Functionalised dextran nanoparticles for drug delivery to the brain

IBEGBU, Madu Daniel

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Biomaterials and Drug Delivery Research Group
School of Pharmacy and Biomedical Sciences
University of Portsmouth
Abstract

Towards the development of drug carriers that are capable of crossing the Blood Brain Barrier, the techniques of emulsion polymerisation and nanoprecipitation have been utilised to produce nanoparticulate carriers from a systematic series of alkylglyceryl dextran (of two different average molecular weights, 6 kDa and 100 kDa) that had been functionalised with ethyl and butyl cyanoacrylates. Also, zero length grafting of polylactic acid to butyl, octyl and hexadecylglyceryl dextran has allowed the preparation of polylactic acid-functionalised nanoparticles. All materials and derived nanoparticles have been characterised by a combination of spectroscopic and analytical techniques. The average size of nanoparticles has been found to be in the range 100-500 nm. Tagging or loading of the nanoparticles with fluorophores or model drugs allowed the preliminary investigation of their capability to act as controlled-release devices. The effects of an esterase on the degradation of one such nanoparticulate carrier have been studied. Testing against bend3 cells revealed that all materials display dose-dependent cytotoxicity profiles, and allowed the selection of nanocarriers that may be potentially useful for further testing as therapeutic delivery vehicles for conditions of the brain.
Dedication

To God Almighty
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Declaration

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Abbreviation list

ACA= Alkyl cyanoacrylates
AFM= Atomic force microscopy
BBB= Blood-brain barrier
BCA= n-butyl cyanoacrylate
CNS= Central nervous system
DCM= Dichloromethane
Dex= Dextran
Dex6= Dextran 6 kDa
Dex100= Dextran 100 kDa
Dex6G4 = Dextran 6 kDa modified with butyl glycidyl ether
Dex6G8 = Dextran 6 kDa modified with octyl glycidyl ether
Dex6G12= Dextran 6 kDa modified with glycidyl lauryl ether
Dex6G14= Dextran 6 kDa modified with dodecyl/tetradecyl glycidyl ether
Dex6G16= Dextran 6 kDa modified with hexadecyl glycidyl ether
Dex100G4 = Dextran 100 kDa modified with butyl glycidyl ether
Dex100G8 = Dextran 100 kDa modified with octyl glycidyl ether
Dex100G12= Dextran 100 kDa modified with glycidyl lauryl ether
Dex100G14 = Dextran 100 kDa modified with dodecyl/tetradecyl glycidyl ether
Dex100G16 = Dextran 100 kDa modified with hexadecyl glycidyl ether
DLS= Dynamic light scattering
DMEM= Dulbecco's Modified Eagle Medium
DMSO= Dimethyl sulfoxide
DS= Degree of substitution
DSC= Differential scanning calorimetry
ECA= ethyl 2-cyanoacrylate
GPC= Gel permeation chromatography.
ICS= Cerebrospinal fluid
ISF= Interstitial fluid
M/Z= mass-to-charge ratio
MALDI/TOF MS = Matrix assisted laser desorption/ionisation time of flight mass spectroscopy
Abbreviations

MIA = N-Methylisatoic anhydride
MRP = Multi Drug resistance protein
MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NPs = Nanoparticles
NTA = Nanoparticle Tracking Analysis
OGE = Octyl glycidyl ether
PACA = Poly (alkyl cyanoacrylates)
PECA-Dex100G4(1:1) = prepared with equal ratio of ECA monomer to modified dextran
PECA-Dex100G16(1:6) = prepared with 6:1 ratio of ECA monomer to modified dextran
PBCA = Poly (butyl cyanoacrylate)
PDI = Polydispersity index
PECA = Poly (ethyl 2-cyanoacrylate)
PES = Polyethersulphone
PLGA = Poly(lactic-co-glycolic acid)
PLA = Poly (lactic acid)
SEM = Scanning electron microscope
t-BuOK = Potassium tert-Butoxide
TEM = Transmission electron microscope
TGA = Thermal gravimetric analysis
ZP = Zeta potential
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1. Introduction and aims

1.1 Drug delivery to the brain

In 2010 it was estimated that 38% of the European population suffered from a brain disorder as compared with 27% in 2005 (1). These disorders include the neurodegenerative diseases (dementia, epilepsy, multiple sclerosis) and mental disorders (depression, schizophrenia, panic disorder, drug dependence and insomnia). Epidemiological studies have shown that about one third of the world population may suffer from some mental disorder at some stage in their lives (2). Schizophrenia, depression, epilepsy, dementia, alcohol dependence and other mental, neurological and substance-use (MNS) disorders constitute 13% of the global burden of disease, surpassing both cardiovascular disease and cancer (3). A recent review has identified several CNS (central nervous system) diseases that may be treatable with biological actives if appropriate therapeutic delivery systems were to be developed (4).

The human brain (Figure 1.1) is irrigated by capillaries the total length of which is estimated at ca. 644 km; corresponding to surface area available for transport of about 20 m² (6), and a thickness of the cerebral endothelial membrane of the order of 0.2-0.3 µm.

Figure 1.1: The structure of the brain (5)
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One of the major inhibiting factors to the efficient treatment of brain disorders is the lack of universally applicable methods for transporting therapeutic agents across the blood-brain barrier (BBB), which has evolved to separate circulating blood from the brain extracellular fluid (BECF) in the central nervous system (CNS) such as to prevent potential neurotoxins from reaching the brain (7).

The BBB, which is formed by capillary endothelial cells (ECs) that are connected by tight junctions with an extremely high electrical resistivity (> 0.1 Ω m), allows the passive diffusion of some gases and that of water and of some lipid soluble molecules, and also the selective transport of certain molecules (glucose, amino acids) that are crucial to neural function. Consequently, therapeutic approaches to circumvent the BBB without altering its integrity are an area of intense activity in drug research and development.

1.1.1 The blood-brain barrier

The acknowledgement of the existence of the BBB is consequent to the work of Ehrlich, Lewandowsky and Goldmann, in the late 1800s and early 1900s, who reported the absence of bile acids, ferrocyanide or trypan blue in the brain and spinal cord following intravenous administration of each of these agents (8-11). The BBB is a highly specialised brain endothelial structure of the fully differentiated neurovascular system (8). It is formed from brain capillary endothelial cells (12) and localized at the level of tight junctions (TJ) between adjacent cells. It has been described as a multicellular vascular structure that isolates the CNS from systemic blood circulation (13), inhibiting the transport into the brain of plasma components, red blood cells and leukocytes. Thus the BBB is crucial for preservation and regulation of the neural microenvironment; the specialised TJs have been described as the core structures responsible for these functions. The endothelial cells of the BBB are characterised by extremely low numbers of transcytotic vesicles and by a restrictive paracellular diffusion barrier (13, 14). The barrier functions by utilising carrier-mediated transport systems to exert a tight control over the movement of nutrient molecules, ions, and oxygen, and to protect the brain from toxins and pathogens (13). The barrier restricts the free flow of hydrophilic compounds, small proteins and charged molecules but relatively small lipophilic molecules (<600 Da) that can form fewer than nine H-bonds are normally capable of permeating the BBB (15, 16). It has been argued that some therapeutic agents may reach the brain if they are capable of
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becoming associated with specific transporters and/or receptors at the luminal side of endothelial cells (9).

The main anatomical structure of the BBB (Figure 1.2) is the cerebral blood vessel formed by the ECs, which is characterised by low concentrations of leukocyte-adhesion molecules and consequently is responsible for the limited immune surveillance inherent to the CNS (17). There is a body of experimental evidence which suggests that attempts to compromise the integrity of the BBB for the purpose of delivering therapeutic agents to the CNS may distort the balance of transport of molecules between the brain and the blood, which in turn may lead to aberrant angiogenesis, vessel regression, brain hypoperfusion, intracerebral haemorrhage, trauma, neurodegenerative processes, inflammatory responses, or vascular disorder; these responses are capable of generating toxic metabolites that could affect synaptic and/or neuronal functions (8, 18, 19, 20).

Figure 1.2: Cellular interplay at the neurovascular unit (capillary level) (13)

The BBB consists of a body of cell types that include pericytes, astrocytes, endothelial cells and microglial cells (21). Brain capillary endothelial cells are characterised by narrow tight junction, by low pinocytic activities and by high metabolic activity. They allow little paracellular and no transcellular transport of high-molecular-weight molecules (22).
Pericytes are important in supporting BBB development since they influence the differentiation and maturation of associated endothelial cells. Their interaction with endothelial cells induces the formation of tight junctions. In addition, they serve as partial foundation for the basement membrane. Also, pericytes play a significant role in cytokines production and in antigens presentation, affect end-foot processes, and support proper neuronal functions (13).

Astrocytes account for about 90% of the overall brain mass, they are involved in regulation of brain homeostasis through K⁺ buffering, in the regulation of neurotransmitter and growth-factor release, and in the regulation of brain immune responses. Astrocytes produce Apolipoprotein E (ApoE), a molecule that has been shown to be beneficial to brain homeostasis (23) and may be important in drug transport through receptor mediated endocytosis (24).

The BBB is part of the Neurovascular Unit (NVU), which presents as an elaborate interplay of central and peripheral cells (13). It creates the extracellular fluid compartment, the content of which is different to that of somatic extracellular fluid since it accommodates both Cerebrospinal (CSF) and Interstitial (ISF) fluids. Also, the BBB acts as neuroprotector in that potentially damaging xenobiotics and metabolites are prevented entry or are removed from the organ through the action of specialised transporters (25).

**Tight junctions**

TJs are located in the apical part of the paracellular space and contain transmembrane proteins (occludin, claudins, and junctional adhesion molecule-1) and cytoplasmic proteins (zonula occludens [ZO]-1, -2, -3 and cingulin) that are bound to the actin cytoskeleton (10).
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TJs are domains of occluded intercellular clefts that are shaped either in grooves (E-face) or in ridges (P-face) (Figure 1.3). The latter face exhibits higher electrical resistance and lower permeability than the former. Particles that associate with tight junctions are found in both faces (14). TJs serve a key function in the regulation of paracellular permeability and in maintaining cell polarity (27). Intermingled with tight junctions are usually found Adherens junctions. The paracellular route of drug delivery occurs via the intercellular space and is mediated by alterations of the tight junction barrier of epithelial cells.

A number of signal pathways have been associated with tight junction regulation, which involves but is not limited to G-proteins, serine, threonine and tyrosine-kinases, extra- and intra-cellular calcium levels, cAMP levels, proteases and cytokines (14).
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Bidirectional transport between brain and systemic circulation

The transcellular bidirectional transport across the BBB has been categorized into carrier-mediated transport, ion transport, active efflux transport, receptor-mediated transport, and caveolae-mediated transport of interstitial fluid to blood (28). The influx transporters (mostly involved in nutrients import) and the efflux transporters (involved in removal of metabolites and neurotoxic compounds from brain) both prevent the entry of xenobiotics to the brain (29). An example is P-glycoprotein (P-gp; a 170 kDa protein member of the ATP-binding cassette transporters), which is located at the luminal membrane of endothelial cells. P-glycoprotein has been suggested to serve as an efflux transporter that functions as a clearance system for metabolites and neurotoxic compounds produced in the brain (28). It has been shown that P-gp is distributed throughout both the luminal and abluminal membranes of the endothelium, and in astrocytes and pericytes, suggesting that the pump may be involved in regulating drug transport processes over the entire CNS and at both the cellular and subcellular levels (30).

The movement of nutrients to the brain (e.g. hexoses, amino acids and monocarboxylic acids, nucleosides, amines and vitamins), which is facilitated by specialised carrier-mediated transporters, is characterised by a concentration gradient of each nutrient as it moves from blood to brain (Figure 1.4). The inflow of nutrients is regulated by the metabolic needs of the brain, but is also affected by the availability of nutrients.

Since the brain depends mainly on glucose for its energy needs, Glucose transporter 1 (GLUT1) is crucial to brain function. The concentration of GLUT1 is higher at the abluminal membrane as compared with that of the luminal membrane (29). Consistent with the need for demand-regulated influx of glucose into the organ, the distribution of the same transporter in the brain is asymmetric. Facilitative amino acid transport systems at the BBB (e.g. L1, y+) provide the brain with all the essential amino acids, while sodium-dependent amino acid transport systems (e.g. A, ASC) allow the movement of non-essential amino acids. Sodium-dependent excitatory amino acid transporters are responsible for the removal from the brain of potentially toxic acidic amino acids. Biotin, pantothenic acid and lipoic acid are transported to the brain by means of sodium-dependent transporter, while others vitamins (B1, B3, B5, and E) are transported by specialised carriers.
Localised at the abluminal side of the brain, the sodium pump serves the sodium-potassium (Na⁺-K⁺, ATPase; Figure 1.4) exchange in the brain. Also, the Na⁺-K⁺-2Cl⁻ co-transporter, which resides predominantly at the luminal side of the brain, facilitates the control of sodium, potassium and chloride ions at the brain endothelium. The intracellular pH of the endothelium is regulated by the co-operative actions of the sodium-hydrogen exchanger and the chloride-bicarbonate exchanger, while calcium efflux is mediated by the sodium-calcium exchanger (8, 31).

Monocarboxylate1 (MCT1) is involved in the regulated transport of ketone molecules to the brain, which serve as energy sources, as do hexoses (8). The efflux of anionic compounds is mediated by multi drug resistance-associated proteins (MRP) transporters, which are known to be involved in the organ-specific efflux of molecules (e.g. breast-cancer-resistance protein, BCRP) and family members of the organic anion transporting polypeptide (OATP) and the organic anion transporter (OAT). These transporters have the capability to work co-operatively to inhibit penetration of many drugs into the brain and to increase their efflux from the brain.
Figure 1.4: A simplified atlas of the BBB (8)
1.1.2 Strategies for delivering drugs to the brain

Several strategies have been proposed towards overcoming the BBB-imposed limitations to the delivery of some drugs and other actives to the brain. These strategies may be categorised into invasive and non-invasive; the former requires specialised expertise, which impacts on costs, while the latter may be readily accessible to patients.

1.1.2.1 Invasive approaches

Due to the limited access of drugs to the brain via the BBB, invasive procedures represent the current method of choice for brain-specific therapeutic delivery. Most of these methods are characterised by intraventricular drug infusion or by disruption of the BBB; procedures that are both time-consuming and highly specialised (32, 33). Implantation of a catheter into the ventricular system for the delivery of drugs directly to brain then bypassing the BBB has been described as the most common invasive procedure for drug delivery to CNS (34).

1.1.2.2 Use of penetration enhancers

Several penetration-enhancing approaches have been adopted towards increased paracellular transport in brain capillaries. These include the administration of osmotic solutions, the use of vasoactive substances, the utilisation of alkylglycerols (35) and the application of physical stimuli (9) to induce an enhancement of drug transport across the BBB (36).

Chemical stimuli and tight-junction modulations have been used for opening the BBB to deliver anti-tumour agents to the brain. Although not without side effects, Mannitol has been claimed to be preferable to Polysorbate 80 and to Bradykinin for this purpose (10). The physical opening of TJs by means of electromagnetic radiation has been documented (37); electromagnetic pulses have also been claimed to affect key TJ-related proteins, including ZO-1, occludin, and claudin-5 (38). In attempts to open TJs, sodium caprate has been utilised as have Claudin modulator (39) and junction Zonula Occludens toxin, Zot (40).

However, disturbance of the BBB as a means for the delivery of therapeutic molecules to the CNS is inhibited by the technological complexity of the approach, by risks of tumour dissemination, neurotoxicity and by inadequate selectivity (10).
1.1.2.3 Prodrugs
The use of prodrugs represents another means of circumventing the BBB in the delivery of actives to the brain (37, 38). This approach involves the chemical transformation of an administered drug to generate an active precursor molecule that is capable of traversing the target biological membrane, as is exemplified by the relationship between heroin and morphine (Figure 1.5): heroin (a diacetyl ester congener of morphine) is capable of penetrating the BBB and subsequently becoming metabolised to produce morphine, which is a molecule that is not capable of penetrating the BBB (41).

![Heroin and Morphine](image)

**Figure 1.5:** Structures of heroin and morphine

Amongst other strategies, the co-administration of drugs with P-gp modulators, the utilisation of drug-loaded biopolymers, the use of drug-transporting peptides and the structural modification of drugs have all been examined as means of overcoming the BBB (33), as has nasal drug delivery (a route that is not impeded by the BBB).

1.1.2.4 Modifications of influx transporters
The formulation of drugs such that they exhibit structural resemblance to a substrate of influx transporters to the brain has been proposed as a strategy towards increased availability of a drug to the brain (28). However, an attempted use of a formulation of a D-glucose-chlorambucil derivative failed to transport this active to the brain consequent to the associated inhibition of the GLUT1 influx pump (42).
Introduction and aims

1.1.2.5 Passive drug targeting
Passive targeting occurs due to extravasation of the nanoparticles at the diseased site where the microvasculature is often described as leaky (43). The molecular design of nanoparticulate therapeutic vehicles is primarily determined by the need to overcome the natural defence mechanisms mononuclear phagocyte system (MPS) that work towards their elimination from circulation (43). Selective accumulation of nanocarriers and drug then occurs by the enhanced permeability and retention (EPR) effect. The EPR effect will be optimal if nanocarriers can evade immune surveillance and circulate for a long period (44).

1.1.2.6 Active drug targeting
It has been suggested that epitopes or receptors that are overexpressed in certain diseased states may be exploited in active drug targeting: if ligands are attached at the surface of the nanocarrier for binding to specified receptors expressed at the target site, targeted receptors may be expressed homogeneously on all targeted cells. Targeting ligands are either monoclonal antibodies (mAbs) and antibody fragments or nonantibody ligands. The binding affinity of the ligands influences the tumour penetration because of the binding-site barrier. The ligand is selected to bind to a receptor that is overexpressed by tumour cells or by tumour vasculature and not expressed by normal cells. The active targeting is particularly attractive for the intracellular delivery of macromolecular drugs, such as DNA, siRNA and proteins (44).

1.1.2.7 Carrier-mediated delivery (Colloidal drug delivery systems)
Commonly used nanocarriers for preclinical and clinical drug delivery studies include liposomes (e.g. lipodox), dendrimers (Figure 1.6) and solid-lipid nanoparticles, since these often combine effective drug delivery with high drug loading capacity (44).
Liposomes and micelles

Although the subject of considerable research efforts, liposome-mediated drug delivery is often limited by: the inherent instability of liposomal dispersions, drug leakage, low activity due to no specific tumour targeting, nonspecific clearance by the mononuclear phagocytic system (MPS) and complications associated with the available large-scale production methods (46). Nonetheless, liposome-loaded Doxorubicin (lipodox) has been shown to be significantly more effective on resistant cell line than free Doxorubicin. Co-administration of Doxorubicin and P-gp inhibitor indicated the capability of the liposomal formulation to inhibit the efflux pump P-gp (47-49). Benefits to drug-delivery applications have been shown by micellar formulations, as is exemplified by the capability of micelles poly caprolactone-b-poly ethylene oxide (PCL-b-PEO) of Doxorubicin to shift the accumulation of this active from the cytoplasm to the nucleus, thereby enhancing efficacy.
Introduction and aims

In terms of structure, liposomes are small vehicles with an aqueous inner core which may be enclosed by unilamellar or multilamellar phospholipid bilayers (50). Polymeric micelles (Figure 1.7) are nanocarriers composed of amphiphilic multi-block copolymers capable of forming a shell structure (50).

![Figure 1.7: Schematic representation of liposomes and micelles (50).](image)

Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are particles made from solid lipids and stabilised by surfactants. The need for the use of SLNs in drug delivery arises from the inability of highly ordered crystal lattices to accommodate large amounts of drug molecules. With reference to parenteral application, SLNs offer physical stability, protection of incorporated labile drugs from degradation, controlled drug release (fast or sustained, depending on the adopted molecular design), good tolerability and the potential for site-specific targeting. However SLN structures often suffer from sub-therapeutic loading capacity, polymorphic transitions during storage that lead to the expulsion of the active and relatively high water content in dispersions (70–99.9%); the removal of excess water from SLN dispersions tends to impact upon particle size. Also, the formulation of SLNs normally necessitates the use of high concentrations of surfactants and co-surfactants (e.g. butanol), which in turn may be undesirable for regulatory purposes. Dependent upon the drug/lipid ratio and solubility, the therapeutic content of SLNs may be preferentially localised at the core of the particles or at the shell or be molecularly dispersed throughout the matrix. The release profile of SLNs may be influenced by
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chemical modifications at the lipid matrix, by the concentration of co-formulated surfactant and by the physical parameters adopted during formulation. For therapeutic applications, SLNs are normally injected (intravenously, intramuscularly, subcutaneously or directly to the target organ). SLN formulations are suitable for therapeutic uses requiring systemic body distribution since their small size minimises the risk of embolism by blood clotting or particle aggregation. If administered subcutaneously or designed to accumulate in the MPS, SLNs offer the opportunity to act as a sustained release depot of the drug which allows the incorporated drug to be released over extended time scales either by the erosion of the SLN matrix (e.g. by means of enzymic degradation) or by diffusion from the particles (46).

Dendrimers

Dendrimer (Figure 1.8) nanocomposites are symmetric hyper-branched, star-shaped structures that are designed such as to produce monodispersed formulations (51). The repetitious nature of chain and branching afford a series of radially concentric layers with increasing crowding, as is exemplified by poly(amidoamine)-based structures. The classification of dendrimers according to generation reflects the exponential increase in the number of branches in each layer: dendrimer growth is typically ca. 1 nm per generation. Since dendrimers are typically symmetric around the core, their extended forms in aqueous media are of spheroidal morphology. The loosely packed core and tightly packed periphery of dendrimers often affords good drug loading capacity (52, 53) but this is counterbalanced by little control over the release mechanism of loaded drugs. Also, because of their branched nature, dendrimers are often amenable to conjugation with pharmaceutically active moieties through functional groups. Notably, studies in cultured cells (colon carcinoma) have shown that Dox-dendrimers are less toxic than free Dox (54).

As compared with linear polymers, dendritic structures have “dendritic voids” that give these molecules important and useful features. Dendrimers with a high surface charge density due to ionisable groups are amenable to non-stoichiometric association with therapeutic molecules through electrostatic interactions. Such dendrimer-drug
interactions may afford to actives enhanced solubility, increased stability and efficient transport through biological membranes (55).

