem cells (ASCs) are a subset of MSCs that can be found in human adipose tissue [222]. The adipose tissue is a highly complex tissue that consists of mature adipocytes (90 % of the total volume) and a stromal vascular part composed by pre-
adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages lymphocytes and ASCs [223]. ASCs are an interesting cell-lineage for regenerative medicine since they present morphological, immunological and phenotypical properties similar to stem cells isolated from bone marrow and umbilical cord blood, with some advantages [222, 225, 226]. In fact, ASCs have the potential to differentiate into bone, cartilage, tendons, skeletal muscle and fat under specific conditions and they are easy to access and abundant in the subcutaneous adipose tissue and isolated by an uncomplicated enzyme-based procedure [226]. The combination of ASCs with biomaterial scaffolds is currently a very promising strategy for restoring tissue functions, but further advances are needed.

Heo et al. [229] loaded ASCs on a photo-curable gelatin-gold nanoparticle (GNPs) hydrogel in order to study the effects of GNPs on stem cells. According to recent studies, GNPs have shown to have a positive effect on osteogenesis of MSCs and MC3T3-E1 osteoblast-like cells, other than presenting low costs and non-toxicity [230, 231]. In Heo’s study, the hybrid hydrogel was obtained by irradiation of the mixture of the photo-initiator and methacrylated gelatin leading to successful loading of the GNPs inside the hydrogel network. Assays showed an increase in viability and osteogenic differentiation of ADSCs in comparison with the hydrogel only and similar results in comparison with BMP loaded hydrogel, both in vivo and in vitro. Moreover, all the studied hydrogels had a positive effect on bone healing in the defect sites and were biologically degradable by collagenase.

Graphene, a two dimensional carbon structure that presents unique physical properties such as high surface area, high mechanical strength, high electrical conductivity and ease of functionalization has been recently reported to be able to form hydrogels and to promote in vitro osteogenic differentiation and proliferation of stem cells [229, 232, 233]. Lyu et al.
examined the osteoinductivity of hADSCs loaded self-supporting graphene hydrogels (SGH) obtained by electrostatic stabilization of graphene and its gelation by filtration [234-236]. They found that in comparison to conventional graphene and carbon fibre films, SGH films had higher mechanical strength, flexibility, cell viability (after one day culture, 95.43 ± 0.96 %), mineralisation and osteoinductivity. This finding opens new avenues in the development of hydrogels for bone regeneration applications.

3.6.3 Stem cells from human exfoliated deciduous teeth

Stem cells from human exfoliated deciduous teeth (SHEDs) have been recently identified as an alternative source of multipotent adult stem cells because of their capacity to differentiate into various cell lineages: neural cells, odontogenic cells, osteogenic cells and adipocytes [235, 236]. Moreover, they were reported to have a higher proliferation rate compared to MSCs and dental pulp stem cells and to be easier and more convenient to isolate [235-237]. Unfortunately, few studies about the loading of SHEDs into hydrogels have been published until now, underling the importance of further research in this field.

Su et al. [236] investigated the effects of chitosan/gelatin/β-glycerophosphate thermosensitive hydrogels containing strontium phosphate or tricalcium phosphate (TCP) on the osteogenic differentiation of SHEDs in vitro. Strontium has been added to the scaffold since it is a molecule that has shown to positively influence bone formation by improving bone formation and inhibiting bone resorption [238, 239]. Scanning electron microscopy analysis revealed all the studied hydrogels presented homogenous porous structures (100 µm-300 µm) with high interconnected channels that had high cytocompatibility. Nevertheless, the presence of bioceramic supplements, allowed cells continuous
proliferation for longer cultivation times (14 days). Strontium hydrogels also significantly increased calcification and expression of osteogenic genes compared to tricalcium phosphate.

3.6.4 Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent stem cells derived from the inner cell mass of blastocysts and they can differentiate to form cell populations derived from ectoderm, mesoderm and endoderm, hence any cell type [240]. They possess a nearly unlimited self-renewal capacity and their growth is not restricted by contact inhibition and proliferative senescence. As reported by Li et al., ESCs are a promising cell source for regenerative medicine, even though some challenges need to be overcome, such as achieving large-scale expansion culture systems, mainly due to the involvement of animal components [241, 242]. Even though the propagation of embryonic stem cells has usually been carried out in two-dimensional (2D) systems, 3D scaffolds would facilitate convenience in transplantation and consistency in cell performance and scalability in number [243]. Hence, the use of natural and synthetic hydrogels for hESC propagation in 3D has been proposed [243-247]. However, the study of ESCs properties and propagations on hydrogels for bone tissue engineering needs to be further explored.

Zur Nieden et al. studied the chemical modification of gelatin hydrogels through glyceraldehyde cross-linking to provide a suitable scaffold for ESC osteogenesis [215]. On one hand, gelatin was used as the main component of the matrix for its desirable properties: low toxicity, easy sterilization and limited higher ordered protein structure. On the other hand, glyceraldehyde is an ideal cross-linker since it is non-toxic and makes gelatin stable at 37°C.
The obtained gel was able to provide an initial soft non-adhesive surface for promoting the formation of embryoid bodies and to provide a harder surface that gradually disappeared as osteoblasts differentiated.

3.7 Concluding considerations

The wealth of research into hydrogels formulations for bone regeneration applications demonstrates the great potential of these systems. Hydrogels fulfil many of the requirements of ideal scaffold such as injectability, biocompatibility, biodegradation, etc.; furthermore, they can easily be chemically modified or co-formulated with other components that bestow further properties such as osteoconduction, osteoinduction and osteogenicity. The great majority of hydrogels are composed of polysaccharides and their capacity of functioning as diffusion barrier for both small and large molecular weight drugs has been widely exploited for controlled drug delivery applications. All proposed systems seem to provide incremental improvements on the performance of the hydrogels but perhaps the development of fundamentally innovative ideas is the key to move to clinically applicable formulations. Approaches such as bioprinting or the development of fundamentally new hydrogels such as those formed by graphene oxide could open novel and more promising avenues towards the successful clinical use of composite hydrogels for bone regeneration.
Chapter 4: Injectable composite gels containing silver nanowires as bone scaffold material

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Authors' contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Marta Roldo coordinated the team, and is responsible for conception and design of experiments as well as data analysis and writing. Arianna De Mori, Roger Draheim and Maria Teresa Conconi designed substantial parts of the experiments and contributed to data analysis and writing. The work was carried out in majority by Arianna De Mori with support from Meena Hafidh and Natalia Mele. Rahmi Yusuf trained A.D.M. for bacterial cell culture. Guido Cerri carried out x-ray diffractometry analysis and relative write up. Eugen Barbu, Gianluca Tozzie and Elisabetta Gavini contributed to the writing and the supervision and guidance of the researchers who carried out the experimental work.
Abstract

Notwithstanding the considerable attention attracted by one-dimensional nanostructures such as silver nanowires (AgNWs) due to their outstanding electrical, thermal and antimicrobial properties, their application in bone tissue regeneration has not been explored yet. Here, we report on the development of an innovative scaffold prepared from chitosan, hydroxyapatite and AgNWs (CS-HACS-AgNWs) having both bioactive and antibacterial properties. In vitro analysis highlighted the antibacterial potential of AgNWs against both gram-positive and gram-negative bacteria. The CS-HACS-AgNWs composite scaffold demonstrated suitable Ca/P deposition, gel strength and gelation time. Further antibacterial studies showed that the composite formulation was capable of inhibiting bacterial growth in suspension and preventing biofilm formation on the scaffold.

Finally, the hydrogels revealed their potential to serve as platforms for cell proliferation and differentiation. In summary, the developed CS-HACS-AgNWs composite hydrogel demonstrated potential as a scaffold material to be employed in regenerative medicine; it allows cell proliferation while decreasing the risk of infections. The results presented justify further investigation into clinical applications.

4.1 Introduction

Over the last few decades, significant advancements in the synthesis and characterisation of one dimensional (1D) nanostructured materials have led to novel applications in various fields such as material science, energy technologies, engineering and biomedical science. A wide range of nanostructures, such as nano-tubes, -fibres, -filaments, -whiskers, -horns, -needles, -ribbons, -wires are classified as 1D nanostructures [100] that are all characterised
by a high aspect ratio[15, 91]. These structures present remarkable variation in electrical, optical and magnetic properties when compared to the bulk materials due to their reduced dimensions, controlled structure and large surface-to-volume ratio[184]. The carbon and metal silver variants are of particular interest[8].

Silver one, two and three-dimensional nanostructures have been synthesised, with many studies focusing on silver nanowires (AgNWs). AgNWs are anisotropically grown to obtain an aspect ratio higher than 10 and are typically 10-200 nm in diameter and 5-100 µm in length[15]. To date, silver nanoparticles (AgNPs) have been much more extensively studied than AgNWs, and their distinctive high thermal and electrical conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and non-linear optical behaviour have been highlighted; as well as their antibacterial activity that has extended their usage into several biomedical fields[89]. They are promising agents against a wide range of fungi, viruses and bacterial species; even though the mechanism of action is still not entirely understood, it is believed that nanosilver serves as a source for Ag+ ions that can rupture microbial cell walls, denature proteins, block cell respiration and eventually induce cell death[248]. AgNPs have been used as antimicrobial agents in wound dressing [249], coating of catheters [250] and of cardiovascular implants[251], bone cements [252] and dental materials [253]. Currently literature on biomedical applications of AgNWs is still limited [91], emerging applications include textiles [31], wound coatings [45], drug release and tissue regeneration[69]. In the present work, for the first time, we explore the potential use of AgNWs in composite materials for bone regeneration. Bone defects remain a major problem in orthopedics given to the deficiencies of current commercially available bone grafts and alternative materials are needed [254]. Hydrogels - 3D, hydrophilic, cross-linked polymeric networks that resemble the extracellular matrix with a porosity and aqueous environment
that allows transportation of substances such as nutrients are very good candidates to overcome these obstacles. The main challenge within the field is to produce a bioactive and non-toxic hydrogel scaffold with sufficient mechanical strength [111, 254]. Chitosan (CS), a polysaccharide that is biocompatible, biodegradable and possesses antibacterial properties has been employed to formulate such scaffolds, however, it has poor mechanical properties and has inferior bone induction capability [255]. Our previous work has focused on the development of reinforced bioactive CS scaffolds containing hydroxyapatite (HA), which favours biomineralisation of the scaffold, and carbon nanotubes that provide mechanical enhancement[255]. The present work aims at substituting carbon nanotubes with other 1D nanostructures, namely AgNWs that afford the two-fold advantage of mechanically reinforcing the hydrogel structure while enhancing its antibacterial properties. In fact, the success of bone-defect surgical treatment can often be compromised by bacterial infection that may result in chronic inflammation, implant failure and even death [3, 256]. To date, few studies have been carried out to evaluate the antibacterial properties of AgNWs alone[91], and very limited work has been done in the investigation of their activity within composite materials. Here, we present the synthesis via the polyol method and full characterization of AgNWs, followed by the in vitro characterisation of the composite hydrogels containing the nanowires with particular emphasis on the antimicrobial, bioactive and biocompatible, properties of the scaffolds.

4.2 Materials and methods
4.2.1 Materials

Yeast extract for microbiology, tryptone enzymatic digest from casein, sodium chloride, agar, glycerol phosphate disodium salt hydrate, poly(vinylpyrrolidone) powder (55 kDa), chitosan from shrimp shells low viscosity (degree of deacetylation ~85%, calculated by $^1$H-NMR), phosphate buffered saline tablets, TRIS base, lysozyme from chicken (hen egg-white), silver standard for AAS, 10% fetal bovine serum, trypsin-EDTA 0.25% solution, antibiotic antymycotic solution, β-glycerophosphate, ascorbic acid, sucrose, haematoxylin/eosin and Eukitt were purchased from Sigma-Aldrich (Irvine, UK). Isopropanol, glycerol 99% and L-(+)-lactic acid 90 % were purchased from Acros Organics (Geel, Belgium). N,N-dimethylformamide, silver nitrate, sodium hydrogen carbonate, potassium chloride, magnesium chloride hexahydrate, hydrochloric acid 37%, calcium chloride dehydrate, sodium sulphate hydrate, sodium dihydrogen phosphate dehydrate and calcium chloride dehydrate were purchased from Fisher (Loughborough, UK). Di-potassium hydrogen phosphate anhydrous was purchased from BDH (VWR, Lutterworth, UK). Glutamax and BCA Protein Assay Reagent Kit were purchased from Thermo Fisher (Basingstoke, UK). Alkaline Phosphatase Assay kit was purchased from Abcam (Cambridge, UK). ATPlite Luminescence ATP Detection Assay System was purchased from PerkinElmer (Coventry, UK). OCT cryostat embedding medium (Tissue Tek®) was purchased from VWR (Lutterworth, UK). The osteoblastic cell line MC3T3-E1 was purchased from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Alpha-minimal essential medium (α-MEM) was from Life Technologies (Baltimore, US) and fetal bovine serum and penicillin/streptomycin were obtained from FBS Sigma (ST. Louis, US).
4.2.2 Synthesis and physicochemical characterisation of Silver Nanowires (AgNWs)

AgNWs were synthesised via the polyol method, using silver nitrate (AgNO₃) as the Ag source [257]. PVP (3 g) was fully dissolved in glycerol (95 ml) by heating to 80 °C. The solution was cooled down and AgNO₃ (0.79 g) was added under vigorous stirring (800 rpm) until the powder was fully dissolved. Subsequently, NaCl (5 mM in final solution) was mixed into 10 ml of glycerol and added to the PVP/glycerol solution. The reaction temperature of the mixture was raised to 210 °C. Once the temperature was reached, it was maintained for further 10 min. Samples (1 ml) were taken at different temperatures, diluted with water and analysed by UV (300-900 nm, Thermo Scientific Nicolet Evolution 100 UV-Visible Spectrophotometer) to follow the formation of the AgNWs. Finally the reaction was cooled down to room temperature, diluted 1:1 with deionized water and centrifuged (4000 rpm, 1 hour), the pellet was then washed twice with isopropanol (4000 rpm, 30 min) and twice with deionized water (4000 rpm, 10 min). The product was stored in deoxygenated purified water at room temperature, protected from light. Freeze dried AgNWs were suspended in chitosan solutions (1%, in lactic acid 0.1 M), to achieve different concentrations (0.5-1 mg/ml), by vortexing and sonicating for several min. These were spread onto SEM specimen stubs and dried for 2 days under vacuum. Samples were gold-coated with a Polaron e500 (Quoram Technologies, UK) sputter coater and analysed with a Jeol JSM-6160L scanning electron microscope with electron backscatter. For TEM Imaging AgNWs were suspended in water (0.1 mg/ml in water) and dropped onto TEM grids (Formvar/Carbon 300 Mesh Copper) (Agar Scientific, UK) and allowed to dry at room temperature and pressure. Dry grids were stored in sealed petri dishes in a desiccator until analysis by JEOL JEM-1400 TEM (JEOL, USA). SEM images were analysed with ImageJ to determine the size distribution of the wires obtained. AgNWs have been measured 3 times each. The crystal structure of AgNWs was determined
with a Bruker D2-Phaser diffractometer. Instrumental parameters were: CuKα radiation, 30kV, 10 mA, LynxEye PSD detector with an angular opening of 5°, 2θ range 6-84°, step size 0.020°, time per step 2 s, spinner 15 rpm. The alignment of the instrument was calibrated using an international standard (NIST 1976b). A low-background silicon crystal specimen holder (Bruker) was used. The analysis was performed at room temperature (25°C). The XRD pattern was evaluated using the software Bruker EVA 14.2 (DIFFRACplus Package) coupled with the database PDF-2 (ICDD). Finally, AgNWs were analysed for size and zeta potential by photon correlation spectroscopy (Malvern Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK).

4.2.3 Formulation and characterisation of hydrogels

Chitosan based hydrogels were formulated according to the composition listed in table 4.1, following the method previously described by Cancian et al. The freeze dried scaffolds were stored in a desiccator until further use. Gelation time was assessed using the inverted tube test, as described by Ganji et al. Formulations were prepared as described above but in 2 ml aliquots; after the addition of GP, the composites were vortexed (2 min) and stored at 4°C for 12 h to remove air bubbles. The vials were then incubated at 37°C in a temperature controlled bath (Grant, SUB Aqua Pro). The sol-gel transition was determined by inverting the vials horizontally; the time at which the gel stopped flowing was recorded as the gelation time.
Table 4.1. Components used in the formulation of each millilitre of hydrogel.

<table>
<thead>
<tr>
<th>Component</th>
<th>CS hydrogel</th>
<th>CS-HACS</th>
<th>CS-HACS-AgNWs</th>
<th>CS-AgNWs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid (0.1 M)</td>
<td>0.9 ml</td>
<td>0.9 ml</td>
<td>0.9 ml</td>
<td>0.9 ml</td>
</tr>
<tr>
<td>Chitosan</td>
<td>20 mg</td>
<td>20 mg</td>
<td>20 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>HACS</td>
<td>--</td>
<td>8.6 mg</td>
<td>8.6 mg</td>
<td>--</td>
</tr>
<tr>
<td>AgNWs (powder)</td>
<td>--</td>
<td>--</td>
<td>4 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td>GP (1.12 g/ml)</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Texture profile analysis was performed by a texture analyser (XT plus, Stable Micro Systems Ltd, UK) depressing a polycarbonate probe (10 mm dia) into the gels at 1 mm/s and to a depth of 5 mm, six measurements were taken at room temperature before and after the sol/gel transition took place. Force/distance curves were obtained and the maximum force value recorded was reported as gel strength (N). Equilibrium of swelling was determined on freeze-dried hydrogels, weighed and allowed to swell in PBS (15 ml, pH=7.4) at 37°C. At different time points, the hydrogels were removed from the buffer and weighed, after removing excess liquid. The experiment (carried out in triplicate) was continued until equilibrium was reached. The swelling ratio \(Q\) was calculated according to equation:

\[
Q(\%) = \left( \frac{W_s - W_d}{W_d} \right) \times 100
\]

where \(W_s\) and \(W_d\) represent the weight of the swollen and dry state samples, respectively. The porosity of the scaffolds was determined on dried gels according to the methods described by Nanda et al.[259]. The \textit{in vitro} degradation of pre-weighed dried scaffolds was
carried out in 5 ml sterile phosphate-buffered solution (PBS, pH 7.4) containing 1.0 mg/ml lysozyme. The PBS/lysozyme solutions was changed every 3-4 days. The 50 ml sterile flasks were incubated at 37°C with gentle mechanical agitation for the period of study (80 rpm). After 7, 14 and 21 days, samples were removed from the medium, rinsed with distilled water, freeze dried and weighed. The experiment was carried out in triplicate. The extent of in vitro degradation was calculated using the following equation:

\[
\text{Mass loss} \, (\%) = \left( \frac{W_i - W_d}{W_d} \right) \times 100
\]

where \( W_i \) is the initial weight of the scaffold and \( W_d \) is the weight of the scaffold after the degradation experiment. In vitro calcification studies were performed to investigate the ability of the composite hydrogels to induce calcium salts deposition. Simulated body fluid (SBF) was prepared according to Kokubo and Takadama [260]. The freeze dried samples were submerged into 15 ml of SBF and incubated at 37 °C for 7, 14 and 21 days; the SBF was changed every 4 days. Finally, samples were freeze dried. Controls were hydrated in deionised water for 5 h and dried as above. All samples were analysed by SEM (as described above), coupled with EDS (Silicon Drift Detector (SDD)—X-MaxN, Oxford Instruments, UK). EDS images for calcium (Ca) and phosphorus (P) were analysed using ImageJ 3D Viewer after being overlaid to the corresponding SEM images. The total area of Ca and P overlapping onto the total sample surface area was used to calculate percentage of Ca and P salt deposition (\( n = 3 \)). In order to further explore the overall salt deposition within the scaffolds micro-CT was used. Non-coated scaffolds were scanned with the microCT scanner (Versa 510, Zeiss, CA) set to a voltage of 40 kV and a current of 76 μA. An isotropic voxel size of 4.077 μm and
exposure of 3 s were used. The images were then analysed using ImageJ (v1.50, NIH, USA) as described by Cancian et al. [255] and Fig. 4.6.

4.2.3.1 Ag⁺ release studies

Silver cation release kinetics was evaluated. Dried AgNWs and freeze-dried hydrogels were soaked in HPLC-grade water. Then, they were transferred to a water bath maintained at 37°C, with shaking (90 rpm). At scheduled times, the AgNWs in water were centrifuged at 2000 rpm for 3 min (this step was not required for CS-HACS-AgNWs) and 1 ml of supernatant was taken and substituted with 1 ml of fresh water. The concentrations of silver ions were determined by a furnace atomic absorption spectro-photometer (Varian SpectrAA 220FS) at a wavelength and spectral bandwidth of 328.1 and 0.2 nm, respectively. The experiment was carried out in triplicate.

4.2.4 Antimicrobial activity of AgNWs

The antibacterial activity of AgNWs was examined by a suspension assay against gram-negative Escherichia coli (ATCC 25922) and gram-positive Staphylococcus aureus (ATCC 25923), Methicillin-resistant Staphylococcus aureus (ATCC 12403) and Staphylococcus saprophyticus (ATCC 15305). The bacteria were transferred from -80°C (15% glycerol) into 5 ml of fresh sterile LB medium by a sterile toothpick and incubated (at 37°C and 200 rpm) until the bacterial suspension was cloudy (1 day for E.coli and S. aureus, 2 days for MRSA and 3
days for *S. saprophyticus* (MaxQ™ 8000, Thermo Scientific). Then, 50 µl of bacterial suspension were transferred into 5 ml of fresh sterile LB medium and the bacteria were further incubated at 37°C until the suspension was newly cloudy (1 day for *E. coli*, *S. aureus* and *MRSA* and 2 days for *S. saprophyticus*). 1x10⁶ CFU/ml of cells (equal to an OD₆₀₀nm of 0.001) were grown in 50 ml of liquid LB medium supplemented with 12.5, 25, 50 and 100 µg/ml of AgNWs. The stock suspensions of AgNWs were prepared the day before the use and stored at 4°C. This was accomplished by firstly weighing the dried AgNWs and finally resuspending them in sterile LB by sonication (40 kHz, CamLab) until the suspension was homogeneous. This step ensured the sterilization of the AgNWs suspension due to high frequency ultrasounds. Pure medium with bacterial cell inoculation and pure LB served as controls. Growth rates and bacterial concentrations were detected by measuring the optical density (OD₆₀₀nm) of the inoculated LB broth medium at 600 nm at different time points. Different concentrations (12.5, 25, 50 and 100 µg/ml) of the stock AgNWs suspension and pure LB were used as blanks. The experiment was carried out into autoclaved 250 ml glass flasks at 37°C and 200 rpm.

4.2.5 Antimicrobial activity of composite hydrogels

The antibacterial activity of CS-HACS and CS-HACS-AgNWs scaffolds was examined by a suspension assay against the micro-organisms listed in section 2.4. The scaffolds were sterilized by firstly submerging them in 70% (v/v) ethanol for 15 min and then rinsing them three times with sterile PBS. Finally, they were autoclaved in LB and sonicated for 15 min at 40 kHz before discarding the LB. All the scaffolds were then placed into 2 ml of 1x10⁵ CFU/ml
cell suspension in 14 ml glass vials and analysed as described below.

4.2.5.1 *Biofilm formation assay*

For the determination of biofilm formation, after incubation at 37°C and 100 rpm for 24 hours, the hydrogels were gently soaked in PBS for 10 seconds to remove loosely adherent bacteria and subsequently fixed in paraformaldehyde (4% in PBS) overnight, before being washed in PBS (3 times) and soaked into 5%, 10%, 20% (2 hours) and 30% (overnight) sucrose solutions. The scaffolds were transferred into aluminum cases and embedded into optimal cutting temperature compound (O.C.T.). Then, they were rapidly frozen into a beaker containing pentane, submerged into liquid nitrogen. The hydrogels were stored at -80°C until cryosectioning. The hydrogels were cut into 7-9 μm slices. The slices were placed onto poly-L-lysine coated slides. The sections were air-dried for 30 min and stored at -20°C until staining. Prior to staining, the sections were washed with deionized water for 1 hour and stained with carbol fuchsin (for gram-negative cells) or crystal violet (for gram- positive cells). Slides were stained for 1 minute and then washed with running deionized water for 5 min. The slides were left to dry and observed with an optical microscope (GXM-L1500BHTG, GTVision, UK).

4.2.5.2 *Antimicrobial testing of the dried hydrogels*

To test the antimicrobial properties of the scaffolds, these were prepared as above but samples of the incubation medium were taken at scheduled times (3, 7 and 24 hours), the
bacterial suspensions were transferred into a sterile 15 ml tube and the scaffolds were washed with sterile PBS (1 ml) vortexing for 30 seconds. The last step was done for collecting the not fully attached bacteria. The PBS solution was then transferred into the 15 ml tube. The total collected bacterial suspension was serially diluted in PBS (from $10^{-1}$ to $10^{15}$) into sterile 2 ml tubes and plated in triplicate on LB agar plates (100 µl per plate). After static incubation for 18 hours at 37 °C (HeraTherm Incubator, Thermo Scientific) the colony forming units (CFUs) were manually counted. Controls were prepared with: LB only; scaffolds and LB; and individual bacteria in LB only.