Figure 1.8: The structure of dendrimer(55)

Nanoparticulates

Since colloidal carriers, particularly biodegradable polymeric nanoparticles, are often amenable to structural modifications that may bestow to them the capability to be transportable through the BBB, many researchers regard these structures as promising vehicles for the delivery of drugs to the brain. Many colloid-forming biopolymers (e.g. dextran, PACA, PLA) have been considered for the purpose. The properties of nanoparticles utilised for the \textit{in vivo} transport and enhanced pharmacokinetic profile of therapeutic compounds originate from their nano-scale size which furnishes them with colloidal stability and allows them to penetrate tissues easily through capillaries and epithelial linings. Amenability to functionalisation, both at the surface and at the core of nanoparticles, has provided opportunities for applications in drug delivery and in molecular imaging (56). The performance of drug-loaded nanocarriers for targeting organs or tissues may be fine-tuned by adjusting the balance between particle size, size distribution, surface charge, surface modification and hydrophobicity (57).
Nanoparticulate drug carriers are nanoscaled solid colloidal structures (nanospheres or nanocapsules) that may be prepared from natural or from synthetic polymers (58-60). Research efforts in nanoparticle-mediated drug delivery have been rationalised in terms of their potential to effect: (i) long blood circulation time that is coupled with a capability to enter the smallest capillaries; (ii) resistance to rapid phagocytic clearance; (iii) capability to reach the target organ by penetrating cells and tissues; (iv) capacity to exhibit controlled release properties that are inherent to the rate of biodegradability or occur in response to a stimulus or a combination of stimuli (pH, temperature, ion sensibility); and, (v) improved target-organ specificity (61).

For potential use in drug delivery nanoparticles must possess properties of biocompatibility and biodegradability to non-toxic and non-immunogenic products (62). Nanoparticles are differentiated from larger size congeners by their active surface area which in turn impacts upon aspects of chemical and biological reactivity (63) through effects at the solid–liquid interface and those at the contact zone with biological substrates (64). Chemical composition, surface function, geometry, porosity, surface crystallinity, size range, heterogeneity, roughness, and degree of hydrophilicity are all considered important in determining the scope and extent of the interactions of nanoparticles with biological systems (65). Of importance are the characteristics of the surface layer (zeta charge, aggregation potential, dispersion state, stability, extent of hydration) as influenced by the characteristics of the surrounding medium (ionic strength, pH, temperature, and presence of organic molecules or surfactants) (66). The surface chemistry of nanoparticles that are amenable to functionalisation offers the possibility to optimise the behaviour of nanoparticles in biological environments (64). It has been suggested that to be usefully applied in drug delivery to the brain (67), nanoparticles must have the following characteristics: diameter around 100 nm, physical stability in blood (no aggregation), non-susceptibility to the mononuclear phagocytic system (MPS), prolonged blood residence time, capacity for BBB-targeted brain delivery (receptor-mediated transcytosis across brain capillary endothelial cells), amenability to a scalable and cost-effective manufacturing process, capacity to accommodate therapeutic agents (small molecules, peptides, proteins, nucleic acids), chemical inertness (chemical degradation/alteration, protein denaturation), and possible modulation of the drug
release profiles. There is experimental evidence to suggest that the size of NP for effective brain drug delivery must be in the range 100-300 nm (68).

Dependent upon their method of formulation and constituent materials, nanoparticles formulations may be divided into two broad types, namely: nanospheres and nanocapsules. The method of choice for the formulation of nanoparticles of either type is interfacial polymerisation (the mixing of an organic phase with an aqueous phase). Differences in the two types of nanoparticle are manifested by their morphology and architecture and also by the patterns characterising the drug distribution profile and the rate of drug release. Drug molecules are localised at the central core of nanocapsules whereas in nanospheres they are dispersed evenly throughout the matrix (Figure 1.9). Nanocapsules offer the possibility for zero order release while nanospheres normally exhibit first order release kinetics (69).

1.1.3 Nanoformulated drugs currently in clinical trials
Amongst the first nanoformulated (Table 1.1) drugs to be employed in CNS-targeted clinical trials were glutathione-decorated liposomes, drug-protein conjugates (e.g. ANG1005) and polyglutamate paclitaxel (70). The initial promise of these formulations has stimulated considerable research activities in the use of nanoparticles as drug carriers, with polyester-based structures finding particular favour amongst researchers because of their molecular-design-determined biocompatibility and tuneable degradation properties (71).
Table 1.1 Representative examples of water-insoluble-drug nanoformulations that are approved for clinical use or are under clinical evaluation (72).

<table>
<thead>
<tr>
<th>Nanonization strategy</th>
<th>Trade name</th>
<th>Drug</th>
<th>Inactive ingredients</th>
<th>Indication</th>
<th>Dosage form</th>
<th>Developer, status</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-pressure homogenization</td>
<td>Triglide®</td>
<td>Fenofibrate</td>
<td>Carboxymethylcellulose sodium, croscarmellose sodium, lecithin, sodium lauryl sulfate</td>
<td>Hypercholesterolemia</td>
<td>Oral tablet</td>
<td>SkyePharma/Sciela, approved in 2005</td>
</tr>
<tr>
<td>Media milling</td>
<td>Rapamune®</td>
<td>Sirolimus</td>
<td>Proviodone, ploxaorn 188</td>
<td>Immunosuppression</td>
<td>Oral tablet</td>
<td>Elan/Wyeth, approved in 2000</td>
</tr>
<tr>
<td></td>
<td>Emerex®</td>
<td>Aprepitant</td>
<td>Hydroxypropyl cellulose, sodium lauryl sulfate</td>
<td>Antiemetics</td>
<td>Oral capsule</td>
<td>Elan/Merck, approved in 2003</td>
</tr>
<tr>
<td></td>
<td>Trico®</td>
<td>Fenofibrate</td>
<td>Hydroxypropyl methylcellulose, sodium lauryl sulfate, crospovidone</td>
<td>Hypercholesterolemia</td>
<td>Oral tablet</td>
<td>Elan/Abbott, approved in 2004</td>
</tr>
<tr>
<td></td>
<td>Megace ES®</td>
<td>Megestrol</td>
<td>Hydroxypropyl methylcellulose, dicocose sodium</td>
<td>Antianorexia, cachexia</td>
<td>Oral suspension</td>
<td>Elan/Parr Pharmaceutical, approved in 2005</td>
</tr>
<tr>
<td></td>
<td>Invega®, Susten®</td>
<td>Paliperidone palmitate</td>
<td>Polyoxybute 20, polyethylene glycol 4000</td>
<td>Schizophrenia</td>
<td>Intramuscular suspension</td>
<td>Elan/Johnson &amp; Johnson, approved in 2009</td>
</tr>
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</table>

<table>
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<tr>
<th>Nanonization strategy</th>
<th>Trade name</th>
<th>Drug</th>
<th>Inactive ingredients</th>
<th>Indication</th>
<th>Dosage form</th>
<th>Developer, status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoemulsion</td>
<td>Estrasorb®</td>
<td>Estradiol</td>
<td>Soybean oil, polyoxybate 80, ethanol</td>
<td>Vasomotor symptoms associated with menopause</td>
<td>Topical emulsion</td>
<td>Novax/Gracovex, approved in 2003</td>
</tr>
<tr>
<td></td>
<td>Flexogran®</td>
<td>Camphor, menthol, methyl salicylate, 5-Amino levulinic acid</td>
<td>Medium chain triglycerides, lecithin</td>
<td>Analgesics</td>
<td>Topical emulsion</td>
<td>AlphaRx, approved in 2001</td>
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<td></td>
<td>BF-200 ALA-gel</td>
<td>Miglyol, lecithin</td>
<td>Actinic keratosis for photodynamic therapy</td>
<td>Topical gel</td>
<td>Biofrontera, Phase III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restasis</td>
<td>Cyclosporine</td>
<td>Castor oil, polyoxybate 80, carboxamid 1342</td>
<td>Chronic dry eye disease</td>
<td>Ophthalmic emulsion</td>
<td>Allergan, approved in 2002</td>
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<table>
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<tr>
<th>Polymeric micelles</th>
<th>Drug</th>
<th>Inactive ingredients</th>
<th>Indication</th>
<th>Dosage form</th>
<th>Developer, status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK911®</td>
<td>Dooxuribin</td>
<td>Polyethylene glycol-copolymer (sparisic acid)</td>
<td>Solid tumors</td>
<td>Lyophilized powders</td>
<td>Nippon Kayaku, Phase II</td>
</tr>
<tr>
<td>NK105®</td>
<td>Paclitaxel</td>
<td>Polyethylene glycol-copolymer (sparisic acid)</td>
<td>Solid tumors</td>
<td>Lyophilized powders</td>
<td>NanoCarrier/Nippon Kayaku, Phase II</td>
</tr>
<tr>
<td>Genevol-PM</td>
<td>Paclitaxel</td>
<td>Polyethylene glycol-copolymer (sparisic acid)</td>
<td>Solid tumors</td>
<td>Lyophilized powders</td>
<td>Samyang, Approved in South Korea in 2007, Phase II in the US</td>
</tr>
</tbody>
</table>

1.2 Polymeric drug delivery systems

Owing to their tuneable biodegradability, many polymers, especially polyesters, have found applications in drug delivery and in tissue engineering. The archetypal example of such a polymer is polycaprolactone, a biocompatible and biodegradable polyester (73). (Polyesters are synthetic materials that are normally prepared through the polycondensation of monomers containing two or more carboxylic acid groups with monomers containing two or more hydroxyl functionalities or by the ring-opening transesterification of lactones). The biodegradability of polyesters is the result of their limited hydrolytic stability which over time results in depolymerisation to the precursor monomers. Linked to the hydrolytic depolymerisation of esters is their biocompatibility which can be designed into the molecular structure by the appropriate choice of the alcohol- and carboxylic acid-functionalised monomers or co-monomers that condense to form the macromolecular backbone.
The polymeric carriers used in drug delivery systems may be categorised into natural and synthetic. The latter materials may be subdivided into biopersistent and biodegradable (Table 1.2) polymers. The former comprise stable materials that maintain their physicochemical features in the physiological environment; they are eliminated from the body without undergoing metabolism (51) and their therapeutic payloads are released by diffusion through the polymeric matrix. By contrast, biodegradable polymers undergo metabolic transformation at the physiological environment, which renders the kinetics for their release highly sensitive to their biodegradation pathway. As long as biodegradable materials are not amenable to biodegradation into cytotoxic products, the use of such polymers is considered preferable to that of biopersistent molecules, in that biodegradable polymeric materials allow for the complete release of the active (51).

The use of biodegradable polymers in drug delivery avoids issues relating to the fate of the body of the depleted drug delivery device. Polymeric nanoparticles are most often prepared by poly(D,L-lactide-co-glycolide), polylactic acid, polycaprolactone, poly-alkyl-cyanoacrylates, chitosan and gelatin (57). The loading of drugs to nanoparticles may reduce the side effects inherent in most therapeutic agents (e.g. Doxorubicin-loaded nanoparticles have been shown to be less toxic than the free drug), and may afford control over the release and biodistribution of the active, increase specificity, prolong bioactivity and inhibit opsonisation or rapid elimination from circulation. It has been claimed that nanoparticles have the capacity to protect anticancer drugs from biotransformation and to delay clearance from the host (74). The thermodynamic stability of nanoparticles renders them preferable drug-host structures to liposomes, both in terms of stability during storage and in biological fluids where they offer \textit{in vivo} protection from proteases (75). Neha \textit{et al.} (76) have outlined the following potentially useful characteristics of polymeric nanoparticles to applications in drug delivery:

- in terms of efficiency and effectiveness, they offer a significant improvement over traditional oral and intravenous methods of administration;
- they deliver a high concentration of pharmaceutical agent to the desired location;
- Tuneable drug-release profiles render polymeric nanoparticles ideal candidate vehicles for cancer therapy and for the delivery of vaccines, contraceptives and targeted antibiotics;
- Polymeric nanoparticles can be easily incorporated into other technologies related to drug delivery, such as tissue engineering;
- Readily accessible nanoparticle formulations are often capable of affording increased stability to volatile pharmaceutical agents.

Table 1.2 Classification of biodegradable polymers (73)

1.2.1 Poly(alkyl cyanoacrylate)s
Alkyl cyanoacrylates (ACA) are low viscosity compounds that are formed by the condensation reaction of alkyl cyanoacetates and formaldehyde. The first report of a cyanoacrylate dates back to 1949, but the importance of this class of molecules as adhesives was not realised until 1959 (77). The physicochemical properties of cyanoacrylates are determined by the length of their alkyl side chains. Considering their proven biocompatibility following extensive use as medical adhesives, alkyl cyanoacrylates have been investigated for their ability to form nanoparticulate vehicles for biomedical applications; especially for the delivery of cancer drugs (78) and for the
transport of therapeutic agents across the BBB (79). Poly(alkyl cyanoacrylate)s are colourless brittle materials that are susceptible to environmental degradation unless stabilised by acidic stabilizers (80). The ease of degradation is inversely proportional to the length of the alkyl chain (81, 82). Methyl cyanoacrylate, ethyl 2-cyanoacrylate, n-butyl 2-cyanoacrylate, isobutyl cyanoacrylate, isohexyl cyanoacrylate and octyl cyanoacrylate, have all been used in the formulation of nanoparticles (Figure 1.10A).

![Figure 1.10: The structures of alkyl cyanoacrylate (R= methyl, ethyl, butyl, isobutyl, isohexyl, octyl etc.) (A) and poly(lactic acid) (B).](image)

Alkyl cyanoacrylates have found diverse practical applications that include use as adhesive products for household repairs and for wound closure (short-chain homologues are preferred for use as adhesives; 77, 83), weed control (84) and detection of latent fingerprints in crime investigations (85, 86). Alkyl cyanoacrylate monomers are highly reactive. They polymerise at room temperature via initiation by nucleophiles (environmental moisture) through a mechanism that involves chain propagation by the repetitive addition of monomer units to the carbanionic end of the growing chain (87). For the longer side-chain homologues, the rate of the polymerisation reaction is sensitive to temperature. The polymerisation reaction is sensitive to pH, with neutral to basic pH resulting in the agglomeration of monomers (83).

The pioneering work of Troster et al. (88), who reported that coating poly(methyl methacrylate) nanoparticles with Polysorbate 80 increased the accumulation of these nanoparticles into the rat brain, prompted much work towards nanoparticulate-assisted drug delivery to the brain. Consequently, biocompatible PACA polymers have been demonstrated to cross the BBB (89), as is exemplified by Polysorbate 80-coated n-butyl-2-cyanoacrylate (79, 90-92). The initially proposed endocytotic mechanism of transport of these nanoparticles, has however been disputed by Olivier et al. (93) who have argued that the mechanism of drug transfer involves the induction of toxicity to the BBB. Nonetheless, the mechanism of nanoparticle-mediated drug transport across the BBB is generally accepted to involve an initial receptor-mediated endocytosis which is followed
by transcytosis into the brain or by drug release within the endothelial cells (94). The use of PBCA nanoparticles that are coated with Polysorbate 80 has received considerable attention in drug delivery across the BBB (41). Significantly, PBCA nanoparticles that had been loaded with Doxorubicin and overlaid with the non-ionic surfactant Polysorbate 80 are reported to be capable of reaching to the brain intact and of releasing their Doxorubicin content following endocytotic uptake by brain blood endothelial cells (91). The use of Polysorbate 80 coatings has been rationalised in terms of earlier work by Kreuter et al. (92), who, on the basis of electron microscopy and fluorescent studies, had suggested that drug-loaded nanoparticles coated with Polysorbate-80 undergo phagocytic uptake by brain blood vessel endothelial cells. However, the matter is still subject to considerable debate since other findings suggest that the uptake of PBCA nanoparticles by the brain is consequent to interaction with the LDL-receptor by mimicking the LDL-protein following association with Apolipoprotein E from blood plasma (95).

1.2.2. Poly lactic acid (PLA)
Poly (lactic acid) (Figure 1.10B), PLA, is amongst the most commonly used biodegradable polymers in therapeutic delivery, especially that of vaccines. The main criticism this polymer has received relates to the generation of acidic micro environments during degradation. PLAs are linear aliphatic thermoplastic polyesters (96) that are commonly synthesised from the condensation of α-hydroxy acids. The basic building block for PLA is lactic acid. PLA is susceptible to degradation by simple aqueous hydrolysis and undergoes thermal degradation above 200°C. The respective glass transition and melt temperatures of the material are at 55°C and 175°C (97). Owing to its excellent biocompatibility and biodegradability (98), PLA has found uses in drug delivery. PLA has been utilised widely as a structural polymer or co-polymer in the preparation of nanoparticles. Its combination with glycolic acid to form poly (lactic-co-glycolic acid) (PLGA) is well documented, as is its zero-length grafting attachment to modified dextran and also its combination with poly ε-caprolactone.

1.2.3. Polysaccharides

Dextran, a hygroscopic polysaccharide, is an odourless and tasteless white amorphous powder which is insoluble in ethanol and diethyl ether but gradually soluble in water. The constitutional repeat unit of dextran is glucose, which forms linear bonds of α-1,6
glycosidic linkages with few branches at the α-1,2, α-1,3 and α-1,4 positions (99, 100). Apart from its common use in density centrifugation (to remove vasculature of a given homogenate) (70, 101); dextran is a useful matrix material for drug delivery applications since it exhibits properties of biocompatibility, degradability and non-immunogenicity (102). The same material is often employed as surfactant or as copolymer in nanoparticles fabrication, especially those of ACA-based structures (103), where it imparts increased hydrophilicity by means of the considerable –OH functionalisation of the pyranose ring. Notably, the α-1,6 polyglucose linkages of dextran are not susceptible to cleavage by most endogenous cellular glycosidases (104). It is a polysaccharide that is present in certain microorganisms, especially bacteria. Dextran, which is primarily utilised by microorganisms as a structural support material and as an energy store, is also integral to the immune-response mechanisms.

![Figure 1.11: The chemical structure of dextran.](image)

Dextran has been used for several decades as a plasma volume expander. This highly water soluble substance can be readily attached to drugs either directly or through linkers. Studies have shown that both the distribution and the elimination of dextran are influenced by the combined effects of molecular weight and surface charge of the polymer. The degree of water solubility of dextran decreases with increased branching, as has been exemplified by the controllable degrees of hydrophobicity that have been obtained through modifications with alkyl glycerols of systematically varied chain lengths (105). An in vitro study involving modified dextran has shown that chemically modified dextran exhibits reduced rates of dextranase-induced depolymerisation as compared with the unmodified material (105).
The use of dextran in such applications has been extended to nanoparticulate formulations that could involve co-formulation with alkyl cyanoacrylates. Native dextran has been used extensively as a surfactant in the formulation of poly(alkyl cyanoacrylate)-containing molecular structures (90, 93, 103, 106). The combined use of these materials has been rationalised in terms of the capability of dextran to impart to alkyl cyanoacrylates flexural strength and an increased capacity to accommodate a therapeutic load for drug delivery applications. However, the observation that PECA-Dex structures are susceptible to aggregation and to becoming brittle over time has stimulated further research activities towards the development of alternative materials with improved long-term stability.

Polymeric nanoparticles prepared from alkyl cyanoacrylates and dextran have shown promise as carrier vehicles for drug delivery to the brain, as is exemplified by the observed delivery of drugs and peptides across the BBB by means of Polysorbate 80-coated formulations of PACA and dextran. The capability of the biodegradable PACA macromolecules to act as carriers for drugs and fluorophores alike has been integral to their utilisation in drug delivery (107). It is common practice to incorporate covalently bonded fluorophore end groups into the polymer structure by initiating the polymerisation reaction by means of nucleophilic fluorophores (108). Alternatively, the fluorescent labelling may be achieved by nanoprecipitation of the preformed polymer (109).

Derived from chitin, chitosan is regarded as a nontoxic, biocompatible and biodegradable cationic polysaccharide. Chitosan is comprised of β(1,4)-linked 2-acetamido-2-deoxy-β-D-glucan and 2-amino-2-deoxy-β-D-glucan. The commercial preparation of chitosan is through alkaline deacetylation of chitin. Chitosan nanoparticles are prepared mainly by the ionic gelation of chitosan through the tripolyphosphate anions method (110, 111). Chitosan has been utilised widely in the development of controlled-release drug delivery systems (51,112). Interestingly, chitosan nanoparticles have been described as an efficient vehicle for the delivery of insulin through the nasal mucosa (113).
1.2.4. Polycyanoacrylates

The chemical stability of PACA has been shown to be sensitive to the nature of the initiator and the method employed for the polymerisation, and also by the length of alkyl side chains. PACAs are susceptible to biodegradation both at the backbone C—C bond (due to the presence of the strongly electron withdrawing cyanate group) and at the ester linkage (by hydrolysis). The ease of hydrolytic degradation to the alkyl alcohol and to poly(alkyl cyanoacrylic acid) has been observed to decrease with increasing length of the side chain. Backbone (C—C) degradation occurs by the unzipping of the polymer chains in a depolymerisation process that produces the precursor monomer (108).

The ease with which ACA monomers undergo polymerisation reactions to form PACA, coupled with the biocompatibility of PACA and its biodegradability to innocuous products, render this polymer a suitable structural material for the fabrication of drug carrier structures (58). Conventionally ACA is polymerised in acidified water using a two phase polymerisation process that affords control over particle size has been argued however that this slow process and yields a particle size distribution that is broader than that which can be obtained with ethanol/water systems (114). The easy accessibility and ready availability of ECA renders this monomer the cyanoacrylate of choice for nanoparticle formulation for anionic emulsion polymerisation reaction via initiation by the hydroxyl group of water or the nucleophilic centre of other initiator molecules such as amines (114). The amino group of proteins, has been utilised as an initiating moiety in the polymerisation of ECA as exemplified by the reaction with Bovine serum albumin (BSA), where the protein molecules are also claimed to serve as stabilizer surfactants (114). Adjustment of the pH affords control over the size distribution of PACA nanoparticles (115) whereas the hydrophobicity of the same nanoparticles may be tuned by adjusting the length of the side chain such that control may be exerted over the capacity of nanoparticles to swell in biological fluids (116). Transmission electron microscopy (TEM) has shown that PECA nanoparticles are structured as a highly porous but dense polymeric matrix with high surface area that facilitates the entrapment of wide range of drug molecules (117). Clinical trials of formulations of PACA for drug delivery applications have not unmasked any metabolites-related toxic effects of notable significance (118).
1.3. Methods for the preparation of nanocarriers
The variants of the two main methods of choice for the preparation of nanoparticles, dispersion of preformed polymer and polymerisation of monomers, have been reviewed by Rao and Geckeler (119). Nanoparticles formation by the dispersion of preformed polymers may involve solvent evaporation, nanoprecipitation, salting out, dialysis, rapid expansion of supercritical solution (RESS) and rapid expansion of supercritical solution into liquid solvent (RESOLV). The formation of nanoparticles by the polymerisation of monomers may utilise the techniques of micro-emulsion, mini-emulsion, surfactant-free emulsion or interfacial polymerisation. The method of nanoparticle preparation is determined by the chemical structure of the adopted matrix, which in turn is selected according to the requirements of size and of the proposed use (120).