4.2.6 Cell culture studies

The osteoblastic cell line MC3T3-E1 was cultured with proliferation medium composed of α-MEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown at 37°C in a humid atmosphere with 5% CO₂. Medium was refreshed three times a week. Freeze-dried hydrogels were hydrated for 5 min, then immersed in 70% ethanol, washed with phosphate buffer saline (PBS) four times, transferred into a 24-well plate, and irradiated with UVA light overnight. Samples were equilibrated in proliferation medium for 1 h, the medium was then removed and $1.8 \times 10^{4}$ cells were seeded in each well in osteogenic differentiation medium which contained α-MEM without nucleosides, 10% FBS, 1% penicillin/streptomycin, 1% glutamax, 10 mM β- glycerophosphate, 50 µg/ml ascorbic acid and 10 nM dexamethasone. After 7 and 14 days from seeding, cell proliferation, and differentiation were evaluated. Cell viability was assessed using the Luminescence ATP Detection Assay according to the manufacturer’s instructions. Briefly, substrate solution was added to cell lysates and the luminescence was measured with VICTOR³ 1420 Multilabel.
Counter (PerkinElmer). Counts per seconds were converted in cell number by using a standard curve previously obtained with known cell numbers (from $10^4$ to $10^5$ cells). The detection of ALP activity was carried out by using the Alkaline Phosphatase Assay kit, following manufacturer’s instructions. Briefly, cell lysates were centrifuged and supernatants were treated with 5mM p-nitrophenyl phosphate in the dark. Optical density (OD) was revealed at 405 nm by using the EL 311 SX microplate autoreader (BioTek Instruments, Inc., Winooski, VT, USA). OD values were converted in nanomoles of ALP by using a standard curve previously obtained with known ALP quantities (from 4 to 20 nanomoles). Protein content of each samples was analyzed with a BCA kit. Finally, results were expressed as nM ALP/µg protein.

4.2.7 Statistical analysis

Statistical analysis has been performed and is detailed in each figure caption.

4.3 Results

4.3.1 Synthesis and physicochemical characterisation of silver nanowires

AgNWs were successfully synthesised using the polyol method. During the synthesis, as the temperature was increased, a typical change in colour from clear to yellow, then orange, red, grey and green was observed (Fig. 4.1A). This correlated to the changes in the UV-Vis spectrum with increasing temperatures (Fig. 4.1B). As can be seen in Fig. 4.1B, below 180°C a peak was present at 410 nm, this is due to the surface plasmon resonance (SPR) signal of
Ag nanoparticles and nanorods that initially form as nucleation sites for the growth of nanowires. As the temperature increases, peaks appear at 350 and 380 nm, these are attributed to the formation of Ag nanowires, as confirmed by SEM (Fig. 4.1C)[261]. UV-vis spectra just show the range The XRD pattern (Fig. 4.1D) confirmed the formation of face-centered cubic metallic Ag. In fact the d-values perfectly matched to PDF Number 04-0783 (Silver-3C, syn); also the intensity of the peaks corresponded, except for (111). In the card 04-0783, the (111)/(200) intensity ratio is 2.5, whereas in our pattern was 3.4 indicating the formation of well-elongated AgNWs [262, 263]. Minimal trace impurity of AgCl (PDF No. 31-1238, Chlorargyrite) can be seen at 2θ 32.2° and 46.2° (Fig. 4.1D), an aspect sometimes reported in the synthesis of AgNWs. Thus, our AgNWs synthesis product was mainly composed of metallic silver, even though small traces of ionic silver were found, indicating that both the synthesis and the washing steps were efficient enough to guarantee a nearly pure product. We also studied the morphology of AgNWs obtained at the end of the synthesis. They had an average length of 5.03 ± 1.85 µm and an average diameter of 99.45 ± 20.20 nm (See Supplementary Fig. S4.1), as determined by analysing SEM images with ImageJ. These dimensions are in good agreement with those reported by Yang et al.[257]. The zeta potential of AgNWs in deionized water was found to be -10.88 ± 1.86 mV, indicating their tendency to aggregate in aqueous media.
Figure 4.1 Physicochemical characterization of AgNWs: (A) Typical colour of the reaction mixture at different temperatures; (B) the corresponding UV spectra; (C) SEM image of AgNWs suspended in a solution of chitosan in lactic acid; (D) XRD pattern of the synthesised AgNWs (red bars: Ag, PDF No. 04-0783; blue bars: AgCl, PDF n. 31-1238).

TEM images highlighted that even though the majority of the reaction product was composed of AgNWs, traces of other nanoparticles (e.g. nanorods and nanospheres) were present, suggesting that longer reaction times would be necessary to fully complete the reaction (Fig. 4.2A and 4.2B).
Figure 4.2 TEM photos of AgNWs. (A) Lower magnification image of AgNWs (scale bar 500 nm); (B) higher magnification image of AgNWs (scale bar 100 nm).

4.3.2 Characterisation of composite gels

AgNWs were used to formulate composite chitosan gels; the thermosensitive gels were composed of chitosan crosslinked with glycerol phosphate and mixed with a chitosan/hydroxyapatite composite (HACS), previously described. A significant reduction in time of sol/gel transition was observed on addition of the HACS composite to the chitosan gel (from 12.0 ± 3.3 min to 3.2 ± 0.4 min; \( p = 0.000007 \); Fig. 4.3A), no further significant change was observed on addition of AgNWs. When only AgNWs were added a non-significant (\( p = 0.582796 \)) reduction to 10.3 ± 2.4 min was observed (see Supplementary Fig. S4.2). AgNWs induced a further significant increase in gel strength (to 0.138 ± 0.005 N, \( p = 0.005068 \)) after the increase initially observed on addition of HACS (from 0.035 ± 0.001 N to 0.089 ± 0.024 N, \( p = 0.002747 \); Fig. 4.3B). Also AgNWs alone were able to significantly increase the strength of the chitosan gel (to 0.136 ± 0.001 N, \( p = 0.000034 \), see Supplementary Fig. S4.3).
Figure 4.3 Physical characterisation of the hydrogels: CS (black), CS-HACS (light grey) and CS-HACS-AgNWs (dark grey). (A) Gelation time calculated by the inverted tube method. Data are reported as mean ± SD (n = 9). One-way ANOVA returned \( p < 0.0001 \). Post-hoc Tukey’s comparison test results are shown in the graph: **** indicates \( p < 0.0001 \) as compared to the control (CS). (B) Gel strength calculated by texture analysis before (empty bars) and after (filled bars) gelation. Data are represented as mean ± SD (n = 3). The \( t \)-test performed on all samples before and after gelation showed significantly different strength values for all gels (\( *** \) indicates \( p < 0.01 \) and \( **** \) \( p < 0.0001 \)). One-way ANOVA to compare the different formulations returned \( p < 0.01 \) before and \( p < 0.0001 \) after gelation. Post-hoc Tukey’s test results showed that before gelation only CS is different from all other gels (\( ^{\#} p < 0.05 \)), while the individual results for after gelation are reported on the graph (\( ** p < 0.01; **** p < 0.0001 \)).

Equilibrium swelling provides an idea of the maximum amount of water a hydrogel can absorb once in contact with a liquid. Addition of HACS significantly increased the total amount of water absorbed at equilibrium from 47.5 ± 8.4 % to 93.5 ± 34.7 % (\( p = 0.019271 \), Fig. 4.4A), this is likely due to the significant increase in porosity from 14.5 ± 3.6 % to 26.3 ± 1.9 % (\( p = 0.002538 \), Fig. 4B) and/or to a higher interconnectivity within the scaffold and/or a higher number of available hydrophilic groups.

Degradation studies have been performed to understand what factors affect the degradation of the composite hydrogels (see Supplementary Fig. S4.4). In the degradation
experiment, control hydrogels were incubated in PBS for three weeks and an initial weight loss was observed at 7 days. This was then maintained ($p > 0.05$) for the duration of the three weeks, indicating that an initial weight loss due to solubilisation of lightly crosslinked chains is likely to happen in the first week after implantation, however in absence of enzymes, this degradation does not proceed (Fig. 4.3C). A smaller weight loss was observed for CS-HACS ($p = 0.876728$) and CS-HACS-AgNWs ($p = 0.003344$) at 7 days, confirming that enhanced crosslinking is present in these composites. An unchanged residual weight was recorded also for these two composites at two and three weeks. When lysozyme was added, CS gels showed significantly increased degradation during the three weeks with a final total weight loss of $91.7 \pm 7.1 \%$. A similar pattern was observed also for the composite gels, for a significant weight loss was observed after three weeks. At all-time points, the weight loss of CS-HACS-AgNWs was significantly lower ($p < 0.01$) than both CS and CS-HACS gels.

Biomineralisation of hydrogels was studied soaking them in SBF for three weeks to evaluate Ca:P deposition by SEM-EDS. A significant increase in Ca/P deposit was observed after 3 weeks of incubation of hydrogels in simulated body fluid (SBF), this was particularly true for CS-HACS hydrogels (Fig. 4.4D and Fig. 4.5). This was expected as the already present hydroxyapatite can function as nucleation site for the deposition of calcium salts [111]. On the other hand, the absence of hydroxyapatite in CS gels lead to a non-significant deposition of salts ($p > 0.05$).
Figure 4.4 Further physical characterization, enzymatic degradation and bioactivity determination of hydrogels: CS (black), CS-HACS (light grey) and CS-HACS-AgNWs (dark grey). (A) Equilibrium swelling (%) data are reported as mean ± SD (n = 6). One way ANOVA returned \( p < 0.05 \), results of the post-hoc Tukey’s multicomparison test are reported in the graph (\( * p < 0.05 \)). (B) Gels porosity determined by gravimetric method; data are reported as mean ± SD (n = 3). One way ANOVA returned \( p < 0.05 \), results of the post-hoc Tukey’s multicomparison test are reported in the graph, (\( * p < 0.05 \) and \( ** p < 0.01 \)). (C) Gels degradation in the presence of lysozyme (full bars), the empty bars represent the relative control gels treated in PBS; data are reported as mean ± SD (n = 3). One way ANOVA returned \( p < 0.05 \), results of the post-hoc Tukey’s multicomparison test are reported in the graph (\( # p < 0.05 \)). A t-test was performed against the control for each gel at each time point; \( * p < 0.05 \), \( ** p < 0.01 \) and \( *** p < 0.01 \). (D) Percentage surface calcification of hydrogels incubated at 37°C in SBF for 7, 14 and 21 days as calculated by image analysis via ImageJ and BoneJ. Controls were only incubated for few hours in deionised water. Results are reported as mean ± SD (n = 3). Samples labelled with the symbol # resulted to be significantly different from their control sample (\( p < 0.05 \)). One way ANOVA returned \( p < 0.05 \) only when testing CS-HACS at different time points, the results of the post-hoc Tukey’s multicomparison test are reported in the graph (\( * p < 0.05 \), \( ** p < 0.01 \)).
For further investigation of salt deposition within the scaffold, dry scaffolds were scanned by micro-CT with low x-ray energy and low applied voltage (40 kV). As reported by Cancian et al., the region of interest (ROIs) characterized by a higher density may be attributed to salts, such as Ca-P [255] (Fig. 4.6). As expected, quantitative analysis of the hydrogels highlighted a significantly higher salt deposition within the scaffolds containing HACS in comparison to the controls. Moreover, an increase of salt deposition by time was noticed for both CS-HACS and CS-HACS-AgNWs hydrogels (Fig 4.7).
Figure 4.6 Reconstructed microCT slices of (A) CS, (B) CS-HACS and (C) CS-HACS-AgNWs after 14 days of soaking in SBF; Otsu threshold of ROI of (D) CS, (E) CS-HACS and (F) CS-HACS-AgNWs: ROI are marked in red.

Figure 4.7 Average % salt deposition on different types of hydrogel samples (from microCT). Data are reported as mean ± SD (n = 3). One-way ANOVA returned p < 0.05 for both gels containing AgNWs, compared to control,
and P <0.001 was obtained for CS-HACS hydrogels both at 14 and 21 days compared to the control. # was used to compare CS-HACS or CS-HACS-AgNWs to CS gels at each time point. * was used to compare CS-HACS to CS-HACS-AgNWs at each time point.

4.3.3 Ag⁺ release

AAS was used to determine the total Ag⁺ release from AgNWs and CS-HACS-AgNWs scaffolds. Silver release was studied over a period of 38 days, using ultrapure water as medium (Fig. 4.8). After 38 days, approximately 11 ppm of Ag⁺ were released from AgNWs, while nearly 1 ppm was released from the hydrogels.

![Cumulative release profile of silver cations from AgNWs (dots) and CS-HACS-AgNWs (square).](image)

**Figure 4.8** Cumulative release profile of silver cations from AgNWs (dots) and CS-HACS-AgNWs (square).

4.3.4 Antimicrobial activity of AgNWs

The antimicrobial activity of AgNWs was tested against four bacterial strains. Three are gram-positive (*Staphylococcus aureus, Staphylococcus saprophyticus* and Methicillin-
resistant *Staphylococcus aureus*) while *Escherichia coli* is gram-negative. Staphylococci comprise nearly two-thirds of all pathogens implicated in orthopaedic implant infections and thus are clinically relevant in testing materials developed for bone regeneration [3]. Antibacterial efficacy was also assessed against *E. coli* as it often serves as a gram-negative bacterial representative and allowed us to estimate the broadness of the potential antibacterial spectrum. Based on these previous results, we monitored bacterial growth in liquid culture medium in the presence of increasing concentrations of AgNWs (*Fig. 4.9*) and determined the lag time before exponential growth (the period when the bacteria are adjusting to the nascent environment), the time required by the bacterial populations to reach stationary phase (when the OD$_{600nm}$ stabilizes due to the rate of cell death equaling the rate of cell division) and the maximum optical density (OD$_{600}$) reached at stationary phase. *S. aureus* growth was completely inhibited by 100 µg/ml AgNWs (see Supplementary Fig. S4.5). The lag phase was found to be statistically increased for the concentration 50 µg/ml ($p = 0.007773$) in comparison to the control. While, the time to reach the stationary phase and the maximum OD$_{600nm}$ were not statistically different from the control (see Supplementary Fig. S4.5). Firstly, the lag phase was prolonged compared to the control for all the studied concentrations ($p < 0.001$ for 12.5 and 25 µg/ml and $p < 0.0001$ for both 50 and 100 µg/ml; see Supplementary Fig. S4.5). Moreover, this effect seemed to increase when higher AgNWs concentrations were employed.
Figure 4.9 Growth curves of different bacteria in suspension in the presence of 0 (grey), 12.5 (green), 25 (blue), 50 (red) and 100 (black) µg/ml of AgNWs.

4.3.5 Antibacterial activity of the composite scaffolds

One of the main objectives of this study was to develop scaffolds imparting antibacterial activity through silver nanowires incorporation and thus we tested the antibacterial activity of CS-HACS-AgNWs hydrogels against CS-HACS scaffolds towards *S. aureus*, *E. coli*, MRSA and *S. saprophyticus*. The analyses were performed incubating the bacterial suspensions with the sterile dried composites. Biofilm formation on CS-HACS and CS-HACS-AgNWs hydrogels was investigated after 24 hours, staining the fixed hydrogels to highlight the presence of bacteria.
Both of the scaffolds were found to suppress the bacterial growth, hence biofilm formation, on the scaffolds, as no bacteria have been imaged on the considered surfaces. Hence, both chitosan and silver nanowires appeared to prevent biofilm formation within the considered period of time (see Supplementary Fig. S4.6). The antibacterial activity of the composites was studied in the suspension, too. As reported in Fig. 4.10, CS-HACS-AgNWs scaffolds showed a remarkable bactericidal activity against all the four bacteria strains, giving an inhibition of the growth of 100% ± 9.6x10^-6 (p = 0.000275) against E. coli, 100 % ± 9.2x10^-7 (p < 0.000001) against S. aureus, 99.99% ±0.0065 (p = 0.02041) against MRSA and of 100% ± 0 (p < 0.000001) against S. saprophyticus, at 24 hours. On the other hand, CS-HACS scaffolds did not show the ability to stop the bacterial growth within 24 hours, except for S. saprophyticus. Interestingly, AgNWs gels showed an inhibitory effect on MRSA viability, whereas AgNWs only did not have any effect on cell growth. On another hand, AAS data suggested that Ag⁺ concentration in CS-HACS-AgNWs medium should have been nearly 10 times lower than from AgNWs, at 24 hours. By the way this trend can be explained considering that the gels were submitted to sterilization procedures prior antibacterial tests: the sterilization procedures may have altered the properties of the gel and partially degraded them, favouring a much higher Ag⁺ release than the in vitro studies conducted in paragraph 4.4.4. Moreover, whereas the antibacterial activity of AgNWs were tested though OD₆₀₀ₙₙm readings which does not differentiate between live and dead bacteria, the cell availability on cements was accessed directly counting living bacteria. Overall these results highlighted that the addition of AgNWs was essential to lead to a bactericidal activity of the hydrogels.
Figure 4.10 Antibacterial activity of hydrogels in suspension. Growth of *S. aureus*, *E. coli*, MRSA and *S. saprophyticus* in suspensions containing CS-HACS (light grey squares), CS-HACS-AgNWs (dark grey triangles) and LB only (black circles). Results are reported as mean ± SD. One-way ANOVA was performed between different samples, when significant a Tukey’s post-hoc multicomparison test was performed, results are reported on the graph. * represents the statistical difference between scaffolds and bacterial suspensions while # represents the statistical difference between CS-HACS and CS-HACS-AgNWs scaffolds.

4.3.6 Proliferation and differentiation of MC3T3-E1

The biocompatibility of the hydrogels was verified by determining the growth of MC3T3-E1 cells through the adenosine triphosphate (ATP) assay, a well-known reproducible and reliable
assay of cell viability (Fig. 4.11A) [264]. The cells responded well to all the hydrogels with an increase in cell number. A significant enhancement of cell growth was detected in HACS containing hydrogels, as shown by the increase in cell number at both day 7 and 14. HACS gels supported cell growth better than the hydrogels containing CS only. At 14 days no significant difference between the CS-HACS and the CS-HACS-AgNWs was detected and both of them showed better cell proliferation than CS only. Furthermore, the differentiation of the pre-osteoblast MC3T3-E1 cells was determined in order to evaluate the potential of the hydrogels to support osteoblast differentiation for bone repair. The alkaline phosphatase (ALP) assay was performed as ALP activity is considered to be a biochemical marker for osteoblast differentiation and an indicator of bone tissue formation [265]. The MC3T3-E1 cells began to differentiate into osteoblasts in all the samples, as confirmed by the presence of ALP activity after 7 days in culture (Fig. 4.11B). ALP activity was higher in CS samples than those observed in the other hydrogels. With the addition of the hydroxyapatite a decrease in ALP was observed at day 7 while at 14 days no difference between CS and CS-HACS gels were detected. At day 14, a decrease in enzyme activity was visible in all cultures; this is expected as the highest concentration of ALP is expressed during the early stages of bone formation [266].
Figure 4.11 Cell proliferation and differentiation. (A) ATP produced by MC3T3-E1 cells grown in the presence of the following hydrogels: CS (black), CS-HACS (light grey) and CS-HACS-AgNWs (dark grey). Data are reported as mean ± SD (n=3). One-way ANOVA returned $p < 0.019$ for 7 and $p < 0.0001$ for 14 days. (B) ALP expression by MC3T3-E1 cells grown in the presence of the same hydrogels. Data reported as mean ± SD (n=3). One-way Anova returned $p < 0.05$ for 7 days and $p = 0.557$ for 14 days. The results of the post-hoc Tukey multi comparison test for (A) and (B) are reported in the graph: ***, $p<0.0001$; **, $p<0.001$; *, $p < 0.01$ compared to the control; #, $p < 0.05$, ##, $p < 0.01$.

4.4 Discussion

Bone fractures are often exacerbated by the occurrence of bacterial infections that can lead to prolonged or even absent bone healing. Therefore, 3D-engineered scaffolds with both bone regeneration and infection control capabilities are urgently needed. In this study, we aimed to develop the first biodegradable, osteoconductive and osteogenic injectable hydrogel enriched with AgNWs with intrinsic antibacterial properties. Thus, we initially explored the physicochemical characteristics of the hydrogels, related to their potential osteoconductivity, or their ability to promote formation of new tissue within and outside the scaffold itself.
Injectable hydrogels are potentially ideal pharmaceutical formulations for bone regeneration as they reduce the discomfort of the patient due to their non-invasive administration. However, a relatively fast gelation time, once administrated, is essential for this type of formulations as this allows an effective entrapment of additives or cells at the site of application. CS-GP hydrogels are known for being thermosensitive as they undergo solution-gel transition in a temperature-dependant manner [254]. Our in vitro studies demonstrated that addition of HACS significantly diminished gelation time, at body temperature, in comparison to CS only gels, whereas AgNWs did not affect this property. This behavior may be attributed to the higher number of functional groups available for ionic interactions in CS-HACS than in CS hydrogels. Even though chitosan-based hydrogels are ideal scaffolds for tissue regeneration as they mimic the ECM, they present inadequate mechanical performance, which makes them too weak for applications in the musculoskeletal system. Multiple approaches have been taken by researchers to overcome this problem, like incorporating nanoparticles [267]. In this study, the addition of HACS significantly enhanced the compressive gel strength in comparison to the controls. This result was further improved by the addition of AgNWs, due to their metallic nature and one-dimensional morphology. A further important property of a hydrogel for tissue regeneration is its swelling capacity: swelling is related to water uptake from the surrounding tissues that favours nutrients and cells migration [149, 268]. Chitosan is known for its hydrophilic nature due to the presence of several hydroxyl and amino groups able to form hydrogen bonding with the surrounding water. The inclusion of HACS into CS-based hydrogels ensured a higher water uptake, due to the higher number of hydrophilic groups but also to the higher porosity of these gels in comparison to the CS only gels. Thus, HACS is not only acting as a crosslinker, as shown by the enhanced mechanical behavior, but also as a spacer between polymeric
chains enhancing the porosity and hydrophilicity of the composites.

During bone repair, the aim is to employ materials that will be resorbed while the new bone is forming and will allow drug release; thus degradation studies have been performed to understand what factors affect the resorption of the composite hydrogels. The *in vitro* degradation studies were carried out in presence of lysozyme, an enzyme that degrades chitosan *in vivo*. Based on gravimetric analysis and observation by SEM, CS-HACS-AgNWs hydrogels showed significantly reduced degradation in comparison to the control. The addition of HACS composites had no effect on the degradation in comparison to chitosan hydrogels, in good agreement with the findings reported by Dhivya *et al.*[269]. This may suggest that the introduction of AgNWs might be used to regulate and delay the degradation of the gels.

A further important step for bone regeneration is mineralization of the tissue. The *in vitro* apatite formation ability of a scaffold can be correlated to its *in vivo* bone-bonding ability and capacity to absorb calcium and phosphate ions from the surrounding body fluids. Indeed, Ca and P are used by human body to form hydroxyapatite, the bone mineral that constitutes nearly the 65% of bone weight. Thus, the presence of hydroxyapatite on the surface of the scaffolds is essential to promote the nucleation and growth of calcium phosphate. Our *in vitro* study showed that more Ca/P deposits where present on the external and internal surface of the hydrogels over time if hydroxyapatite was present in the composite. The data presented support the key role played by hydroxyapatite in the bioactivity of the composite hydrogels and at the same time demonstrate that the addition of AgNWs did not interfere with Ca/P deposition.

As mentioned, bacterial infections are a burden after bone fracture surgeries. Controlled release of silver cation is a key factor for preventing and treating infections. Indeed, Ag⁺ can
interact with electron donor groups in biological molecules containing sulphur, oxygen or nitrogen, causing damages of microorganism functions[91]. Silver is present in AgNWs in the metallic state, this has no antibacterial properties, however it is able to react with moisture and O₂ in the culture medium, producing ionized Ag⁺. This is highly reactive as it directly interacts with proteins and promotes structural changes in the bacterial cell wall, leading to cell distortion and death. As reported by Kumar et al., steady and prolonged release of silver from scaffolds at a minimum concentration level of 0.1 part per billion (ppb) can provide effective antimicrobial activity [97]. In our study we have explored the antibacterial activity of AgNWs and CS-HACS-AgNWs. The cumulative release profiles of silver cations from AgNWs and hydrogels indicate that the total silver ion release, for all the points, is well within the potential toxic limit mentioned for human cells which is 10 ppm (µg/ml), but high enough to have the potential to inhibit bacterial growth.

The antibacterial properties of AgNWs against suspension cultures of E. coli, S. aureus, MRSA and S. saprophyticus were evaluated. AgNWs were able to prevent the growth of S. aureus and S. saprophyticus at concentrations higher than 50 µg/ml, they affected the lag phase of S. aureus at concentrations higher than 50 µg/ml and of E. coli for all the studied concentrations. Moreover, the OD₆₀₀ of E. coli stationary phase was statistically different for the concentration 50 µg/ml. Our results are in good agreement with Cui and Liu [63]: they carried out a similar experiment for E. coli showing that AgNWs were able to affect the lag phase for concentrations equal or higher than 12.5 µg/ml. However, in their work, concentrations of AgNWs equal to 50 µg/ml were able to prevent bacterial growth. In agreement with our study, Hong et al. reported a 6 hours delay of E. coli growth for the AgNWs concentration 50 µg/ml, without any inhibition[61]. Finally, in our work, E. coli exhibited greater sensitivity to AgNWs than S. aureus. This trend has been reported for silver
nanoparticles, too[270].

The antimicrobial properties of the composite hydrogels were evaluated both in suspension and on the scaffold. On one hand, chitosan is known to exhibit antimicrobial properties against fungi, yeasts, viruses and bacteria, including *E. coli* and *S. aureus*, inhibiting DNA transcription and mRNA synthesis[271]. On the other, the introduction of AgNWs was expected to enhance these antibacterial properties mainly due to the release of Ag+ from the scaffolds. Indeed, CS-HACS-AgNWs could reduce the bacterial growth of over 99% for all the studied species over a period of 24 hours in suspension, in comparison to CS- HACS hydrogels. Moreover, biofilm formation was prevented on the hydrogels with or without AgNWs, over a period of 24 hours. The ability of CS-HACS hydrogels to inhibit bacterial growth on the scaffold, may be explained by the direct interaction of positively charged chitosan with negatively charged bacterial cell surface, which caused cell membrane disruption leading to death[272]. These results are in good agreement with previous literature studies, regarding the ability of low viscosity chitosan to prevent *Staphylococcus* and *E. coli* growth[273, 274] dependently on the amount of chitosan added. A further important property of a scaffold for bone tissue engineering is osteogenicity. Researchers define as osteogenic a scaffold that favours the adhesion and proliferation of osteoprogenitor cells, therefore proliferation of pre-osteoblasts (MC3T3-E1) was studied. Cell number significantly increased on CS-HACS and CS-HACS-AgNWs more than in gels with CS only over a period of 14 days. This indicates a good biocompatibility of the studied biomaterials. Cells differentiation into osteoblasts was confirmed by ALP assay that is a marker of early stages of osteoblast differentiation. ALP plays a role in the conversion of inorganic pyrophosphate into inorganic phosphate, a process that promotes the formation of bone matrix, before the mineralization process [275]. Over a period of 14 days, all the studied hydrogels promoted
cell differentiation at the same level. However, there was a decrease for all the studied cultures between 7 and 14 days. ALP decrease over time might be explained due to the cells entering a new development stage and not because the hydrogels are unable to sustain osteogenic differentiation. Further studies should be carried out in order to evaluate osteoblast differentiation through the expression of markers such as Runx2, osteocalcin, Osterix and collagen II. In particular, Runx2 is a critical transcription factor for osteoblast differentiation and chondrocytic maturation, whereas Osterix provide specificity in osteoblast differentiation. Osteocalcin is a bone matrix protein, released by osteoblasts, and it is a marker for bone production [276, 277].

4.5 Conclusions

AgNWs were incorporated into the scaffolds in order to provide antibacterial properties to the chitosan-hydroxyapatite based hydrogels. The addition of silver nanowires did not interfere with the rapid sol/gel transition at body temperature, enhancing the mechanical properties as well as the bioactive properties of the scaffolds. Furthermore, the presence of AgNWs induced a reduction of the degradation rate of the hydrogels. The scaffolds demonstrated antibacterial properties, both preventing bacterial cell growth in suspension and onto the scaffolds for the considered time-span. Therefore, the use of this type of hydrogel into clinic should potentially decrease the risk of infections. Furthermore, the gels proved to be bioactive and biocompatible allowing in vitro cell attachment and proliferation. Further investigation should be carried out to confirm differentiation of MC3T3-E1 into osteoblasts. Overall, results suggest that CS-HACS-AgNWs hydrogel developed in this work have proven bioactive, antimicrobial and biocompatible properties.
Acknowledgments: The authors wish to thank the University of Portsmouth Research and Innovation Development Fund and the Institute of Biology and Biomedical Science (IBBS) for financial support.

Competing interests statement: The authors declare no competing interests.

Data Statement: All data generated or analysed during this study are included in this published article (and its Supplementary Information).

4.6 Supplementary information

Figure S4.1 AgNWs size distribution obtained by SEM image analysis. (A) Length (mean 5.03 ± 1.85 µm; n = 28) and (B) diameter (mean 99.45 ± 20.20 nm, n = 28). Shapiro-Wilk normality test returned p > 0.05 for both length and diameter.
Figure S4.2 Physical characterisation of the hydrogels: CS (black), CS-HACS-AgNWs (black background with dark grey lines), CS-HACS (light grey) and CS-HACS-AgNWs (dark grey). Gelation time calculated by the inverted tube method. Data are reported as mean ± SD (n = 9). One-way ANOVA returned p < 0.0001. Post-hoc Tukey’s comparison test results are shown in the graph: **** indicates p < 0.0001 and ** indicates p < 0.001.

Figure S4.3 Gel strength calculated by texture analysis before (empty bars) and after (filled bars) gelation. Data are represented as mean ± SD (n = 3). The t-test performed on all samples before and after gelation showed significantly different strength values for all gels ($$ indicates p < 0.01 and $$$$ p < 0.0001). One-way ANOVA to compare the different formulations returned p < 0.01 before and p < 0.0001 after gelation. Post-hoc Tukey’s test results showed that before gelation only CS is different from all other gels (# p < 0.05), while the individual results for after gelation are reported on the graph (** p < 0.01; **** p < 0.0001).
**Figure S4.4** SEM images (200× magnification) of the degradation process in PBS and in lysozyme for CS, CS-HACS and CS-HACS-AgNWs gels at 7, 14 and 21 days.
**Figure S4.5** Bacterial growth lag time, time to reach the stationary phase, and O.D. at stationary phase for different bacteria in suspension treated with 0 (grey), 12.5 (green), 25 (blue), 50 (red) and 100 (black) µg/ml of AgNWs. Data are reported as mean ± S.D. (n=3). One-way ANOVA was performed between different AgNWs concentrations against the same bacteria, when significant a Tukey’s post-hoc multicomparison test was performed, results are reported on the graph. The symbol* indicates statistical difference when compared to 0 µg/ml, the symbol # indicates comparison with 100 µg/ml, the symbol $ is used for specific comparisons as indicated in the graph.
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**Figure S4.6** Microscopy images of biofilm formation on CS-HACS, CS-HACS-AgNWs hydrogels and controls after fuchsin (*E. coli*) or crystal violet (*S. aureus*, MRSA and *S. saprophyticus*) staining. Magnification 100x.
Chapter 5: Novel antibacterial PMMA composites as alternative bone cements with tunable thermal and mechanical properties

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Authors’ contributions

Marta Roldo and Gianluca Tozzi conceived the work and the experiments. Arianna De Mori is responsible for designing and carrying out the experiments as well as data analysis and writing. Alexander Peter Kao scanned the samples by micro-CT. Anita Sanghani isolated and cultured cells from sheeps. Emanuela Di Gregorio gave support to carry out some of the experiments. Eugen Barbu and Roger Draheim contributed to writing.

Abstract

PMMA-based cements are the most used bone cement in interventions such as vertebroplasty, but they present several drawbacks, including lack of antibacterial properties, monomer leakage toxicity and highly exothermic polymerization reactions which can damage the surrounding tissues. Postoperative infections of orthopaedic implants are a serious problem that can negatively affect the complete fracture repair. Moreover, an increasing number of bacteria are developing resistance against commonly
used antibiotics. Silver nanowires (AgNWs) have been attracting increased attention due to their antibacterial activity and lower toxicity compared to cationic Ag and silver nanoparticles (AgNPs). Furthermore, several additives, such as chitosan (CS), can be included in the PMMA cements in order to reduce the polymerization temperature, without reducing the mechanical performances. Finally, the introduction of a methacrylic group on chitosan (CSMCC) may promote crosslinking with MMA and reduce the quantity of MMA required for polymerisation. The purpose of this study, therefore, was to combine AgNWs (0 and 1% wt/wt) and CS or CSMCC at various ratios (0, 10, 20 and 30% wt/wt) into PMMA-based cements, testing two different ratios of powder and MMA (P/L) and to evaluate the mechanical, thermodynamic, antibacterial and cytotoxic properties of the resulting composite cements. Only cements with concentrations of chitosan higher than 10% presented a significantly reduced polymerization temperature. The mechanical performances were affected for concentrations higher than 20% with a P/L concentration equal to 2:1. Concentrations of AgNWs as low as 1% wt/wt conferred antimicrobial activity against *S. aureus*, whereas biofilm formation on the surface of the cements was increased when chitosan was included in the preparation. The combination of chitosan and AgNWs allowed a higher concentration of Ag⁺ to be released over time than just PMMA-AgNWs. Inclusion of AgNWs did not affect cell availability on the scaffolds. Our work demonstrates that a combination of chitosan and AgNWs may be beneficial for both reducing polymerization temperature and biofilm formation, without significantly affecting mesenchymal stem cells proliferation on the scaffolds. No advantages have been noticed as a result of reducing P/L ratio or using methacryloylchitosan instead of chitosan.

5.1 Introduction
Osteoporosis is a progressive medical condition characterized by osteopenia and bone mass loss [278]. This disease makes the bones increasingly weak and prone to fractures, amongst the most common are vertebral compression fractures (VCFs); if multiple thoracic fractures occur, they can lead to lung and heart problems. The most used treatment to reduce morbidity is percutaneous vertebroplasty that consists in the administration of a filler through a needle to the vertebrae cavity [279]. This therapy can restore the vertebral body and reduce the pain in the majority of the patients.

To date, poly(methyl methacrylate) or PMMA is the most used bone cement in vertebroplasty with the aim of both reinforcing and stabilising the vertebral body destroyed by osteoporosis or tumours and for providing local antineoplastic effects, such as delivering anticancer drugs [280]. Moreover, PMMA is used in total hip replacement as a grout to fix a prosthesis or implant against the bone. PMMA beads and the liquid monomer (methyl methacrylate, MMA) are mixed to form a slurry that, once injected, conforms to the shape of its surrounding, allowing distribution of implant loads and formation of strong mechanical bonds with bone following monomer polymerization. However, if the cement mantle becomes loose (this may be due by bacterial infection among other causes), the surrounding bone may resorb and ultimate failure of the implant may occur [281]. Further drawbacks related to PMMA cements are: lack of bioactivity, stiffness mismatch between the bone and the cement, high exothermic reaction temperature that can cause tissue necrosis and monomer toxicity that is characterized by a sudden drop in blood pressure [2, 282]. Moreover, it has been reported that MMA can cause allergic reactions through direct contact of the MMA liquid with the skin (dermatitis) or through inhalation [283]. Finally, an important risk related to any biomaterial implantation is the occurrence of infections that can lead to biofilm formation and implant failure [284].
One strategy employed to promote better PMMA-bone integration, is to increase the cement porosity with the addition of biodegradable materials. For instance, chitosan is a biodegradable material that can degrade, over time, leaving a rough and porous 3D structure in which the bone can grow, promoting a more stable fixation thanks to the improved interlocking between bone and cement. A further advantage of the use of additives in bone cements is their potential to dissipate the heat generated from the polymerization reaction throughout the bone cement material, minimizing the risks of bone necrosis; glycidyl methacrylate (GMA) and trimethoxysilyl propyl methacrylate (3MPMA) have been used to reduce polymerization temperature. Alternatively, the functionalisation of chitosan with glycidyl methacrylate (GMA) to promote crosslinking with MMA can have a similar effect [285].

As mentioned above, bone implant surgeries can be complicated by the occurrence of infections. Osteomyelitis is a bacterial infection of the bone mostly caused by the bacterium *Staphylococcus aureus* [286]. This infection can lead to destruction of the bone and consequently to increased rates of treatment failure, delayed osseous-union and even amputation [253]. Furthermore, more and more bacteria are becoming resistant to antibiotics and new treatments are needed [287].

Silver nanowires (AgNWs) present antibacterial properties [279] and can offer a prolonged silver ion release. However, their potential use in biomedical applications has been poorly explored and, unlike for Ag nanoparticles, no studies have been carried out on their integration in bone cements. Oleic acid capped silver nanospheres (5.3±2.3 nm) were loaded into PMMA-based cements and the samples showed antibacterial activity against a wide variety of microorganisms such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and *S. aureus*, at a NPs concentrations as low as 0.05% wt/wt[288]. Slane et al. formulated
a PMMA bone cement loaded with silver NPs (30-50 nm) functionalized with polyvinylpyrrolidone at concentrations 0.25%-0.5% and 1% wt/wt. No antibacterial activity was found in suspension, but all concentrations of AgNPs were able to significantly reduce biofilm growth on the scaffold[289]. However, all these systems presented a critical problem related to the extremely poor biodegradability and interconnectivity of PMMA. If the plastic does not degrade, the silver contained inside its mass, will not make in contact with the human fluids, will not oxidize and will not interact with bacterial cells.

In the present work, we report the preparation of novel cement composites formulated with PMMA, methacryloyl chitosan (CSMCC) and AgNWs, and discuss the results of our investigation into their cytocompatibility and antibacterial and mechanical properties.

5.2 Materials and methods

5.2.1 Materials

Agar, ammonium hydroxide (28-30%), ammonium sulphate, calcium chloride, chitosan from shrimp shells low viscosity (degree of deacetylation ~85%, calculated by $^1$H-NMR), dimethyl sulfoxide cell culture tested, methacryloyl chloride (≥97.0%), methane sulfonic acid (≥99.0%), poly(vinylpyrrolidone) powder (55 kDa), silver standard for AAS, tryptone enzymatic digest from casein, sodium chloride and yeast extract for microbiology were purchased from Sigma Aldrich (Irvine, UK). DePuy SmartSet MV Medium Viscosity Bone Cement (see Table 5.1 for composition) was purchased from eSutures.com (Mokena, IL, USA). DMEM high glucose with glutaMAX and phenol red, glycerol, heat inactivated fetal bovine serum, isopropanol, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), PBS pH 7.4, trypan blue stain, silver nitrate, trypsin 0.25% EDTA with phenol red
and x100 penicillin streptomycin were from Fisher (Loughborough, UK). ATPlite Luminescence ATP Detection Assay System was purchased from PerkinElmer (Coventry, UK). Deuterium chloride (D, 99.5%, DCL 20%), deuterium oxide (D, 99.9%), dimethyl sulfoxide-d6 (D, 99.9%) + 0.05% v/v TMS (tetramethylsilane) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

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<th>Table 6.1. Chemical composition of SmartSet MV cement.</th>
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<td><strong>Bone cement powder</strong></td>
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<td>Poly(methyl methacrylate) (%wt/wt)</td>
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<td>Poly(methyl methacrylate-co-styrene) (%wt/wt)</td>
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<td>Benzoyl peroxide (%wt/wt)</td>
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<td>Barium sulphate (%wt/wt)</td>
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| **Bone cement liquid**                                 |
| Methyl methacrylate (%wt/wt)                           | 98.00 |
| N,N-Dimethyl-p-toluidine (%wt/wt)                      | ≤2.00 |
| Hydroquinone (ppm)                                    | 75    |

**5.2.2 Synthesis and characterization of cement components**

**5.2.2.1 Synthesis of CSMCC**

Chitosan (CS) (2 g) was dissolved in concentrated methanesulfonic acid (11 ml) over 2 h at 0°C with overhead stirring. Methacryloyl chloride (12 ml) was then added dropwise into the chitosan gel, and the light brown reaction mixture was further stirred for 4 h at 0 °C, under protection from light. The solution was poured into cold water and a light cream
precipitate was obtained after neutralization with aqueous ammonia (35% v/v). The precipitate was dialyzed against deionized water for 1 day and the solid was recovered by centrifugation at 2880 g for 20 min (three times); the pellet was dried under vacuum, (40°C) and then pulverized to afford a fine powder.

5.2.2.2 Synthesis of AgNWs

AgNWs were synthetized according to literature [257] with some minor modifications. PVP (3 g) was fully dissolved in glycerol (95 ml) by heating to 80 °C. The solution was cooled down and AgNO₃ (0.79 g) was added under vigorous stirring (800 rpm) until the powder was fully dissolved. Subsequently, NaCl (5 mM in final solution) was mixed into 10 ml of glycerol and added to the PVP/glycerol solution. The reaction temperature of the mixture was raised to 210 °C. Once the temperature was reached, it was maintained for further 10 min.

5.2.2.3 Physicochemical characterization of CSMCC

FT-IR spectra were recorded using a Varian FT-IR 640-IR Instrument (Agilent, Santa Clara, CA, USA) and spectra were processed using Agilent Resolutions Pro software. For ¹H-NMR analysis: CSMCC was dissolved in DMSO-d6 containing 0.5% TMS standard; chitosan was dissolved in D₂O:DCI (98:2); methacryloyl chloride (200 µl) was mixed with 800 µl of CDCl₃. All the samples were analysed using a JEOL Eclipse+ 400 MHz NMR instrument (Oxford Instruments, Oxford, UK). The degree of substitution in the reaction product (DS%) was determined according to the following formula:
DS(%)=\[\frac{\left(\left(I_{v1} + I_{v2}\right)\right)}{2}\] \times \left(\frac{l_{glucopyranose\ ring}}{l_{gp}}\right) \times 100

where, \( I_{v1} \) and \( I_{v2} \) are the integral intensities of the signals assigned to the two vinylic protons present in methacryloyl chloride, while \( l_{gp} \) is the integral intensity of protons belonging to glucopyranose ring of chitosan.

The powder particle size was determined by laser diffraction using a Helos particle sizer coupled with a RODOS dry dispersion unit and ASPIROS micro dose module (Sympatec GmbH, Clausthal-Zellerfeld, Germany); a pressure of 2 bar was used for the measurements.

5.2.2.4 Antibacterial properties of CS and CSMCC against S. aureus in suspension

CS and CSMCC 10 mg/ml stock suspensions, in sterile lysogeny broth (LB) were prepared and sterilized under UV light overnight and sonicated for 20 min at 40 Hz before testing. Antibacterial studies were carried out in suspension according to Ardila et al. with some modifications [252]. S. aureus (ATCC 25923) were transferred from -80°C (30 % glycerol) into 5 ml of fresh sterile LB by a sterile toothpick and incubated (at 37°C and 200 rpm) until the bacterial suspension was cloudy. Then, the suspension (50 µl) was transferred into 5 ml of fresh sterile LB and the bacteria were further incubated at 37°C until the suspension was cloudy again. The experiment was carried out into 8 ml autoclaved sealed bottles in which the bacteria (10^6 CFU/mL) were added to CS and CSMCC suspensions (0.2, 0.4, 0.8, 1, 2, 5 and 10 mg/ml) and the volume was brought to 2 ml with sterile LB medium. The experiment was carried out in triplicate (n=3). After incubation at 37°C and 200 rpm for 12 hours (MaxQ™ 8000, Thermo Scientific), bacterial concentration was determined by measuring the optical density (OD_{600nm}) of the suspension. Different concentrations of the stock CS and CSMCC suspensions, as well as and pure LB were used as blanks.
5.2.3 Preparation and physicochemical testing of composite PMMA cements

Different cement formulations have been tested, by changing the concentrations of additives and/or powder to liquid monomer ratio (Table 5.2). The general method to prepare the cements is here reported. Powders were uniformly mixed, using a vortex. The cements were prepared by manually combining the liquid monomer with the powders, at the required P/L ratio, until the powders were fully wet. If AgNWs were included in the preparation, freeze-dried AgNWs were suspended in MMA by sonication for at least 20 min at 40 Hz. When the mixture became dough-like and easier to handle, the mass was poured into a custom made 10 well PTFE mould (6 mm diameter, 12 mm height) and manually pressed. The cements were allowed to cure for 1 hour, removed from the mould and stored in a desiccator until further use.

Table 5.2. Formulation of composite cements and average weight loss and water uptake after 4 weeks of incubation in PBS. Weight loss and water uptake are reported as mean ± SD (n≥3). Dunnett’s multiple comparison test was carried out to compare the results to the control (PMMA_2:1): (p < 0.05 and b p<0.01, c p<0.001, d p<0.0001). PMMA= poly(methyl methacrylate); CS=chitosan; CSMCC = methacryloyl chitosan; AgNWs = silver nanowires. PMMA= poly(methyl methacrylate); CS=chitosan; CSMCC = methacryloyl chitosan; AgNWs = silver nanowires.

<table>
<thead>
<tr>
<th>CEMENT TYPE</th>
<th>PMMA (g)</th>
<th>MMA (ml)</th>
<th>CS (g)</th>
<th>CSMCC (g)</th>
<th>AgNWs (wt/wt%)</th>
<th>Weight loss (%)</th>
<th>Water uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA_1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.09±0.09</td>
<td>2.84±1.03</td>
</tr>
<tr>
<td>PMMA_0.8</td>
<td>2</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.12±0.12</td>
<td>3.44±0.71</td>
</tr>
<tr>
<td>PMMA_AgNWs_1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.23±0.15</td>
<td>2.76±0.66</td>
</tr>
<tr>
<td>PMMA_AgNWs_0.8</td>
<td>2</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.13±0.22</td>
<td>2.77±0.73</td>
</tr>
<tr>
<td>PMMA_CS 10%_1</td>
<td>1.8</td>
<td>1</td>
<td>0.2</td>
<td>-</td>
<td>0.16±0.25</td>
<td>3.86±1.40</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
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<td>-----</td>
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<td>-----------</td>
<td></td>
</tr>
<tr>
<td>PMMA_CS 10%_0.8</td>
<td>1.8</td>
<td>0.8</td>
<td>0.2</td>
<td>-</td>
<td>0.21±0.24</td>
<td>3.99±3.93</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CS10%_AgNWs</td>
<td>1.8</td>
<td>1</td>
<td>0.2</td>
<td>1</td>
<td>0.16±0.01</td>
<td>2.02±1.31</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CS10%_AgNWs</td>
<td>1.8</td>
<td>0.8</td>
<td>0.2</td>
<td>-</td>
<td>1.03±0.65(a)</td>
<td>9.14±2.43(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CS20%</td>
<td>1.6</td>
<td>1</td>
<td>0.4</td>
<td>-</td>
<td>0.62±1.28</td>
<td>8.70±2.44(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CS20%</td>
<td>1.6</td>
<td>0.8</td>
<td>0.4</td>
<td>-</td>
<td>0.93±0.95(a)</td>
<td>9.76±2.10(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CS20%_AgNWs</td>
<td>1.6</td>
<td>1</td>
<td>0.4</td>
<td>-</td>
<td>1.10±2.07</td>
<td>10.30±2.80(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CS20%_AgNWs</td>
<td>1.6</td>
<td>0.8</td>
<td>0.4</td>
<td>-</td>
<td>3.66±1.03(b)</td>
<td>13.36±5.00(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CS30%</td>
<td>1.4</td>
<td>1</td>
<td>0.6</td>
<td>-</td>
<td>3.66±1.03(b)</td>
<td>13.36±5.00(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CS30%</td>
<td>1.4</td>
<td>0.8</td>
<td>0.6</td>
<td>-</td>
<td>4.00±1.54(a)</td>
<td>8.65±2.96(a)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CS30%_AgNWs</td>
<td>1.4</td>
<td>1</td>
<td>0.6</td>
<td>-</td>
<td>2.61±0.25(c)</td>
<td>11.59±4.92(b)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CS30%_AgNWs</td>
<td>1.4</td>
<td>0.8</td>
<td>0.6</td>
<td>-</td>
<td>3.29±0.51(a)</td>
<td>8.42±3.71(a)</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CSMCC10%</td>
<td>1.8</td>
<td>1</td>
<td>-</td>
<td>0.2</td>
<td>0.27±0.27</td>
<td>3.41±3.09</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CSMCC10%</td>
<td>1.8</td>
<td>0.8</td>
<td>-</td>
<td>0.2</td>
<td>0.21±0.30</td>
<td>6.33±4.29</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:1_CSMCC10%_AgNWs</td>
<td>1.8</td>
<td>1</td>
<td>-</td>
<td>0.2</td>
<td>0.21±0.16</td>
<td>5.49±1.79</td>
<td></td>
</tr>
<tr>
<td>PMMA 2:0.8_CSMCC10%_AgNWs</td>
<td>1.8</td>
<td>0.8</td>
<td>-</td>
<td>0.2</td>
<td>0.20±0.07</td>
<td>2.18±1.57</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3.1 Polymerisation temperature and time

The temperature changes during the setting reaction were measured according to the method of Chen et al. with minor adjustments [290]. The MMA was added to the powders and mixed until the dough was fully wet (total powder mass = 3 g). This was poured into plastic containers (3.5 cm diameter x 1 cm height) under ambient conditions. Three thermocouples were inserted few mm under the surface of the dough and the temperature was measured and recorded at 5 s intervals for 25 min, using data acquisition software, Pico Log from Pico Technology. Temperature was plotted versus time to determine the peak temperature and setting time. The maximum temperature within the dough was assigned to the peak temperature during polymerization of the composite cement. The setting time was the time point when the exothermic temperature rises to the midpoint.
between the ambient and the peak temperature. The setting temperature was determined using the equation below:

\[ T_{set} = \frac{(T_{max} + T_{amb})}{2} \]

where, \( T_{set} \) is the setting temperature, \( T_{max} \) is the maximum temperature reached during curing, and \( T_{amb} \) is the ambient temperature prior to mixing. The experiment was carried out at ambient temperature (21–22°C). The experiment was carried out once for each cement type.