Other methods that have been utilised for the preparation of nanoparticles for drug delivery include: (i) freezing-induced phase separation (121); (ii) emulsion solvent evaporation (122); (iii) single emulsion–solvent evaporation technique, which has been claimed to be the best general method of encapsulating hydrophobic molecules into nanoparticles; and, (iv) miniemulsion, which has been utilised for the co-formulation of paclitaxel and PBCA (123).

Bertholon et al. (124) in their studies of redox and anionic emulsion polymerisation of nanoparticulate PACA have shown that low (highly acidic) pH leads to the formation of higher molecular weight and higher average particle size than corresponding nanoparticles prepared at higher pH. The same group of workers speculated that the higher molecular weight polymers formed as a result of the highly acidic pH making fewer OH groups available for the initiation of polymerisation.

1.4. Characterisation techniques
Integral to the characterisation of nanoparticles is the determination of size, polydispersity index (PDI), zeta potential, texture morphology and thermal stability. Particle size and its distribution influence such key properties as stability in suspension, viscosity, surface area, and parking density. For biomedical applications, size also impacts on the capability of therapeutic agents-loaded nanoparticles to penetrate deeply into tissues through the narrow capillary route and further to penetrate cells; amongst other
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techniques, the sizes of nanoparticles may be determined by DLS, TEM, NTA, SEM, and AFM.

Zeta potential provides a measure of the stability of nanoparticles in a given medium. Zeta potential values above 30 mV (-ve or +ve) are normally considered indicative of good colloidal stability as it indicates that the electrostatic repulsion between neighbouring nanoparticles is greater than the antagonistic van der Waals forces of attraction, preventing aggregation or precipitation (125); zeta potential may be determined by dynamic electrophoretic mobility. The preparation of nanoparticles of narrow size distribution, as measured by the PDI, is prerequisite to the reproducible behaviour of vehicles intended for systemic use.

1.4.1. Elemental analysis
The technique used for the determination of elemental C, H, and N and S is based on the quantitative “dynamic flash combustion” method (Figure 1.12). The samples are held in a tin capsule, placed inside the autosampler drum where they are purged by a continuous flow of helium and dropped at pre-set intervals into a vertical quartz tube maintained at 900°C. When the samples are placed inside the furnace, the helium stream is temporarily enriched with pure oxygen, the sample melts and the tin promotes a violent reaction (flash combustion); under these conditions even thermally resistant substances are oxidised fully. Quantitative combustion is then achieved by passing the mixture of gases over a catalyst layer. The mixture of combustion gases is then passed over copper to remove the excess oxygen and to reduce the nitrogen oxides to elemental nitrogen. The resulting mixture is then directed to the chromatographic column where the individual components are separated and eluted with the help of a thermal conductivity detector (TCD) whose signal feeds the automatic EAGER300™ workstation as nitrogen, carbon dioxide, sulphur dioxide and water. The instrument is calibrated with the analysis of standard compounds using the K factors calculation or linear regression method incorporated in the EAGER300™ software.
1.4.2. MALDI-TOF MS
MALDI-TOF MS, a technique developed by Hillenkamp and Karas in 1988 (126), is widely utilised for the mass spectroscopic characterisation of proteins and other biomacromolecules (127).

Principles of MALDI
Unlike conventional mass spectroscopy techniques (electron impact, chemical ionisation), in MALDI-TOF MS (Figure 1.13) (a technique normally applied in the characterisation of complex molecules) ionised molecules (cationic) or molecular fragments that have the same energy travel at different velocities, with the implication that fragments of different masses reach the detector at different times; larger ions travel with a lower velocity than smaller cations. Since the time of travel depends on charge, mass and kinetic energy of the ion, delay extraction (DE) has been shown to be very helpful in improving resolution in the application of MALDI-TOF; DE is employed to delay the travel of ions by 150 ns from formation, such that most of their excess energy is dissipated, before the accelerating voltage is applied. However the resolution benefit of DE decreases with samples of increasing molecular weight.
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**Sample preparation**

The sample preparation stage is critical in the quality of the spectrum produced in MALDI-TOF spectrometry. Amongst the methods of sample preparation, the dried-droplet method has become the most widely utilised: a saturated matrix solution is mixed with the analyte at the ratio of 5000:1, and an aliquot of about 0.5 µl is placed on the sample receptor where it is dried under vacuum before ionisation. Other approaches to sample preparation include: thin and thick layer (127), fast-vaporisation, electrospray, matrix-precoated layer, particle-doped (two phase liquid) and chemical-liquid (128) methods.

![Figure 1.13: Schematic representation of the MALDI instrument (128).](image)

The advent of MALDI has offered many advantages in the analysis of macromolecules as compared with conventional chromatographic techniques. These include the capability to determine absolute molecular weight irrespective of macromolecular structure, the ease of use (the technique imposes few demands in terms of instrument- and sample-preparation procedures), and the capability to characterise molecules of <20 kDa (128, 129). However, the technique is regarded as complementary to chromatographic methods since it is not suitable for the study of macromolecules with PDI >1.6 or for the analysis of complex mixtures of surfactants. Most importantly, MALDI is of little usefulness in the analysis of macromolecules that do not possess chemical functionalities that are capable of stabilising the prerequisite cationic structure at the gaseous phase (128).
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The vaporisation and ionisation technique is widely used in the characterisation of biomolecules by MALDI-TOF. This is a non-destructive technique that vaporises and ionises large and small biomolecules without promoting fragmentation (Figure 1.14). To this end, the sample to be analysed is incorporated first into a matrix (e.g. 2,5 dihydroxybenzoic acid (130, 131), which is in excess of the analyte and functions by absorbing strongly the energy of a laser source thereby facilitating for vaporisation of the analyte. In doing this, it also serves as proton donor for the analyte thereby facilitating ionisation and subsequent analysis by the detector (linear time-of-flight (TOF) analyzer, TOF reflectron or fourier transformed mass analyzer).

![Diagram](image)

**Figure 1.14: Non-destructive vaporisation and ionisation of biomolecules by MALDI TOF (126)**

The usefulness of mass spectrometry methods in the identification and characterisation of unknown compounds is twofold, in that it allows the:

a. Determination of the molecular mass of the compound under study; mild direct ionisation techniques allow the detection of the highest m/z ratio ion which often corresponds to an isotopic form of the intact, ionised molecule, while chemical ionisation techniques (e.g. ESI, MALDI) typically identify the protonated or deprotonated form of the molecule.
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b. Detection of the functional groups present in the molecule on the basis of specific fragment ions and/or fragment ion series present in the mass spectrum, and characteristic fragment ions formed by loss of neutral molecules from the intact ionised molecule.

Synthesis of the spectral information regarding the molecular ion and the identified molecular fragments allows the determination of the structure of the molecule under study.

The use of high mass resolution instruments allows the minimisation of issues relating to peak broadening and of those due to presence of any interfering or isobaric ions. The technique advantages have been put at low cost, high sensitivity, large mass range and recording a whole spectrum in a single acquisition step (132).

1.4.3. Nanoparticle Tracking Analysis (NTA)
Nanoparticle Tracking Analysis (NTA) was developed by Carr et al; in this technique, particles under Brownian motion are recorded by software-guided video designed to identify and track the centre of each particle on a frame-by-frame basis (Figure 1.15). The image analysis software determines the distance moved by each particle, which in turn allows the determination of the corresponding diffusion coefficient. The hydrodynamic diameter ($d$) of particles is calculated (133) by inputting the temperature ($T$) and viscosity ($\eta$) of the medium into the Einstein-Stokes equation ($k = $ Boltzmann constant).

$$d = \frac{kT}{3\pi \eta D} \quad \text{(eq. 1.1)}$$

![Schematic representation of the Nanoparticle Tracking Analysis (NTA)](image-url)
1.4.4. Zeta potential determination
There exist two layers around a particle that is suspended in an electrolytic solution, namely: the Stern layer and inner region, where the ions are bound strongly; and the outer, diffuse region, where the ions are bound less strongly (Figure 1.16). The net surface charge of particles determines the distribution of ions surrounding it, effecting the formation of an electrical double layer around each particle. Zeta potential is used to determine the magnitude of the interaction between charges in a system, and provides a measure of surface charge. Thus, zeta potential is the energy difference that exists at this boundary. Colloidal systems with a zeta potential between +30 and -30 are normally considered stable (134).

![Figure 1.16: Schematic representation of particles and surround charges](image)

1.4.5 TGA
Thermogravimetric analysis TGA (135) is a method of thermal analysis in which changes in the physical and chemical properties of materials are monitored either as a function of increasing temperature (at constant heating rate), or as a function of time (at constant temperature and/or constant mass loss). TGA is used to study the decomposition and thermal stability of materials under specified conditions and to examine the kinetics of the physicochemical processes occurring within a sample. The technique is commonly
Introduction and aims

employed to determine heat-induced changes that involve either mass loss or mass gain due to decomposition, oxidation or loss of volatiles (such as moisture). Factors that influence the nature of the TGA curve (Figure 1.17) include sample mass, volume and physical form, the shape and nature of the sample holder, the nature and pressure of the atmosphere in the sample chamber, and the scanning rate. The principal applications of TGA in polymers are the determination of the thermal stability of polymers, compositional analysis, and identification of polymers from their decomposition pattern.

Figure 1.17 Representative TGA curve.

1.4.6 SEM

The SEM is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. This complements optical images with the traditional microscope in that it offers large depth of field (which allows more of a specimen to be in focus at one time), and high resolution.
The electron beam scans the surface line by line to produce the image (Figure 1.18); since metals are conductive, their samples do not require pre-imaging treatment but non-metals need treatment that renders them conductive; this may involve coating with gold, graphite or other electrically conducting materials in a sputter coater. The sample to be coated is fixed on a stub and placed under vacuum. The interaction of electron with the sample produces many signals which include secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence) (CL), specimen current and transmitted electrons. The signals result from interactions of the electron beam with atoms at or near the surface of the sample. Secondary electrons and backscattered electrons are commonly used for imaging samples.
1.5. Aim of the study
Amongst the approaches towards the delivery of therapeutic agents to the brain without compromising the integrity of the BBB, the utilisation of polymeric nanoparticulate drug-carrier vehicles – the focus of this work – has gained considerable following amongst researchers. The objectives of this study are to:

i) synthesise amphiphilic dextran derivatives through functionalisation with alkyl glycidyl ethers;

ii) use these modified dextrans in combination with alkyl cyanoacrylates (ACA) for the preparation of well-defined nanoparticles;

iii) study the capability of these nanoparticles to act as carriers for fluorescent-marker molecules and for small molecule drugs; and,

iv) investigate the biodegradability and the potential toxicity of these nanoparticles, and assess any associated interactions for the purpose of evaluating promise in therapeutic applications.
2. Chemical modifications of dextran

The chemical modification of dextrans has been rationalised in terms of the capability of dextran to impart to alkyl cyanoacrylates flexibility and an increased capacity to accommodate a therapeutic load for drug delivery applications. However, the observation that PECA-Dex structures are susceptible to aggregation and to becoming brittle over time has stimulated further research activities towards the development of alternative materials with improved long-term stability. The functionalisation of dextrans with alkyl glyceryl moieties represents one of the approaches towards that goal.

2.1 Dextran

Dextran is an odourless and tasteless hygroscopic white powder that is insoluble in ethanol or diethyl ether but dissolves gradually in water. It is a polysaccharide that is present in certain microorganisms, especially bacteria. Dextran, which is primarily utilised by microorganisms as a structural support material and as an energy store, is also integral to the immune-response mechanisms.

The chemical structure of dextran (Figure 2.1) is that of a neutral polysaccharide that has glucose as its sole constitutional repeat unit. The glucose moieties form linear bonds at the α-1, 6 glycosidic linkages (more than 95% of the chain) with few branches at the α-1,2, α-1,3 and α-1,4 linkages (99, 100). Owing to its glucose-based structure, dextran is characterised by biocompatibility, degradability and non-immunogenic properties which render it a useful structural material for biomedical applications (102). The use of dextran in such applications has been extended to nanoparticulate formulations that often involve co-formulation with alkyl cyanoacrylates (24, 93, 137).
Chemical modification of dextrans

Figure 2.1: The chemical structure of dextran

The –OH functionalities render dextran a molecule that is readily amenable to structural modification (138). Consequently, a number of modified dextrans have been prepared in which aromatic rings or aliphatic chains are attached to the molecular structure (139-144). For example, dextran that had been modified by esterification with benzoic acid and valeric acid has been used in protein partitioning (145). In other examples, Rouzes et al. (98) utilised hydrophobically modified dextran as a surfactant for the preparation of nanoparticles with well-defined surface properties, while Sadtler et al. (146) reported its use in the stabilisation of oil-in-water emulsions. Dextran derivatives with good chemical stability at extreme pH environments have been achieved through functionalisation with 1,2-epoxydodecane (144). The chemical modifications of dextran have been extended to attempts to obtain amphiphilic polymers which are capable of forming micellar structures that can associate with organic solutes at the hydrophobic domain. Rotureau et al. (141) have shown that lipophilic modification of dextran through linkage with aromatic or aliphatic hydrocarbons results in macromolecular structures of controlled amphiphilicity. The amphiphilic nature of modified dextrans renders these materials suitable components for the fabrication of nano-sized structures of well-defined size, as is exemplified by the work of Rouzes et al. (147) who utilised such materials in combination with PLA to produce nanomaterials in the 150-200 nm range, and that of others who utilised amphiphilic dextrans as surface-modification components of both nano- and micro-sized drug delivery systems (147, 148). Native dextran has been used extensively as
Chemical modification of dextrans

a surfactant in the preparation of poly(alkyl cyanoacrylate)-based structures (24, 90, 93, 103). The combined use of these materials has been rationalised in terms of the capability of dextran to impart to alkyl cyanoacrylates flexibility and an increased capacity to accommodate a therapeutic load for drug delivery applications. However, the observation that PECA-Dex structures are susceptible to aggregation and to becoming brittle over time has stimulated further research activities towards the development of alternative materials with improved long-term stability. The functionalisation of dextrans with alkyl glyceryl moieties represents one of the approaches towards that goal.

2.1.1 Chemical structure of oxiranes for synthesis of alkyl-glyceryl dextrans
Various chain lengths of oxiranes were used in the modification of dextran, these includes butyl glycidyl ether, octyl glycidyl ether, glycidyl lauryl ether, tetradecyl glycidyl ether, and hexadecyl glycidyl ether (Figure 2.2)

![Figure 2.2 Chemical structure of alkyl glycidyl ether where n=1, butyl; n=5, octyl; n=9, lauryl; n=11, tetradecyl; n=13 hexadecyl.](image)

2.2 Materials and instrumentation
Dextrans from *Leuconostoc spp.* (MW 6 kDa and 100 kDa), dimethyl sulfoxide (DMSO, anhydrous, ≥ 99.9%), potassium tert-butoxide (t-BuOK; reagent grade > 97 %), butyl glycidyl ether, octyl/decyl glycidyl ethers, glycidyl lauryl ether, tetradecyl glycidyl ethers and hexadecyl glycidyl ether were all obtained from Sigma-Aldrich UK.

TGA analysis was carried out with TG 209 F1 Libra (TGA from Netzsch Instruments), with temperature ranged from 25 to 500°C, heating at 10K/min. under Nitrogen atmosphere.

A Büchi Rotovapor (R-200) powered with a Sogeval Saskia PIZ 100 vacuum pump equipped with a liquid nitrogen trap was used to remove solvents under reduced pressure during the process of modified dextrans purification, Dialysis was performed using Visking membrane tubing (Medicell International Ltd, UK) with cut-off either 12-14 kDa or 3.5 kDa in a discontinuous system (10.0 L deionised water (15.0 mΩ); exchanged 3
Chemical modification of dextrans

The alkylglyceryl derived dextrans were characterised by $^1$H- and $^{13}$C-NMR spectroscopy using a JEOL Eclipse 400+ instrument (JEOL, UK; 400 MHz for $^1$H- and 100 MHz for $^{13}$C-NMR); samples were dissolved in either D$_2$O or DMSO-d$_6$ employing 0.2 % tetramethylsilane (TMS) as a reference. The spectra were processed using the JEOL Delta v 5.0.2 software or Advanced Chemistry Development (ACD) software. FT-IR spectra were recorded using a Nicolet 6700 instrument (Thermo Scientific, UK) equipped with an ATR Smart Orbit accessory with diamond crystal, at 64 scans and 4 cm$^{-1}$ data spacing. The analysis of spectra was performed using the Omnic Specta 8.0 software; for the FTIR analysis, dried samples were used, and analysed by placing the sample on top of the diamond crystal plate and reading taken with the Omnic Specta 8.0 software. The molecular weights of alkylglyceryl dextrans were estimated using a GPC (waters 2000) with a refractive index detector, under thermostatted conditions (temperature 30°C), 80/20 solution of water/methanol was used as eluent at a flow rate of 0.6 mL/min). The calibration was performed with Pullulan standards (Shodex Denko) of MW 0.6 × 10$^4$, 1 × 10$^4$, 2.17 × 10$^4$, 4.88 × 10$^4$ and 11.3 × 10$^4$, 21 × 10$^4$, 36.6 × 10$^4$, 80.5 × 10$^4$ g/mol.

MALDI-TOF MS experiments were performed on a Micromass MALDI MicroMX instrument operating in positive reflectron mode in the m/z range of 400-1600; a 337nm nitrogen laser used for ionisation (pulse setting 1800V; detector setting 2300V; flight tube 12Kv; reflectron 5.2Kv). α-Cyano-4-hydroxycinnamic acid (CHCA) solution at a concentration of 10 mg/mL in acetonitrile:water (50:50) provided the matrix for the experiment. CHCA was mixed in the 1:1 ratio with the sample (dissolved in 50:50 v/v acetonitrile:water) and deposited onto the MALDI plate.

2.3 Methods

Dextran (2g; 12.35 mmol), dissolved in anhydrous DMSO (150 mL), was allowed to stir under nitrogen for 2 h before gradual addition of potassium tert-butoxide (t-BuOK) (1.385 g; 12.35 mmol) that had been dissolved in anhydrous DMSO (50 mL) (t-BuOK was dissolved in DMSO by allowing it to stir for 30 min in anhydrous DMSO under Nitrogen atmosphere at room temperature). Stirring was continued for another 2 h before the gradual addition of a solution of specified alkyl glycidyl ether (G4, G8, G12, G14, G16) at 40°C and further stirring for 24 h. After this time, the reaction mixture was transferred into a dialyzing membrane vessel (MWCO 3.5 or 12-14 kDa) and dialysed against
Chemical modification of dextrans
deionised water (10.0 L; exchanged 3x per day) over five days. The content of the dialysis vessel (ca. 400 mL) was washed with diethyl ether or with DCM (3x 150 mL) to remove impurities and any unreacted ether. Traces of volatile solvents were removed using the rotary evaporator (reduced pressure, 50°C) and the sample was again subjected to the dialysis procedure (48 h) before lyophilisation with a Virtis freeze dryer (<-85°C, for 48h) to yield the corresponding alkylglyceryl dextrans, which were characterised using FTIR, $^1$H- and $^{13}$C-NMR spectroscopy, GPC, MALDI-TOF MS, SEM and TGA. Yields were in the range 40-60%.

2.4 Results and Discussion

2.4.1 Synthesis of alkylglyceryl dextrans
The modification of dextrans with alkyl glycidyl ethers (Figure 2.3) was carried out by attaching alkylglyceryl side chains to the hydroxyl groups through two-step reactions which involved the conversion of the hydroxyl groups of the pyranose rings of dextran into the more reactive alcoholates and subsequent reaction with specified alkyl glycidyl ethers (149). The synthesis was carried out, at 40°C, in anhydrous DMSO and under a nitrogen atmosphere for 24 h; similar modification of dextran are well documented in the literature (139, 141, 144, 150). The reactivity of the –OH pyranose ring of the glucose unit is in the order 2>4>3.
Native dextrans with a molecular weight of either 6 kDa or 100 kDa were modified by covalent attachment of alkylglyceryl pendent chains to their hydroxyl groups. This was carried out by means of a series of commercially available alkyl glycidyl ethers of different chain lengths: butyl glycidyl ether (G4; $^1$H NMR, $^{13}$C-NMR; Appendix 1), octyl glycidyl ether (G8), glycidyl lauryl ether (G12), tetradecyl glycidyl ether (G14; $^1$H-NMR, $^{13}$C-NMR; Appendix 2), and hexadecyl glycidyl ether (G16; $^1$H-NMR, $^{13}$C-NMR; Appendix 3). The reactions produced a range of alkylglyceryl dextrans: butylglyceryl dextrans (Dex100G4 or Dex6G4), octylglyceryl dextrans (Dex100G8 or Dex6G8), laurylglyceryl dextrans (Dex100G12 or Dex6G12), tetradecylglyceryl dextrans (Dex6G14 or Dex100G14) and hexadecylglyceryl dextrans (Dex100G16 or Dex6G16); the numbers 6 and 100 represent the molecular weight of the dextrans while 4, 8, 12, 14, 16 represent the chain lengths of alkyl glycidyl ethers used for the modification of dextrans.

Hexadecyl glyceryl dextran was prepared in two-step reactions which involved conversion of OH group to more reactive alcoholate with t-BuOK and subsequent reaction with the specified glycidyl ether; other dextran derivatives were synthesised through the same procedure.
Chemical modification of dextrans

2.4.2 Characterisation of the alkylglyceryl dextrans

2.4.2.1 NMR and FTIR

The modified dextran structures were confirmed by FTIR and NMR spectroscopy. The modification of the dextran was assessed by comparing the $^1$H-NMR spectrum of the modified dextrans (examples in Figures 2.4 and 2.5) with that of the native material (Figure 2.6): the presence of resonances at 0.87 ppm, 1.3 ppm and 1.4 ppm confirmed the successful functionalisation of dextran. Similar results were obtained for butyl, octyl, lauryl and tetradecyl dextrans. Table 2.1 summarises the DS% of the modified dextrans.

The successful synthesis of alkylglyceryl dextran was consistent with FTIR spectroscopy data: the grafting of alkyl glyceryl chains resulted in the formation of a new secondary alcohol. The band at 1012 cm$^{-1}$ is assigned to the vibrational mode of C–O. In crystalline dextran, two absorption peaks at 851 and at 914 cm$^{-1}$ were observed, respectively assigned to (C–C) and (C–H) bending modes. It has been proposed that the absorption bands in the spectral range between 1200 and 1500 cm$^{-1}$ are mainly due to CH deformation vibrations and (COH) bending vibrations (151). The bands at 2921 cm$^{-1}$ and at 3215 cm$^{-1}$ are respectively consistent with (C–H) and (O–H) stretching vibrations (Figure 2.7 and Figure 2.8).

SEM images of native dextran and of Dex100G4 visualised the morphology of these materials (Appendix 13).

The enumeration of the degree of substitution (Table 2.1), as expressed by the percentage of substituted saccharide units carrying an alkylglycerol group, was achieved by $^1$H-NMR through the comparison of the ratio of integral of the intensity of the terminal methyl group of alkyl glycerol ($I_A$) with that of the anomeric proton of the glucopyranose ring ($I_B$); (equation 2.1; Table 2.1).
Chemical modification of dextrans

Figure 2.4: $^1$H-NMR spectrum of alkyl glyceryl dextran (Dex100G16)

Figure 2.5: $^1$H-NMR spectrum of butyl alkyl glyceryl dextran (Dex100G4)
Chemical modification of dextrans

Figure 2.6: $^1$H-NMR of native dextran

$$DS\% = \frac{I_1}{3I_s} \times 100 \quad \text{(eq. 2.1)}$$

Table 2.1: The degree of substitution of the synthesised glyceryl dextrans.

<table>
<thead>
<tr>
<th>DexnGm</th>
<th>DS (%)</th>
</tr>
</thead>
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<tr>
<td>Dex6G4</td>
<td>87.70</td>
</tr>
<tr>
<td>Dex100G4</td>
<td>68.49</td>
</tr>
<tr>
<td>Dex6G8</td>
<td>114.94</td>
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<td>64.91</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>Dex6G16</td>
<td>55.56</td>
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<tr>
<td>Dex100G16</td>
<td>144.92</td>
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</table>
Figure 2.7: FTIR of butyl glyceryl dextran (Dex100G4)
2.4.2 GPC

GPC was used to determine the molecular weight distribution profile of some of the synthesised alkylglyceryl dextrans and that of the starting material (native dextrans, Dex 6 kDa and Dex 100 kDa). Owing to differences in the experimental protocol, the average molecular weights of commercial dextrans that served as starting materials were higher than those given by the manufacturer (Table 2.4). It was found that Dex6G4 had an average molecular weight that was lower than that of the native precursor dextran (Dex6), while Dex100G16 had an average molecular weight that was higher than that of its precursor macromolecule (Dex100). Since longer chain length should influence the GPC-determined average molecular weight, a study was conducted to compare Dex100G16 with its progenitor molecule, Dex100, (Table 2.4). The calibration curve of pullulan standard, calibration data and the equation of the calibration curve are respectively presented in Figure 2.9, Table 2.2 and Table 2.3, while the chromatograms of the analysed dextran and alkylglyceryl dextran are reproduced in Figures 2.10-2.13.
Chemical modification of dextrans

Figure 2.9 GPC calibration curve (Pullulan standards)

Table 2.2: GPC calibration data using Pullulan standards

<table>
<thead>
<tr>
<th></th>
<th>Retention Time /min</th>
<th>Elution Volume /mL</th>
<th>Mol Wt</th>
<th>Log (Mol Wt)</th>
<th>Calculated Weight</th>
<th>% Residual</th>
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</table>

Table 2.3: Equation calculated for the GPC calibration curve

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>R^2</th>
<th>Standard Error</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.999902</td>
<td>0.999804</td>
<td>1.407507 e-002</td>
<td>Log Mol Wt = 2.24e+001 - 3.87e+000 T^1 + 3.08e-001 T^2 - 8.84e-003 T^3</td>
</tr>
</tbody>
</table>
Figure 2.10 GPC chromatogram of native dextran (Dex6)

Figure 2.11: GPC chromatogram of native dextran (Dex100)
Chemical modification of dextrans

Figure 2.12: GPC chromatogram of Dex6G4

Figure 2.13: GPC chromatogram of Dex100G16
### Table 2.4: Mn, Mw and PDI of the dextrans and alkylglyceryl dextrans

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn</th>
<th>Mw</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native dextran 6 kDa</td>
<td>4223</td>
<td>9811</td>
<td>2.3</td>
</tr>
<tr>
<td>Native dextran 100 kDa</td>
<td>24919</td>
<td>109399</td>
<td>4.4</td>
</tr>
<tr>
<td>Dex6G4</td>
<td>3640</td>
<td>5469</td>
<td>1.5</td>
</tr>
<tr>
<td>Dex100G16</td>
<td>186810</td>
<td>188308</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Where: Mn = number average molecular weight  
Mw = weight average molecular weight  
PDI = Polydispersity index

#### 2.4.3 MALDI-TOF MS

![MALDI-TOF spectrum of Dex6G4](image)

Peak-to-peak mass differences of 130.2 and 146.2, observed between the main peaks that form the spectral pattern, are likely due to fragmentation at the O-C bond level (as suggested in Figure 2.14, insets); this is indicative of a fragmentation pathway that is characterised by the loss of C\textsubscript{7}H\textsubscript{14}O\textsubscript{2} and C\textsubscript{7}H\textsubscript{14}O\textsubscript{3} (MW 130.18 and 146.18 Da, respectively), which in turn confirms the successful chemical grafting of native dextran with butylglyceryl pendent chains; no cross-ring fragments could be identified.
Chemical modification of dextrans

The isotopic ratios (discernible in Figure 2.15) are indicative of primarily singly charged ions. Since the CHCA matrix used (a strong acid in the gaseous phase) tends to induce extensive fragmentation (Karas et al. 1995, Harvey 1999; (152, 153), the detection of molecular ions was not possible. This is consistent with suggestions that 2,5-dihydroxybenzoic acid (2,5-DHB) is a better matrix for the MALDI-TOF MS study of high molecular weight polysaccharides (such as dextran) that require a high matrix-to-analyte ratio to give enhanced signals (153, 154). The mass/charge (m/z) fragment loss and nature of fragment loss are presented in Table 2.5.

Table 2.5: Identified m/z fragment mass loss in the MALDI-TOF spectra of the modified dextrans.

<table>
<thead>
<tr>
<th>sample</th>
<th>m/z fragment loss</th>
<th>Nature of fragment loss identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex6G4</td>
<td>130.1</td>
<td>C$<em>7$H$</em>{14}$O$_2$</td>
</tr>
<tr>
<td>Dex100G4</td>
<td>130.1; 146</td>
<td>C$<em>7$H$</em>{14}$O$_3$</td>
</tr>
<tr>
<td>Dex100G8</td>
<td>~186</td>
<td>C$<em>{11}$H$</em>{22}$O$_2$</td>
</tr>
<tr>
<td>Dex6G12</td>
<td>242.2</td>
<td>C$<em>{15}$H$</em>{30}$O$_2$</td>
</tr>
<tr>
<td>Dex100G12</td>
<td>242.3</td>
<td>C$<em>{15}$H$</em>{30}$O$_2$</td>
</tr>
</tbody>
</table>

Figure 2.15 MALDI TOF spectrum of Dex100G4

Similar spectra have been obtained for the grafted 100kDa or 6kDa dextran (Appendix 7).
2.4.5 TGA

TGA was carried out to determine the thermal stability of samples; the thermograms of analysed samples are collated in Appendix 6.

![TGA results for Dex6](image)

Figure 2.16: TGA results for Dex6

Figure 2.16 is the TGA plot from a sample that had been heated under nitrogen to 500°C. There are 2 mass loss steps: the first of these corresponds to a mass loss of -8.91% and exhibits a maximum mass loss rate (peak in DTG curve) at 82.5°C, while the second step (maximum mass loss rate at 315.0°C) is associated with a -74.25% loss relative to the original mass.
Figure 2.17 shows the TGA profile of the sample that was heated under nitrogen atmosphere to 500°C. The thermogram is again associated with two mass-loss steps, the first of which corresponds with a mass loss of -9.01% and is characterised by a mass-loss rate maximum at 66.6°C; the second step (mass loss -86.6%) exhibits its maximum mass loss rate at 327.1°C.

The thermograms of other samples are presented in Appendix 6: the data of the thermograms in appendices are summarised in Table 2.6; the tabulated temperature values were determined from the first derivative (point of inflection) of the corresponding thermogram.
Table 2.6: TGA data of first and second mass loss for modified and unmodified dextrans

<table>
<thead>
<tr>
<th>sample</th>
<th>First mass loss/temperature, °C</th>
<th>Second mass loss/temperature, °C</th>
<th>Total mass loss %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% mass loss</td>
<td>Temperature /°C</td>
<td>mass loss</td>
</tr>
<tr>
<td>Dex6</td>
<td>-8.90</td>
<td>82.5</td>
<td>-74.25</td>
</tr>
<tr>
<td>Dex6G4</td>
<td>-9.01</td>
<td>66.6</td>
<td>-86.58</td>
</tr>
<tr>
<td>Dex6G8</td>
<td>-3.54</td>
<td>69.0</td>
<td>-77.81</td>
</tr>
<tr>
<td>Dex6G12</td>
<td>-5.36</td>
<td>59.7</td>
<td>-86.17</td>
</tr>
<tr>
<td>Dex6G14</td>
<td>-4.39</td>
<td>61.7</td>
<td>-86.49</td>
</tr>
<tr>
<td>Dex6G16</td>
<td>-2.85</td>
<td>58.1</td>
<td>-87.70</td>
</tr>
<tr>
<td>Dex100</td>
<td>-9.38</td>
<td>84.2</td>
<td>-70.63</td>
</tr>
<tr>
<td>Dex100G4</td>
<td>-9.70</td>
<td>66.0</td>
<td>-76.63</td>
</tr>
<tr>
<td>Dex100G8</td>
<td>-6.41</td>
<td>61.6</td>
<td>-77.30</td>
</tr>
<tr>
<td>Dex100G12</td>
<td>-4.95</td>
<td>59.1</td>
<td>-90.60</td>
</tr>
<tr>
<td>Dex100G14</td>
<td>-4.82</td>
<td>74.4</td>
<td>-86.00</td>
</tr>
<tr>
<td>Dex100G16</td>
<td>-3.12</td>
<td>24.3</td>
<td>-90.08</td>
</tr>
</tbody>
</table>

All thermograms exhibit two decomposition stages: the first mass loss, which occurs below 100°C, is attributed to water loss from the sample while the second mass loss, which occurs in the temperature range 200-350°C, is due to the degradation of the alkylglyceryl dextran structure (155, 156).

To determine the trend of water content loss across the range of materials, percentage mass losses were plotted against chain lengths.
In accord with expectation, the first mass loss (loss of water) varies with chain length, with more hydrophilic samples exhibiting correspondingly higher percentage mass loss (Figures 2.18 and 2.19): the longer the chain length, the more hydrophobic the sample and the lower its water content. Accordingly, for samples of Dex100 kDa (Figure 2.18), the plot reveals the gradual decrease in water content. A similar behaviour was observed for samples of Dex6kDa (Figure 2.19), with Dex6G8 presenting the only anomaly.
2.4.6 Discussion on synthesis and characterisation techniques

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) has been developed as a soft ionisation and as a sensitive method that allows the rapid detection of macromolecules and biopolymers (157). MALDI can generate singly or multiply charged ions ([M+nH]⁺), the prevalence of which depends on the nature of the matrix, the laser intensity and/or the voltage used. While it is generally accepted that the MS analysis of polysaccharides is of lower sensitivity than that of peptides and proteins, it has been shown that MALDI-TOF MS may be usefully applied to the measurement of the molecular mass of carbohydrates up to 10⁶ Da (158); it has been recently acknowledged however that this becomes more difficult for higher molecular weights as neutral polysaccharides show poor ionisation efficiency (154). Figure 2.15 presents a typical MALDI TOF spectrum of the modified dextran polymers considered in this study. The spectra are characterised by the lack of a molecular ion and of patterns that are characteristic of natural glycans, and a series of glycosidic ions. The observed pattern is attributed to the loss of butylglyceryl chains, which is consistent with the successful chemical modifications of dextran.

The mechanism for the attachment of alkyl glycerol chains to dextran involves the t-BuOK-mediated deprotonation of the alcohol moieties of dextran to the strongly nucleophilic butoxide ion, which in turn attacks the strained glycidyl ether ring (149).

Since the viscosity of the reaction medium, which is influenced by both the length of the alkyl chains and the concentration of t-BuOK, appeared to impact upon the fate of the reaction, preliminary experiments were conducted in order to determine optimised mixing ratios for the reactants. An attempt to reduce the viscosity of the reaction medium by increasing the amount of solvent (DMSO) by 30% did not produce the desired effect. By contrast, modulation of the ratio of t-BuOK to alkyl glycidyl ether increased the extent of the functionalisation. For optimal functionalisation, the ratio 1:3:4 (dextran: t-BuOK: alkyl glycidyl ether) was used for the shorter chain butyl- and octyl- glycidyl ethers; the corresponding ratio of 1:2:1 was selected for the modification of dextrans with lauryl glycidyl ether; the ratio of 1:1:1 was used for the reaction involving the tetradecyl glycidyl and hexadecyl glycidyl ethers.
Chemical modification of dextrans

As expected, GPC experiments showed that the higher the degree of substitution or the chain length of the sample the higher the corresponding molecular weight. All other $^1$H- and $^{13}$C-NMR signals were in good agreement with those expected on the basis of published work (159).

2.5 Conclusions
Alkylglyceryl dextrans have been synthesised by the reaction of commercially available dextrans and a range of alkyl glycidyl ethers in the presence of a strong base (t-BuOK) in anhydrous DMSO and under an atmosphere of nitrogen. The crude products were washed with organic solvent, subjected to dialysis, and freeze-dried to a final yield of 40-60% before characterisation with NMR and FTIR. NMR spectroscopic investigations showed that the degree of substitution was in the range 50 - 145%.

Thermograms of the alkylglyceryl dextrans have shown a two-step mass loss profile that corresponds to the initial loss of water and the higher-temperature thermal decomposition of the dextran core. TGA measurements revealed that the water content of each dextran and modified dextrans is influenced by the length of the hydrophobic side chain.

GPC data showed that butylglyceryl dextran (Dex6G4) has an average molecular weight that is lower than that of the 6kDa native dextran. By contrast, Dex100G16 exhibits an average molecular weight that is higher than that of the native dextran (Dex100), which is attributed to the contribution of the pendent alkylglycerol chain.

Due to extensive fragmentation of the modified dextrans and to the absence from the spectra of molecular ion peaks, MALDI-TOF MS did not prove suitable for the determination of the molecular weight of samples.
3. Preparation and characterisation of nanoparticles

The preparation of nanoparticles of poly(alkyl cyanoacrylate) was carried out by anion-propagated emulsion polymerisation and solvent-displacement nanoprecipitation, whereas the preparation of nanoparticles of PLA-alkylglyceryl dextran was by means of the dialysis method.

3.1 Mechanism of ACA polymerisation

The method of choice for the chemical synthesis of alkyl cyanoacrylates (ACA) is the Knoevenagel condensation reaction (73). ACA molecules polymerise readily at room temperature in the presence of any nucleophile, base or even trace moisture to yield PACA polymers. In solution, PACA polymers are highly susceptible to rapid degradation into species of lower molecular weight, which is witnessed as anomalous solution-viscosity behaviour (160). The low ceiling temperature of PACA has been linked to retro-Knoevenagel depolymerisation (161).

The polymerisation of ACA has been examined in depth by Ryan and McCann, who have made the following observations: (i) the ACA monomer and polymer are in rapid, dynamic equilibrium; (ii) a very high concentration of monomer relative to that of the initiator favours the production of long chains; (iii) chain-termination occurs by the anionic end-group becoming protonated by water or other acid functionalities to form a species that is not capable of carrying the reaction; (iv) the use of base effects a deprotonation of this dormant chain end, allowing the reactivation of the polymerisation reaction; (v) the monomer that is present during the equilibrium adds rapidly to any nucleophiles present to initiate new-chains formation; (vi) since the concentration of the monomer in the new equilibrium is much lower than that during polymerisation, the new chains that form have drastically lower molecular weights (implying that longer-chain formation is favoured by the distortion of the monomer to polymer ratio through the continuous addition of monomer into the polymerisation vessel); and, (vii) the addition of acid into the reaction mixture inhibits the deprotonation of the chain ends, preventing the establishment of the equilibrium (160).
The most widely used alkyl cyanoacrylate monomer is ethyl cyanoacrylate (ECA). As with other alkyl cyanoacrylates, anionic species or a Lewis base are both capable of initiating the rapid polymerisation of this monomer to give a high molecular weight polymer; amines and phosphines are adequately nucleophilic substances for the purpose. The degree of substitution in amines affects reactivity, with tertiary amines initiating an exothermic polymerisation that results in the formation of high molecular weight polymers; primary and secondary amines give polymers of lower molecular weights (162).

The high reactivity of alkyl cyanoacrylates is consequent to the electron withdrawing effect of the ester and nitrile functionalisation of the monomer unit. The effective utilisation of alkyl cyanoacrylates in nanoparticles formulation involves polymerisation, which requires a nucleophile or base for the reaction initiation; water, amines, alcohols and phosphines are employed commonly. The combination of the monomer with the initiating nucleophile results in the formation of a chain-carrying species that is stabilised by the ester and nitrile moieties, while the associate polarisation of the double bond activates further nucleophilic attack. Chain propagation occurs by the repetitive addition of electron deficient monomer to the anionic end of the growing chain and further to the biomedically useful polymer (Figure 3.1). The hydrogen bonds between ammonium and cyano groups that become established during the polymerisation of ACA coupled with van der Waals interactions represent the forces of attraction that are responsible for the cohesion of PACA nanoparticles that are important in their use as biomedical materials (163).
Dissociation of water

\[ 2\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + \text{OH}^- \]

Initiation

\[
\begin{align*}
\text{Initiation} & : \quad \begin{array}{c}
\text{O} \quad \text{R} \\
\text{H} \quad \text{CN} \\
\text{H}_2\text{O}^+ \\
\text{H} \\
\end{array} & \rightarrow \quad \begin{array}{c}
\text{CN} \\
\text{H} \quad \text{CO}_2\text{R} \\
\text{H} \quad \text{CO}_2\text{R} \\
\end{array} + \quad \text{H}_2\text{O} \\
\end{align*}
\]

Propagation

\[
\begin{align*}
\text{Propagation} & : \quad \begin{array}{c}
\text{HO} \\
\text{H} \quad \text{CN} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\end{array} & \rightarrow \quad \begin{array}{c}
\text{CN} \\
\text{H} \quad \text{CO}_2\text{R} \\
\text{H} \quad \text{CO}_2\text{R} \\
\end{array} \\
\end{align*}
\]

Chain transfer

\[
\begin{align*}
\text{Chain transfer} & : \quad \begin{array}{c}
\text{HO} \\
\text{H} \quad \text{CN} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\end{array} + \quad \text{H}_2\text{O} & \rightarrow \quad \begin{array}{c}
\text{CN} \\
\text{H} \quad \text{CO}_2\text{R} \\
\text{H} \quad \text{CO}_2\text{R} \\
\end{array} + \quad \text{OH} \\
\end{align*}
\]

Termination

\[
\begin{align*}
\text{Termination} & : \quad \begin{array}{c}
\text{HO} \\
\text{H} \quad \text{CN} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\text{H}_2 \quad \text{CO}_2\text{R} \\
\end{array} + \quad \text{H}_2\text{O} & \rightarrow \quad \begin{array}{c}
\text{CN} \\
\text{H} \quad \text{CO}_2\text{R} \\
\text{H} \quad \text{CO}_2\text{R} \\
\end{array} + \quad \text{H}_2\text{O} \\
\end{align*}
\]

Figure 3.1: Mechanism for the anionic emulsion polymerisation of ACA, as initiated by mild nucleophiles (164).

3.2 Materials and instrumentation

Ethyl-2-cyanoacrylate, dextrins (100 and 6 kDa), ethyl-2-cyano-3-ethoxyacrylate (Sigma-Aldrich), butyl cyanoacrylate (donated by Henkel Ireland) and dimethyl sulfoxide (DMSO; anhydrous, ≥ 99.9 %) were sourced from Sigma-Aldrich, UK. Dicyclohexylcarbodiimide (DCC, ≥ 99 %,) was obtained from Fluka. Poly(lactic acid) with free carboxyl group (PLA, MW 15 kDa) was obtained from Purac, Netherlands. Ultrapure water (18.2 mΩ) was obtained using a Triple Red instruments. All reagents were used as obtained. The dialysis purification step of PLA-alkylglyceryl-dextrans was performed in a discontinuous system.
Preparation and characterisation of nanoparticles

(deionised water, 15.0 mΩ, 10.0 L; exchanged 3 times/day) using Visking membrane tubing (Medicell International Ltd, UK; cut-off either at 12-14 kDa or at 3.5 kDa). Alkylglyceryl dextran of different chain lengths. Low speed centrifugation was performed by means of a Rotofix 32A Hettich (Zentrifugen-Germany). Ultracentrifugation experiments utilised a Beckman Ultracentrifugation XL-90 instrument. The Z-average diameter (nm), PDI and zeta potential (mV) were determined by Dynamic Light Scattering experiments (Malvern Zetasizer Nano instrument, Worchester, UK) using Whatman filter paper of specified pore size for filtration. Freeze drying involved the use of either a Virtis (Advanced Sentry 2.0 Controller for Virtis® Benchtop™ or a ‘K’ Freeze Dryer (SP Industries-Warminster, USA) instrument. Sonication was by means of a Grant ultrasonic bath XB3 (Farnell, UK).

TGA analysis was carried out with TG 209 F1 Libra (TGA from Netzsch Instruments), with temperature ranged from 25 to 500°C, heating at 10K/min. under Nitrogen atmosphere. Nanoparticle Tracking Analysis (NTA) was carried out at 25 °C using a Nanosight instrument that was equipped with a thermostatted LM-14 unit and with a 532 nm (green) laser. The image capture (length 60 s) was by means of a CCD Marlin camera, data were analysed using NTA 3.0 software.