5.2.3.2 Degradation studies

The degradability of the composite cements was evaluated by determining weight loss, water absorbance and porosity changes following incubation in PBS. Cement specimens were immersed in PBS (pH=7.0) and placed in an incubator at 37°C for 4 weeks, shaking at 90 rpm (Grant, Cambridge, UK); the PBS medium was changed every week. At scheduled time points, samples were removed from PBS and weighed (after removal of any excess liquid). Samples were then dried in a vacuum oven at 37°C for 3 days before measuring their final weight. Water absorption and weight loss were determined applying the equations reported by Kim et al.\(^9\). Total porosity and pore size were examined using X-ray micro-CT (Versa 520, Carl Zeiss Ltd., CA) and the 3D data was then analysed with Avizo (9.3.0, FEI company). The images were collected with the X-ray tube operating at a peak voltage of 60 kV, a power of 5 W, a tube current of 84 \( \mu \)A and with a Zeiss LE1 filter in place. An isotropic voxel size of 6.11 \( \mu \)m was achieved using the 0.4x objective and a total of 1601
projections were collected over 360 degrees of rotation. Each projection was acquired using a 3 s exposure time. The 2D X-ray projection images were reconstructed using the filtered back projection algorithm within the manufacturer’s reconstruction software. Reconstructed volumes of each sample were cropped (edges were cropped to eliminate edge artefacts and air from all around the samples) and a threshold was applied to highlight the regions of interest. Then, border kill tool was used to discard the pores on the edges. Finally, the volumes were analysed by the tools ‘Label Analysis’ and ‘Volume Fraction’ to obtain the values of volume pore size and porosity, respectively. Six samples per type were tested for weight loss and water uptake. One sample per type was analysed three times for porosity and pore size determination.

5.2.3.3 Mechanical tests of cements

Compressive tests were carried out to evaluate ultimate compressive strength (UCS), Young’s modulus (E) and yield point. A servohydraulic Test System (MTS Systems Corp., Eden Prairie, MN) was used to compress the cements. The cement sample faces were sanded and then, the diameter and length of each sample were measured using a Vernier caliper. Cements were inserted into metallic end caps on both sides (epoxy resin glue was used to stick the endcaps to the cements). Then, samples were compressed at a compression speed of 0.02 mm/sec to 25% strain. Six samples per cement type were tested, before and after degradation. Stress and strain were calculated and stress versus strain curves were plotted for each sample. The stress (MPa) was calculated using the equation below:
Stress ($\sigma$) = \[ \frac{F}{\text{cross sectional area}} \]

where, $F$ (N) is the load applied to the specimen to cause a displacement in the sample and the cross sectional area was expressed in mm$^2$. The strain was calculated using the equation below:

\[ \text{Strain} = \frac{\Delta L}{L} \]

where, $\Delta L$ (mm) is the displacement and $L$ (mm) is the original length of the sample. A typical stress strain curve is reported in Fig. 5.1.

![Stress-strain curve](image)

**Figure 5.1** Representative stress-strain curve of PMMA cement. The initial region (violet region) was discarded; the slope (orange curve) of the elastic region represented the elastic modulus. A second line parallel to the elastic region and with 0.2%-strain offset (orange dashed line) was used to intersect the stress-strain curve and to identify the yield point. The intercept between the equation describing the elastic region and the plateau region (yellow curve) was identified as ultimate compressive strength. The two images of a PMMA cement represent the specimen before (left) and after (right) compression.
5.2.3.4 Morphological characterization

The external surfaces of cements before and after degradation were investigated using a high resolution scanning electron microscope (SEM, Jeol JSM-6160L). Samples were gold-coated using a Polaron e500 instrument (Quoram Technologies, UK). ImageJ (version 1.8.0) software was used to determine the superficial pore size. Results are reported in Supplementary information (Fig. S5.2A-F).

5.2.3.5 Silver release studies

The release of silver cations was studied by soaking the cements in 5 ml of HPLC-grade water at 37°C, with shaking (90 rpm). At scheduled times, 1 ml of supernatant was taken and substituted with 1 ml of fresh water. The concentration of silver ions was determined by a furnace atomic absorption spectrophotometer (VarianSpectrAA 220FS) at a wavelength and spectral bandwidth of 328.1 nm and 0.2 nm, respectively. A calibration curve was prepared by diluting a silver standard (1 g/l) in deionized water (1, 2.5 and 5 mg/l).

5.2.3.6 Biofilm formation on cement surfaces

Cements were directly prepared in 96-well plates. The day after, they were soaked in 70% EtOH for 30 min and washed three times in sterile water. Then, they were irradiated with UV light in a laminar flow for 1 h. One hundred microliters of a 1x10⁶ CFU/ml suspension of S. aureus in LB medium were plated in each well. The plates were then incubated for 24 h
at 37 °C, under aerobic conditions. Then, the medium was removed and each well was gently washed once with PBS in order to remove loosely adherent bacteria. An MTT assay was carried out in order to quantify the total bacteria on cement surfaces: 100 μl of 0.5 mg/ml of MTT solution in PBS was added to each well and the plates were incubated for 1 hour in the dark. The supernatant was removed and DMSO was added to dissolve the formazan salts inside the cells. Then, the violet solutions were transferred to a new 96- well plate and the absorbance was read at 570 nm using DMSO as blank. The experiment was performed in triplicate.

5.2.4 Cytotoxicity studies

5.2.4.1 Cell culture preparation

Primary sheep mesenchymal stem cells were obtained under project licence number PPL70/8247, ethical approval was granted by the animal welfare and ethical review board (AWERB) at the Royal Veterinary College, London, UK. Cells were used between passage 2 and passage 5. Cells were grown in high glucose DMEM supplemented with 1% penicillin/streptomycin and 10% heat inactivated FBS. Cells were grown until 70% confluence in an incubator at 37°C and 5% CO₂ and then detached with 0.25% Trypsin-EDTA. The cell suspension was centrifuged at 400 x g for 5 min (Eppendorf Centrifuge 5702, UK) and the pellet was resuspended. Finally, cells were stained with Trypan Blue and visually counted.

5.2.4.2 Cytotoxicity studies of the cement extracts

Cements were prepared as reported in paragraph 5.2.3. After 1 day since manufacturing,
Cements were submerged in complete DMEM at a weight to volume ratio 1:5 and incubated at 37°C for 24 h [291]. The solutions were then filtered through a 0.22 μm filter and frozen until use. Cells were grown overnight at a cell density of 5x10^3 cells/well in a 96-well plate. Then, the medium was removed and 100 μl of cement extracts were added to each well and the plates were incubated for 24 and 48 h. At these scheduled time points, the medium was removed and an MTT assay was performed. The cells were plated for 4 h with 0.5mg/ml MTT in complete medium. Then the medium was removed and 100 μl/well of DMSO was added. Absorbance was read at 570 nm using DMSO as blank. The experiment was performed in triplicate.

5.2.4.3 Cell proliferation studies

Cell proliferation was determined using an ATP bioluminescence assay. The cements were prepared in the same manner as described for the biofilm studies. Cells (5000 cells/well) were seeded and their proliferation was measured with an ATP assay, according to the manufacturer’s instructions (ATPlite, Perkinelmer). Luminescence readings were taken in a 96 well plate (SpectraMax i3x, Molecular DEVICES, USA). Six replicates per type were tested.

5.2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.03 software. Details of the different statistical analyses used are reported where relevant.
5.3 Results and discussion

5.3.1 Physicochemical characterization of CSMCC

The product was synthetized by substituting the proton of the primary hydroxyl group in chitosan with a methacryloyl moiety, via a nucleophilic acyl substitution reaction carried out in a strongly acidic environment (pkₐ of methanesulfonic acid = -1.9) (Fig. 5.2 A). The unreacted methacryloyl chloride was removed during the washing steps as the ammonium salt of methacrylic acid [292]. The formation of CSMCC was confirmed by FTIR (Fig. 5.2 B): the new peak at 1710 cm⁻¹ may be attributed to the C=O bond in the newly formed methacryloyl group. The chemical structure of CSMCC was also confirmed by ¹H-NMR (Fig. 5.2 C), with signals of vinyl protons appearing at δ 5.71 and 6.02 ppm, and the three methyl protons of the methacryloyl group at δ 1.01 ppm. The ¹H-NMR spectrum also shows a signal
at 2.24 ppm from the three methyl H atoms (N-acetyl glucosamine), a signal at δ 1.48 ppm from H2 (glucosamine), several overlapping signals (from δ 1.7 to 2.0 ppm) from H3–H6 connected to the non-anomeric C3–C6 carbons in the glucopyranose ring, and δ 2.3 ppm from the anomeric proton. The degree of substitution of CSMCC was ca. 25.7%.

For the bone cement preparation, the particle size is important in order to guarantee a homogenous dispersion of the powders into the liquid monomer. The average size of PMMA, CS and CSMCC particles is reported in Table 5.3. CS and CSMCC particles presented an irregular shape (Fig. S 5.1) and had a significantly higher particle size than those in the PMMA powders. The particle size of the employed PMMA was in the range 30–150 μm, as typically found in commercial bone cements [293]. When comparing the VMD (volume median diameter) values of CS and CSMCC, a statistically significant difference was found, indicating that CSMCC powder was slightly bigger than CS powder (Table 5.3).

**Table 5.3.** Average particle size, as determined by laser diffraction, expressed as Sauter mean diameter (SMD) and volume median diameter (VMD) of CS, CSMCC and PMMA powders. The values are reported as mean ± SD (n≥3). Dunnnett’s multicomparison test was used to compare SMD and VMD of CS and CSMCC with the ones of PMMA: \(^a p<0.05\). T-test was used to compare CS and CSMCC: \(^b p<0.05\).

<table>
<thead>
<tr>
<th>Material</th>
<th>SMD (μm)</th>
<th>VMD (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA</td>
<td>34.6 ± 19.6</td>
<td>290.5 ± 171.6</td>
</tr>
<tr>
<td>CS</td>
<td>120.3 ± 21.8(^a)</td>
<td>533.0 ± 32.1(^a)</td>
</tr>
<tr>
<td>CSMCC</td>
<td>138.7 ± 53.9(^a)</td>
<td>630.3 ± 39.9(^a, b)</td>
</tr>
</tbody>
</table>

5.3.1.1 *Antibacterial properties of CS and CSMCC powders*

The bacteriostatic potential of CS and CSMCC powders were tested against *S. aureus* by measuring the absorbance of the bacterial suspension after 12 h of incubation in LB (Fig. 5.2).
The compounds were studied in suspension as this is the physical form in which the chitosan and modified chitosan were incorporated in the poly(methyl methacrylate) cements. While bacterial growth was detected for all concentrations studied, results showed that concentrations equal or higher than 2 mg/ml were required to significantly affect bacterial growth. In this respect, no differences were noticed between chitosan and modified chitosan.

**Figure 5.3** Antibacterial properties of CS and CSMCC. Optical density (OD$_{600nm}$) of *S. aureus* suspension in presence of medium as control (black bars), CS (light grey bars) and CSMCC (dark grey bars) in suspension at different concentrations. Data are reported as a mean ± SD (n=3). One-way ANOVA returned $p<0.05$, results of the Dunnett’s multiple comparison test are reported in the graph (*$p$=0.047; **$p$=0.0027; ***$p$=0.003; ****$p<0.0001$). The t-test performed between CS and CSMCC at different concentrations revealed no statistical difference ($p>0.05$).

The poor antibacterial activity observed for all chitosan powders studied in comparison to other literature reports can be possibly explained considering the relatively high pH of the suspension (in our study the pH was neutral, around 7.0), while the majority of studies using chitosan have been carried out at a pH lower than the pK$_a$ of chitosan; in an acidic environment chitosan is protonated, and thus capable of interacting with the negatively
charged bacteria surface). For example, Erdem et al. tested the antibacterial activity of chitosan against *S. aureus* at different pH values [294]. The higher activity was observed with lower pH values and it was significant for pH lower than 6. Tsai and Su have shown that at pH 7.0, chitosan reduced cell number by 3 log, while at pH 9.0 no bactericidal activity was found [295].

5.3.1.2 Experimental peak temperature and setting time of cements

Commercially available acrylic bone cement is typically activated by mixing a powder containing PMMA, benzoyl peroxide (radical initiator) and barium sulphate (radiopaque agent), with a liquid composed of MMA (monomer), hydroquinone (stabilizer) and *N,N*-dimethyl-p-toluidine accelerator that encourages the polymer and monomer to polymerise at room temperature (cold curing cement).
When the two components are mixed together, the liquid monomer polymerizes around the pre-polymerized PMMA particles (Fig. 5.4 C) to form hardened PMMA (Fig. 4.4 A-B) [296].

One of the major problems with the clinical use of PMMA cements is that the exothermic radical process initiated by mixing induces a risk of local tissue necrosis. According to ISO 5833:2002 the maximum temperature for acrylic resin cements should be ≤ 90°C, where temperatures lower than 56°C would ensure a reduced tissue necrosis (e.g. collagen) at the interface between the cement and the natural tissue [297]. Thus, in this study we registered the temperature, within the cements, during the polymerization process. For each bone cement investigated in this study, the maximum temperature decreased compared to PMMA 2:1 control (66.7±8.7°C) (Fig. 5.4 A and B). In particular, concentrations of CS/CSMCC equal to or higher than 20% showed a significant reduction in polymerization temperature (e.g. CS20%_0.8 resulted in \( p < 0.01 \) and CSMCC20%_1 resulted in \( p < 0.05 \)). On the other hand, when the P/L ratio decreased to 2:0.8, the exothermic reaction temperature decreased, even though it was not statistically different for any of the samples (\( p > 0.05 \)). The lowest polymerization temperatures of CS enriched cements were recorded for PMMA CS30%_0.8 (45.4±3.4°C) and PMMA CS30%_AgNWs_0.8 (44.2±1.4°C), whereas for CSMCC for PMMA CSMCC30%_0.8 (44.9±7.6°C) and PMMA CSMCC30%_AgNWs_1 (45.0±2.5°C). It was previously shown that the introduction of nanoparticles, such as MgO, HA, CS, BaSO\(_4\) and SiO\(_2\) reduced the peak polymerization temperature. This behavior may be due to the potential of homogeneously distributed additives to dissipate the heat.
generated by polymerization reactions throughout the bone cement material [298]. Furthermore, Haas et al. suggested that by decreasing the ratio between the initiator and MMA, the exothermic temperature can decrease due to the incomplete polymerization occurring in the system [299].

According to ISO 5833:2002, the ideal setting time for acrylic resin cements, depending on the usage, should be between 3 to 15 min. In our work, we found that the setting time increased with the addition of increasing concentrations of CS/CSMCC (Fig. 5.5 C and D), from nearly 667.17±28.1 sec (ca. 11 min) of PMMA_1 to 843.8±2.9 sec (ca. 14 min) of PMMA CS30%_1. These findings are in good agreement with what was reported in literature for the reaction rate of free radical homo polymerization reactions. Indeed, it has been shown that the polymerization rate is proportional to the monomer concentration and to the square root of initiator concentration. Thus, the lower the concentration of initiator (benzoyl peroxide in our formulation), the longer the polymerization time required [300]. Moreover, we observed that the setting time of PMMA decreased with increasing the P/L ratio. The faster setting times with an increased ratio between initiator and MMA could be explained by a faster radical formation.
Figure 5.5 Setting properties of cements. Peak polymerization temperatures of PMMA cements containing CS (A) or CSMCC (B) and setting time of PMMA based cements, containing CS (C) or CSMCC (D), with different ratios of P/L. Data are reported as a mean ± SD (n=6). One-way ANOVA returned p <0.05, results of the Dunnett’s multi comparison test, used to compare all the samples with the PMMA control, are reported in the graph (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.3.1.3 In vitro degradation studies.

The porosity and pore size distribution of composite cements was evaluated by X-ray micro computed tomography (X-ray micro-CT). During the polymerization process, voids and bubbles are inevitably created due to the entrapment of air that cannot be released mainly due to the cement paste viscosity. Moreover, in cements containing biodegradable materials, such as chitosan, it is expected that a degree of degradation would take place over time with an increase in porosity. Micro-CT allows to distinguish between different
materials according to their different X-ray attenuation. In this study, samples of PMMA control and cements containing 20% CS or CSMCC were scanned.

PMMA-CS sample analyzed before degradation seemed to have a significantly higher porosity than PMMA only \( (p<0.05) \), whereas the PMMA_2:1_CSMCC20% was not statistically different. Furthermore, the cement containing CS \( (p<0.05) \) and CSMCC underwent degradation during a 4-week incubation period in PBS, but not significant degradation was shown in the PMMA only cements (Fig. 5.6 A).

Figure 5.6 Pore volume and pore size of cements. Percentage pore volume (A) of PMMA (black), PMMA after 4 weeks (grey), CS_20% (red), CS_20% after 4 weeks (orange), CSMCC_20% (dark green) and CSMCC_20% after 4 weeks (light green). Data are reported as a mean \( \pm \) SD \( (n=3) \). The t-test was performed between the same cement type before and after soaking \( (p=0.029) \). One-way ANOVA returned \( *p=0.040 \) when comparing the % pore volume of CS_20% or CSMCC_20% to PMMA before degradation in PBS; results of the Dunnett’s multiple comparison test are reported in the graph. One-way ANOVA returned \( \&p=0.0062 \) when comparing the % pore volume of CS_20% or CSMCC_20% to PMMA after degradation in PBS; results of the Dunnett’s multiple comparison test are reported in the graph. (B) Diameter distribution of pore diameter (\( \mu \text{m} \)) with relative frequency for PMMA (black), PMMA after 4 weeks (grey), CS_20% (red), CS_20% after 4 weeks (orange), CSMCC_20% (dark green) and CSMCC_20% after 4 weeks (light green).
Results from pore size analysis (Fig. 5.6 B) show PMMA_2:1 cements presented an average pore size of 12.2 ± 10.1 μm before degradation; the pore size was statistically not different after incubation; PMMA_2:1_CS20% average pore size was 11.6 ± 13.4 μm before and 13.1 ± 10.9 μm after degradation and the degree was degradation was statistically different between the two time points; similarly PMMA_2:1_CSMCC20% had a pore size of 11.59±9.22 μm before and 13.59±12.42 μm after degradation, but it was not statistically different (p>0.05). PMMA cements before and after degradation had a narrower pore size distribution in comparison to the cements containing CS or CSMCC both before and after degradation.

In order to further assess the composite cements, *in vitro* degradation was evaluated by a gravimetric method. This method is considered the golden standard for *in vitro* wear assessment [250]. This provides information about global mass loss, though it does not give any information about the distribution of wear within the material. As shown in Table 5.2, PMMA-only cements showed a poor weight loss (0.09±0.09% and 0.12±0.12%, for PMMA_2:1 and PMMA_2:0.8, respectively), whereas CS and CSMCC based cements presented higher weight loss, that increased with the increase of the polysaccharides concentration, as expected. For instance, the calculated weight loss for PMMA_CS_2:1 samples increased from 0.16±0.25 to 0.68±0.57 and finally 3.66±1.03%, for 10, 20 and 30% chitosan content, respectively. No statistical difference between the two MMA concentrations was found. These results indicate that the degradation rate of these cements can be controlled by changing the additives concentration.

Water absorbance increased with the increase of CS or CSMCC up to nearly 13% (Table 5.2). Similar trends are reported in literature for chitosan-based polyester and poly(methyl methacrylate) cements[249]. This trend can be justified in two different ways: the presence
of chitosan that is hygroscopic promoted the water absorption by the scaffold, while this was not possible in PMMA only cements that are hydrophobic. Alternatively, a higher porosity and/or interconnectivity of the material, due to chitosan degradation, could promote water retention within the scaffold (as suggested from micro-CT scans). No statistical difference was determined between CS and CSMCC based cements ($p<0.05$).

5.3.1.4 Mechanical properties

The evaluation of the mechanical properties of PMMA cements is crucial for determining their long-term stability in their conventional application where they are required to transfer loads between bones and implants[248]. One of the major problems related to standard PMMA cements is the mismatch between the mechanical properties of the cement and the bone, especially in the case of osteoporotic bone. Standard PMMA cements have an elastic modulus of about 3 GPa, while trabecular bone has a value between 20 and 800 MPa [301-303]. Compressive strength of PMMA cement and vertebral cancellous bone are around 100 MPa and 1–15 MPa, respectively. Consequently, vertebral augmentation with PMMA has been shown to increase the risk of fracture of the adjacent vertebral bodies [302]. Therefore, it is important to reduce both the stiffness (elastic modulus) of the scaffold and its compressive strength, without compromising the long-term functionality of the prosthetic fixation. Knets et al. reported that increasing the porosity of the PMMA based bone cements, it was possible to reduce the flexural modulus of elasticity [304], without affecting the flexural strength. Another solution to reduce the Young’s modulus of the PMMA cements is to load the cements with additives: for instance, Tan et al. added hydroxypropyltrimethyl ammonium chloride chitosan (HACC) into PMMA.
bone cement at different mass ratios, showing a significant reduction of the elastic modulus when mass ratios ≥ 20% were used [305]. Thus, in this study we incorporated increasing mass ratios of CS/CSMCC in order to study whether the mechanical properties of PMMA cements could be improved.

Surgical PMMA cement is brittle in nature. Like all brittle materials, it is weak under tension but quite strong in compression, and is capable of yielding under uniaxial compression. Therefore, the yield strength was determined in compression. Another reason for this choice is that the main direction of load on bone cement in a total hip implant is compression.

The compressive strength and elastic modulus of PMMA loaded with CS and CSMCC at different mass ratios are shown in Fig. 5.7. The compressive strength and elastic modulus generally decreased increasing the mass ratio of CS or CSMCC in the mixture. This trend is in good agreement with what reported by Dunne et al. for PMMA bone cements (Palacos R) loaded with chitosan, at lower concentrations (1, 3 and 5 % wt/wt) [306]. or by Tan et al. which incorporated 20% chitosan into PMMA bone cements (CMW Endurance Bone Cement) [307]. When AgNWs were included in the formulation, no statistical differences were reported from the corresponding cement. This trend has been also reported by Slane et al., who incorporated 1.0% wt/wt silver nanoparticles into acrylic bone cements [289].

When the ratio between powder and liquid was 2:1, the compressive modulus and elastic modulus were significantly decreased with the inclusion of 30% CS. The decreased mechanical properties when CS or CSMCC were incorporated can be attributed to the presence of less chemical links inside the cements and possibly the presence of loose powder. On the other hand, when the ratio between powder and liquid was 2:0.8, the compressive strength was significantly different for lower concentrations of CSMCC (20%).
The mechanical performance was also evaluated after the in vitro degradation study carried out for 4 weeks, however no significant differences were determined with an unpaired t-test (p>0.05) (data not shown).

![Graph](image)

**Figure 5.7** Compressive strength (MPa) and Young’s modulus (MPa) of composites cements, based A) CS and B) CSMCC. Results are reported as a mean ± SD (n=6). One-way ANOVA returned p<0.05, results of the Dunnett’s multicomparison test (used to compare all the samples with the PMMA_2:1) are reported in the graph (*p<0.05). Unpaired t-test was performed to compare each formulation containing a weight to volume ratio of 2:1 with the respective formulation containing a weight to volume ratio of 2:0.8 († p<0.05). Young’s modulus (MPa) of C) CS and D) CSMCC. One-way ANOVA returned p<0.05, results of the Dunnett’s multicomparison test, used to compare all samples with PMMA control, are reported in the graph (*p<0.05). Unpaired t-test was performed to compare each formulation containing a weight to volume ratio of 2:1 with
the respective formulation containing a weight to volume ratio of 2:0.8 ($^2 \ p<0.05$). Results are reported as a mean ± SD (n=6).
5.3.1.5 Silver release studies from cements

A release study of silver ions was performed in deionized water for a period of 21 days, and the cumulative release profiles are presented in Fig. 5.8. The bone cements loaded with AgNWs continuously released Ag\(^+\) ions to the surrounding water (the amount of silver increased significantly within the first day, but then it reached a plateau phase). No statistical difference was found between the different cements studied (\(p>0.05\)), even though the cements containing CS/CSMCC presented a higher Ag\(^+\) release at any time point. The total cumulative release was well below the potential toxic limit of 10 ppm for human cells [308].

![Figure 5.8 Cumulative release of silver ions from bone cements: PMMA_AgNWs_1 (black), PMMA_AgNWs_0.8 (red), PMMA_CS20%_AgNWs_1 (blue), PMMA_CS20%_AgNWs_0.8 (green) PMMA_CSMCC20%_AgNWs_1 (yellow), PMMA_CSMCC20%_AgNWs_0.8 (purple). Data are reported as a mean ± SD (n=3). One-way ANOVA, at 21 days, returned \(p>0.05\).](image-url)
The antimicrobial properties of the bone cements were determined using an MTT assay after 24 h of immersion in a suspension of *S. aureus*. PMMA only cements presented lower bacteria attachment than the cements containing also CS or CSMCC powders (Fig. 5.9). In particular, when CS or CSMCC were used at concentrations higher or equal to 20%, the bacterial attachment was statistically higher than PMMA only. The lack of antibacterial activity of chitosan powders into PMMA cements was already highlighted by Dunne et al. [309], which incorporated chitosan at concentrations up to 5%. Indeed, they reported that the adherence of *S. aureus* was similar to the controls both at 6 h and 72 h. On the other hand, our results are in contrast with what reported by Shi et al, which showed a significant reduction in *S. aureus* adherence to a PMMA cement (Smartset without gentamicin) loaded with a CS to bone powder ratio of 15% [310]. However, Shi et al, studied the bacterial attachment at 3 h, whereas in our study, we used an attachment time of 24 h to allow a higher bacterial adhesion. Furthermore, they have used a different bacterial strain (ATCC 25923) from our study (ATCC 12403).