The hydrodynamic diameter of the nanoparticles was determined with DLS using a Malvern Zetasizer Nano instrument, UK, equipped with a 633 nm He-Ne laser (173° back scattering angle detection; Zetasizer software v.7.01 software). Samples analysis was done in triplicate (25 °C, 2 min equilibration) using clear polycarbonate disposable cuvettes. Data are presented as Z averages (Z-av.) and the associated polydispersity index (PDI).

The autotitrations of colloidal material were performed to simultaneously determine Z-average diameter (Z-av) in (nm), and ZP (mV) as a function of pH; an MTP-2 (Multi-Purpose Titrator-2, Malvern, UK) equipped with a solvent degasser was employed. The sample (10 mL) was titrated automatically against aqueous NaOH solutions (5 mM or 50 mM) and against aqueous HCl solutions (5 mM or 50 mM). Prior to each experiment, solutions were filtered through 0.2 μm PES filters (Whatman).
3.3 Methods

3.3.1 Attempted NP formulation with ethyl 2-cyano-3-ethoxyacrylate
Since there are few reports regarding the capability of ethyl 2-cyano-3-ethoxyacrylate (Figure 3.2) to undergo co-polymerisation to nanoparticulate structures, an attempt was made to formulate nanoparticles by means of an anionic emulsion polymerisation protocol (24, 93). To this end, ethyl 2-cyano-3-ethoxyacrylate (1 g) was dispersed in acetone/water (1:1 v/v; 1 mL) and added dropwise to a solution of dextran (100 kDa; 1% w/v, 100 mL) in KH₂PO₄ buffer (pH 2.5). The mixture was allowed to stir overnight (room temperature), after which time it was neutralized with NaOH solution (1 N), filtered through 0.8 µm filter paper and centrifuged (90000 g, 1 h).

![Figure 3.2: Structure of ethyl 2-cyano-3-ethoxyacrylate](image)

Since no solid product could be isolated, attempts were made to repeat the experiment at increased pH, in water-free acetone and in the absence of phosphate buffer. All these attempts failed to produce polymeric structures, suggesting that this molecule is not amenable to polymerisation under the selected experimental conditions. Hence, ethyl 2-cyano-3-ethoxyacrylate was excluded as a co-monomer in further nanoparticle-formation experiments, limiting the choice of cyanoacrylates to the ethyl and butyl homologues.

3.3.1.1 Preparation of PECA-Dex100 nanoparticles
Method: PECA-Dex100 was prepared using a standard literature method (24,93). Ethyl 2-cyanoacrylate (1 mL) was added dropwise into dextran solution (1% (w/v), 100 mL, pH 2.5; 100 KDa) and the solution was allowed to stir at room temperature overnight. After this time, the mixture was neutralised with NaOH solution (1 N), centrifuged (Rotofix; 3500 rpm, 20 min), filtered (0.8 µm Whatman filter paper), isolated by ultracentrifugation (18000 g, 30 min; Beckman ultracentrifuge), rinsed with deionised water and re-suspended in deionised water (5 mL; sonication in water bath, 10 min).
size and zeta potential of the thus suspended nanoparticles was determined using a Malvern instrument before the water was removed (freeze dryer, 24 h). The synthesis of PECA repeated in phosphate buffer medium resulted in the formation of NPs that were characterised by poor yield, low zeta potential and high conductivity.

### 3.3.2 Preparation of poly (ethyl 2-cyanoacrylate)–alkylglyceryl-dextran NPs

**Method 1** (24, 93): Ethyl 2-cyanoacrylate (ECA) (600 µL) was added dropwise to alkylglyceryl-dextran (DexnGm; 100 mg; n=6 or 100; m=4, 8, 12, 16) dispersed in water (100mL; pH 2.5, HCl) and stirred at room temperature for 4 h (Figure 3.3). After this time the solution was neutralised with NaOH solution (1 N), centrifuged for 15 min (3000 rpm; Rotofix) and further ultra-centrifuged for 30 min at 12,200 rpm (18000 g; Beckman ultracentrifuge). The isolated sample (pellet) was rinsed, dispersed in water and sonicated for 10 min before freeze drying to a batch-dependent yield in the range 20-40%.

![Figure 3.3: Schematic representation of the protocol for the synthesis and characterisation of PACA-alkylglyceryl dextran.](image-url)
**Method 2** (69): ECA (600 µL) was dissolved in acetone (100 mL) and gradually added (room temperature) to a stirring solution of alkylglyceryl dextran (DexnGm; 100 mg) in water (100 mL); stirring was continued overnight at room temperature to allow the evaporation of most of the acetone. Following centrifugation at 40000 rpm for 30 min, the pellet was rinsed, re-suspended in water and subjected to sonication for 10 min before isolation by freeze drying to a batch-dependent yield in the range 50-70%.

**Method 3** (24): ECA (1 mL) was added dropwise to DexnGm (1% w/v; pH 2.5, HCl) and was allowed to stir for a minimum of 4 h at room temperature, before neutralisation with NaOH solution (1 N), filtering (P3 glass filter), and isolation by centrifugation (12200 rpm, 30 min). The crude product was rinsed, dispersed in water (10 mL), sonicated (10 min) and freeze dried to a yield of 30-40%. SEM image of PECA-Dex100G4 is in Appendix 4.

### 3.3 Preparation of PLA15-Dex100G8-PECA

Alkylglyceryl dextran, PLA and ECA were all incorporated into a single multi-block structure through the simple polymerisation procedure of nanoprecipitation solvent displacement.

Dex100G8 (0.05 g) was dispersed (by sonication) into an acetone (50 mL) solution of ECA (0.265 g) and PLA (0.265 g). This mixture was then added dropwise in water (100 mL; 18.2 mΩ) under continuous stirring. After overnight stirring at room temperature, the mixture was dialysed in a discontinuous manner (10 L container; 3x a day for 2 days), filtered (Whatman paper, 90 mm; 1 qualitative circle) placed over sintered glass (P3) and the filtrate was freeze dried before being subjected to characterisation by DLS (Table 3.8), TGA (Figure 3.18), DSC (Figure 3.19) and $^1$H-NMR (Figure 3.17).

### 3.3.4 Effect of filtration on size of PECA-Dex100G8 NPs

ECA (100 mg) in acetone (100 mL) was gradually added to stirring Dex100G8 (100 mg) that had been dispersed in water (100 mL) and was allowed to stir at room temperature overnight. After this time, NaOH solution (2 mL, 0.1 N) was added to the resulting mixture and allowed to stir for a further 30 min before filtration over sintered glass (P3), centrifugation (40000 rpm, 30 min), rinsed and re-dispersion in water (10 mL). Following filtration (yield, 15-25%) the isolated product was characterised using DLS (Table 3.3) and NMR (Figure 3.5).
3.3.5 Preparation of PECA-Dex6G16 nanoparticles by nanoprecipitation

ECA (600 µL) in acetone (100 mL) was added gradually to stirring Dex6G16 (100 mg) in water (100 mL; 18.2 mΩ). Stirring was continued overnight (room temperature) after which time the product was isolated by centrifugation (12200 rpm, 30 min), rinsed with deionised water, dispersed in water (10 mL) and sonicated (ultrasonic bath).

3.3.6 Preparation of PLA-derived nanoparticles by zero length crosslinking

Three different alkylglyceryl dextrans were employed for the preparation of PLA-alkylglyceryl dextran derived nanoparticles (Figure 3.4; Scheme 1) by zero length grafting of PLA to alkylglyceryl dextran with N,N’-dicyclohexylcarbodiimide (DCC) (165, 166) and DMAP. PLA15 respectively grafted onto Dex100G4, Dex100G8 and Dex6G16 produced PLA15-Dex100G4, PLA15-Dex100G8 and PLA15-Dex6G16.

The apparatus was dried (vacuum) and flushed with Nitrogen before use. DCC (0.045 g, 0.2 mmol) dissolved in anhydrous DMSO (10 mL) was added with stirring to a solution of PLA (1.75 g, 0.11 mmol) in anhydrous DMSO (100 mL) contained within a round-bottomed flask that had been equipped with a Nitrogen inlet tube. After 30 min, the solution was transferred into a stirring solution (Nitrogen atmosphere) of octylglyceryl-dextran (Dex100G8, 0.85 g) and 4-(dimethylamino)-pyridine (DMAP; 0.026 g, 0.22 mmol) in anhydrous DMSO (100 mL) that had been stirred for an hour. The reaction mixture was allowed to stand at room temperature for 24 h, after which time it was filtered through Whatman paper over P3 sintered glass (to remove dicyclourea (a byproduct of the reaction) and dialyzed for 48 h (MWCO 12-14 kDa) against deionised water, before freeze drying. The dried powder was dispersed in acetone (50 mL), filtered through Whatman filter paper (to remove unreacted PLA) and the isolated pellet was dried under vacuum at room temperature. The thus dried pellet was washed with water, dispersed in deionised water (100 mL) and freeze dried to yield (20-30%) of the product. This was used to purify PLA15Dex100G8.

Purification (method 2): The preparation of PLA15-Dex100G16 and PLA15Dex100G4 nanoparticles followed the protocol presented in Figure 3.4. The zero-length grafting (165, 166) of PLA to alkylglyceryl dextran was carried out with N,N’-dicyclohexylcarbodiimide (DCC). After dialysis and freeze drying, the powder was dispersed in acetone (100 mL),
spun at 22,000 rpm for 30 min (to remove unreacted PLA) and the pellet was dried overnight under vacuum. The dried pellet was suspended in deionised water (300 mL) and placed on a shaking platform overnight (room temperature) before freeze drying to yield a pellet of the material labelled PLA15-Dex100G16. To the supernatant (acetone solution containing mostly unreacted fraction of PLA) was added water (500 mL) to precipitate any unreacted PLA, which was removed by filtration. The water-acetone mixture was allowed to stir gently at room temperature for 48 h (to remove excess acetone), and the solid mass that formed (yield 50-70%) was labelled PLA15-Dex100G16-acetone fraction.

Figure 3.4 scheme for the synthesis of PLA-alkylglyceryl dextran nanoparticles

3.3.7 Preparation of PBCA-Dex100G4 nanoparticles
BCA (200 mg) was added gradually into a stirred suspension of Dex100G4 (200 mg) in water (100 mL) and the mixture was allowed to stir for a further 4 h at room temperature. After this time, the mixture was neutralised with NaOH solution (1 N), filtered through sintered glass, centrifuged (40000 rpm, 30 min) and the nanoparticles that separated were rinsed with water, sonicated, dispersed in water (20 mL) and freeze dried to a yield of 26%. This product was characterised with DLS and NTA.

3.3.8 Degradation study
The esterase-induced study of the degradation of PECA-Dex100G4 was conducted according to a standard literature method (167, 168): ECA (2.5 g, 2.36 mL) was added
dropwise to water (250 mL; 18.2 mΩ, pH 3) that contained Dex100G4 (250 mg; 0.1% w/v) and maintained at 25°C (water bath) and stirred for 3 h. After this time, specified concentrations (1-5 mg/mL) of an esterase solution in phosphate buffer (pH7) were added to each NPs suspension (3.5 mL) contained in 30 mL of Ringer’s solution. The resulting mixtures were incubated at 37°C under shaking (50 cycles/min). Aliquots were withdrawn after 0.5 h, 1 h, 2 h, 3 h and 4 h and size and PDI were determined using DLS. The same procedure was repeated with samples at a constant esterase concentration of 2 mg/mL and also with an esterase-free mixture (which served as control).

The effect of increased enzyme concentration on the degradation pattern of PECA-Dex100G4

The methods adopted for the formulation of nanoparticles and for the study of their degradation were identical to those described previously (167, 168); with the only variations for the latter study being the duration of the experiment, which was prolonged and the concentration of enzyme that was increased (Table 3.1). A mass of 31 mg of PECA-Dex100G4 contained in a 3.5 mL volume of each suspended population of nanoparticles was used as substrate for the degradation study. A stock solution of the enzyme (180 mg in 15 mL; equivalent to 12 mg/mL) was made and distributed amongst the samples under test as indicated in Table 3.1. Samples were incubated at 37°C (Grant OLS 200 orbital/linear shaking water bath) under shaking (50 rpm/min); the extraction of aliquots at premeditated time points allowed the measurement of size and the determination of PDI by DLS.
Table 3.1: protocol for the degradation study of PECA-Dex100G4 NPs

<table>
<thead>
<tr>
<th>sample</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme (mg)</td>
<td>-</td>
<td>7.06</td>
<td>12.94</td>
<td>24.71</td>
<td>36.47</td>
<td>71.75</td>
</tr>
<tr>
<td>Units of Enzyme</td>
<td>-</td>
<td>120</td>
<td>220</td>
<td>420</td>
<td>620</td>
<td>1220</td>
</tr>
<tr>
<td>Enzyme Vol. (mL; conc. 12mg/mL)</td>
<td>-</td>
<td>0.588</td>
<td>1.078</td>
<td>2.059</td>
<td>3.04</td>
<td>5.98</td>
</tr>
<tr>
<td>Ringer’s Solution (mL)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Nanoparticles (mL)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

3.4 Results and discussion

3.4.1 Preparation of nanoparticles of ethyl 2-cyanoacrylate

3.4.1.1 Preparation of PECA-Dex100 NPs
The yield of PECA-Dex100 NPs (2.41%), was very poor as compared with that reported by Han et al. (108) who, using a similar method, reported a yield of over 93%. Three batches of NPs were prepared, all of which produced nanoparticles of reproducible size in the Z-average diameter range 250-400 nm and PDI of 0.364.

3.4.1.2 Preparation of poly (ethyl 2-cyanoacrylate)–alkylglyceryl-dextran NPs

3.4.1.2.1 Preparation of PECA-Dex6G16 NPs by nanoprecipitation
This method combines the emulsion polymerisation, solvent evaporation and nanoprecipitation methods into a one-pot synthesis. As the acetone evaporates the acetone-soluble ECA is allowed to react via an anionic mechanism leading to instantaneous nanoprecipitation. The presence of acetone appears to play an important role in regulating the size and size distribution of the formed nanoparticles (Table 3.2), which is very small (153.3 nm) as compared with that of nanoparticles obtained from conventional anionic polymerisation.
Table 3.2: DLS Characterisation PECA-Dex6G16 nanoparticles (n=1)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Av. diameter</td>
<td>153.3±0.4 nm</td>
</tr>
<tr>
<td>PDI</td>
<td>0.132±0.006</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-27.6±0.7 mV</td>
</tr>
</tbody>
</table>

3.4.1.2.2 Effect of filtration on size of PECA-Dex100G8 NPs

A study was conducted in an effort to evaluate the effect of filtration on the size, PDI and zeta potential of the nanoprecipitation-solvent-displacement-method formulated PECA-Dex100G8; nanoparticles were characterised by DLS (Table 3.3) and NMR (Figure 3.5).

To evaluate the effect of filtration, poly(ethersulfone) (PES) filter membrane (0.45 µm) was used for the filtering of a portion of the dispersed sample before DLS analysis (Table 3.3) – no significant differences were observed between the filtered and unfiltered samples.

Table 3.3: Effect of filtration on NPs size, PDI and zeta potential (±SD; n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Un-filtered</th>
<th>Filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (Z-av.d)nm</td>
<td>120.94±30.57</td>
<td>110.77±29.12</td>
</tr>
<tr>
<td>PDI</td>
<td>0.22±0.02</td>
<td>0.18±0.00</td>
</tr>
<tr>
<td>Zeta (mV)</td>
<td>-38.37±1.12</td>
<td>-31.39±0.90</td>
</tr>
</tbody>
</table>
Figure 3.5: $^1$H NMR spectrum of PECADex100G8 nanoparticles

NMR spectrum PECA-Dex100G8 (Figure 3.5) shows that at least two of the characteristic resonances of PECA are well defined, namely those at 1.3 ppm and at 4.2 ppm. The spectral features of alkylglyceryl dextran are less pronounced (Figure 3.5), as is consistent with the high ratio of PECA to alkylglyceryl dextran. This confirms data from elemental analysis determinations, which estimated the ratio of PECA to alkylglyceryl dextran at $ca$. 90:10. The $^1$H- and $^{13}$C-NMR spectra of pure PECA are respectively presented in Figures 3.6 and 3.7.
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Figure 3.6: $^1$H-NMR spectrum of pure PECA

Figure 3.7: $^{13}$C-NMR spectrum of pure PECA
3.4.2 Elemental analysis results
The method for the formation of nanoparticles appeared to be of little influence on the elemental composition and hence to the ratio of PECA to alkylglyceryl dextran in the nanoparticulate formulation. The nanoparticles evaluated for elemental composition and ratio of PECA to alkylglyceryl dextran had been formulated by: (i) anionic emulsion polymerisation (samples 1-8); or (ii) the nanoprecipitation-solvent-diffusion method (samples 9-10; Table 3.4).

Elemental analysis enabled the determination of the ratio of PECA to alkylglyceryl-dextran in complex nanoparticles (Table 3.4). The percentage ratio of alkylglyceryl dextran and PECA of the polymer obtained in the experiment was deduced by considering their percentage composition of carbon, nitrogen, oxygen, and hydrogen following the equation presented in Appendix 11. Experimental elemental compositions were in agreement with theoretically calculated values. Across the range of synthesised nanoparticles, PECA was the higher percentage constitutional unit (over 90% in some samples) while alkylglyceryl-dextran accounted for a small percentage (3.9% to 19.62%) of the structural component of nanoparticles (Table 3.4).

Elemental analysis reported for the respective composition percentage weight per weight (w/w) of dextran and poly (isobutyl cyanoacrylate), PIBCA, in a polymer obtained from the emulsion polymerisation of isobutyl cyanoacrylate (IBCA) is 22% and 78% (169), which is consistent with current findings in that the ratio of dextran to alkyl cyanoacrylate is consistently low. This observation is in agreement with that from comparative (alkylglyceryl-dextran to PECA) NMR experiments which were characterised by the low intensity of signals from alkylglyceryl-dextran moieties as compared with those from alkyl cyanoacrylate groups (Figure 3.5). Since it has been observed that sequential washing and centrifugation of nanoparticles from dextran and BCA reduces the PBCA:dextran ratio to 9:1, it may be reasonably assumed that a significant amount of dextran is simply physisorbed onto the nanoparticles (170).
### Table 3.4: Percentage elemental composition of PECA-alkylglyceryl dextran NPs (experimentally found and calculated)

<table>
<thead>
<tr>
<th>S/No</th>
<th>Material</th>
<th>g</th>
<th>%DS</th>
<th>X</th>
<th>%PECA (w)</th>
<th>%Dex-G (w)</th>
<th>Found (av%C)</th>
<th>Cal. C.%</th>
<th>Found (av%H)</th>
<th>Cal. (H%)</th>
<th>Found (av%N)</th>
<th>Cal.(%N)</th>
<th>av%O(100-CHN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PECA Dex6G4</td>
<td>4</td>
<td>87.7</td>
<td>26.29</td>
<td>92.25</td>
<td>7.75</td>
<td>55.94</td>
<td>57.22</td>
<td>5.77</td>
<td>5.83</td>
<td>10.33</td>
<td>10.33</td>
<td>27.96</td>
</tr>
<tr>
<td>2</td>
<td>PECA Dex100G4</td>
<td>4</td>
<td>68.49</td>
<td>26.66</td>
<td>92.99</td>
<td>7.01</td>
<td>56.07</td>
<td>57.17</td>
<td>5.81</td>
<td>5.79</td>
<td>10.41</td>
<td>10.41</td>
<td>27.71</td>
</tr>
<tr>
<td>3</td>
<td>PECA Dex6G8</td>
<td>8</td>
<td>114.94</td>
<td>10.21</td>
<td>80.38</td>
<td>19.62</td>
<td>56.31</td>
<td>58.03</td>
<td>6.34</td>
<td>6.51</td>
<td>8.65</td>
<td>8.65</td>
<td>28.70</td>
</tr>
<tr>
<td>4</td>
<td>PECA Dex100G8</td>
<td>8</td>
<td>64.91</td>
<td>46.35</td>
<td>95.92</td>
<td>4.079</td>
<td>56.22</td>
<td>57.51</td>
<td>5.73</td>
<td>5.78</td>
<td>10.67</td>
<td>10.67</td>
<td>27.38</td>
</tr>
<tr>
<td>5</td>
<td>PECA Dex6G12</td>
<td>12</td>
<td>54.35</td>
<td>31.04</td>
<td>94.34</td>
<td>5.66</td>
<td>56.31</td>
<td>57.61</td>
<td>5.89</td>
<td>5.88</td>
<td>10.41</td>
<td>10.41</td>
<td>27.39</td>
</tr>
<tr>
<td>6</td>
<td>PECA Dex100G12</td>
<td>12</td>
<td>67.56</td>
<td>49.07</td>
<td>96.09</td>
<td>3.91</td>
<td>56.35</td>
<td>57.69</td>
<td>5.82</td>
<td>5.83</td>
<td>10.63</td>
<td>10.63</td>
<td>27.19</td>
</tr>
<tr>
<td>7</td>
<td>PECA Dex6G16</td>
<td>16</td>
<td>133.33</td>
<td>19.84</td>
<td>88.09</td>
<td>11.91</td>
<td>56.95</td>
<td>59.36</td>
<td>6.45</td>
<td>6.61</td>
<td>9.13</td>
<td>9.133</td>
<td>27.47</td>
</tr>
<tr>
<td>8</td>
<td>PECA Dex100G16</td>
<td>16</td>
<td>144.92</td>
<td>17.32</td>
<td>86.07</td>
<td>13.93</td>
<td>57.22</td>
<td>59.78</td>
<td>6.58</td>
<td>6.80</td>
<td>8.78</td>
<td>8.78</td>
<td>27.42</td>
</tr>
<tr>
<td>9</td>
<td>PECA Dex10G4A</td>
<td>4</td>
<td>68.49</td>
<td>36.92</td>
<td>94.84</td>
<td>5.159</td>
<td>56.81</td>
<td>57.29</td>
<td>5.67</td>
<td>5.75</td>
<td>10.62</td>
<td>10.62</td>
<td>26.90</td>
</tr>
<tr>
<td>10</td>
<td>PECA Dex100G16A</td>
<td>16</td>
<td>144.92</td>
<td>18.85</td>
<td>87.05</td>
<td>12.95</td>
<td>57.99</td>
<td>59.63</td>
<td>6.35</td>
<td>6.72</td>
<td>8.94</td>
<td>8.94</td>
<td>26.73</td>
</tr>
</tbody>
</table>

**g** = no of C atoms in the alkyl group present in the pendent chain (in substituted dextran)

**%DS** = the degree of substitution of dextran with pendent chains (as calculated by NMR)

**X** = molar ratio (ECA monomer : dextran glucopyranose units)

**ECA**  
**Dex**  
**G**

\[(\text{C}(g+3)\text{H}(2g+6)\text{O}2)\]  
(equations in Appendix 11)
For the $^1$H-NMR determination of the percentage degree of substitution (％DS) of modified dextran used for nanoparticles formulation, the ratio of the intensity of the resonance integral of the terminal methyl of the alkylglycerol chain (A) was divided by three-times (corresponding to the ratio of attaching protons) the intensity of the integral of the signal due to the anomeric proton of the pyranose ring of the glucose unit of dextran (equation 3.1). As expressed, ％DS is defined as the number alkylglycerol chains attaching per 100 glucose units of dextran (171) normalised with reference to the maximum theoretical percentage of 300 for the fully –OH substituted pyranose groups structure.

$$DS\% = \frac{A}{3B} \times 100 \quad (eq. \ 3.1)$$

### 3.4.3 MALDI-TOF-MS of PECA-alkylglyceryl dextran NPs

![MALDI-TOF spectrum of PECADex6G4](image)

PECA-Dex6G4 NPs Peak to peak mass differences between neighbouring peaks was ca. 130 Da, which may be explained in terms of the mass loss corresponding to the PECA
Preparation and characterisation of nanoparticles

fragment. The spectrum exhibits the characteristic features of carbon-oxygen bond fragmentation (Figure 3.8).