We have hypothesized that this behavior may be due to the physical form of CS or CSMCC powders that act as physical supports for the attachment of bacteria, as previously reported for chitosan microspheres [311] or powders/flakes [312]. Attachment of bacteria to chitosan is due to electrostatic interactions between the positively charged chitosan with the negatively charged cell surface.

On the other hand, when AgNWs were incorporated within the formulation they induced a significant reduction of the viable bacteria attached to the cement for all the studied formulations (*p*<0.05), except for CS/CSMCC 10%. These results re in good agreement with what reported by Prokopovich et al. which showed significant reduction of bacterial
attachment on PMMA cements loaded with 0.05% silver nanospheres (5.3±2.3 nm)[288].

Our findings show that also AgNWs loaded PMMA cements can reduce biofilm formation on bone cements. Future studies should focus on testing lower concentrations of AgNWs in order to understand the extent of this antibacterial activity.

Figure 5.9 Biofilm inhibition formation. Absorbance relative to number of bacterial cells on cements (P/L=2/1) surfaces obtained by MTT assay after 24 hours. (A) PMMA based cements containing CS. (B) PMMA based cements containing CSMCC. Data are reported as mean ± SD (n=4). One-way ANOVA returned $p<0.05$, results of the post-hoc Tukey’s multicomparison test are reported in the graph (*$p<0.05$). Unpaired t-test was performed to compare each formulation with and without AgNWs. * represents $p<0.05$, ** represents $p<0.01$, *** represents $p<0.001$ and **** represents $p<0.0001$.

5.3.2 Evaluation of the cytocompatibility of the composite cements

In this study, we explored the indirect effect of cement eluates (Fig. 5.10 A and B) and the direct toxicity of PMMA cements (Fig. 5.10 C and D).

The use of eluates has the advantage of simulating the postsurgical release of toxic leachables into the surrounding bony environment. Moreover, through serial dilutions it is possible to evaluate the dose-dependent effect. To investigate the toxicity of the extracts, cements were soaked in complete medium, using a liquid to solid ratio of 5:1, according to
EN ISO 10993-12 standard (1996). The extracts were sterilized by filtration using a 0.2 μm pore membrane to avoid artefacts due to any other sterilization process. Herein, the cytotoxicity of cement extracts was evaluated on sheep mesenchymal stem cells. Data show the percentage cell viability after 1 and 2 days of incubation with extracts. No statistical difference was found for the same formulation after 24 and 48 hours of exposure (p>0.05), except for PMMA_AgNWs_1 (p=0.0119). The cell viability at 24 h was higher than 80% for all cements with a P/L 2:1 at 24 hours, except for the cements containing 20% of CS or CSMCC and AgNWs that seemed to significantly decrease the cell viability since the first day of exposure, e.g. PMMA_CS20%_AgNWs_1 (51.6±31.5%), PMMA_CSMCC20%_AgNWs_1 (55.6±36.6%), PMMA_CS20%_AgNWs_0.8 (52.4±9.3%) and PMMA_CSMCC20%_AgNWs_0.8 (45.2±30.9%). Instead, the diluted extracts (50%) presented a cell viability that was always superior to 80%, indicating a dose-dependent effect (data not shown). When comparing the two different concentrations of MMA at different time points, no statistical difference was found (p>0.05). Overall these results suggest that with the addition of CS/CSMCC (and consequently reduction of bone cement powder) in the formulation, less liquid cement could react with the initiator and more toxicants seemed to be released from the cements over time. Future studies are needed to confirm this hypothesis, through the characterization of the eluents using mass spectrometry techniques.

Adenosine triphosphate (ATP) is a common marker for cell viability and cell proliferation as it is present in all metabolically active cells. The amount of light produced during the assay is proportional to the ATP concentrations within the cells. In this work, this assay has been employed to assess the cell proliferation after 1 and 3 days of seeding cells onto the cement surfaces. It was found that there was a significant difference in ATP levels during
the incubation time for samples containing PMMA only with \( p = 0.021 \) and without AgNWs \( p < 0.0001 \) and CS/CSMCC in low amounts: PMMA_CS10% \( p < 0.0001 \), PMMA_CS10%_AgNWs \( p = 0.0170 \), PMMA_CSMCC10% \( p = 0.0203 \) and PMMA_CS20% \( p = 0.0125 \). For higher concentrations, the increase of ATP was not statistically significant \( p > 0.05 \). Moreover, it can be seen that an increase of CS/CSMCC lead to a significantly reduced cells number in comparison to the control at any time point. These results are in good agreement with what reported by Tan et al. that loaded PMMA-based cements with chitosan powder at 20% wt/wt (PMMA-C)\(^{34}\). Compared with PMMA, PMMA-C cements presented a lower proliferation rate. These results are in agreement with the cytotoxicity studies on the extracts: the increase of the ratio between bone cement liquid and bone cement powder decreases cell viability, over time.

On the other hand, the inclusion of AgNWs did not interfere with cell proliferation in comparison to their controls.
Figure 5.10 Cytotoxicity of cements on mesenchymal stem cells. Cytotoxicity was tested against the cements eluates (A and B) and by direct contact of cells with cements (C and D). Toxicity of extracts from cements of P/L ratio 2:1 (A) and 2:0.8 (B) at 24 (left column) and 48h (right column). Data are reported as mean ± SD (n≥3). One-way ANOVA returned \( p < 0.05 \), results of the post-hoc Tukey’s multicomparison test are reported in the graph (* \( p < 0.05 \)). Unpaired t-test was performed to compare each formulation with or without AgNWs. ATP assay for MSCs after 1 day and 3 days of incubation on CS cements (C) and CSMCC cements (D) One-way ANOVA was carried out to compare ATP levels of each cement to PMMA 2:1 at day 1 and day 3 and showed statistical difference (\( p < 0.05 \)). Results of Dunnett’s multi comparison are reported in the graph; in particular, $ represents \( p < 0.05 \), $$ represents \( p < 0.01 \), $$$ represents \( p < 0.001 \) and $$$$ represents \( p < 0.0001 \), at day 1. £ represents \( p < 0.05 \), ££ represents \( p < 0.01 \), £££ represents \( p < 0.001 \) and ££££ represents \( p < 0.0001 \), at day 3. The t-test was performed to compare each type of cement at 1 day and 3 day (* represents \( p < 0.05 \), **** represents \( p < 0.0005 \)).

5.4 Conclusions

CS and CSMCC were added to PMMA cements in concentrations ranging from 10 to 30% wt/wt. Reduction of the polymerization temperature was found to be dose-dependent and
to be significantly reduced for concentrations higher than 10%.

An increased water uptake and weight loss was found in the cements containing chitosan or modified chitosan in comparison to the controls; the effect was more noticeable with an increase in their concentration. The compressive strength and elastic modulus generally decreased with the increasing mass ratio of CS or CSMCC in the mixture; the same trend was observed for cytotoxicity. No statistical differences were observed between the materials containing either chitosan or methacryloyl chitosan.

The results of the in vitro studies have demonstrated that the incorporation of AgNWs in PMMA-chitosan cements in a concentration of 1% wt/wt can be a viable approach to prevent S. aureus infections on the scaffold, while not affecting their mechanical properties and cytocompatibility. These systems demonstrated a long term release of Ag\(^+\) ions in an aqueous medium, suggesting potential antimicrobial activity over an extended period of time.

This study suggests that the inclusion of CS/CSMCC (between 10 and 20%) and AgNWs (1%) in the existing commercial materials can provide bone cements with good results in terms of cytocompatibility combined with appropriate thermal, mechanical, and antibacterial properties.

Future studies should focus on testing methacryloyl chitosan with higher degrees of substitutions in order to evaluate whether mechanical properties and toxicity properties may be improved.

**Acknowledgments**

The authors would like to thank Mr. Colin Lupton (School of Mechanical Engineering of
Portsmouth) for his precious advice on mechanical and thermal studies. Moreover, the authors would like to thank Marta Peña Fernández (School of Mechanical Engineering of Portsmouth) for her suggestions on samples preparation for mechanical studies. This research has been funded by the Institute of Biological and Biomedical Sciences of Portsmouth (IBBS).

5.5 Supplementary information

5.5.1.1 Imaging of powders

As part of the study, photos of powders were taken using a light microscope (Fig. S5.1).

![Figure S5.1](image)

**Figure S5.1** Light microscope images of (A) PMMA, (B) CS and (C) CSMCC particles. Magnification 10X. Scale bar 84.46 μm.

5.5.1.2 SEM characterization of cement surfaces

SEM images of cement surfaces were taken before and after degradation in PBS for 4 weeks. The SEM images (Fig. S5.2) revealed the presence of PMMA beads (arrow), incorporated into a homogeneous polymerized MMA cement interspersed with uneven holes of different diameters. These features can be attributed to the manual powder mixing method and cylinder preparation in comparison to the clinical one. According to previous
studies, some studies have shown better osteoconductivity for substitutes with the pore size between 200 and 600 μm, whereas other reports have shown no significant difference in osteoconductivity among different pore sizes. Images showed an increased porosity on the cements surfaces before and after degradation, whenever CS/CSMCC were present in the formulation.

**Figure S5.2** SEM images of cements surfaces. X200 magnification. (A) PMMA_2:0.8, (B) PMMA_2:0.8_CS20 and (C) PMMA_2:0.8_CSMCC20. Arrow indicates PMMA beads within the cement.
Conclusions and Future work

6.1 Conclusions

Bone-related infections are common complications that can follow repair of a traumatic bone fracture of the surgical intervention for joint replacement. They can be exacerbated by osteomyelitis, which involves the inflammatory destruction of the joint and the bone with consequent implant failure, and as such they have a big impact on patience quality of life and cost of treatment [167]. Furthermore, the increased number of bacteria developing resistance to commonly used antibiotics further complicates the problem. As such, current treatment strategies for device-related infections are limited, often causing significant patient morbidity, at high economic cost. Thus, the development of alternative strategies to prevent bacterial bone infections is a topical and timely need. As an alternative to antibiotics, silver nanoparticles have been attracting increasing attention since they have shown potential antibacterial properties against a wide range of microorganisms combined with optical and electrical properties that make them interesting for a variety of biomedical uses [69, 91].

The aim of this project was to develop and characterize novel composite materials with inherent antibacterial properties for bone repair and bone regeneration applications. In particular, we formulated a hydrogel and a bone cement containing AgNWs as antibacterial agents.

In the first part of the work, we successfully synthesized AgNWs, in presence and absence of CNTs and observed that the two materials presented similar physiochemical characteristics. CNTs were found to guide the formation of AgNWs on their external surface and promote AgNWs formation at lower temperature. Both nanomaterials presented
bacteriostatic and bactericidal activity against four different bacterial strains (E. coli, S. aureus, MRSA and S. saprophyticus) in a dose dependent manner. The antibacterial activity seemed to depend on the specific bacteria tested rather than on whether they were Gram positive or Gram negative. Both ROS generation and membrane damage were found to effect the toxicity observed. Also cytotoxicity of AgNWs against mammalian cells ((human foreskin fibroblasts (Hs27), human colon adenocarcinoma (Caco-2) and human osteoblasts (hFOB 1.19)) was found to be dependent on cell type, dose and incubation time with ROS formation playing a major role. Preliminary studies on osteoblasts also showed that AgNWs were efficiently internalized by the cells.

In the second part of the work, we formulated a thermosensitive chitosan-based hydrogel enriched with HACS and AgNWs to promote both remineralization of the implant and to prevent biofilm formation. The inclusion of AgNWs showed to improve the hydrogel strength without interfering with gelation time and Ca/P deposition. Further antibacterial in vitro studies showed that the composite formulation was capable of inhibiting bacterial growth in suspension and preventing biofilm formation on the scaffold against E. coli, S. aureus, MRSA and S. saprophyticus. Finally, in vitro studies showed the scaffold’s ability to promote MC3T3 cells proliferation.

In the third part, AgNWs were added in commercial PMMA bone cements containing different weight ratios of CS/CSMCC, ranging from 10 to 30 % wt/wt. Initially, CSMCC was successfully synthesised with a degree of substitution of ca. 25.7%. Increasing the concentration of additives in cements significantly reduced the polymerization temperature, possibly decreasing the risk of tissue necrosis in vivo. Furthermore, the elastic
modulus was reduced with increasing concentrations of chitosan, with potential effect in decreasing the mechanical mismatch between the cement and the trabecular bone. AgNWs (1% wt/wt) significantly reduced the number of viable S. aureus cells on the scaffold, while not affecting the mechanical properties of the composite materials. AgNWs loaded cements demonstrated a long term release of Ag· ions in water, suggesting potential antimicrobial activity over an extended period of time. Cytotoxicity studies showed the higher the concentrations of CS/CSMCC included in the cements, the higher the cytotoxicity of the cements’ extracts and the lower the proliferation on the cements, probably due to an increased amount of unreacted reagents, such as MMA. This study suggests that the inclusion of CS/CSMCC (between 10 and 20%) and AgNWs (1%) in the existing commercial materials can provide bone cements with good results in terms of cytocompatibility combined with appropriate thermal, mechanical, and antibacterial properties. No statistical differences were recorded between CS and CSMCC.

Overall, this project shows that the inclusion of AgNWs, at appropriate concentrations, could be beneficial for preventing biofilm formation in soft and hard scaffolds for bone regeneration, without significantly affecting cell viability.

6.2 Future work

1) Evaluation of antibacterial and cytotoxicity properties of silvernanowires and their composites with carbon nanotubes for biomedical applications.

Future work will focus on more in depth exploration of AgNWs mechanism of action in bacteria and mammalian cell assessing: 1) the contribution of Ag· ions release; 2) possible
DNA damage; 3) AgNWs internalization through TEM and fluorescence microscopy; 4) alteration of mitochondrial membrane potential ($\Delta \Psi_m$) in eukaryotic cells and 5) activation of apoptosis pathways.

2) Injectable composite gels containing silver nanowires as bone scaffold material

Future work will focus on confirming osteoblasts differentiation through the observation of expression of other markers which are up-regulated during later stages of bone formation, such as RUNX2 (Runt-related transcription factor 2), osteocalcin and osteopontin [313]. RUNX2 should show an increase at about 14 days, whereas osteocalcin and osteopontin should increase through 14-21 days in culture. Further in vitro studies should be carried out, implanting the scaffold into rat calvarial bone tissue defects. New bone formation can be evaluated over two months via micro-CT and histology. Hydrogels can also be loaded with growth factors (e.g. BMP-2) to improve their osteogenic properties, as it has been demonstrated that chitosan-based hydrogels are able to release macromolecules in a controlled manner [254].

3) Novel antibacterial PMMA composites as alternative bone cements with tunable thermal and mechanical properties

Future work should focus on testing in vitro biocompatibility of the cement in an animal model infected with S. aureus. X rays, microbiological and histopathological analyses should be carried out after two months to evaluate bone formation and efficacy of the antimicrobial effect.
Bibliography

37. Galveston, Structure, in Medical microbiology, B. S., Editor. 1996: University of Texas Medical Branch, U.S.A.


110 | Schultz, D. and R. Kishony, Optimization and control in bacterial Lag phase. Vol. 11. 2013. 120.


Appendix I: Carbon nanotubes play an important role in the spatial arrangement of calcium deposits in hydrogels bone regeneration

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This paper has been published in:

Abstract

Age related bone diseases such as osteoporosis are considered among the main causes of reduced bone mechanical stability and bone fractures. In order to restore both biological and mechanical function of diseased/fractured bones, novel bioactive scaffolds that mimic the bone structure are constantly under development in tissue engineering applications. Among the possible candidates, chitosan-based thermosensitive hydrogel scaffolds represent ideal systems due to their biocompatibility, biodegradability, enhanced antibacterial properties, promotion of osteoblast formation and ease of injection, which makes them suitable for less invasive surgical procedures. As a main drawback, these chitosan systems present poor mechanical performance that could not support load-bearing applications. In order to produce more mechanically-competent biomaterials, the combined addition of hydroxyapatite and carbon nanotubes (CNTs) is proposed in this study. Specifically, the aim of this work is to develop thermosensitive chitosan hydrogels containing
stabilised single-walled and multi-walled CNTs, where their effect on the mechanical/physiochemical properties, calcium deposition patterns and ability to provide a platform for the controlled release of protein drugs was investigated. It was found that the addition of CNTs had a significant effect on the sol–gel transition time and significantly increased the resistance to compression for the hydrogels. Moreover, in vitro calcification studies revealed that CNTs played a major role in maintaining a stable 3D-structure, allowing a more homogeneous deposition of salts essential for the correct repair of fragile or fractured bones.

8.1 Introduction

Bone is a dynamic, mineralised and highly vascularised tissue whose major functions are body support, motion and organ protection. Major alterations in its structure, due to injuries or metabolic diseases, can cause long-term pain, infection, inflammation and loss of mobility that can lead to a general decrease in functionality and quality of life [1]. Current therapies for complex bone fractures are mainly based on auto- and allo- bone grafts, or on the use of inert materials such as metals and ceramics that replace bone. These invasive strategies have, however, many serious limitations such as an inadequate revascularisation and mineralization of the bone grafts, risk of infection, rejection and immunoreaction [2].

Bone tissue engineering (BTE) provides an interdisciplinary approach to promote the physiological regeneration of bone tissues, overcoming the downfalls of current methods. BTE research focuses on developing bioactive scaffolds that, mimicking the bone structure, work as temporary matrices for cell proliferation, extracellular matrix deposition and
vascularization of newly formed tissue [3, 4]. In order to reach these targets, an ideal scaffold for BTE applications should possess the following properties: have a porous structure; be biocompatible; have good mechanical properties to support tissue ingrowth; be fully biodegradable; resorb while bone formation occurs, eliminating the need for a revision surgery; not form toxic products; and finally deliver bioactive molecules or drugs in a controlled manner where needed [5].

Among the possible scaffolds for tissue engineering, hydrogels represent ideal systems due to their biocompatibility and biodegradability [6, 7]. These gels can be formulated from different natural polymers such as collagen, chitosan, fibrin, hyaluronic acid and alginate. Chitosan is a linear polysaccharide derived from N-deacetylation of chitin, which is commonly found in the exoskeletons of crustaceans and insects [10]; its biocompatibility, antibacterial properties and structure amenable to simple chemical modifications, make chitosan a very interesting polymer for biomedical applications [11]. Chitosan only hydrogels present poor bioactive and mechanical properties; however, we have previously demonstrated that the combined addition of hydroxyapatite (HA) and carbon nanotubes (CNTs) can improve gels properties [12]. HA is a natural, nontoxic and bioactive ceramic, shown to enhance osteoconduction, bone bonding and stiffness in scaffolds. Moreover, HA favours deposition of calcium phosphate, improving the bone-matrix interface strength [13, 14].

Carbon nanotubes are cylindrical molecules of carbon atoms in sp2 conformation with outstanding chemical, electrical, mechanical and thermal properties [15]. They have recently been shown to have a role in the differentiation of osteoblasts suggesting that they might have a double function in scaffolds for BTE as structural reinforcement and osteoinductive materials [16]. However, their application is dependent on their successful dispersion in
physiological conditions. In fact, CNTs have a tendency to form large bundles in aqueous media, resulting from Van der Waals interaction and π-π stacking [17-19]. Such bundles may cause slippage between nanotubes, become stress concentrators or initiate cracks under applied forces. Many covalent and non-covalent modifications of CNTs have been proposed in order to increase their water dispersability and hence their biocompatibility. Non-covalent modifications, in particular, are based on the absorption or the wrapping of various functional molecules, such as biomolecules, polymers, surfactants and phospholipids around the carbon nanotubes [20]. In a previous study, we have demonstrated the possibility of efficiently stabilising SWCNTs with the amphiphilic and self-assembling chitosan derivative N-octyl-O-sulfate chitosan (NOSC) [21].

In the present study, we developed thermosensitive chitosan hydrogels containing NOSC stabilised CNTs (SWNTs or MWNTs). Our aim was to develop a more flexible formulation strategy by using non-covalent CNTs stabilisation instead of chemical grafting and studying the effects of this strategy on the mechanical and physicochemical properties of the formulations. Image analysis was used to qualitatively and quantitatively assess the effect of CNTs on salts deposition within the formulated hydrogels. The ability of the gel to provide a platform for the controlled release of protein drugs was also evaluated.

8.2 Materials and methods

8.2.1 Materials

All salts and solvents, unless otherwise stated, were obtained from Fisher Scientific (UK). Sodium phosphate monobasic monohydrate, low viscosity chitosan from shrimp, glycerol phosphate disodium salt hydrate, tris(hydroxymethyl)amino-methane ((HOCH 2 ) 3 CNH 2 ),
albumin from bovine serum (BSA), albumin from chicken egg white (OVA) and QuantiPro™ Bicinchoninic Acid Assay Kit were obtained from Sigma-Aldrich (UK). L (+)–lactic acid 90% solution in water was obtained from Acros Organics (USA). Dialysis membranes (size 10, MWCO 12-14 KDa) were obtained from Medicell International Ltd (UK). MWCTs and SWCTs were purchased from Cheap Tubes Inc. (USA). The MWCTs used in the study were 8-15nm in diameter and 10-50µm in length. The SWCTs used were 1-4nm in diameter and 5-30µm in length. Di-potassium hydrogen orthophosphate anhydrous (K₂HPO₄) was acquired from British Drug Houses (UK) and troclosene sodium dehydrate from Guest Medical (UK). Water used in all experiments was purified water. N-octyl-O-sulfate chitosan and chitosan modified hydroxyapatite (HACS) were prepared as described previously [12, 21].

8.2.2 Formulation of hydrogels

Clear solutions of chitosan were prepared by dissolving chitosan (200 mg) in lactic acid (0.1 M, 7 ml), 86 mg of HACS were then added and dispersed by vigorous mixing and sonication. CNTs loaded formulations were prepared by addition of stable CNTs suspensions in NOSC (2 ml), water was added to the control. NOSC suspensions of CNTs were obtained by adding CNTs (2.5 mg) to a solution of NOSC (1.25 mg/ml) sonicating for 2 hours, followed by overnight rest and further sonication for 1 h. A glycerol phosphate solution (1 ml, 1.12 g/ml) was then added to the vials and stirred for 10 min. Gelation was obtained by incubating the formulations at 37°C. The gels prepared were labelled CS (chitosan control gel), CS-HACS-SWNTs and CS-HACS-MWNTs (gels containing HACS and SWNTs or MWNTs, respectively). All gels were prepared in triplicate; gelation time was evaluated visually by the inversion method [22].
8.2.3 Texture analysis of the gels

A Texture Analyzer (XT plus, Stable Micro Systems Ltd, UK) was used to determine syringeability of the suspensions before gelation as well as the texture profile of the formed gels. The methods employed have been described previously by Yasmeen et al. [12]. Briefly, for syringeability the liquid formulations were loaded onto 5 ml plastic syringes fitted with 19 G, 25 mm long needles. The vertically clamped syringe was actioned by the instrument probe (10 mm diameter) that compressed the barrel (1 mm/s) to a distance of 40 mm and the initial glide force, dynamic glide force and maximum force were measured. Compressibility tests were performed by depressing a polycarbonate probe (10 mm dia) into the gels at 1 mm/s up to a depth of 5 mm, six measurements were taken before and after the sol–gel transition took place. Gels were formed and tested in 14 ml glass vials. Force/displacement curves were obtained and used to calculate the gel compressibility (N-mm). All measurements were performed at room temperature.

8.2.4 Characterisation of the freeze dried hydrogels

Freeze dried hydrogels were prepared from the suspension described above and then transferred into 24 well-plates (2 ml per well), before incubation at 37°C. Once formed, the gels were flash-freeze and freeze-dried (Edwards Micro Modulyo RV3, UK). The samples were characterized by FT-IR (Varian Spectrometer 640, UK), DVS (Surface Measurement Systems DVS Advantage, UK) and SEM (JEOL JSM-6060 LV Scanning Electron Microscope, UK). In the DVS analysis the mass change of the gels subjected to a changing water vapour partial pressure at 25°C was recorded. The partial pressure was increased from 0% to 90 % at incremental steps of 10%, where the next step was reached either after equilibrium or
after a maximum time of 360 min. A full adsorption/desorption cycle was performed and the data collected were used to calculate the adsorption/desorption isotherms.