![MALDI TOF spectrum of PECA-Dex6G12](image)

**Figure 3.9: MALDI TOF spectrum of PECA-Dex6G12**

PECA-Dex6G12 NPs characteristic peak-to-peak mass differences was *ca.* 125 Da, indicating that PECA was eliminated by means of carbon-oxygen bond fragmentation (figure 3.9). Other spectra were appended in (Appendix 8).

The most characteristic fragment mass loss was that of PECA, which is consistent with the 80-90% proportion of PECA that was calculated from elemental analysis data. This large percentage of PECA structural units in nanoparticle structures may account for the mass loss of 125 Da observed for most nanoparticles except those from PECA-Dex100G4 and PECA-Dex6G4 that had peak differences of 130 Da (Table 3.5) and those from PECADex6G8 (Table 3.5) which did not have consistent characteristic of 125 Da peak-to-peak fragment mass difference (172).
Table 3.5: m/z fragment mass loss of PECA-alkylglyceryl dextran NPs

<table>
<thead>
<tr>
<th>sample</th>
<th>m/z fragment mass</th>
<th>Nature of fragment loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECA-Dex6G4</td>
<td>130.1,114.2</td>
<td>C_7H_{14}O_2</td>
</tr>
<tr>
<td>PECA-Dex100G4</td>
<td>130.2</td>
<td>C_7H_{14}O_2</td>
</tr>
<tr>
<td>PECA-Dex6G8</td>
<td>125.1</td>
<td>C_6H_{11}NO_2</td>
</tr>
<tr>
<td>PECA-Dex100G8</td>
<td>125.1</td>
<td>C_6H_{11}NO_2</td>
</tr>
<tr>
<td>PECA-Dex6G12</td>
<td>125.1,16</td>
<td>C_6H_{11}NO_2</td>
</tr>
<tr>
<td>PECA-Dex100G12</td>
<td>125.1</td>
<td>C_6H_{11}NO_2</td>
</tr>
<tr>
<td>PECA-Dex6G16</td>
<td>125.1</td>
<td>C_6H_{11}NO_2</td>
</tr>
<tr>
<td>PECA-Dex100G16</td>
<td>125.1</td>
<td>C_6H_{11}NO_2</td>
</tr>
</tbody>
</table>

3.4.4 Autotitration of PECA-alkylglyceryl dextran nanoparticles

To evaluate the stability of the nanoparticles, there was carried out an automatic pH titration of PECA and PECA-alkylglyceryl nanoparticles (Figures 3.10-3.14) against aqueous HCl (0.005/0.05M). Data show that the zeta potential of PECA nanoparticles and PECA-alkylglyceryl dextran nanoparticles decreases with decreasing pH.

PECA-alkylglyceryl dextran nanoparticles (PECA-Dex6G4 (1:1), PECA-Dex6G4 (1:6), PECA-Dex6G16 (1:1), PECA-Dex6G16 (1:6); respectively presented in Figures 3.11; 3.12, 3.13 and 3.14) and PECA nanoparticles (Figure 3.10) were all assessed for their stability at different pH environments. The Z-averaged diameter (nm) and zeta potential (mV) were determined (MPT-2 Malvern instruments) as a function of pH over the range of ca. pH 3-8 using HCl solutions as titrants (0.005 M and 0.05 M). Protonation/deprotonation processes may explain the slight variations in the size of nanoparticles under different pH conditions.

The observed pH-induced decreases in the size of PECA-NPs (Figure 3.10) and PECA-Dex6G4 (1:1) NPs (Figure 3.11) are not statistically significant (p<0.05, ANOVA; Table 3.6). Similarly, the average sizes of PECA-Dex6G4 (1:6) NPs (Figure 3.12), PECA-Dex6G16 (1:1) NPs (Figure 3.13) and PECA-Dex6G16 (1:6) NPs (Figure 3.14) were all little influenced by pH. The pH-induced differences in the measured zeta potentials of PECA NPs (Figure 3.10) and PECA-Dex6G4 (1:1) were not statistically significant (p<0.05) but those of other NPs exhibited considerable variation (Table 3.6).
Figure 3.10: Effect of pH on PECA-nanoparticles (0.25mg/mL) MPT-2 Titration-[0.005M/0.5M HCl] (n=2, ±SD)

Figure 3.11: Effect of pH on PECA-Dex6G4 (1:1) nanoparticles (0.25mg/mL); MPT-2 Titration (0.005/0.5M HCl; n=3 ±SD)
Figure 3.12: Effect of pH on the characteristics of PECA-Dex6G4(1:6) nanoparticles (0.25mg/mL); MPT-2 Titration (0.005/0.5M HCl; n=3 ±SD).

Figure 3.13: Effect of pH on PECA-Dex6G16(1:1) nanoparticles (0.25mg/mL); MPT-2 Titration (0.005/0.5M HCl; n=3 ±SD).
Figure 3.14: Effect of pH on PECA-Dex6G16 (1:6) nanoparticles (0.25mg/mL); MPT-2 Titration (0.005/0.5M HCl; n=3 ±SD).

Table 3.6: Comparison of effects of pH on the size and zeta potential of titrated nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANOVA (p)</td>
<td></td>
</tr>
<tr>
<td>1. PECA</td>
<td>0.262</td>
<td>0.193</td>
</tr>
<tr>
<td>2. PECA-Dex6G4(1:6)</td>
<td>0.785</td>
<td>0.022</td>
</tr>
<tr>
<td>3. PECA-Dex6G4(1:1)</td>
<td>0.056</td>
<td>0.184</td>
</tr>
<tr>
<td>4. PECA-Dex6G16(1:1)</td>
<td>0.685</td>
<td>0.001</td>
</tr>
<tr>
<td>5. PECA-Dex6G16(1:6)</td>
<td>0.158</td>
<td>0.000</td>
</tr>
</tbody>
</table>

(p < 0.05 significant)
3.4.5 Preparation of PBCA-Dex100G4 nanoparticles

PBCA-Dex100G4 nanoparticles were characterised for size, PDI and zeta potential with DLS and NTA (Table 3.7), NTA (Figure 3.15) and further with FTIR (Figure 3.16). The functional group of interest were noted as: C=O stretch at 1743.88 cm\(^{-1}\); C–O stretches in the range 1300 - 1000 cm\(^{-1}\); hydrocarbon functionalities CH, CH\(_2\) and CH\(_3\), respectively at 2954, 1450 and 1374 cm\(^{-1}\); OH absorption at 3500–3200 cm\(^{-1}\) (168); and CN band at 2242 cm\(^{-1}\) (173).

Table 3.7: Size, PDI and zeta potential of PBCA-Dex100G4 NPs (n=1)

<table>
<thead>
<tr>
<th></th>
<th>DLS</th>
<th>NTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-av.diameter(nm)</td>
<td>149.8±1.2</td>
<td>120.3±35.5</td>
</tr>
<tr>
<td>PDI</td>
<td>0.394±0.018</td>
<td></td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-47.8±2.0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.15: Size concentration intensity distribution of PBCA-dex100G4 NPs
Preparation and characterisation of nanoparticles

3.4.6 Preparation of PLA15-Dex100G8-PECA
Alkylglyceryl dextran, PLA and ECA were all incorporated into a single multi-block structure through the simple polymerisation procedure of nanoprecipitation solvent displacement.

PLA-Dex100G8-PECA was prepared (n=3) in nanoparticulate form with narrow size distribution (Table 3.8).

Table 3.8: Size and PDI of PLA15-Dex100G8-PECA NPs (±SD, n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-av.diameter(nm)</td>
<td>143.13±1.70</td>
</tr>
<tr>
<td>PDI</td>
<td>0.19±0.10</td>
</tr>
</tbody>
</table>

The combination of alkylglyceryl dextran, PLA and ECA was used in an attempt to formulate PLA15-Dex100G8-PECA nanoparticles that are both stable in suspension and flexible. ECA was employed as the crosslinker that connects covalently the polymer chains. Since available OH groups in both PLA and alkylglyceryl dextran could serve as the
initiating site for the polymerisation of ECA, the covalent bond formed by PECA served as a link between the alkylglyceryl dextran chain on one side and the PLA on the other. Other intermolecular forces (H-bond and van der Waals interactions) are however assumed to be of significance in maintaining the nanoparticulate structure in suspension.

The NMR spectrum of PLA15Dex100G8PECA (Figure 3.17) shows the characteristic methyl and methylene resonances of PLA at 1.45 ppm and 5.19 ppm, respectively; the 1.2 ppm signal in the spectrum of PECA is attributed to methyl protons while that at 4.2 ppm is assigned to the methylene proton of PECA. The anomic proton of the pyranose ring of dextran is manifested at 4.67 ppm. Protons corresponding to the alkylglyceryl chain methyl group are observed at 0.87 ppm.

![NMR spectrum of PLA15-Dex100G8-PECA](image)

Figure 3.17: \(^1\)H-NMR spectrum of PLA15-Dex100G8-PECA (400 MHz).

### 3.4.7 TGA and DSC thermograms of PLA-Dex100G8-PECA

Figure 3.18 shows the TGA plots of a sample of PLA-Dex100G8-PECA which had been heated to 600°C under a nitrogen atmosphere and then subjected to an oxidative treatment (synthetic air) to 1000 °C.
In the temperature range up to 600 °C there are at least three mass loss steps. The major mass loss (51.4%) occurs at a rate (peak in DTG curve) at 168.6 °C. This step is marked by a shoulder (at 192.4°C) in the DTG signal, which indicates the complexity of the mechanism of degradation. The second step is associated with a mass loss of 42.4% and is characterised by a rate maximum at 333.3 °C. The final (third) step exhibits a mass loss of 5.5 %. Exposure of the sample to synthetic air causes a further mass loss of 1.2%.

**DSC results for the sample of PLA-Dex100G8-PECA.**

In (Figure 3.19) the sample was heated from -100 °C up to 100 °C at a heating rate of 10 K/min. Indicative of a glass transition, the first heating cycle (blue curve) identifies a thermal event at 54.2 °C (midpoint) which is accompanied by a change in specific heat capacity of 0.444 J/g*K. The shift in heat capacity is partially overlapped by a relaxation event that is centred at 60.1 °C. The same plot shows a small endothermic effect, which is most likely associated with the onset of evaporation or that of oxidative degradation of some sample components.

The second heating cycle (green curve) confirms the glass transition temperature of the sample at 49.6 °C, with a change in the specific heat capacity of 0.232 J/(g*K).
endothermic relaxation effect observed during the first heating was reproduced during the second heating run.

**Figure 3.19:** DSC of PLA-Dex100G8-PECA NPs

### 3.4.8 Preparation of PLA-derived nanoparticles by zero length crosslinking

**Figure 3.20:** $^1$H-NMR spectrum of PLA15-Dex100G8 nanoparticles.
Figure 3.21: H/H-COSY NMR of PLA15-Dex100G8 nanoparticles.

Figure 3.22: $^{13}$C-NMR of PLA15-Dex100G8 nanoparticles
The grafting of PLA to alkylglyceryl dextran (PLA-Dex100G8) has been successful in that it yielded nanoparticles that are readily dispersible in water. The nanoparticles were characterised with NMR spectroscopy, $^1$H (Figure 3.20), H/H-COSY (Figure 3.21) and $^{13}$C-NMR (Figure 3.22). The mixing of alkylglyceryl dextran and PLA in the 1:2 ratio results in insufficient crosslinking as witnessed by the significant amounts of unreacted PLA that are removed during purification. Consequently the mixing of these materials in the 1:1 ratio, which will result in considerably lower loses of PLA during purification, was suggested for subsequent reactions.

PLA15-Dex100G16(or4) nanoparticles were synthesised successfully, but their purification required several steps (166) and was conducted in a way that two fractions of the material were obtained. While both fractions of nanoparticles exhibited the characteristic $^{13}$C- and $^1$H-NMR resonances of glyceryl-dextran and PLA, it was the $^1$H NMR spectra of the acetone fraction that showed PLA and alkylglycerol chain resonances that were of notably higher intensity than that of dextran, as is desirable. The acetone fraction was associated with the dominant yield of 50-70%.

The chemical shifts of $^{13}$C-NMR and $^1$H-NMR of dextran were in agreement with published data (159, 172, 174-177), with characteristic features in their ranges 65 ppm to 99 ppm for $^{13}$C NMR, and 3 ppm to 5.2 ppm for $^1$H NMR. Slight variations in the observed chemical shifts are attributed to solvent effects (178). The chemical shifts of PLA both in $^{13}$C- and $^1$H-NMR were consistent with the structure of PLA, exhibiting $^{13}$C-NMR multiplets at 169.53-169.75 ppm (–CO), 69.23 ppm (–CH), and 17.00 ppm (–CH$_3$), and $^1$H-NMR resonances at 5.19 ppm (CH) and 1.46 ppm (CH$_3$); the observed chemical shifts correlate well with published data (179, 180).

3.5 Enzymatic degradation of PECA-alkylglyceryl dextran nanoparticles
The degradation study of PECA-Dex100G4 nanoparticles, along with that of a control sample, was conducted at constant enzyme concentration and variable enzyme concentration. The use of heat to stop the degradation process resulted in the formation of cloudy solutions, which could not be utilised effectively for further analysis.
The measurement of size and PDI by DLS did not reveal any statistically significant differences in the degradation behaviour of control and enzyme-containing PECA-Dex100G4 nanoparticles (Figure 3.23, 3.24 and 3.25). It is possible that the amount or activity of the enzyme utilised was not sufficient to bring about the significant degradation of the nanoparticles within the timescale of the experimental protocol, especially since the degradation of PECA-Dex100G4 NPs was found to be dependent on both time and enzyme concentration: the longer the time of the experiment or the higher the concentration of the enzyme, the faster the degradation. Notably, PECA-nanoparticles are known to be susceptible to surface degradation (181).

Figure 3.23: Effect of time on size and PDI of PECA-Dex100G4 nanoparticles (n=3, ±SD; control, no enzyme)
Figure 3.24: Effect of constant enzyme concentration on size and PDI of PECA-Dex100G4 nanoparticles (n=3, ±SD).

Figure 3.25: Effect of enzyme concentrations on size and PDI of PECA-Dex100G4 nanoparticles (n=3, ±SD).
Preparation and characterisation of nanoparticles

The effect of increased enzyme concentration on the degradation pattern of PECA-Dex100G4 NPs

Figure 3.26: Enzyme-mediated degradation of PECA-Dex100G4 nanoparticles during the first 4h of the extended study (0, 1 and 4 h time points) for different enzymatic activity (A= 0 units; B= 120 Units; C= 220 Units; D= 420 units; E=620 units; F=1220 units).

Consideration of the data for the first 4 h of the 84 h degradation study, for all the sample sets there was an apparent decrease in size during the first hour of the experiment (Figure 3.26), and also between aliquots of A, B and C sampled at the first- and fourth-hour periods; this is in agreement with published data on PBCA nanoparticles (168). Aliquots of D, E and F sampled at four hours from the onset of the experiment exhibited increased sizes relative to those sampled at one hour. The increases in the size of D, E, and F after four hours of incubation with the enzyme were witnessed as initiation of aggregation. The increase appears to be directly related to enzyme concentration. In accord with the findings of Muller et al. (181), who reported the NaOH-induced surface degradation of PECA-nanoparticles, the degradation pattern observed with samples A, B and C could be consequent to surface degradation whereby PECA becomes depleted over time; the increase in size observed for D, E and F after the expected initial decrease may be explained in terms of agglomeration that is consequent to the complete degradation.
of the nanoparticles (Figure 3.27); considering the high concentration of the enzyme, it is possible that depleted NPs plays a significant role in this process. The esterase enzyme degradation study showed that the degradation of nanoparticles is directly proportional to the amount of enzyme, which is in agreement with published data (182), and that the higher the enzyme concentration the faster the degradation.

![Graph](image)

**Figure 3.27:** Enzyme degradation of PECA-Dex100G4 over time, for different enzymatic activity (A= 0 units; B= 120 Units; C= 220 Units; D= 420 units; E=620 units; F=1220 units).

### 3.6. Conclusions

In our hands, ethyl 2-cyano-3-ethoxyacrylate on its own could not be formulated into stable nanoparticles. The synthesis of PECA in phosphate buffer resulted in the formation of NPs that were characterised by poor yield, low zeta potential and high conductivity.

PBCA-Dex100G4 NPs was prepared at an average size of 149.8 nm and zeta potential of -47.8 mV. FTIR studies confirmed the presence of the cyanoacrylate group since the spectra exhibit the characteristic stretching vibrations of C=O (1743.88 cm\(^{-1}\)), CN (2242 cm\(^{-1}\)) and C-O (1300 - 1000 cm\(^{-1}\)).

The dispersion of nanoparticles in water and its subsequent filtration through a PES membrane showed that the filtering process does not influence the DLS-determined characteristics of nanoparticles.
Preparation and characterisation of nanoparticles

Alkylglyceryl dextran was successfully reacted with PLA through the zero-length grafting of PLA to alkylglyceryl dextran in the presence of DCC. As determined by $^1$H NMR, the degree of grafting of PLA to alkylglyceryl dextran was 163.9%.

An esterase-induced degradation study of samples of PECADex100G4 showed that the higher the enzyme concentration the faster the degradation, as evidenced by the rate of the agglomeration process that accompanies nanoparticle depletion.
4. Loading of nanoparticles with drugs, peptides and fluorophores

The labelling of drug delivery vehicles with fluorescent dyes is rationalised in this work in terms of the capability to track the progress of the label through the host environment by means of the many fold array of fluorescence-detection technologies. To facilitate cell interaction studies, fluorophores, peptides and fluorescence drugs were loaded or tagged to the synthesised NPs.

4.1. Actives and fluorophores

Rhodamine B (Figure 4.1) is an organic dye that has been used traditionally as a tracer dye in water to determine the rate and direction of flow. Owing to its highly fluorescent nature and solubility in water, methanol and ethanol, Rhodamine B has found extensive use as a tracer molecule in biomedical research.

![Figure 4.1: Chemical structure of Rhodamine B](Image)

Curcumin, found in rhizomes of curcuma longa or turmeric, is a low molecular weight diphenol-functionalised bis-α,β-unsaturated β-ketone that exists in equilibrium with its enol tautomer (183). Curcumin (structure in Figure 4.2) is soluble in both polar and non-polar organic solvents. Although highly insoluble in water (11 ng/mL), it is soluble in alkaline or extremely acidic aqueous solvents; the pH-dependent solubility is assumed to be due to ionisation of the phenolic or enolic groups and/or due to degradation. This is
Loading of nanoparticles with drugs peptide and fluorophores

witnessed by the solvent-dependent UV-vis absorption profile of Curcumin. Toluene solutions of the material fluorescence strongly, exhibiting maximum absorption at 418 nm (184). The main degradation products of Curcumin (185) are ferulic acid, feruloylmethane, vanilline and acetone. Curcumin is known to possess many therapeutic effects but is rapidly eliminated from systemic circulation (186).

![Chemical structure of Curcumin](image)

Figure 4.2: Chemical structure of Curcumin

It has been suggested that the low bioavailability of Curcumin limits its capability to reach target tissues where it can exert its claimed pleiotropic effects. Hence, the covalent attachment of Curcumin to nanoparticles represents one approach towards overcoming this limitation.

Because of its low aqueous solubility and its amphiphilic nature, the fluorescence intensity of Curcumin decreases with increasing water volume and also decreases as the acidity of its liquid host varies from neutral to extremely acidic or to basic (pH >8.0) (185).

The absorption and fluorescence capacity of Curcumin may be used to both locate and quantify the molecular distribution in normal and tumour cells (187). The fluorescence of the molecule allows the visualisation of the distribution of Curcumin while UV absorption measurements provide a means for the quantification of this molecule within a specified sample size, rendering the molecule suitable for the assessment of the fate of Curcumin-loaded nanoparticles for delivery through the BBB. However, the determination of the fate of Curcumin in living tissues is limited by its amphiphilic nature and susceptibility to degradation: deprotonation of Curcumin effects spectral changes, both in terms of the extinction coefficient and in terms of the spectral profile (184) while degradation is manifested by colour change. Furthermore, the steady-state absorption and
Loading of nanoparticles with drugs peptide and fluorophores

Fluorescence characteristics of Curcumin are sensitive to the immediate environment due to keto-enol isomerism. The loading of Curcumin amphiphilic NPs may therefore lead into structural changes that need to be taken into consideration in attempts to follow the fate of such nanoparticles. These potential complications are balanced by the capability of Curcumin-labelled nanoparticles to fluoresce in the blue laser channel, which renders those nanoparticles amenable to direct visualisation under the optical microscope. Also, the use of Curcumin fluorophores is complementary with the concomitant use of DAPI (4′6-diamino-2-phenyl indole) nuclear stain. Although the spectral characteristics of Curcumin are sensitive to solvent polarity (188), the molecule displays an intense absorption band in the region 350-480 nm in all media of interest for this work.