8.2.5  *In vitro* calcification assay

Calcification studies were performed to investigate the ability of the composite hydrogels to induce calcium salts deposition. Simulated body fluid (SBF), prepared according to Kokubo and Takadama [23], was used to mimic the in vivo environment, as it contains ions at very similar concentrations to those of human blood plasma. The freeze dried samples were inserted in a 50 ml tube to which 15 ml of SBF were added. Samples were incubated at 37°C for 7 or 14 days. The SBF was changed every 4 days. Finally, samples were dried under vacuum at 40°C overnight. Controls were hydrated in deionised water for 5 hours and dried as above. All samples were gold-coated with sputter coater (Polaron e500, Quoram Technologies, UK) and analysed by SEM, coupled with EDS (Silicon Drift Detector (SDD)–X-MaxN, Oxford Instruments, UK) and microCT (XTH225, Nikon Systems, UK). The microCT scanner was set to a voltage of 55 kV and a current of 75 μA. With an isotropic voxel size of 10.6 μm and exposure of 2 s, the image acquisition was performed at a rotational step of 0.23° over 360° for 90 min approximately. The images were then analysed using ImageJ (1.48v, NIH, USA) as described below. To quantify calcium deposition, image thresholding (global) based on the grey scale histogram was carried out to distinguish between the different material densities (different attenuation). Total volume of the sample and total volume of the denser material in the sample (i.e. the deposits of calcium salts) were obtained and the percentage of calcium deposition was calculated for each sample (n=5). EDS images for calcium (Ca) and phosphorus (P) were also analysed after being overlaid to corresponding
SEM images. The total area of overlapping for Ca and P onto the total sample surface area was used to calculate percentage of calcium and phosphorus salt deposition (n=3).

8.2.6 Release of model drugs

Release studies were performed including bovine serum albumin (BSA, 1mg/ml) in the hydrogels (5 ml). After the gelation of the samples, prepared in a 15 ml tubes (n=4), PBS (5ml) was added. The samples were then incubated at 37°C and at set time points 2 ml of PBS were sampled and replaced with 2 ml of fresh buffer. Release studies were also performed including ovalbumin (OVA, 1mg/ml) in the hydrogels. The suspensions were transferred (1g per sample) into a Flot-A-Lyzer G2 dialysis device (cut off 50 KDa ) and PBS (15 ml) was added to them. The samples were subsequently incubated at 37°C and at set time points the sampled buffer was replaced with fresh buffer. Determination of both BSA and OVA concentrations was carried out with a QuantiPro™ Bicinchoninic Acid Assay Kit (Sigma, UK) and samples prepared according to manufacturer’s instructions, reading the UV absorbance at 570 nm. Unloaded gels were used as a control to eliminate any false response due to chitosan interaction with the assay kit. The two drugs were tested with slightly different methods due to limitations imposed by their molecular weight. In order to compare the release profiles, these were calculated as percentage of drug release per unit of surface area of the gel in contact with the release medium.

8.3 Results

8.3.1 Texture analysis of the thermosentive hydrogels
A chitosan only formulation was used as a control to evaluate the effect of the addition of HACS and CNTs suspensions on the physical properties of gels before and after formation. The chitosan only formulation underwent sol/gel transition at 37 °C in 60 min, whereas the addition of HACS and NOSC suspensions of SWNTs and MWNTs reduced this time to 7 and 9 min, respectively. The syringeability of the suspensions was also studied. A force of 30 N was considered as the maximum acceptable force for an injection to be performed without particular patient discomfort [24]; none of the samples tested presented values above 10 N (Table 8.1) [25]. Furthermore, texture analysis was performed to determine gel compressibility (Fig. 8.1A). All suspensions tested showed significant difference in strength before and after gelation at 37 °C (at least p = 0.0004, two tailed, t-test with Welch’s correction) apart from the chitosan control. In particular, after gelation the formulation containing SWNTs showed significantly higher values compared to all other formulations (0.273 ± 0.001 N-mm).

Table 8.1 Syringeability of composite formulations

<table>
<thead>
<tr>
<th></th>
<th>Stiction (N)</th>
<th>Plateau Force (N)</th>
<th>End capture (N)</th>
</tr>
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<tbody>
<tr>
<td>Chitosan</td>
<td>8.36 ± 0.33</td>
<td>8.56 ± 0.29</td>
<td>8.92 ± 0.13</td>
</tr>
<tr>
<td>Chitosan-HACS</td>
<td>8.86 ± 0.39</td>
<td>8.56 ± 0.34</td>
<td>9.09 ± 0.34</td>
</tr>
<tr>
<td>Chitosan-HACS-(NOSC-SWNTs)</td>
<td>6.69 ± 0.25***</td>
<td>6.40 ± 0.24***</td>
<td>6.56 ± 0.21***</td>
</tr>
<tr>
<td>Chitosan-HACS-(NOSC-MWNTs)</td>
<td>7.03 ± 0.15***</td>
<td>6.92 ± 0.15***</td>
<td>7.51 ± 0.22***</td>
</tr>
</tbody>
</table>

Stiction, plateau force and end capture are expressed as mean ± SD (n = 3). One way ANOVA, P < 0.001; Tukey–Kramer multiple comparison test *** P < 0.001, compared to the chitosan control.
Fig. 8.1a Compressibility of the different formulations before (grey) and after (black) sol-gel transition. Results are reported as a mean ± SD (n=3). One way ANOVA (a = 0.05) on the values after gelation returned $p= 0.0131$; Tukey’s multiple comparison test: *$p < 0.05$, compared to chitosan, # $p<0.05$ compared to CS-HACS. Water sorption and desorption isotherms of the freeze dried hydrogels; b chitosan; c CS-HACS-(NOSC-SWNTs); d CS-HACS-(NOSC-MWNTs). Data are reported as a mean ± SD (n=3).

8.3.2 Characterisation of the freeze dried hydrogels

The morphology of the freeze-dried hydrogels was evaluated by SEM (Fig. 8.2). The chitosan only gel appeared to be scaly and rough while the gels containing CNTs showed a more defined porous structure with evidence of coated CNTs filaments bridging the structure; this was previously observed by Ormsby et al. who observed CNTs bridging sub-micron voids and preventing pore coalescence [26].
Fig. 8.2 SEM of chitosan, CS-HACS-(NOSC-SWNTs) and CS-HACS-(NOSC-MWNTs) freeze dried hydrogels. 

*Arrows* indicate coated carbon nanotubes within the hydrogel matrix.

### Table 8.2 Kinetic parameter values for water penetration in freeze-dried hydrogels.

<table>
<thead>
<tr>
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<th>$n_p$</th>
<th>$K_p$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS control</td>
<td>1.67 ± 0.15</td>
<td>0.000369 ± 0.000314</td>
<td>0.968 ± 0.015</td>
</tr>
<tr>
<td>CS-HACS-(NOSC-SWNTs)</td>
<td>1.79 ± 0.23</td>
<td>0.000260 ± 0.000335</td>
<td>0.973 ± 0.016</td>
</tr>
<tr>
<td>CS-HACS-(NOSC-MWNTs)</td>
<td>1.65 ± 0.16</td>
<td>0.000461 ± 0.000298</td>
<td>0.983 ± 0.007</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD (n = 3)

8.3.3 Dynamic vapour sorption
DVS analysis was performed to evaluate the amount and kinetics of water sorption of the hydrogels. Previous work showed that the addition of chitosan grafted carbon nanotubes to chitosan hydrogel significantly decreased the hydrophilicity of the formulation [12]; this was not the case when the added CNTs were non-covalently coated with NOSC (Fig. 8.1 B-D). Applying Korsmeyer-Peppas model to the absorption vs. time curves, the mechanism of water penetration described by the value of $n_p > 0.89$ was determined to be super case-II transport (Table 8.2). Hence, it seems that the dynamic swelling behaviour of hydrogels is dependent on the contribution of both the penetrant (water) diffusion and polymer relaxation.

8.3.4 In vitro calcification studies

Bioactivity of a scaffold proposed for bone regeneration can be evaluated by studying the calcium salts deposition on its surface and internal structure [28, 23]. Samples were soaked in SBF for 7 and 14 days, at 37 °C, and superficial Ca/P co-deposition was evaluated by EDS coupled SEM (Fig 8.3) while deposition, in the whole volume, of a denser material was also visualised and quantified by microCT. The quantitative analysis of the EDS data (Fig. 8.4 A) revealed that no calcium-phosphate deposits were found in the chitosan control samples, both at 7 and 14 days. Samples containing CNTs also contained HACS and similar values of co-localised Ca and P at time zero were expected. This was indeed confirmed by the quantitative analysis. However, the SEM images showed that HACS is more homogeneously distributed in the samples containing SWNTs. In both samples an increase in the concentration of co-localised Ca and P was observed, with significantly higher values for the gel containing SWNTs (7.8 ± 0.1%). Salts deposition was also observed by micro CT (Fig. 8.4).
were volumes of denser material were identified and quantified, these may represent the deposition of other salts as well as calcium phosphate salts that possess similar x-ray attenuations. The microCT analysis evidenced differences in the microarchitecture of the hydrogel, were chitosan alone gels presented a less homogenous and more porous structure (Fig. 8.5). This may be attributed to the drying process, during which the migration of water from the core to the periphery of the gel might have dragged the chitosan to condense on the edge leaving a porous structure in the core. This phenomenon is much less noticeable in the gels containing CNTs, confirming how CNTs have an important role in the formation and preservation of the 3D gel structure. As a consequence, a marked difference in the distribution of the salts deposition was observed and resulted in a much more homogeneous distribution in gels containing CNTs. Quantitative analysis (Fig. 8.4 B) also revealed a higher deposition of salts in CNTs containing gels, with no significant difference between SWNTs and MWNTs (34.6 ± 2.6 and 36.0 ± 1.1% salt deposition over total sample volume, respectively) [29]. These data are in good agreement with the EDS/SEM data and confirm that calcification is notably more pronounced in samples containing HACS and CNTs.
Fig. 8.3 Overlay images of SEM pictures with EDS data: Ca (*green*), P (*red*) and co-localisation of Ca/P (*yellow*).
Fig. 8.4  

(a) Quantitative analysis of surface calcium deposition presenting % of surface covered by co-localised Ca and P (from SEM). Data are reported as a mean ± SD (n=3). One-way ANOVA (α = 0.05) p<0.0001 for both gels containing CNTs; Tukey’s multicomparison test, ***p<0.001 compared to control at time zero, ###p<0.001 compared to a specific sample as indicated in figure.  

(b) Average % salt deposition on different types of hydrogel samples (from microCT). Data are reported as a mean ± SD (n=5). One-way ANOVA (α = 0.05) p< 0.0001 for both gels containing CNTs, Tukey’s multicomparison test, all samples presented p<0.001 compared to control, and P<0.001 was obtained for all comparisons between 7 and 14 days. Release profile of c BSA d OVA from (black closed circle) chitosan; (red closed square) CS-HACS-(NOSC-SWNTs); (blue closed triangle) CS-HACS-(NOSC-MWNTs) gels. Data are reported as a mean ± SD (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-HACS-(NOSC-SWNTs)</td>
<td>1.45 ± 0.54</td>
<td>1.32 ± 0.04</td>
</tr>
<tr>
<td>CS-HACS-(NOSC-SWNTs)</td>
<td>1.55 ± 0.30</td>
<td>1.55 ± 0.15*</td>
</tr>
</tbody>
</table>

* P < 0.05, compared to the HACSncludedinthegelswhichhadac Ca/P ratio of 1.34 ± 0.06.
8.3.5 Release of model drugs

The release of two macromolecular model drugs, albumin form chicken egg (OVA, Mw 45 kDa, Fig. 8.4c) and bovine serum albumin (BSA, Mw 66 kDa, Fig. 8.4d), were studied for 14 days. All the hydrogels tested showed the potential to provide controlled release within the 2 weeks of the experiment. Analysis of the release during the first 5 h revealed very similar release profiles for all gels, indicating that the initial release is governed by the behavior of the chitosan component of the gel. The addition of HACS and CNTs decreased the amount of BSA release over 14 days, but this did not have a significant effect on the release of OVA. This suggests that the mechanism of release for OVA is mainly dependent on the interaction of the protein with the chitosan network. Also gel degradation has an effect, indeed a marked change in the gel structure was noticed after 14 days of BSA release study. Hydrogels before the release study presented a typical porous structure, with well-defined pores of different size; while hydrogels after the release presented a scaly appearance.
8.4 Discussion

Composite thermosensitive chitosan hydrogels were formulated. The addition of CNTs was confirmed to have a significant effect on the sol–gel transition time; these results are in accordance to those obtained by addition of chitosan grafted CNTs [12], indicating that both covalently and non-covalently coated CNTs present similar thermal behaviour and lead to
similar effects on the gelation time of the formulations [30, 31]. Unexpectedly, the formulations containing NOSC suspensions of CNTs had better syringeability performance than chitosan only or chitosan with HACS formulation, while previous studies showed that the addition of chitosan grafted CNTs increased the force needed to expel the suspension from the syringe [12]. These data show that covalent and non-covalent coating of CNTs can have very different effect on the viscosity of the suspensions and their syringeability. In our previous work, we demonstrated that NOSC tends to stretch and align along the surface of CNTs [32], reducing the opportunity for formation of physical entanglements between NOSC and the free chitosan chains present in the gel. It is postulated that under the shear stress applied during injection non-covalently coated CNTs are able to align within the flowing liquid formulation therefore present a reduced resistance compared to a crosslinked system. Conversely, CNTs afforded a significant increase in resistance to compression for the composite hydrogels. In comparison with published data, where chitosan grafted CNTs were added instead of non-covalently coated ones, no statistical difference was noticed in the hydrogels containing MWNTs, while SWNTs produced statistically stronger gels ($P < 0.05$) when suspended in NOSC solutions [12]. The use of NOSC for the stabilisation of CNTs reduced the hydrophobicity of the nanotubes when compared to chitosan grafted CNTs. This phenomenon can be explained by considering the shielding effect that NOSC has on the surface of the nanotubes. In fact, five chains of NOSC arrange themselves along the entire surface of CNTs, thus resulting in a net decrease of surface hydrophobicity [21]. Reduced hydrophobicity has been linked with better in vivo outcomes [26, 33, 34]; furthermore, previous studies demonstrated the cytocompatibility of CNTs stabilised in NOSC (data not shown). The in vitro calcification studies revealed that CNTs played a major role in the spatial arrangements of newly formed calcium deposits in the composite materials studied,
suggesting that they may have a role in the way the repair of fragile and/or fractured bones occurs in vivo. Finally, in model drug delivery studies, the composite gels demonstrated potential as delivery platforms for macromolecular drugs with minimum interference by CNTs in the determination of the mechanism of drug release.

In conclusion the present study reports of a simple non-covalent method for the inclusion of CNTs into hydrophilic chitosan hydrogels. The non-covalent stabilisation of the nanotubes is advantageous as it facilitates the injectability of the formulation while affording gels with higher compressibility. CNTs also proved to be key in guaranteeing a homogeneous deposition of salts suggesting that they can be used for guided mineralisation of scaffold for bone regeneration. Finally the gels have potential also as platforms for prolonged drug delivery.

Acknowledgments

This research was co-funded by the Institute of Biomedical and Biomolecular Science Quality Research fund and the Research Development Fund of the University of Portsmouth, UK.

8.5 References


Appendix II: 3D Printing and Electrospinning of Composite Hydrogels for Cartilage and Bone Tissue Engineering

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²Zeiss Global Centre, School of Engineering, University of Portsmouth, Portsmouth PO1 3DJ, UK

Authors’ Contributions

Arianna De Mori reviewed the literature on electrospinning and Marta Peña Fernández reviewed the literature in 3D printing. They contributed equally to the literature search and writing of the paper. Gordon Blunn and Gianluca Tozzi revised the paper and provided expertise in tissue engineering and 3D printing, respectively. Marta Roldo coordinated the team, contributed to the development and planning of the work and its revision with particular focus on electrospinning and hydrogel formulation.

Abstract

Injuries of bone and cartilage constitute important health issues costing the National Health Service billions of pounds annually, in the UK only. Moreover, these damages can become cause of disability and loss of function for the patients with associated social costs and diminished quality of life. The biomechanical properties of these two tissues are
massively different from each other and they are not uniform within the same tissue due to the specific anatomic location and function. In this perspective, tissue engineering (TE) has emerged as a promising approach to address the complexities associated with bone and cartilage regeneration. Tissue engineering aims at developing temporary three-dimensional multicomponent constructs to promote the natural healing process. Biomaterials, such as hydrogels, are currently extensively studied for their ability to reproduce both the ideal 3D extracellular environment for tissue growth and to have adequate mechanical properties for load bearing. This review will focus on the use of two manufacturing techniques, namely electrospinning and 3D printing that present promise in the fabrication of complex composite gels for cartilage and bone tissue engineering applications.

**Keywords:**

composite hydrogels; electrospinning; 3D printing; bone; cartilage

9.1 Tissue Engineering

Defects that affect tissues such as cartilage or bone can be irreversible and become a clinical challenge. This is particularly true when these lesions are associated with conditions such as osteoarthritis and osteoporosis. Currently, it is estimated that 75 million people suffer of osteoporosis in Europe, USA and Japan [1], while 27 million are affected by osteoarthritis in USA only [2]. These numbers are certainly due to increase, as the population is aging, leading to higher healthcare costs. Current treatments for bone
(i.e., autografts and allografts) present drawbacks such as limited availability of donor tissue, risk of infection and unsatisfactory lesion repair [3], whilst the use of bone graft substitutes are not as reliable as gold standard autograft. For small cartilage defects autologous chondrocyte implantation, mosaic pasty and autologous matrix-induced chondrogenesis can be used with variable success but for larger cartilage defects, often joint replacement is the only solution.

Therefore, new strategies are needed to repair damaged cartilage and bone tissue [4]. Tissue engineering is a promising interdisciplinary approach in this field: it aims at developing temporary 3D multicomponent scaffolds that mimic the natural tissue, working as a porous framework for the migration, adhesion and growth of cells to replace the damaged biological material. Ideally, a scaffold for tissue regeneration should have a highly interconnected porous network for the diffusion of nutrients and gases, have good mechanical properties in loadbearing conditions and degrade without producing toxic products with increasing formation of new tissue [3].

9.2 Challenges in Tissue Engineering of Soft and Hard Tissues

Tissue engineering has become one of the most commonly used approaches for cartilage and bone tissue repair [5,6,7,8]. Even though these two tissues are important constituents of the skeletal system, their structure and mechanics differ considerably. Bone is a hard and rigid tissue, whereas cartilage is soft, viscoelastic and flexible and these two tissues are different in several aspects [9]. In addition, according to the anatomic location and function, the same type of tissue can be heterogeneous displaying different anisotropic properties, biochemistry and cellular activity [10]. These characteristics complicate the
fabrication of scaffolds that successfully mimic the structural and mechanical characteristics of the target tissue.

9.2.1 Cartilage

Cartilage is a strong and elastic connective tissue that covers the articulating surface of the bone in diarthrodial joints (articular cartilage) and is a structural component of the rib cage, ear, nose and other body components [11]. Three types of cartilage exist, according to the extracellular matrix (ECM) composition: elastic cartilage (if elastic fibres are present in the ECM), fibrous cartilage (if the matrix is rich in collagenous fibres) and hyaline cartilage if the matrix is predominantly composed of glycosaminoglycans, (GAGs). The latter, also known as articular cartilage, if found at the interface between the gliding bony surfaces in the articular synovial joints, provides a deformable low-friction surface that facilitates the movement of articulating bones and supports high dynamic compressive loads [12]. Mechanically, human cartilage presents the following characteristics: compressive modulus of 0.7–0.8 MPa, shear modulus of 0.7 MPa and tensile modulus of 0.3–10 MPa [13]. At the microscopic scale, human cartilage consists of an ECM, which can be mineralized and is produced and maintained by chondrocytes embedded within it. The hydrated ECM is composed of proteoglycans consisting of a core protein with covalently attached GAGs, mainly chondroitin sulphates, and collagen type II [14]. The GAGs are responsible for the cartilages’ ability to support high compressive loads, whereas the collagen II fibrils contribute to its high tensile strength and ability to tolerate shear stresses. Cartilage composition includes 80% of water, drawn into the collagen II fibrils by the hydrophilic proteoglycan complexes; this plays an important role in defining the tissue
load-bearing function [12]. As cartilage is compressed, the extracellular matrix is compacted causing the efflux of water. As the cartilage is compacted more, the flow of water is reduced due to increasing drag increasing the hydrostatic pressure, which withstands the load. It is important to note that cartilage is avascular, which means that nutrients and cells infiltration is poor and wound healing, after injury or trauma, is hindered. In particular, fibrocartilage is formed to replace the native cartilage and this new tissue is functionally and biomechanically inferior to the native one. Current therapies to facilitate cartilage regeneration include autografting, microfractures and autologous chondrocyte implantation (ACI); however, they all present drawbacks and are unable to fully restore the functional hyaline cartilage, making long-term prognosis uncertain [15]. For instance, autografting causes donor site morbidity and is limited by donor tissue availability. Microfracture treatments can extend the damage to the surrounding tissue and stem cells implantation can still induce the formation of fibrocartilage. In this scenario, cartilage tissue engineered (CTE) scaffolds could be a turning point.

9.2.2 Bone

Bone is the main constituent of the musculoskeletal system [16] and differs from other connective tissues (i.e., cartilage, ligaments and tendons) in rigidity and hardness [17] due to high mineralization of its ECM [18]. Bone provides stiffness to the skeleton allowing for the shape of the body to be maintained, plays a role in the transmission of muscular forces for movement, affords protection to soft tissues within the cranial, thoracic and pelvic cavities as well as the bone marrow [19,20]. At the microscopic scale, bone is arranged in
two architectural forms: trabecular and cortical bone. Trabecular bone represents 20% of the skeletal mass and forms the inner part of the bone, it presents high porosity (50–90%) and contributes to mechanical support in bones such as the vertebrae. Cortical bone, the dense outer layer of bone, comprises 80% of the weight of the human skeleton, and its function is to provide mechanical strength and protection \[16\]. Mechanically, the bone presents the following characteristics: the compressive strength for cortical bone ranges from 170 to 193 MPa and its elastic modulus is found to be in the range of 7 –20 GPa. Trabecular bone, however, has a compressive strength ranging from 2 to 12 MPa and its modulus is in the range of 0.1–5 GPa \[21,22\]. Bone tissue presents a highly complex and hierarchical structure \[23\] which can be defined as a nanocomposite consisting of inorganic nanocrystalline hydroxyapatite (HAp), organic components (mainly type-I collagen) and water \[24\]. The nanocomposite structure is essential to provide the required compressive strength and high toughness of the bone \[25\]. Collagen fibres reinforced by HAp crystals form a tough and flexible nanostructured extracellular matrix, which supports adhesion, proliferation, and differentiation of bone cells (osteoblasts, bone lining cells, osteocytes, and osteoclasts) \[19,26,27\]. Bone constantly undergoes remodelling during life to help it adapt to loading conditions; remove old, microdamaged bone replacing it with new, mechanically stronger bone; and help to preserve bone strength \[28\]. This remarkably dynamic structure of bone displays exceptional regenerative properties \[29\]; however, non-union fractures, tumour resections and some musculoskeletal diseases can lead to critical size bone defects \[30\] that cannot heal spontaneously and require additional treatment before they can regenerate \[31\]. Bone is the most commonly transplanted tissue after blood \[32\]. Several therapeutic approaches including bone grafting procedures \[33\], implantation of different biomaterials \[34\] and
application of hormones or growth factors have been investigated [35]. However, there is still no effective treatment for most cases [36,37]. The limitations of autografting and allografting were previously addressed [38,39]. Metal implants provide immediate mechanical support, but present limitations due to poor integration with the tissue, infection and fatigue fracture [40,41]. Furthermore, ceramic bone graft substitute materials present very low tensile strength and are brittle, restricting their use in locations of significant torsion, bending or shear stress [42]. In this perspective, bone tissue engineering (BTE) has emerged as a promising approach for bone reconstitution, overcoming the limitations of traditional implants [43,44,45].

9.2.3 Osteochondral Tissue

Osteochondral (OC) tissue is located at the interface between the osseous and the chondral tissue and it promotes their interplay; its role and location require a complex composition that includes cartilage, calcified cartilage and subchondral bone. The mechanical, structural and biochemical characteristics of the osteochondral tissue vary throughout. For instance, from a biochemical point of view, mineral content increases from cartilage to bone, while collagen and water concentration diminishes. Structurally, pore size, porosity and vascularization increase from cartilage to bone face. Mechanically, compressive modulus increases from cartilage to osseous tissue [46]. Osteochondral defects (OCDs) seem to play an important role in the genesis of joint diseases, such as osteoarthritis or osteochondritis dissecans. Moreover, subchondral bone includes unmyelinated free nerve endings which may cause pain in case of OC degeneration, due to the applied forces from surrounding tissues [47]. This said, it is fundamental to repair osteochondral defects to prevent joint destruction. However, OCDs are extremely difficult
to treat due to the widely different features between articular cartilage, calcified cartilage and subchondral bone [48]. Osteochondral scaffolds should be designed to concurrently rehabilitate these three tissues all together. Several approaches have been studied to promote OC regeneration, such as debridement, bone marrow stimulation techniques, and the use of osteochondral allografts. However, these present strategies are affected by many drawbacks. For instance, microfractures may lead to degeneration of the repaired tissue and formation of the non-functional fibrocartilage. In the case of grafting techniques, there is lack of donor tissue or immunoreactions. Considering the complexity of this tissue, more research into osteochondral engineering is required [46,49].