![Figure 4.3: Chemical structure of Doxorubicin hydrochloride](image)

Doxorubicin (Figure 4.3) has been described as a very potent anti-cancer agent (189, 190) that has great potential for CNS chemotherapy but lacks the ability to cross the BBB because it is a substrate for the ATP-dependent efflux pump P-glycoprotein (P-gp) at the base of the BBB (191, 192). Prerequisite to the transport of Doxorubicin to the brain is the development of a suitable carrier that can cross or circumvent this barrier (193). Studies with P-gp suppressors (e.g. Cyclosporine A) have shown that significant amounts of Doxorubicin may be capable of penetrating the BBB following administration (191, 194). Also, PACA nanoparticles have been associated with the transport of Doxorubicin to the brain in significant amounts (91, 195). In another example, co-administration of Doxorubicin and Curcumin that had been loaded into PACA/chitosan nanoparticles has been suggested to increase the penetration of Doxorubicin across the BBB (196). An additional benefit associated with the use of nanopolymeric PACA carriers is the claimed lowering of the cardiotoxicity of Doxorubicin, which is a known side effect that limits its
use for therapeutic purposes (193, 197, 198). The change in the biodistribution profile of Doxorubicin NPs may be responsible for the relatively low cardiotoxicity of this agent.

The ratio of copolymer to PECA in Doxorubicin nanoparticles is reported to influence the initial burst release of the drug from the nanoparticulate matrix, as is exemplified by the observation that a structure of PECA-PLGA in the 1:1 ratio exhibits reduced losses during the initial burst release of Doxorubicin (199). The burst release behaviour seen in this study may be due to the adopted ratio (1:6) of alkylglyceryl dextran to PECA. Notably, emulsifier-free PBCA loaded with Doxorubicin is reported to release its drug content very slowly, exhibiting a half-life of over 100 h in PBS (200).

4.2 Methods and instrumentation
Dialysis membrane (10000-MWCO) and PBS tablets were sourced from Sigma-Aldrich. Other equipment used included a: Perkin Elmer UV-Vis spectrophotometer, Grant water bath, Varian Cary Eclipse Fluorescence spectrophotometer, Falcon tubes (50 mL). The Perkin Elmer UV-Vis spectrophotometer was zeroed before use, with the wavelength set between 400-750nm. Samples were measure individually by placing the sample-containing cuvette against that of the reference sample. A Varian Cary Eclipse spectrophotometer recorded fluorescence measurements after placing the sample-containing cuvettes in the pre-zeroed sample reading chamber; the wavelength was set according to absorbance wavelength of the sample to be measured (excitation-emission) and data were processed using Cary Eclipse software. Mini-spin Eppendorf centrifuge.

4.2.1. Loading of PECA-Dex, PECA-Dex100G4 and PECA-Dex100G8 NPs with Rhodamine B
Rhodamine B solution (10 µl, 10 µg/µl) was added to a stirred suspension of PECA-Dex, PECA-Dex100G4 and PECA-Dex100G8 nanoparticles each (1 mL, 20 mg/mL) and stirring was continued for 3h at room temperature. After this time, the nanoparticles where separated by centrifugation (18000 g) and re-suspended in deionised water (5 mL) characterised by DLS (Figure 4.6).

For the preparation of Polysorbate 80-coated nanoparticles, Polysorbate 80 (100 µl, 10% w/v) was added into the stirred suspension of PECA-Dex100 nanoparticles (tagged with Rhodamine B) to a final concentration of 1% (w/v) and stirring was continued for a further
30 min. The particles were separated by centrifugation using the Eppendorf centrifuge (12200 rpm, 30 min) and re-dispersed following rinsing with deionised water and re-suspension in deionised water (5 mL) under sonication (10 min) and characterised with DLS (Table 4.1); the NTA fluorescing video record is placed in (Appendix 5).

Monitoring the release of Rhodamine B

PECA-dex, PECA-Dex100G4 and PECA-Dex100G8 nanoparticulate samples (2 mg) that had been loaded with Rhodamine B were each dispersed in PBS (10 mL, pH 7.4), and sonicated for 10 min before 1.5 mL of each solution was introduced into an 1.5 mL Eppendorf tube and incubated in a water bath (Grant thermostat temperature shaker, thermostatted at 37°C). Samples were removed sequentially at specified time intervals and centrifuged (12200 rpm, 15 min). To a specified volume of the supernatant (1 mL) was added to PBS (2 mL) and the absorbance was recorded over the UV range 650 nm to 450 nm using an Elmer Perkin instrument; the variation in the intensity of the absorption maximum (564.76 nm) was monitored at specified time intervals. Since the release of Rhodamine B from nanoparticles was found to be very limited, this study was discontinued.

4.2.2.2 PECA-Dex100G4 and PLA-Dex100G8PECA Nanoparticle-loading with Curcumin: the EtOH/H2O (1:1) method

Curcumin (0.1 mg) was loaded onto PECA-Dex100G4 NPs and PLA-Dex100G8PECA NPs (100 mg) by stirring (overnight, room temperature) nanoparticles in ethanol-water (1:1 v/v). After this time, the samples were spun at 12200 rpm for 15 min using the mini-spin Eppendorf and the absorbance of the supernatants (1 mL aliquot to which 2 mL of DMSO had been added) were taken to determine the degree of loading. Concentration was determined using the equation of the calibration curve of Curcumin (Appendix 12).

4.2.2.1 Determination of Curcumin content of PECA-Dex100G4 NPs and PLA-Dex100G8-PECA NPs

Freeze-dried nanoparticles (20 mg) of PECA-Dex100G4 and PLA-Dex100G8-PECA that had been loaded with Curcumin were each dissolved in DMSO (3 mL) and their absorbance were measured to allow the determination of the concentration of released Curcumin, which corresponds with that loaded onto the specified weight of nanoparticles. Again, loading was determined with reference to the standard plot of Curcumin in DMSO.
4.2.2.2 Loading of Curcumin into PECA-Dex100G4 nanoparticles: cross linking with acetic acid and TPP (Tripolyphosphate)

The loading of PECA-Dex100G4 NPs with Curcumin was conducted according to the method of Rejinold et al. (201) in which acetic acid and Tripolyphosphate (TPP) were employed to promote entrapment to the nanoparticles. A release study was conducted in which a portion (0.008 g) of the pellet was dispersed in PBS (10 mL) before being distributed into 9 Eppendorf tubes that were subsequently incubated at 37°C in a Grant water bath. Aliquots were withdrawn at specified time periods and each sample was spun for 5 min at 5000rpm; the method was not suitable because the released Curcumin was either sticking to the container or washed off during the process of supernatant removal.

4.2.2.3 Loading and release of Doxorubicin hydrochloride from PECA-Dex6G4 NPs and PECA-Dex6G8 NPs by nanoprecipitation-solvent evaporation.

Doxorubicin was loaded by nanoprecipitation using acetone as the organic solvent for ECA.

Preparation of the NPs was by the monomer polymerisation method. ECA (150 µl) in acetone (25 mL) was gradually added to a stirring mixture of Dex6G4 or Dex6G8 (25 mg) in water (25 mL, 18.2mΩ) followed by Doxorubicin hydrochloride (2 mg) in acetone (concentration 2 mg/mL). The reaction mixture was allowed to stir overnight (room temperature) and the colloid that formed was separated by centrifugation at 40000 rpm for 30min. The amount of unloaded Doxorubicin hydrochloride in the supernatant was determined fluorimetrically. The pellet was dispersed in deionised water (10 mL) and after sonication (10 min) an aliquot (5 mL) of each sample was extracted and used for the release study. To this end, the aliquot (5 mL) of either PECA-Dex6G4-Dox or PECA-Dex6G8-Dox was placed in a dialysis membrane bag and immersed in 50 mL capacity falcon tubes containing PBS (20 mL). The calculated amount of the free Doxorubicin hydrochloride equivalent contained in the nanoparticles was used as control. The samples were incubated in water bath (37°C) at an oscillating speed of 100 rpm. At specified time intervals, an aliquot (1 mL) of each sample was withdrawn and replaced with fresh media. Fluorescence intensity was measured using a Varian Cary Fluorescence spectrophotometer (Excitation and Emission wavelengths respectively at 480 nm and 590 nm) and the amount of drug released at each time point was quantified by comparison against the Doxorubicin hydrochloride calibration curve (Appendix 14).
4.2.4 The preparation of PECA-Dex100G4-Dox

Method: The preparation of PECA-Dex100G4-Dox was by the anionic emulsion polymerisation method, which is widely employed for the formulation of cyanoacrylate monomers into nanoparticles. ECA (100 µl) was gradually added to Dex100G4 (100 mg) in water (50 mL; 18.2 mΩ, pH 2.5) containing Doxorubicin hydrochloride (1.5 mg) and allowed to stir at room temperature for 4 h before neutralisation with aqueous NaOH (1 N). The colloid that formed was filtered through P3 sintered glass. An aliquot of specified volume was taken and subjected to centrifugation (40000 rpm, 30 min). The pellet was dispersed in water (10 mL, 18 mΩ) and sonicated and thereafter characterised by DLS. Drug loading was calculated by subtraction following the determination of the drug content of the supernatant by UV-Vis measurements (400 nm-750 nm; Doxorubicin exhibits its absorbance maximum at 480nm). The Doxorubicin loading efficiency was determined to be 68.7%.

4.2.5 Loading of Doxorubicin hydrochloride to PECA-Dex6G16 NPs

The monomer polymerisation method via nanoprecipitation by solvent evaporation was used. To a flask (stirring) containing Dex6G16 (100 mg) and Doxorubicin (1.4 mg) in deionised water (100 mL) was added gradually and in sequence a solution of ECA (ethyl 2-cyanoacrylate; 600 µl) in acetone (100 mL). Stirring was continued overnight (room temperature), after which time the mixture was spun (40000 rpm, 30 min; XL-90 Beckman Ultracentrifuge) and the pellet was dispersed in deionised water, sonicated (10 min) and freeze dried.

4.2.6 Determination of EGFP enhanced green fluorescence protein (EGFP)

PECA-Dex100G8-EGFP NPs were dispersed (1573 µg/mL) in PBS (pH7.4) and diluted to specified concentrations before the measurement of absorbance at each dilution to determine the concentration of EGFP through comparison with a BSA calibration curve (Appendix 15).

4.2.7 Tagging of PECA-Dex100G8 with MIA

The tagging of MIA to NPs was accomplished according to a published procedure (202). To a dispersion of PECA-Dex100G8 NPs (ca. 20 mg) in ethanolic sodium borate buffer (5 mL) was added under sonication/vortexing a solution of MIA (20 mg) in ethanol (0.2 mL); a UV lamp facilitated the detection of MIA (Figure 4.4; the MIA excitation and emission wavelengths are at 350nm and 445nm respectively). The mixture was dialysed (72 hr) and
Loading of nanoparticles with drugs peptide and fluorophores

freeze dried. Nanoparticle size was determined (DLS and Nanosight). MIA attachment to NPs was assessed further through visualisation by confocal microscopy (Appendix 9).

Figure 4.4: MIA reaction with PECA-Dex100G8

Purification of MIA-tagged nanoparticles with Sephadex

Column chromatography (Sephadex 50 medium) was used in an effort to increase the purity of MIA-tagged NPs. After packing, the column was washed repeatedly with water (18.2 mΩ), (the eluent) before the sample was introduced. Fractions were collected in 6mL aliquots. The second and third fractions were identified as those containing the fluorophore-tagged NPs, with the second fraction containing the highest proportion. Further fractions (up to nine 6mL aliquots) did not contain any fluorophore, as indicated with aid of a UV lamp.
4.2.8 Tagging of MIA to PECA-Dex6G16 NPs and loading of Curcumin
Method: PECA-Dex6G16 NPs (200 mg) was dispersed in sodium borate/ethanol (1:1; 40mL) before being added to MIA (100 mg) in ethanol (20 mL). After sonication, the mixture was shaken in Bio-Rad (2h, room temperature) before purification by dialysis (72 h). To the dialysed product was added Curcumin (1 mg) in acetone (2 mL) and stirring was continued for 3h (room temperature). After filtering (P3 sintered glass), the determination of Curcumin and MIA content in MIA-Curcumin-tagged nanoparticles was carried out by utilising the dual wavelength capability of the fluorimeter (Cary Eclipse, Varian Fluorescence Spectrophotometer). The excitation-emission wavelength of Curcumin was set at 420nm-480nm and that of MIA was set at 350nm-445nm.

4.2.9 Tagging of Tetramethyl Rhodamine-5-carbonyl azide (TMRCA) to PECA-Dex6G12 nanoparticles
Method: PECA-Dex6G12 nanoparticles (50 mg) that had been dispersed in anhydrous toluene (20 mL) and mixed with TMRCA (0.6 mg) were allowed to stir at 80°C for 5h under an atmosphere of nitrogen (Figure 4.5) (203-205). After this time, the mixture was placed in a dialysis-membrane vessel (3500MWCO) and first dialysed against DMF (HPLC grade, Fisher) for 72 h before further dialysis against double-distilled water for 4 days. The aqueous-medium-derived Tetramethyl Rhodamine-nanoparticles were freeze dried.

4.2.10 Evaluation of Evans blue retentive capacity
To evaluate the retentive capacity of the nanoparticles for the dye, after loading Evans Blue to PECA-Dex6G4 NPs, the colloidal mixture that formed was centrifuged twice and after each centrifugation cycle, the absorbance of supernatant was measured. After the second centrifugation, the degree of loading was evaluated at 0.016% and the entrapment efficiency at 2.034%. An attempt to load Evans blue into PECA-Dex6G12 nanoparticles yielded similar results; the calibration curve for Evans blue is appended (Appendix 16).
Figure 4.5: Covalent linkage of Tetramethyl Rhodamine to PECA-Dex6G12 nanoparticles
4.3 Results and discussion

4.3.1 Rhodamine

4.3.1.1 Rhodamine B loading into nanoparticles
The size distribution of nanoparticles tagged with Rhodamine B coated with Polysorbate 80 was within the range 400-500nm and exhibited low zeta potential (see example for Rhodamine B-loaded PECA-Dex100 NPs in Table 4.1). The size distribution of NPs tagged with Rhodamine B alone were within the size range 300 nm to 500 nm (Figure 4.6).

Table 4.1: Size, PDI and zeta potential of Rhodamine B-loaded, Polysorbate 80-coated PECA-Dex100 NPs

<table>
<thead>
<tr>
<th>parameter</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (Z average) /nm</td>
<td>402.9±3.70</td>
</tr>
<tr>
<td>PDI</td>
<td>0.35±0.012</td>
</tr>
<tr>
<td>Zeta potential /mV</td>
<td>-18.0±0.1</td>
</tr>
</tbody>
</table>

Figure 4.6: Size of Rhodamine B-loaded nanoparticles prepared from PECA-Dex, PECA-Dex100G4 and PECA-Dex100G8 (n=3)
4.3.2 Curcumin

4.3.2.1 PECA-dex100G4 and PLA-dex100G8PECA Nanoparticle-loading with Curcumin: the EtOH/H2O (1:1) method

In accord with expectation due to the very low solubility of Curcumin in water, Curcumin loading into nanoparticles was highly efficient (Table 4.2).

Table 4.2: The absorbance and concentration of Curcumin in the supernatant (EtOH:H2O), (1:1) from the loading of NPs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance</th>
<th>Concentration (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-Dex100G8-PECA-Curcumin</td>
<td>0.47308</td>
<td>0.00285</td>
</tr>
<tr>
<td>PECA-Dex100G4-Curcumin</td>
<td>0.52779</td>
<td>0.00319</td>
</tr>
</tbody>
</table>

4.3.2.2 Determination of Curcumin content of PECA-Dex100G4 Nps and PLA-Dex100G8-PECA NPs

Freeze-dried nanoparticles of PECA-Dex100G4-Curcumin NPs and PLA-Dex100G8-PECA-Curcumin were evaluated for their Curcumin content (Table 4.3).

Table 4.3: concentration of Curcumin content of dissolved Curcumin loaded NPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance</th>
<th>Concentration (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-Dex100G8-PECA-Curcumin</td>
<td>0.64525</td>
<td>0.00392</td>
</tr>
<tr>
<td>PECA-Dex100G4-Curcumin</td>
<td>0.45075</td>
<td>0.00271</td>
</tr>
</tbody>
</table>

4.3.3. Doxorubicin

4.3.3.1 The preparation of PECA-Dex100G4-Dox

The preparation of PECA-Dex100G4-Dox by the anionic emulsion polymerisation method yielded nanoparticles of low PDI and high zeta potential (Table 4.4).

Table 4.4: Size, PDI and zeta potential of PECA-Dex100G4-Dox nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>PDI</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECA-Dex100G4-Dox</td>
<td>281.0±6.7 nm</td>
<td>0.153±0.022</td>
<td>-35.7±2.7 mV</td>
</tr>
</tbody>
</table>
4.3.3.2 PECA-Dex6G16-Doxorubicin (PECA-Dex6G16-Dox) nanoparticles

To assess the effects of purity on size, PDI and zeta potential, the characterisation of PECA-Dex6G16-Dox was carried out by DLS both before (as prepared) and after centrifugation (by resuspending the pellet and sonicating for 10 min). As compared with their as-prepared congeners, the purified NPs exhibit smaller size, lower PDI and higher zeta potential (Table 4.5), all of which highlight the importance of the purification step.

Table 4.5: Size, zeta potential and PDI characterisation of Doxorubicin-PECA-Dex16 (Dox-PD16) nanoparticles (DLS)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before centrifugation</td>
<td>268.2±2.9</td>
<td>0.540±0.021</td>
<td>-26.2±0.5</td>
</tr>
<tr>
<td>Dispersed pellet</td>
<td>254.0±4.6</td>
<td>0.389±0.047</td>
<td>-39.4±3.3</td>
</tr>
</tbody>
</table>

4.3.3.3 Loading and release of Doxorubicin hydrochloride from PECA-Dex6G4 NPs and PECA-Dex6G8 NPs by nanoprecipitation-solvent evaporation

The chain length of the Dex6G4 or Dex6G8 used as copolymer in the formation of nanoparticles of PECA-alkylglyceryl dextran that were loaded with Doxorubicin hydrochloride appears to affect loading capacity. Dex6G4, which has a shorter chain length as compared with Dex6G8, had a smaller amount of Doxorubicin hydrochloride loaded to its nanoparticulate formulation (PECA-Dex6G4) than Dex6G8 (PECA-Dex6G8). The more hydrophobic nature of Dex6G8 could have promoted the adsorption of the Doxorubicin hydrochloride [Doxorubicin base is hydrophobic whereas its HCl salt is water soluble (206)]. The drug is assumed to be entrapped into PECA-Dex6G8 NPs in its hydrophilic Doxorubicin.HCl form, which probably enables efficient diffusion from the nanosphere matrix. Of the two nanoparticulates investigated, those of PECA-Dex6G8 NPs, which are associated with the higher loading capacity for Doxorubicin.HCl, were seen to exhibit the faster rate of release of this active.
The freeze dried nanoparticles drug content (Table 4.6) was determined by dissolving the NP-drug in DMSO and the values were determined from the calibration curve (presented in Appendix 14). Samples of nanoparticles that had been subjected to freeze drying, contained drastically reduced amounts of entrapped drug relative to those that had not been subjected to a freeze drying protocol. This was particularly pronounced with PECA-Dex100G4 NPs. The release of the Doxorubicin content of PECA-Dex6G8 or PECA-Dex6G4 nanoparticles is influenced by polymer-chain hydrophilicity, which in turn is influenced by the liquid environment and by the amount of loaded drug that is retained in the nanoparticle matrix. The complex binding and releasing mechanisms are yet to be deconvoluted (207).

Doxorubicin release from PECA-Dex6G8-Dox nanoparticles was very fast (similar to that of the free Doxorubicin from the dialysis-membrane bag; Figure 4.7). PECA-Dex6G4.Dox nanoparticles had about 40% of their drug content released within about 8 h from the start of the release study, which also saw about 95% of the drug released from PECA-Dex6G8 nanoparticles within the same timescale (Figure 4.7). The release profile (Figure 4.7) of Doxorubicin from the nanoparticulate matrix, especially that of PECA-Dex6G8-Dox,
Loading of nanoparticles with drugs peptide and fluorophores

Suggested that almost all loaded Doxorubicin had been released within few hours. It is possible that Doxorubicin release from these nanoparticles may have been better controlled if structures containing different ratios of the constitutional polymers had been used, or by the use of Doxorubicin in its free base form. The release profiles demonstrated that the nanoparticles exhibited limited affinity for the drug at the temperature and pH of the adopted experimental protocol.

Table 4.6: Percentage Doxorubicin loading to the nanoparticles (loading efficiency)

<table>
<thead>
<tr>
<th>sample</th>
<th>% drug loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECA-Dex100G4</td>
<td>45.8%</td>
</tr>
<tr>
<td>PECA-Dex100G8</td>
<td>58.3%</td>
</tr>
</tbody>
</table>

4.3.3.4 Loading of both Doxorubicin and Curcumin into PECA-Dex100G12 nanoparticles: The monomer polymerisation method provided the means for the loading of PECA-Dex100G12 nanoparticles with equal proportions of Curcumin and Doxorubicin (208). Since for both molecules OH functionalities may become involved in the initiation of the polymerisation of ECA, the formulation cannot be regarded as a mere combination of Doxorubicin and Curcumin but rather as a molecular complex of these actives with the nanoparticles. Accordingly, release studies involving this nanoparticulate complex have shown that Doxorubicin is released from the complex at a faster rate than Curcumin. In the absence of detailed spectroscopic characterisation data the relative contributions of the binding forces that operate between the individual components of the complex cannot be deconvoluted. However, the observed release patterns suggest that these nanoparticles may be amenable to employment for therapeutic purposes; Table 4.7 summarises the DLS characterisation data of Doxorubicin-Curcumin nanoparticles.

Table 4.7: Size, PDI and zeta potential of PECA-Dex6G12 nanoparticles loaded with Doxorubicin-Curcumin.