9.3 Hydrogels as Tissue Engineering Scaffolds

Hydrogels have been widely investigated not only for tissue engineering applications, but also for drug delivery and wound dressing [4,50,51]. They are insoluble hydrophilic polymeric networks that can swell without disintegrating and absorb a high degree of water, up to several times their dry weight [52]. Their fully hydrated 3D structure resembles the extracellular matrix of native tissues, both physico-chemically and biologically. Moreover, their porous structure enables the transfer of nutrients and metabolites that are fundamental for cell growth. Hydrogels can be formulated from different natural and synthetic polymers such as alginate, chitosan, fibrin, hyaluronic acid (HA), poly(ethylene glycol) (PEG) and poly(ethylene oxide) (PEO). However, hydrogels often show inadequate mechanical performance, due to the interstitial liquid and its plasticizing effect, which make them too weak for applications in the musculoskeletal system [53]. For instance, most hydrogels have an elastic modulus ranging from kPa to MPa, whereas native bone has a modulus of ~1–20 GPa. Matching these properties is
fundamental for two main reasons: (1) scaffolds must support loads and movements; and (2) cells respond differently to different stresses, such as compression, tension and shear [54]. Luckily, hydrogels are tuneable materials; their chemical modification and differential crosslinking allow achieving the desired properties for the proposed application. For example, it is possible to increase the hydrogel elastic modulus increasing crosslinks density inside the gel or combining two or more independent networks, known as interpenetrating networks. Numerous researchers have worked on finding ways to formulate hydrogel constructs with optimized mechanical properties [55,56,57], and several techniques have been employed in order to fabricate them [58]. Microfabrication techniques such as electrospinning and 3D printing have emerged as promising strategies for manufacturing complex hydrogel structures for tissue engineering applications.

9.4 Electrospinning

Electrospinning is a versatile, efficient, cheap and reproducible technique that can be used to produce 1D fibrous materials or composites with a wide range of diameters (from nm to mm), by applying an electrostatic force to a solution. Applications in tissue/organ repair and regeneration [59], drug delivery [59], medical diagnostic, protective fabrics against environmental [60] and infectious agents, and dental materials have all been studied [61]. A general electrospinning apparatus (Figure 9.1) consists of three parts: a high voltage power supply device; a syringe/capillary tube with a metallic needle; and a grounded metallic collector.
In a typical electrospinning process, a polymeric solution (or melt) is loaded into a syringe and ejected at a controlled rate, forming a drop (**Figure 9.2a**). Simultaneously, a high voltage (up to 2–30 kV, depending on the solution used) is applied, and a charged jet of the polymeric solution or melt is formed (**Figure 9.2b–c**). When the electrostatic repulsion starts to overcome the surface tension of the fluid, the hemispherical surface of the liquid will deform into a conical shape, called Taylor cone, at the tip of the needle (**Figure 9.2d**). Finally, the solution jet starts to evaporate, and the polymer solidifies creating a thin fibre jet that deposits on the grounded collector.
The electrospinning of fibres is relatively complex, as the product characteristics can be influenced by several parameters classed into three categories:

1. Solution characteristics (viscosity, surface tension and conductivity): the electrospinning technique relies on the uniaxial stretching of a charged jet, which, in turn, is significantly affected by changing the concentration of the polymeric solution. Generally, by reducing the polymer concentration, the fibre diameter is decreased. However, when the concentration of the polymeric solution is lowered to a critical value, known as entanglement concentration (Ce), beaded fibres are produced. If the concentration is too high no fibres are produced due to the excessive viscosity [63]. Solution conductivity is fundamental to optimize both fibre diameter and stability of the Taylor cone. If the solution has a low conductivity the fluid surface cannot be charged, and no Taylor cone can be formed. By increasing the charge on the surface of the droplet a Taylor cone is formed and the fibre diameter is decreased [64]. The conductivity of a polymeric solution can be controlled by the addition of salts. Moreover, the solvent has a
crucial role in determining the characteristics of the solution; an ideal solution must be able to solubilize the polymer at the required concentrations and be sufficiently volatile to evaporate in the space between the needle and the collector. However, if the boiling point is too low, the solvent will evaporate too quickly causing the drying of the jet at the needle tip [65].

2. Process parameters (applied voltage, flow rate and tip to collector distance): these parameters influence the diameter and morphology of the fibres. As the feed rate increases, the charge density will decrease. Thus, by increasing the flow rate the diameter of fibres is increased and beaded morphology can be observed [64]. Fibres are formed just when the applied voltage is higher than the threshold voltage (the value depends on the solution). Generally, by increasing the voltage there is an increase of the electrostatic force on the solution and thus, a reduction of the fibre diameter. A critical distance between tip and collector is needed for the solvent evaporation and for the preparation of smooth and uniform electrospun fibres. Generally, the longer the distance, the thinner the fibres will be.

3. Environment conditions (humidity and temperature) [66,67]. Ideal environmental conditions must be found for better and improved fibre production. Ambient humidity and temperature can affect both the morphology and diameter of the fibres. Depending on the chemistry of the polymer [68], the fibre diameter can increase or decrease and no definitive comparisons with experimental data can be currently made.

To date, many electrospun scaffolds made of polymers and inorganic nanoparticles have been produced for tissue engineering applications. However, these electrospun scaffolds are in the form of 2D mats (Figure 9.3b) with tightly packed fibres that negatively impact cell infiltration and growth throughout the scaffold. Alternatively, scaffolds obtained by
the amalgamation of fibres and hydrogels can be used to achieve better properties, such as a well-interconnected porous structures [69]. Different composite structures have been employed to fabricate fibrous hydrogels: laminated composites (Figure 9.3c), encapsulating fibres into the hydrogels (Figure 9.3d), injectable hydrogels, composite coatings and dual electrospun/electrospray composites [70].

Figure 9.3. (a) Architectural framework of a native extracellular matrix (ECM); (b) electrospun 2D mat; (c) laminated hydrogel; and (d) fibres encapsulated into a hydrogel. Reproduced from [71] with permission. Copyright (2011) PMC.

9.4.1 Fibrous Hydrogels for Cartilage Tissue Engineering via Electrospinning
Articular cartilage plays an important role in load-bearing joints during dynamic loading. When damaged, it cannot heal naturally, and clinical treatments are necessary. Tissue engineering aims at temporarily replacing damaged articular cartilage with 3D scaffolds. Hydrogels are promising materials for cartilage regeneration when strategies to reinforce their structure are applied, for example combining them with electrospun fibres. Indeed, these scaffolds can emulate the natural ECM (for its porosity and water content) and possess improved mechanical properties due to the fibres ability to reorient under deformation, stiffening, strengthening and toughening the system. Thus, researchers have tried to optimize the mechanical properties of the hydrogels, studying different parameters such as the polymers used, fibre diameter and alignment, porosity and mono or multi layered scaffolds.

Many synthetic and natural polymers have been used for electrospun fibres for cartilage regeneration, such as polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(l-lactic acid) (PLLA), poly(vinyl alcohol) (PVA), Bombyx mori silk fibroin and chitosan. Chitosan, an amino saccharide that is biodegradable and cytocompatible, with antibacterial and wound healing activities as well as tissue-adhesive features has been one the most studied polymers. This polymer can be easily obtained by alkaline treatment of chitin, a naturally occurring polysaccharide obtained from the exoskeleton of crustaceans. To overcome chitosan poor mechanical properties, Mirahmadi et al. [72] developed a chitosan hydrogel enriched with electrospun silk fibroin fibres. They fabricated two gels, one with homogeneously dispersed chopped degummed silk fibres (SC/GP-D) and one as a three-layered composite with a layer of electrospun fibres sandwiched between two layers of chitosan gel (SC/GP-L). Results showed that the mechanical properties were generally enhanced by silk and that the laminated gel
presented both better compressive and Young’s moduli compared to chitosan only (3.1 times stiffer) even though the mechanical performances were not as good as the natural cartilage. On the other hand, the SC/GP-D scaffold was the best scaffold for cartilage formation (as shown by proteoglycan and collagen II content) among the studied hydrogels [72]. For future studies, a combination of both degummed fibres and nanofiber sheets could be examined to obtain improved mechanical properties. A second possibility to improve the mechanical characteristics of the gel is to incorporate individual and short electrospun nanofibers in hydrogel scaffolds, positioning them in a random way to favour an irregular orientation of the chondrocytes. Mohabatbour and his group [73] fabricated PLA fibres fragmented through aminolysis reaction to improve their hydrophobicity and cell-interaction abilities. They fabricated an alginate grafted hyaluronic acid (Alg-HA) incorporating fragmented PLA nanofibers. The nanofiber incorporated hydrogels had higher compressive modulus and lower swelling ratio than Alg-HA hydrogel alone. In this case, the composite was cytocompatible and the chondrocytes were able to maintain their functional properties producing GAGs and other extracellular molecules. This research highlighted that, to control gel fracture and strength, fibres can be differently oriented into a hydrogel.

Other studies have focused on introducing biological signals such as chondroitin sulphate (CS), hyaluronic acid, and collagen, into tissue-engineered scaffolds to encourage tissue specificity. Coburn et al. [74], for instance, fabricated poly(vinyl alcohol)-methacrylate (PVA-MA) fibrous scaffolds with or without chondroitin sulphate, a signal that has been shown to enhance chondrogenesis of mesenchymal stem cells. These hydrogels allowed for immediate cell infiltration, cartilaginous tissue formation and chondrogenic differentiation (as indicated by a higher cartilage specific gene expression).
Finally, the addition of CS increased type II collagen deposition compared to PVA fibres alone [74].

More recently, solution electrospinning has been further developed into Melt Electrospinning Writing (MEW), this exploits a layer-by-layer process similar to other 3D printing technologies affording highly organised fibrous 3D structures in the micron scale. This process eliminates the need for organic solvents that can induce cell toxicity and avoids mechanical and electrical coiling of the fibres simplifying the manufacturing process. Using this technique, Bas et al. [75] produced a negatively charged proteoglycan matrix with a star-shaped poly(ethyleneglycol)/heparin hydrogel combined with wet melted PCL fibres and deposited a 0°–90° crosshatch architecture with different network spacing. The best electrospun matrix had 600 μm spacing; its negative charge density and strong water retention capacity, provided by PEG crosslinked heparin, accurately mimicked the natural cartilage tissue in terms of electrochemical, mechanical and viscoelastic properties. The constructs presented high chondrocyte viability and allowed for cell differentiation under physiologically relevant loading.

Even though the most common strategy is to form fibrous scaffolds with embedded fibres within hydrogels, multilayer scaffolds constructed with fibres in different orientations have been investigated. For instance, Tonsomboon et al. [76] studied how different designs of laminated and non-laminated electrospun gelatin nanofibers in an alginate hydrogel could mimic the mechanical characteristics of the collagenous ECM. In particular they have fabricated single layer composites (a) with or (b) without a random orientation or multilayer composites with (c) unidirectional (where fibres had the same orientation), (d) cross-ply (where alternating layers were perpendicular) or (e) angle-ply
orientation (where there were four different fibres orientations). Firstly, this work showed that nanocomposite hydrogels were stronger and tougher than single polymer hydrogels. Secondly, aligned fibres increased tensile strength without improving toughness. Thirdly, multilayer arranged nanofibers increased the toughness by two orders of magnitude when compared to the controls [76]. Therefore, this paper demonstrated that, by tuning different architectures of fibre reinforced and laminated composite hydrogels, it is possible to resemble the mechanical properties of the native tissue. Literature on hydrogels reinforced with electrospun fibres for cartilage regeneration is summarised in Table 9.1.

Table 9.1. Publications on electrospun fibres reinforced hydrogels for cartilage regeneration.

<table>
<thead>
<tr>
<th>Fibre(s)</th>
<th>Hydrogel</th>
<th>Fabrication method</th>
<th>Mechanical properties</th>
<th>Cytocompatibility</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL or CSMA/PVAMA</td>
<td>PEG-diacylate</td>
<td>Fibres mixed with the hydrogel</td>
<td>Not reported</td>
<td>Chondrogenic differentiation</td>
<td>[71]</td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>Alginate-polyacrylamide</td>
<td>Sandwich-like structure</td>
<td>Young modulus: 3.4 MPa</td>
<td>Not reported</td>
<td>[77]</td>
</tr>
<tr>
<td>PDLA/PLLA or PDLA/PCL</td>
<td>Chitosan</td>
<td>Fibres infiltrated with hydrogels</td>
<td>Compressive modulus: 2-12 MPa</td>
<td>Cartilage ECM deposition</td>
<td>[78]</td>
</tr>
<tr>
<td>PCL</td>
<td>GelMA and GelMA/HAMA</td>
<td>Fibres infiltrated with hydrogels</td>
<td>Compressive modulus: 20-1500 kPa</td>
<td>Not reported</td>
<td>[79]</td>
</tr>
<tr>
<td>PCL</td>
<td>PEG-heparin</td>
<td>Fibres infiltrated with hydrogels</td>
<td>Compressive modulus: 20-1500 kPa</td>
<td>Cell viability &gt; 80% Chondrogenic differentiation</td>
<td>[75]</td>
</tr>
<tr>
<td>PLA</td>
<td>Alginate-graft-hyaluronate</td>
<td>Hydrogel mixed fibres and gelled</td>
<td>Compressive modulus: 3.5-4 kPa</td>
<td>Cell viability &gt; 85% Chondrogenic differentiation</td>
<td>[73]</td>
</tr>
<tr>
<td>Silk</td>
<td>Chitosan</td>
<td>Sandwich-like structure</td>
<td>Compressive modulus: 0.5-0.6 kPa</td>
<td>Cell viability &gt; 90% Chondrogenic differentiation</td>
<td>[72]</td>
</tr>
<tr>
<td>PCL</td>
<td>Alginate or alginatesulphate</td>
<td>Hydrogel pipetted onto the scaffold</td>
<td>Shear modulus: 0.5-5 kPa</td>
<td>Cartilage ECM deposition</td>
<td>[80]</td>
</tr>
</tbody>
</table>
9.4.2 Fibrous Hydrogels for Bone Tissue Engineering via Electrospinning

Hydrogels have been suggested as possible scaffolds for bone regeneration, but their poor mechanical properties and low bioactivity make them inappropriate for hard tissue. One strategy is to reinforce the gel with electrospun fibres, but only a few studies have been reported on fibrous scaffolds for application in bone tissue engineering. Mehdi-Sahad et al. fabricated a 3D cell-laden three-layered hybrid scaffold (Figure 9.4), incorporating a 2D mat layer of poly(hydroxybutyrate) (PHB) and nano-hydroxyapatite fibres (diameter 2.0 ± 0.2 μm) between two layers of methacrylated gelatin/HAp. As expected, the introduction of PHB/HAp fibres enhanced the mechanical strength (tensile modulus 7.0 ± 1.2 MPa, tensile strength 329 ± 18 kPa for the hybrid scaffold) and matrix mineralisation while the hydrogel provided a biocompatible scaffold for cell penetration and proliferation. Even though the mechanical properties were improved in comparison to the hydrogel only, they were still inferior to the natural tissue. Thus, Sadat-Shojai and his group [69] suggested increasing the thickness of the electrospun mat located at the centre of the scaffold, however this approach would lead to a denser electrospun mat, with decreased porosity that would potentially hindered cell penetration into the electrospun centre.
A further problem encountered in bone grafting procedures is the development of infection, inflammation and pain, intrinsically linked to any invasive procedure. However, due to poor vascularity of the bone tissue, osteomyelitis is often difficult and costly to treat. For this reason, injectable systems for minimally invasive procedure (MIP) have been developed. Calcium phosphate cements are among the most used cements (e.g., Hydroset Accell 100™) for bone regeneration as they are bioactive, and they have the ability of self-hardening. However, they are brittle, difficult to inject and present limited porosity, therefore research has been focusing on replacing the cements with injectable hydrogels. Liu et al. [81] have produced a biomimetic bone substitute made of chopped poly(ε-lactide-co-ε-caprolactone) nanoyarns manually incorporated in a collagen hydrogel before gelation. Interestingly, to obtain well aligned nanoyarns, a water vortex was used as collector, instead of more traditional systems as rotating drums or dual metal collection rings (Figure 9.5). As a result, they obtained massive continuous nanoyarns with homogenous diameters (16 ± 4 μm). Furthermore, the cut nanoyarns were short enough
to avoid the formation of entanglements when they were mixed with collagen solution. Results showed again that the incorporation of nanoyarns improved the mechanical properties of the hydrogel, without interfering with the cell proliferative ability of collagen.

Another approach for promoting osteogenesis in comparison to just polymeric hydrogels is to include growth factors inside the matrix. The scaffold needs to work as a delivery system promoting a sustained release and improved local retention. Kolambkar et al. [83] introduced electrospun nanofiber mesh tubes as a guide for rat bone regeneration in a segmental bone defect to deliver recombinant bone morphogenetic protein-2 (rhBMP-2) (Figure 9.6G). The PCL nanofibers (Figure 9.6A) had diameters ranging from 51 to 974 nm,
with high porous meshes (80–90%). The thick nanofiber meshes were able to be wrapped tightly around a steel mandrel and glued to form a tube (of 5 and 13 mm length) that was finally put in a mouse bone defect (Figure 9.6D,E), as they were (Figure 9.6B) or after being perforated (1 mm diameter perforations) (Figure 9.6C). Then, 125 μL pre-gelled 2% alginate with or without 5 μg rhBMP-2 were injected in the tube lumen. As control groups, they examined the nanofiber mesh alone and in combination with alginate hydrogel. Results showed that the systems containing meshes + hydrogel + rhBMP-2 produced substantial bone formation and complete defect bridging, while the controls did not exhibit any significant bone repair response. Indeed, defects were bridged by 12 weeks with densely packed, cellular mineralized tissue for both perforated and not perforated meshes containing alginate loaded with rhBMP-2. However, micro-computed tomography (μCT) revealed that perforations in mesh tubes enhanced bone formation at earlier stages in comparison to the scaffolds without perforations. Moreover, samples implanted with both perforated mesh tube and rhBMP-2 containing alginate were the only one presenting mechanical performances, in extracted femora at 12 weeks, statistically similar to the ones of natural bone. They attributed this phenomenon to the fact that the perforations allow sufficient vascularization to develop, while limiting soft tissue ingrowth [83].
Figure 9.6. (A) SEM images of electrospun nanofiber mesh; (B) tubular implant without perforations; (C) tubular implant with perforations; (D) stabilized femur defect with implant; (E) bone defect, after placement of a perforated mesh tube; (F) alginate hydrogel was still present after 1 week, in vivo; and (G) release of rhBMP-2 from alginate hydrogel. Reproduced from [83] with permission. Copyright (2010) Elsevier.

9.4.3 Fibrous Hydrogels for Osteochondral Engineering via Electrospinning

The challenge in the development of OC scaffolds is that they should be able to replicate the complexity of this tissue, and therefore restore cartilage, intermediate calcified cartilage and bone tissues, all together. To achieve this result, composites scaffolds should be able to recruit mesenchymal cells from the bone marrow. Moreover, they must present a stratified structure in order to mimic the three different functional layers of OC tissue [46]. Hydrogel/fibre 3D composites have a great potential to mimic this complexity; however, to date, literature on graded or non-graded hydrogels with electrospun fibres
for osteochondral regeneration is scarce. Single-phase composites have been used for instance by Coburn et al.; they fabricated poly(vinyl alcohol)-methacrylate (PVA-MA) fibrous scaffolds with or without chondroitin sulphate, a signal that has been shown to enhance chondrogenesis of mesenchymal stem cells. These hydrogels, implanted into rat osteochondral bone defects, allowed for immediate cell infiltration, cartilaginous tissue formation and chondrogenic differentiation, as indicated by a higher cartilage specific gene expression. Furthermore, the addition of chondroitin sulphate increased type II collagen deposition compared to PVA fibres alone [74]. Filovà et al. prepared a PVA/liposomes blend that was electrospun and finally incorporated into a fibrin/type I collagen/fibrin composite hydrogel. Compressive tests showed the addition of nanofibers improved the mechanical properties of the composite gel as predicted. Moreover, once implanted into osteochondral defects of miniature pigs, the composite scaffold had better osteochondral regeneration towards hyaline cartilage and/or fibrocartilage compared with the controls that were mainly filled with fibrous tissue [84].

9.5 3D Printing

Three-dimensional (3D) printing refers to manufacturing techniques in which 3D models are built in a computer-controlled layer-by-layer process [85]. It should be clarified that it is common to use the term “3D printing” in literature and mainstream media when referring to all Rapid Prototyping (RP) techniques; however, 3D printing also refers to a particular RP inkjet-based technology. The main advantage of 3D printing techniques in tissue engineering relies on the possibility of generating 3D scaffolds with a precise control over the internal architecture [86,87]. Furthermore, fabrication of scaffolds with
a complex, subject-specific geometry by modelling data acquired using different imaging
techniques such as magnetic resonance imaging (MRI) or computed tomography (CT) scans
可以 be achieved [88]. Around 20 different 3D printing techniques have been applied
to the biomedical field [86]; however, not all of them are compatible for the processing
of hydrogels. For an extensive review in the hydrogel-rapid prototyping for tissue
engineering the reader can refer to a recent publication by Billiet et al. [89].

Briefly, 3D printing of hydrogels can be divided in three main methods: laser-based, nozzle-
based, and inkjet printer-based systems, depending on the stimuli employed to assist the
printing process and deposition of the material. Despite the differences in material
deposition mechanism employed in the different techniques, the typical apparatus includes
a hydrogel reservoir from which the material is transferred in a controlled manner to an
ejection system and a collection platform. Nozzle- and inkjet printers sequentially deposit
material, while laser-based or laser-assisted systems are based on photopolymerization of
the pre-deposited material irradiated by light energy in specific predefined patterns
[90,91,92]. Nozzle-based or extrusion systems rely on a 3D
dispensing process in which the hydrogel is extruded through a nozzle driven by
compressed air or a piston/rotating screw [93,94,95]. Electrical signals are used to control
the ejection of individual droplets and/or direction of a sequence of droplets [96,97].

These conventional 3D printing techniques are used to print cell-free 3D scaffolds for
use in surgery [98]. In recent years, 3D bioprinting has gained popularity in tissue
engineering to allow the direct one-step fabrication of 3D scaffolds containing biomaterials,
cells and other biochemicals in the same structure. The working principles of 3D bioprinting
are similar to conventional 3D printing techniques (Figure 9.7). The main
difference lies on the deposition of the hydrogels together with small units of cells. For a thorough review on the basic principles of bioprinting, readers can refer to Mandrycky et al. [99].

**Figure 9.7.** Illustration of 3D bioprinting technologies based on the mechanism used to assist the deposition of the bioinks and its main components; (Left) Inkjet bioprinters eject small droplets of cells and hydrogel sequentially to build up the scaffold; (Middle) Laser bioprinters use a laser to generate the local ejection of small droplets from a donor ribbon coated with the bioink; (Right) Extrusion bioprinters uses pneumatic of mechanical forces to continuously extrude the bioink through a nozzle. Reproduced from [100] with permission. Copyright (2015) Wiley-VCH.

Hydrogel inks used in 3D printing fabrication methods can be formulated from injectable, shear-thinning hydrogels [101], as they are required to flow under low pressures, gel quickly, and maintain their shape after build up [102]. When the hydrogel inks contain cells and/or biochemicals for the use in bioprinting, they are referred as bioinks [103]. The design of hydrogel inks for 3D printing starts with the formulation of a polymer solution that forms a connected network soon after printing. The printed network can be physically or chemically cross-linked as a response to external stimuli (e.g., temperature, light, and ion concentration) [104]. The development of hydrogel inks suitable for bioprinting (both fabrication and cell culture), remains a challenge [105]. Whereas stiff hydrogels containing high concentration of polymer are needed for optimal shape fidelity, these highly dense
networks limit cell migration, growth and differentiation \([106,107]\). Conversely, cells grow better in soft hydrogels, which are too watery to maintain the desired shape (Figure 9.8). Maintaining shape conformity may compromise the biological competence and the clinical outcomes of the printed structures. Therefore, despite the advances in 3D printing technologies that allow researchers to design and fabricate complex structures, the lack of suitable bioinks for tissue engineering is restricting the progress in the field and its translation to clinical practice. Initially, 3D printing technologies focused on the use of pure polymers; however, as the technology advances, the development of novel composite-hydrogels for 3D printing are becoming increasingly popular, aiming at enhancing properties such as printability, mechanics and bioactivity \([108,109,110,111]\).

Figure 9.8. Schematic of the challenges for engineering bioinks suitable for 3D printing. Optimal shape fidelity can be typically achieved with stiff hydrogels (top right), however, this dense network limits cell viability. Contrarily, cell survive best in soft hydrogels, but shape fidelity cannot be achieved (bottom left). Therefore, a compromise between biological and fabrication properties must be done (middle). Novel strategies aimed at obtaining high shape fidelity with cytocompatible hydrogels. Reproduced from \([105]\) with permission. Copyright (2009) Wiley-VCH.
9.5.1 3D Printing of Hydrogels for Cartilage Tissue Engineering

Current scaffolds of 3D printing of hydrogels for CTE are mainly based on two different approaches: direct printing from hydrogels and hybrid printing from composite-hydrogels [112]. The advantage of using bioinks composed of a unique hydrogel is based on their simpler printability process compared to hybrid bioprinting and their physiological crosslinking conditions. Once again, when a high-level of printability is needed from the bioink, the mechanical properties of the 3D scaffolds are commonly weak [113], but the use of composite-hydrogels or the combination of a polymer network with bioinks can offer enough mechanical performance to support the 3D structure, although it may reduce the bioactivity.

Both natural and synthetic hydrogels have been used for CTE applications using 3D printing, where stem cells and chondrocytes are among the most common cell sources used in cartilage bioprinting [114]. Alginate has been extensively used as a bioink due to its rapid crosslinking. You et al. [113] successfully printed a porous cell-laden hydrogel scaffold using sodium alginate impregnated with ATDC5 chondrogenic cell lines or primary chick chondrocytes as a bioink. The resulting scaffolds supported cell survival (85% cell viability), proliferation and ECM deposition of chondrogenic cells in vitro, however the compressive modulus was considerably low (20–70 kPa) compared to human cartilage (700–800 kPa). The compressive modulus was enhanced (75–250 kPa) in Markstedt et al. [115] by combining cellulose nanofibers with alginate. As a result, the printed complex scaffolds supported the culture of human chondrocytes (73–86% viability after one and seven days) as shown in Figure 9.9. The same bioink formulation (nanofibrillated cellulose/alginate) in combination with human chondrocytes and MSCs was found to
promote in vivo chondrogenesis after subcutaneous implantation of the printed constructs in mice [116, 117], suggesting the potential of 3D bioprinting of human cartilage for clinical applications. On the other hand, cell viability (80–96%) and cartilage ECM deposition was improved by Kesti et al. [118] by developing a cartilage-specific bioink based on a blend of gellan and alginate and incorporating cartilage ECM particles and seeded with bovine articular chondrocytes. 3D scaffolds were successfully printed with good mechanical properties (tensile modulus: 116–230 kPa) and complex shapes (i.e., meniscus, intervertebral disks and nose).

Figure 9.9. 3D printed constructs made of a composite hydrogel (alginate + nanofibrillated cellulose) seeded with human chondrocytes. (A) 3D printed small grids (7.2 × 7.2 mm²). Deformed grid during (B), and after
Hyaluronic acid (HA) is gaining popularity as a bioink for CTE because of its viscoelastic and bioactive properties [119]. However, HA on its own has poor mechanical properties and it is therefore necessary to add other materials to improve printability and performance. Muller et al. [120] and Pescosolido et al. [121] demonstrated increased viability of chondrocyte cells by adding acrylated Pluronic and a dextran derivate to HA.

Gelatin has also shown excellent biocompatibility, but due to its low viscosity it is hard to print [122]. Therefore, gelatin is usually modified with an acrylate or methacrylate agent [123,124]. Gelatin-methacrylamide hydrogels (GelMA) have been extensively used to produce bioinks for CTE [125,126,127]. Schuurman et al. [127] demonstrated that introducing HA increased the printability and bioactivity of the 3D printed scaffolds, with high chondrocyte cells viability (82% after 3 days). Costantini et al. [126] showed that incorporating HA-methacrylate also enhanced the mechanical properties (compressive modulus ranging from 48 kPa for GelMA to 100 kPa for the composite bioink). On the other hand, Levato et al. [125] investigated different cell-sources to impregnate the GelMA-based hydrogels. They concluded that the use of articular cartilage-resident chondroprogenitor cells (ACPCs) as an alternative or in combination with chondrocytes and mesenchymal stromal cells (MSCs) supported the formation of 3D cartilage scaffolds in vitro.

Poly(ethylene-glycol) (PEG) is one of the most common synthetic hydrogels used for 3D printing in CTE, showing higher mechanical properties compared to natural hydrogels. Cui
et al. [128] successfully 3D printed PEG-dimethacrylate (PEGDMA) with human chondrocytes reaching a compressive modulus of 396 kPa and high cell viability (89%). In addition, Gao et al. [129] combined PEG with GelMA and both hydrogels were printed together with human MSCs, demonstrating an improvement of the mechanical properties after chondrogenic differentiation.

The relative weak mechanical properties of all the above-mentioned hydrogel constructs considerably limit their application. To overcome this problem, alternating the printing of bioinks and thermoplastic polymers fibres is becoming more popular in CTE [130]. 3D printed scaffolds have been created by combining the deposition of a stiff polymer (polycaprolactone, PCL) and cell-laden hydrogel (alginate) [130,131,132], with chondrocyte cell viability varying from 70% to 85%. In vivo studies by Kundu et al. [131] showed enhanced cartilage ECM deposition by addition of transforming growth factor-β (TGFβ). Furthermore, Schuurman et al. [130] reported a compressive modulus of 6000 kPa of the printed hydrogels constructs.

9.5.2 3D Printing of Hydrogels for Bone Tissue Engineering

Natural biopolymer hydrogels are excellent bioinks for 3D printing, due to their easily adjustable materials characteristics such as viscosity or gelation kinetics as well as their capacity to provide biocompatibility resulting in a consistency similar to the soft tissue matrix [133], however their weak mechanical properties limits the support of osteogenic differentiation and therefore their use for BTE [134,135]. Adjusting the scaffold composition is essential for the fabrication of bone tissue constructs. Composite hydrogel-based materials consisting of an hydrogel phase mimicking the organic part of the bone
(mainly collagen type I) and a mineral phase representing the mineral content of bone (mainly hydroxyapatite) [136,137,138] can enhance the mechanical properties of the 3D scaffolds and their regenerative potential [139,140]. Natural (e.g., collagen, alginate, chitosan, HA, gelatin, and agarose) and synthetic (PEG) hydrogels have been used as bioinks for BTE applications, with the addition or not of inorganic particles (e.g., HAp).

HA is abundantly present in bone ECM, where it gives mechanical support. When it is modified with methacrylate groups, resulting in methacrylated HA (MeHA), printability and rigidity improve while maintaining good biocompatibility [141]. Poldevaart et al. [141] showed that human bone marrow derived MSCs survival (64% after 21 days) and osteogenic differentiation, measured by quantification of calcium deposition, were successfully achieved in 3D printed MeHA scaffolds; however, the elastic modulus of the hydrogel was very low (10.6 kPa) compared to bone tissue. MSC osteogenic differentiation and mechanical properties were enhanced [142] by combining agarose hydrogels with collagen type I. Different combinations of agarose-collagen were tested showing high cell viability (over 98%) and a compressive modulus ranging from 18 to 89 kPa. The less stiff hydrogel exhibited a higher MSC osteogenic differentiation in vitro. In vivo bone matrix formation was observed [143] after implantation of 3D printed alginate-gelatin scaffolds seeded with human adipose-derived stem cells (hASCs). Despite the good biocompatibility and osteogenic differentiation, using 3D printed hydrogel scaffolds without the addition of any inorganic particle notably affects the mechanical properties.

Early attempts to enhance the performance of the printed hydrogels by incorporating HAp particles by Ang et al. [144] used a chitosan-HAp composite and showing cell biocompatibility. However, osteogenesis and mechanics were not evaluated, and cells
were not printed together with the hydrogels. More recently, 3D printed cell-laden scaffolds using different hydrogel formulations (e.g., chitosan, alginate, and gelatin) with HAp particles have been studied \[145,146\]. The incorporation of HAp particles significantly improved the mechanical strength of the hydrogels and promoted osteogenic differentiation in vivo \[140,145,146,147\], making them suitable for repairing bone tissue defects (Figure 9.10).

![Figure 9.10](image)

**Figure 9.10.** 3D bio-printed constructs made of alginate/gelatin (AG) and alginate/gelatin/nano-HAp (AGH) mixed with human adipose-derived stem cells (hASCs) before and after implantation showing osteoinduction. Constructs were implanted into the back sub-cutaneous area of nude mice and harvested eight weeks after surgery. Larger bone formation was observed in the constructs containing HAp. Reproduced from \[140\] with permission, Copyright (2016) Royal Society of Chemistry.
Although hydrogels and composite-hydrogels have shown to be suitable for application in low load-bearing bone defects, adequate mechanical properties are still needed. In this perspective, hybrid scaffolds combining synthetic polymer scaffolds and cell-laden hydrogels are a promising area to explore. PCL is commonly used for bone scaffolds due to its high mechanical strength [98], but it presents limited cell affinity [148]. To improve cell proliferation and enhance the osteogenesis of PCL 3D printed scaffolds, Dong et al. [149] integrated them with bone marrow MSCs-laden chitosan hydrogel, achieving a compressive strength of the hybrid scaffolds of about 6.7 MPa (similar to trabecular bone). Osteogenesis was enhanced by incorporating chitosan into the PCL scaffolds and in vivo bone formation was found after implantation of the seeded scaffolds in a mice model [149]. Vascularization of 3D printed bone constructs was studied by Kang et al. [150] and Kuss et al. [151]. Cell laden hydrogels were hybrid bioprinted together with a PCL frame. Bone and vessel formation was observed in vitro and in vivo, resulting in promising results for the regeneration of bone defects.

Works involving the use of 3D printing in the fabrication of scaffolds for bone and cartilage regeneration are summarised in Table 9.2 and Table 9.3. Even if these are not exhaustive lists of publications, they highlight the progress done on the front of providing viable cells for implantation. However, in vivo work is so far limited and needs to be explored more widely to bring these strategies closer to clinical translation.

Table 9.2 Overview of publication on 3D printing of hydrogels for cartilage tissue engineering.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Cell source(s)</th>
<th>Printing method</th>
<th>Mechanical properties</th>
<th>Cytocompatibility</th>
<th>Reference</th>
</tr>
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</table>

274
<table>
<thead>
<tr>
<th>Material</th>
<th>Cell Type/Line</th>
<th>Bioprinting Method</th>
<th>Mechanical Property</th>
<th>Cell Viability</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>Sodium alginate</td>
<td>ATDC5 chondrogenic cell line</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 20 – 70 kPa</td>
<td>~ 85% cell viability</td>
<td>Cartilage ECM deposition</td>
</tr>
<tr>
<td>Alginates with cellulose nanofibers</td>
<td>Human nasoseptal chondrocyte</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 75 – 250 kPa</td>
<td>73-86% cell viability</td>
<td></td>
</tr>
<tr>
<td>Alginates with cellulose nanofibers</td>
<td>Human nasoseptal chondrocyte</td>
<td>Extrusion bioprinting</td>
<td>Compressive modulus: 15 – 88 kPa</td>
<td>Cartilage ECM deposition</td>
<td></td>
</tr>
<tr>
<td>Alginates with cellulose nanofibers</td>
<td>Human nasoseptal chondrocytes and MSCs</td>
<td>Extrusion bioprinting</td>
<td>Compressive modulus: 80-96% cell viability</td>
<td>Chondrogenic differentiation Chondrocytes proliferation</td>
<td></td>
</tr>
<tr>
<td>Alginates with gellan</td>
<td>Bovine articular chondrocytes</td>
<td>Extrusion bioprinting</td>
<td>Tensile modulus: 116-230 kPa</td>
<td>80-96% cell viability</td>
<td>Cartilage ECM deposition Chondrocytes proliferation</td>
</tr>
<tr>
<td>Methacrylate d HA with diacrylated Pluronic</td>
<td>Bovine articular chondrocytes</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 1.5 – 6.5 kPa</td>
<td>62-86% cell viability</td>
<td></td>
</tr>
<tr>
<td>HA with dextran derived</td>
<td>Equine articular chondrocytes</td>
<td>Extrusion bioprinting</td>
<td>Ultimate compressive stress: 100-160 kPa</td>
<td>&gt;75% cell viability</td>
<td></td>
</tr>
<tr>
<td>GelMA with HA</td>
<td>Equine articular chondrocytes</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 5 – 180 kPa</td>
<td>&gt;73% cell viability</td>
<td></td>
</tr>
<tr>
<td>GelMA with HA- methacrylate</td>
<td>Human bone marrow MSCs</td>
<td>Extrusion bioprinting</td>
<td>Compressive modulus: 48 – 100 kPa</td>
<td>85-95% cell viability</td>
<td></td>
</tr>
<tr>
<td>GelMA</td>
<td>Equine ACPCs/Chondrocytes/MSCs</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 100 – 187 kPa</td>
<td>&gt;75% cell viability</td>
<td></td>
</tr>
<tr>
<td>PEGDMA</td>
<td>Human articular chondrocytes</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: ~ 400 kPa</td>
<td>89% cell viability</td>
<td>Cartilage ECM deposition</td>
</tr>
<tr>
<td>PEG-GelMA</td>
<td>Human MSCs</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 1 MPa</td>
<td>~ 80% cell viability</td>
<td>Cartilage ECM deposition Chondrogenic differentiation</td>
</tr>
<tr>
<td>Embryonic chick chondrocytes</td>
<td>Inkjet bioprinting</td>
<td>Not reported</td>
<td>Cartilage ECM deposition</td>
<td>77-85% cell viability</td>
<td></td>
</tr>
</tbody>
</table>
Alginate reinforced with PCL

<table>
<thead>
<tr>
<th>Materials</th>
<th>Cell source(s)</th>
<th>Printing method</th>
<th>Mechanical properties</th>
<th>Cytocompatibility</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeHA</td>
<td>Human BM MSCs</td>
<td>Inkjet bioprinting</td>
<td>Elastic modulus: ~11 kPa</td>
<td>~65% cell viability, Osteogenic differentiation</td>
<td>[141]</td>
</tr>
<tr>
<td>Agarose with collagen type I</td>
<td>Human BM MSCs</td>
<td>Inkjet bioprinting</td>
<td>Compressive modulus: 18-90 kPa</td>
<td>~95% cell viability, Osteogenic differentiation</td>
<td>[142]</td>
</tr>
<tr>
<td>Alginate-gelatin</td>
<td>hASCs</td>
<td>Inkjet bioprinting</td>
<td>Not reported</td>
<td>Osteogenic differentiation</td>
<td>[143]</td>
</tr>
<tr>
<td>Chitosan-HAp</td>
<td>Human osteoblasts</td>
<td>Extrusion bioprinting</td>
<td>Not reported</td>
<td>Good cell attachment and proliferation</td>
<td>[144]</td>
</tr>
</tbody>
</table>

Table 9.3 Overview of publications on 3D printing of hydrogels for bone tissue engineering.
<table>
<thead>
<tr>
<th></th>
<th>Cells/Cells Source</th>
<th>Printing Method</th>
<th>Elastic Modulus/Compressive Modulus</th>
<th>Cell Viability/Cell Viability</th>
<th>Osteogenic Differentiation</th>
<th>Bone Matrix Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan-Hap/Alginate-Hap</td>
<td>MC3T3-E1 cells</td>
<td>Inkjet bioprinting</td>
<td>Elastic modulus: 4.6-15 kPa/3.5-19 kPa</td>
<td>&gt; 90% cell viability</td>
<td>Cell proliferation</td>
<td>Bone matrix formation</td>
</tr>
<tr>
<td>MeHA with Hap or GelMA with Hap</td>
<td>hASCs</td>
<td>Extrusion bioprinting</td>
<td>Not reported</td>
<td>Osteogenic differentiation</td>
<td>Bone matrix formation</td>
<td></td>
</tr>
<tr>
<td>Alginate-gelatin/alginate-gelatin with nano-Hap</td>
<td>hASCs</td>
<td>Extrusion bioprinting</td>
<td>Not reported</td>
<td>&gt; 88% cell viability</td>
<td>Osteogenic differentiation</td>
<td></td>
</tr>
<tr>
<td>Alginate-PVA with Hap</td>
<td>MC3T3-E1 cells</td>
<td>Extrusion bioprinting</td>
<td>Compressive modulus: 2.4-10.3 kPa</td>
<td>77-95% cell viability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan reinforced with PCL</td>
<td>Rabbit BM MSCs</td>
<td>Extrusion bioprinting</td>
<td>Compressive strength: 6.7 MPa</td>
<td>Osteogenic differentiation</td>
<td>Bone matrix formation</td>
<td></td>
</tr>
<tr>
<td>HA and Gelatin reinforced with PCL/TCP</td>
<td>Human amniotic fluid-SCs</td>
<td>Extrusion bioprinting</td>
<td>Not reported</td>
<td>91% cell viability</td>
<td>Osteogenic differentiation</td>
<td>Bone matrix formation</td>
</tr>
<tr>
<td>MeHA and GelMA reinforced with PCL/HAp</td>
<td>Stromal vascular fraction derived cells</td>
<td>Extrusion bioprinting</td>
<td>Not reported</td>
<td>Osteogenic differentiation</td>
<td></td>
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</table>
3D printing of hydrogels has shown great potential for the production of customized scaffolds in cartilage and bone tissue engineering, as previously described. Because of its ability to fabricate 3D constructs with complex shapes by depositing cell laden hydrogels at desired locations, 3D printing also results in a promising technique for the fabrication of gradient scaffolds with hydrogels stacked in a multilayer manner [152]. This unique capability enables to expand the use of 3D printing to the efficient regeneration of osteochondral tissue, providing a scaffold that favours integration between the chondral and the osseous phases for osteochondral defects repair. 3D printed constructs for osteochondral tissue regeneration are usually built in a bilayer fashion, by employing different bioink formulations for the subchondral bone and the cartilage zone. Early attempts were done by Fedorovick et al. [153] encapsulating human chondrocytes and osteogenic progenitors in alginate hydrogel, biofabricating 3D scaffolds with different parts for both cell types. Distinctive tissue formation at defined locations was observed both in vitro and in vivo, however, the scaffolds presented low mechanical strength (Young’s modulus < 7.6 kPa) and a limited height of the construct could be achieved. Anatomically relevant size bilayered scaffolds were fabricated by Levato et al. [154] combining two different bioinks: GelMA-Gellan Gum with PLA microcarriers (MCs) seeded with MSCs for the bone and without MCs for the cartilage layer. The MC laden region improved the compressive modulus (25–50 kPa) of the hydrogel constructs and supported osteogenic differentiation and bone matrix deposition by MSCs, suggesting the potential of the use of MCs-based biofabrication for osteochondral tissue engineering. An efficient osteochondral gradient scaffolds was fabricated using a novel laser-based 3D printer by Castro et al. [155] and human MSCs osteogenic and chondrogenic differentiation was
enhanced through the incorporation of tissue specific nano-HAp with different concentrations of PEG-diacylate (PEG-Da) for the porous osseous layer and the transitional calcified cartilage layer, and TGF-β1 added to the PEG-Da for the solid cartilage layer. It is known that hydrogels derived from natural ECM can enhance tissue regeneration by providing biochemical signals inducing cellular differentiation and migration [156,157]. In this sense, HA and collagen type I (Col-I), which are the major organic ECM components of cartilage and bone, respectively, were combined in a 3D printed scaffold fabricated by Park et al. [158]. A bilayer construct was 3D printed encapsulating chondrocytes in a HA hydrogel for the cartilage zone and osteoblast in a Col-I hydrogel for the bone area within a PCL printed framework. Viability and function of each cell type were well maintained up to 14 days in vitro. A validation of the potential of a 3D printed bilayer construct for osteochondral tissue regeneration using an in vivo animal model was reported by Shim et al. [159]. A subchondral bone layer was fabricated by dispensing a solution of atelocollagen with human turbinate-derived MSCs onto a PCL framework, whereas a solution of cucurbit [6] uril-HA and 1,6-diaminohexane-conjugated HA was dispensed into the PCL matrix for the superficial cartilage layer. 3D printed scaffolds were implanted in the knee joints of rabbits. Neo-cartilage was observed in the cartilage region and new bone formation in the subchondral bone region at eight weeks post implantation.

9.6 Future Trends

Electrospinning and 3D printing both have a great potential in the fabrication of complex structures such as those required for tissue engineering of bone, cartilage and
osteochondral tissue. Combining the two could also overcome some of the limitations of the individual methods such as the tight intertwining of electrospun fibres that limits cell migration and the limited resolution of some 3D prototyping methods. The first reports of materials obtained by combining these two fabrication methods have only emerged in recent years [160]. More specific reports on their combined use in the manufacturing of tissue engineering scaffolds for bone, cartilage and osteochondral tissues are still limited. Yu et al. prepared PCL printed meshes that were infused with homogenised electrospun PCL/gelatine fibres crosslinked with glutaraldehyde [161]. The work showed that combination of the mechanically competent 3D printed mesh with the biocompatible nanofibers can be exploited to obtain the ratio of porosity and the pore size that is most advantageous for cell migration and proliferation. Naghieh et al. fabricated scaffolds with alternating layers of 3D printed PLA mesh and gelatin/forsterite electrospun fibres [162]. These hierarchical structures presented appropriate mechanical behaviour for applications in bone tissue engineering with additional bioactive properties. These first reports demonstrate the potential of using a combinatorial approach, where 3D printing and electrospinning can afford additive advantages to the use of a single technique. The translational potential of this strategy must be now explored.

9.7 Conclusions

Hydrogels and their combination with other biomaterials are very attractive in tissue engineering applications targeting both soft and hard tissue replacement, with specific relevance to orthopaedics. To achieve complex hydrogel constructs with the necessary requirements to substitute the specific tissue and help regeneration, two manufacturing
techniques are currently considered the most promising in the field: electrospinning and 3D printing. This review considers the most recent advances of hydrogel-based scaffold production using such techniques applied to cartilage and bone tissue engineering. As a result, a variety of materials can be used to fine-tune composite implants to achieve the desired mechanical and biological properties. However, as it stands, both electrospinning and 3D printing of hydrogel composites is still limited by the inability of selectively assign materials to reproduce tissue geometry and properties with required resolution and further work is needed in this sense, particularly to target tissue transition at the interface between bone and cartilage.

Acknowledgments

The authors thank the University of Portsmouth Research and Innovation Development Fund, the Institute of Biology and Biomedical Science (IBBS) and the Faculty of Technology at University of Portsmouth for financial support.

Conflicts of Interest

The authors declare that there is no conflict of interest.

9.8 References


73. Mohabatpour, F.; Karkhaneh, A.; Sharifi, A.M. A hydrogel/fiber composite scaffold for chondrocyte encapsulation in cartilage tissue regeneration. *RSC Adv.* 2016, 6, 83135–83145. [Google Scholar] [CrossRef]


82. Teo, W.-E.; Gopal, R.; Ramaseshan, R.; Fujihara, K.; Ramakrishna, S. A dynamic liquid support system for continuous electrospun yarn fabrication. *Polymer (Guildford)* 2007, 48, 3400–3405. [Google Scholar] [CrossRef]


98. Chia, H.N.; Wu, B.M. Recent advances in 3D printing of biomaterials. J. Biol. Eng. 2015, 9, 4. [Google Scholar] [CrossRef] [PubMed]


102. Stanton, M.M.; Samitier, J.; Sanchez, S. Bioprinting of 3D hydrogels. Lab Chip 2015, 15, 3111–3115. [Google Scholar] [CrossRef] [PubMed]


109. Osterbur, L.W. 3D Printing of Hyaluronic Acid Scaffolds for Tissue Engineering Applications; University of Illinois at Urbana-Champaign: Champaign, IL, USA, 2013. [Google Scholar]


120. Müller, M.; Becher, J.; Schnabelrauch, M.; Zenobi-Wong, M. Nanostructured Pluronic hydrogels as bioinks for 3D bioprinting. Biofabrication 2015, 7, 35006. [Google Scholar] [CrossRef] [PubMed]


130. Schuurman, W.; Khristov, V.; Pot, M.W.; van Weeren, P.R.; Dhert, W.J.; Malda, J. Bioprinting of hybrid tissue constructs with tailorable mechanical properties. *Biofabrication* 2011, 3, 21001. [Google Scholar] [CrossRef] [PubMed]


140. Wang, X.-F.; Lu, P.-J.; Song, Y.; Sun, Y.-C.; Wang, Y.-G.; Wang, Y. Nano hydroxyapatite particles promote osteogenesis in a three-dimensional bio-printing construct consisting of alginate/gelatin/hASCs. *RSC Adv.* **2016**, *6*, 6832–6842. [Google Scholar] [CrossRef]


142. Duarte Campos, D.F.; Blaeser, A.; Buellesbach, K.; Sen, K.S.; Xun, W.; Tillmann, W.; Fischer, H. Bioprinting Organotypic Hydrogels with Improved Mesenchymal Stem Cell Remodeling and


162. Naghieh, S.; Foroozmehr, E.; Badrossamay, M.; Kharaziha, M. Combinational processing of 3D printing and electrospinning of hierarchical poly(lactic acid)/gelatin-forsterite scaffolds as a
Appendix III: UPR16

**FORM UPR16**

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information).

<table>
<thead>
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<th>Postgraduate Research Student (PGRS) Information</th>
<th>Student ID: 792644</th>
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<td><strong>PGRS Name:</strong> Arianna De Mori</td>
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<tr>
<td><strong>Department:</strong> PHBM</td>
<td></td>
</tr>
<tr>
<td><strong>First Supervisor:</strong> MARTA ROLDO</td>
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<td><strong>Start Date:</strong></td>
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<td><strong>Study Mode and Route:</strong></td>
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If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study.

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

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| b) Have all contributions to knowledge been acknowledged? YES NO |
| c) Have you complied with all agreements relating to intellectual property, publication and authorship? YES NO |
| d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration? YES NO |
| e) Does your research comply with all legal, ethical, and contractual requirements? YES NO |

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I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s).

**Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):** ETHIC-2018-1038

If you have not submitted your work for ethical review, and/or you have answered ‘No’ to one or more of questions a) to e), please explain below why this is so:

Signed (PGRS): [Signature]  
Date: 02/10/2019

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