<table>
<thead>
<tr>
<th></th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLS</td>
<td>NTA</td>
<td>Mode</td>
</tr>
<tr>
<td>PECA-Dex100G12-Dox-Cur</td>
<td>211.4±0.1</td>
<td>152±66</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.249±0.022</td>
</tr>
</tbody>
</table>
4.3.3.5 PECA-Dex100G12-Dox TGA and DSC results

TGA performed under an atmosphere of nitrogen indicated that the main degradation step for PECA-Dex100G12-Dox is characterised by a mass loss of 91.4% (Figure 4.8), with a maximum rate of mass loss at 192.0 °C. The second step of the same thermogram is associated with a mass loss of 7.7% and exhibits a peak mass loss rate at 327.5 °C. Exposed to a synthetic air atmosphere the sample exhibited an additional mass loss of 1.1%.

Figure 4.8: TGA plots from PECA-Dex100G12-Dox nanoparticles.

In (Figure 4.9) the first heating curve (blue curve) of this sample, there is observed a glass transition at 52.4 °C (midpoint) which is accompanied by a change in specific heat capacity of 0.059 J/g*K. Immediately after the glass transition, a broad endothermic event is observed which is assumed to be associated with the onset of evaporation or with the oxidative degradation of a component of the sample. The second heating curve (green curve) confirms the glass transition at 52.3 °C and enumerates the change in specific heat capacity at 0.041 J/g*K. Notably, the endothermic event did not occur during the second heating cycle, which is indicative of a possible relaxation effect in the polymer structure.
Loading of nanoparticles with drugs peptide and fluorophores

Figure 4.9: DSC of PECA-Dex100G12–Doxorubicin nanoparticles.

Since the extraction of Doxorubicin-free base with methanol and triethylamine has been shown to be an effective means of increasing Doxorubicin entrapment in the PECA-DexnGm nanoparticles (199), it is likely that the use of Doxorubicin as its hydrochloride salt would have affected the extent of entrapment to PECA-DexnGm nanoparticles. The interactions between PACA and Doxorubicin have been indicated to involve the establishment of hydrogen bonds between the ammonium N-H function and the cyano group, while the cohesion of PACA nanoparticles have been indicated by the same technique to be due to the combined effects of electrostatic forces, H-bonds and dipolar interactions (163).

4.3.4 Other fluorescent markers and model drugs

4.3.4.1 Enhanced green fluorescence protein (EGFP) determination in NPs

Determination of EGFP at various concentrations of PECA-Dex100G8-EGFP NPs EGFP loading was studied over a range of concentrations of nanoparticles that had been dispersed in PBS. This was to confirm whether the fluorescence protein has been loaded unto the nanoparticles. The various concentrations of the NPs-loaded EGFP confirmed the
presence of the fluorescence marker in the NPs (Table 4.8). The nanoparticles were further analysed with SEM. SEM image of PECA-Dex100G8-EGFP loaded nanoparticles is appended in (Appendix 10).

### Table 4.8: Determination of concentrations of EGFP in PECA-Dex100G8-EGFP NPs

<table>
<thead>
<tr>
<th>Initial Conc. of NPs (µg/mL)</th>
<th>Absorbance (nm)</th>
<th>Conc. of EGFP (µg/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.0558</td>
<td>1.294</td>
</tr>
<tr>
<td>200</td>
<td>0.0770</td>
<td>13.760</td>
</tr>
<tr>
<td>300</td>
<td>0.1084</td>
<td>32.240</td>
</tr>
<tr>
<td>400</td>
<td>0.3335</td>
<td>164.558</td>
</tr>
<tr>
<td>500</td>
<td>0.3481</td>
<td>173.240</td>
</tr>
</tbody>
</table>

### 4.3.4.2 N-methyl-isatoic anhydride (MIA)

Dispersion of freeze dried MIA-NPs in a range of media demonstrated the medium-influenced variation in the size of nanoparticles (Table 4.9).

### Table 4.9: The effect of different liquid media on the size distribution of NPs of PECA-Dex100G8 that had been tagged with MIA (DLS)

<table>
<thead>
<tr>
<th>Liquid medium</th>
<th>Z-av.d (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>141.4±1.0</td>
<td>0.241±0.006</td>
</tr>
<tr>
<td>NaCl (10mmol)</td>
<td>133.2±1.6</td>
<td>0.224±0.009</td>
</tr>
<tr>
<td>PBS (7.4)</td>
<td>226.0±5.3</td>
<td>0.467±0.011</td>
</tr>
<tr>
<td>PBS (filtered 0.2µm)</td>
<td>134.6±0.4</td>
<td>0.221±0.007</td>
</tr>
</tbody>
</table>

In an experiment designed to establish the optimum method of co-loading MIA and Curcumin, it was observed that the tagging of MIA before loading the sample with Curcumin was more efficient than that involving loading with Curcumin before tagging with MIA (Table 4.10); DLS results of sizes and zeta potentials are presented in (Table 4.11).
Table 4.10: Fluorescence intensity of MIA-Curcumin nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tagged with MIA before loading with Curcumin</th>
<th>Loaded with Curcumin before tagging with MIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence intensity (Curcumin)</td>
<td>56.9538</td>
<td>20.5744</td>
</tr>
<tr>
<td>Fluorescence intensity (MIA)</td>
<td>507.3941</td>
<td>547.0530</td>
</tr>
</tbody>
</table>

Table 4.11: DLS analysis of MIA-Curcumin NPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tagged with MIA before loading with Curcumin</th>
<th>Loaded with Curcumin before tagging with MIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Av (nm)</td>
<td>634.5±16.1</td>
<td>430.1±8.0</td>
</tr>
<tr>
<td>PDI</td>
<td>0.159±0.023</td>
<td>0.248±0.009</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-27.6±0.1</td>
<td>-23.0±0.5</td>
</tr>
</tbody>
</table>

4.3.4.3 Tetramethyl Rhodamine-5-carbonylazide

The synthesis of Tetramethyl Rhodamine-nanoparticles by covalent bond formation involved reaction between the hydroxyl group of constituent macromolecules and the isocyanate group formed from the acyl azide functionality of TMRCA on heating at 80°C. The conjugation of these molecules was evidenced by NTA, and by the difference between the fluorescence intensities of the blank and Tetramethyl Rhodamine nanoparticles dissolved in DMSO as measured with Microplate Reader. Proton NMR (205) was used to determine the molar ratio of the attached Tetramethyl Rhodamine to the nanoparticles by comparing the signal intensity or the peak area of its methyl proton to the anomic proton of the pyranose ring of the dextran or, the methylene protons (or methyl protons) of the alkyl glyceryl group attaching to the dextran.

Evidence for the conjugation of Tetramethyl Rhodamine to the nanoparticles was obtained by considering the difference in fluorescence intensities of nanoparticles with reference to a blank sample, which had been dissolved in DMSO and measured (Microplate Reader) at the respective excitation and emission wavelengths of 544 nm and 590 nm; fluorescence intensities obtained were 21902 for Tetramethyl Rhodamine tagged nanoparticles and 1952 for empty nanoparticles. The linking of Tetramethyl Rhodamine to the nanoparticles was further evidenced with NTA, in that fluorescing Tetramethyl
Rhodamine nanoparticles could be observed when the filter was shut. The sizes of tagged nanoparticles, as measured by DLS and NTA, are presented in (Table 4.12). Since the size distribution of nanoparticles was high, as reflected by the associated PDI, the separation of nanoparticles to narrower size samples was attempted by filtration.

<table>
<thead>
<tr>
<th></th>
<th>DLS</th>
<th>NTA(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mode</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NP-TMRCA       879.6±42.8 0.525±0.030 735±264 752

4.3.4.4 Evans Blue

Loading of PECA-Dex100G4 NPs with Evans blue

Evans blue dye (structure in Figure 4.10) was loaded into the nanoparticles by means of the monomer polymerisation method. The degree of loading and the efficiency of entrapment were determined by measuring the Evans blue content of the supernatant. The system exhibited a poor loading efficiency and an associated low degree of loading. Evans blue is a negatively charged dye; it is possible that the negative zeta potential of PECA-DexnGm nanoparticles is responsible for the poor affinity of this dye for these nanoparticles.

![Evans blue structure](image)

Figure 4.10: Chemical structure of Evans blue
4.4 Conclusions

Rhodamine B was successfully loaded into the nanoparticles but since the release of Rhodamine B from nanoparticles was poor this study was discontinued.

To promote the entrapment of Curcumin into nanoparticles, the loading of PECA-Dex100G4 NPs with Curcumin was carried out in the presence of acetic acid and TPP. However, inherent to this method was the inefficient study of the release profile.

The study of the release of Doxorubicin hydrochloride indicated that nanoparticles of PECA-Dex6G4 NPs can sustain the release of Doxorubicin hydrochloride more efficiently than those formulated from longer chain homologues (e.g. PECA-Dex6G8 NPs).

Towards the optimisation of methods of tagging MIA to nanoparticles, it was observed that the tagging of MIA prior to loading with Curcumin was preferable to the tagging of MIA after Curcumin had been loaded to the nanoparticles.

EGFP was successfully loaded into the nanoparticles as confirmed by absorbance measurements at specified concentrations.

The synthesis of Tetramethyl Rhodamine-containing nanoparticles by covalent bond formation was achieved through reaction between the hydroxyl group of constituent macromolecules and the isocyanate group formed from the acyl azide functionality of TMRCA. The successful tagging of TMRCA to the nanoparticles was indicated by NTA and by the difference in fluorescence intensities of nanoparticles with reference to those of the blank as determined using the microplate reader.

Evans blue had very low affinity for the nanoparticles as evidence by the ease with which the dye washed away from their nanoparticulate hosts.
5. In vitro studies - Cytotoxicity

The cytotoxicity of nanoparticulate formulations was assessed by measuring the viability of mouse brain endothelial cells (bend3) following incubation with selected nanoformulations.

5.1 Methods and instrumentation

The cytotoxicity evaluation protocol involved the following materials and equipment: incubator (37°C, 5% CO₂), Plate reader PolarStar Optima (BMG Labtech) (570nm Absorbance filter), 96-well plate (Greiner bio-one) Fisher, inverted microscope, haemocytometer, pipettes (1 mL, 100 µl, 50 µl, 20 µl), class II safety cabinet, sterile pipette tips, sterile pipette (10mL, 3mL) disposable, sterile 0.4 µm polyethersulfone (PES) filter, electric pipette (Fisher), T25 culture flask (Fisher), centrifuge, alkyl cyanoacrylate-alkylglyceryl dextran NPs, alkylglyceryl dextrans, MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), DMEM (media), trypan blue, Triton-X100, PBS, Tryple Express, mouse-brain endothelial cells (bend3), DMEM (500 mL), L-Glutamine (5 mL), Sodium pyruvate (5 mL), 2-mercaptoethanol (0.5 mL), NEAA (5 mL), FBS (50mL), Pen/Strep (5 mL).

The cytotoxicity study was conducted by means of the MTT (3-(4, 5(dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (209, 210): MTT metabolism is known to show a good correlation between cell viability and conversion of MTT tetrazolium salt (yellow) to MTT formazan (purple) since only metabolic active cells have the capacity to effect the conversion of this salt to its purple metabolite.

Various alkyl cyanoacrylate-alkylglyceryl dextran NPs and alkylglyceryl dextrans were assessed at 1 mg/mL concentration.

To assess the concentrations of nanoparticles that induce cytotoxicity to bend3 cells, a dose-response study employing a MTT assay were conducted for PECA-Dex100G4 NPs (1:1), PECA-Dex100G4 NPs (1:6) and PBCA-Dex100G4 NPs samples at four specified concentrations (10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL).
**In vitro studies**

**MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay protocol**

The MTT working solution was prepared by allowing a solution of MTT in DMEM (5 mg/mL; kept in the dark at 2-8°C until needed) to reach 37°C and a steady state concentration of 5% CO₂ before dilution (1:5) with DMEM. Bend3 cells (20,000) were seeded (in triplicate) into each of a 96 well plate containing 200 µl of normal media (DMEM containing 10% fetal bovine serum, FBS) and incubated for 24h. After this time, in each well were added 20 µl of each nanoformulation and of alkylglyceryl dextran formulations dispersed in PBS at specified concentrations prior to incubation for 24h at 37°C (5% CO₂). After this time, to each well was added MTT working solution (50 µl) and the mixture was incubated for 2h at 37°C (5% CO₂). The media was then removed from each well, replaced with 100 µl of DMSO and this mixture was agitated gently until the formazan crystals had dissolved. Each plate was inserted into the plate reader and the absorbance was read at 570 nm. The viability of cells was calculated (Equation 5.1) as percentage relative to PBS (negative control); Triton-X100 provided the positive control.

\[
\text{Relative cell viability} \% = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100 \quad (\text{eq. 5.1})
\]

**5.2 Results and Discussion**

Tested against bend3 cells at a relatively high concentration of 1 mg/mL (MTT assay), PECA and PECA-alkylglyceryl dextran nanoparticles exhibited significant cytotoxicity (p<0.05) as compared with the PBS control (Figure 5.1).
**In vitro studies**

Figure 5.1: Relative toxicity of PECA and PECA-alkylglyceryl dextran nanoparticles (1mg/mL; p<0.05, n=3)

Figure 5.2: Relative cytotoxicity of alkylglyceryl dextrans (1mg/mL; p<0.05, n=3)
In vitro studies

To account for these results, alkylglyceryl dextrans were also evaluated using the same assay, and the results (Figure 5.2) exhibited variable behaviour in that Dex100G4 was cytotoxic relative to the PBS control (p<0.05) while Dex100G16, Dex6G4 and Dex6G16 did not display any statistically significant differences at the p<0.05 level with reference to the same control. It appears that a decrease in the molecular weight of dextran reduces cytotoxicity, though the degree of substitution with alkylglyceryl chains is clearly another contributing factor.

Figure 5.3: Relative viability as a function of dose response of alkyl cyanoacrylate NPs against bend3 cells, each incubated at specified concentrations (10 µg/mL, 25 µg/mL 50 µg/mL 100 µg/mL) for 24 h; MTT assay; Triton –X100 and media blank (n =3; ±SD; Triton-X100 positive control).

To identify a range of safe concentrations, a dose response study has been carried out; the apparent toxicity induced by PECA was also compared to that of poly(butyl cyanoacrylate), by testing nanoparticles that were prepared from the same materials but where ECA was replaced by BCA. We found that PBCA-Dex100G4 NPs at concentrations of up to 50 µg/mL were well tolerated by bend3 cells (Figure 5.3, Table 5.1) while NPs of PECA-Dex100G4 (1:1) and PECA-Dex100G4 (1:6) began to exhibit toxic effects at concentrations >25 µg/mL.
In vitro studies

Table 5.1: Cytotoxicity dose response of alkyl cyanoacrylate-alkylglyceryl dextran NPs against bEND3 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>viability (Abs)</th>
<th>percentage viability</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>1.8002</td>
<td>100.0000</td>
<td>0.0294</td>
</tr>
<tr>
<td>PBCA-10</td>
<td>1.6036&lt;sup&gt;+&lt;/sup&gt;</td>
<td>89.0808</td>
<td>0.2972</td>
</tr>
<tr>
<td>PBCA-25</td>
<td>1.5912&lt;sup&gt;+&lt;/sup&gt;</td>
<td>88.3901</td>
<td>0.2988</td>
</tr>
<tr>
<td>PBCA-50</td>
<td>1.4338&lt;sup&gt;+&lt;/sup&gt;</td>
<td>79.6504</td>
<td>0.3314</td>
</tr>
<tr>
<td>PBCA-100</td>
<td>0.5609*</td>
<td>31.1613</td>
<td>0.0096</td>
</tr>
<tr>
<td>PECA(1:1)10</td>
<td>1.5958&lt;sup&gt;+&lt;/sup&gt;</td>
<td>88.6475</td>
<td>0.1781</td>
</tr>
<tr>
<td>PECA(1:1)25</td>
<td>1.3793&lt;sup&gt;+&lt;/sup&gt;</td>
<td>76.6211</td>
<td>0.2434</td>
</tr>
<tr>
<td>PECA(1:1)50</td>
<td>0.4315*</td>
<td>23.9714</td>
<td>0.2047</td>
</tr>
<tr>
<td>PECA(1:1)100</td>
<td>0.3808*</td>
<td>21.1569</td>
<td>0.0213</td>
</tr>
<tr>
<td>PECA(1:6)10</td>
<td>1.4633&lt;sup&gt;+&lt;/sup&gt;</td>
<td>81.2891</td>
<td>0.2183</td>
</tr>
<tr>
<td>PECA(1:6)25</td>
<td>1.3477&lt;sup&gt;+&lt;/sup&gt;</td>
<td>74.8657</td>
<td>0.1566</td>
</tr>
<tr>
<td>PECA(1:6)50</td>
<td>0.3850*</td>
<td>21.3902</td>
<td>0.0421</td>
</tr>
<tr>
<td>PECA(1:6)100</td>
<td>0.3507*</td>
<td>19.4811</td>
<td>0.0292</td>
</tr>
<tr>
<td>0.1%T-X100 Media</td>
<td>0.2697</td>
<td>14.9853</td>
<td>0.0295</td>
</tr>
<tr>
<td>0.1%T-X100 (water)</td>
<td>0.2046</td>
<td>11.3654</td>
<td>0.0030</td>
</tr>
<tr>
<td>1%T-X100 (media)</td>
<td>0.2716</td>
<td>15.0890</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

*significant difference (P<0.05) compared to media;
<sup>+</sup>significant difference (P<0.05) compared to 0.1% TritonX100

Legend:
PBCA-10 = PBCA-Dex100G4 NPs at dose of 10 µg/mL
PBCA-25 = PBCA-Dex100G4 NPs at dose of 25 µg/mL
PBCA-50 = PBCA-Dex100G4 NPs at dose of 50 µg/mL
PBCA-100 = PBCA-Dex100G4 NPs at dose of 100 µg/mL
PECA(1:1)10 = PECA-Dex100G4,(equal ratio of PECA:Dex100G4) at dose of 10 µg/mL
PECA(1:1)25 = PECA-Dex100G4,(equal ratio of PECA:Dex100G4) at dose of 25 µg/mL
PECA(1:1)50 = PECA-Dex100G4,(equal ratio of PECA:Dex100G4) at dose of 50 µg/mL
PECA(1:1)100 = PECA-Dex100G4,(equal ratio of PECA:Dex100G4) at dose of 100 µg/mL
PECA(1:6)10 = PECA-Dex100G4,( ratio 1:6 of Dex100G4:PECA) at dose of 10 µg/mL
PECA(1:6)25 = PECA-Dex100G4,( ratio 1:6 of Dex100G4:PECA) at dose of 25 µg/mL
PECA(1:6)50 = PECA-Dex100G4,( ratio 1:6 of Dex100G4:PECA) at dose of 50 µg/mL
PECA(1:6)100 = PECA-Dex100G4,( ratio 1:6 of Dex100G4:PECA) at dose of 100 µg/mL

Literature reports indicate that, when tested against a L929 fibroblast cell culture, ethyl and isobutyl cyanoacrylate-derived nanoparticles were found to be more toxic than the methyl derivative, which showed intermediate toxicity, while isohexyl cyanoacrylate was the least cytotoxic of the materials tested (81). A further study involving cyanoacrylate nanoparticles (PMCA, PECA, PBCA and PIBCA) and mouse peritoneal macrophages showed identical cellular damage for all tested cyanoacrylates particles (211), indicating that contrary to earlier reports, chain length influences toxicity. Nemati et al. have reported that the toxicity of cyanoacrylates on P388 cells is time dependent and that the rate and
In vitro studies

amount of uptake is determined by the cell types against which the evaluation is conducted (212).

The cytotoxicity of poly(cyanoacrylate) nanoparticles seems to be more complex than the direct contributions of each of the released degradation products, stimulation of inflammation mediators and cell adhesion (107, 213): concentration and time appear to play an important role. For example, literature indicates that PBCA nanospheres did not induce any cytotoxicity at a concentration of 75 µg/mL while membrane damage was observed at a concentration of 150 µg/mL (107). This in turn indicates that PBCA may become cytotoxic at a specified concentration >75 µg/mL. In another example, poly(isobutyl cyanoacrylate) nanospheres at 100 µg/mL exhibited no cell toxicity within the 7 h timescale but showed notable mortality beyond 24 h (81). Other reports on the cytotoxicity of cyanoacrylates are available in the literature, but those are limited to the concentration level of 0.1 mg/mL (214).

Although retro-Knoevenagel to poly(acrylic acid) and the corresponding alcohol is assumed to be the major degradation pathway of cyanoacrylate polymers, the cellular cytotoxicity of cyanoacrylate polymers has been shown to be directly related to the release of formaldehyde (215), which is produced though a minor pathway during the degradation of PACA (214). The rate of degradation is primarily determined by the length of the side chain, with shorter chain homologues degrading more readily (presumably due to the steric hindrance effects exerted by the longer-chain materials; 216). Due to its relative high propensity to adhere to cells (81, 107) PECA would be expected to exhibit higher toxicity than its shorter-chain homologue PMCA. Contrary to expectation, when tested against fibroblasts, PMCA was found to be more toxic than PECA (214). A study of the cytotoxicity of PBCA and POCA (10 µg/mL) against human foreskin fibroblasts showed that the former material is more toxic (217).

As compared with the PBS control, the PECA and PECA-derived nanoparticles evaluated in this study at concentration of (1 mg/mL) exhibited marked toxic effects against all cells tested (bend.3) (Figure 5.1). Complementary dose-response experiments that included PBCA-derived nanoparticles have demonstrated that the respective toxicities of PBCA-Dex100G4 NPs, and PECA-Dex100G4 (both 1:1 and 1:6 ratio samples) nanoparticles are notable at the 100 µg/mL and the 50 µg/mL levels, respectively (Figure 5.3 and Table 5.1). With reference to the PBS control, Dex100G4 appeared significantly more toxic but no
significant difference in toxicity could be identified (P<0.05) with Dex100G16, Dex6G16 or Dex6G4 (Figure 5.2).

Consistent with the findings of Wiegand et al. who reported the molecular-weight-dependent toxic effects of chitosan on human keratinocyte cell lines (218), the percentage viabilities (214) determined for alkylglyceryl dextran samples of different molecular weights imply that similar molecular-weight-dependent effects operate with the latter class of molecules. Notably however, tested against CT-26 cells, dextran derived micelles did not appear to exhibit a significant molecular weight-related effect (219).

5.3. Conclusions
Since complementary studies have indicated that the chain length of PACAs influences their rate of degradation, it is assumed that there is a relationship between chain length and toxicity: the shorter the chain, the faster the degradation and the more toxic the parent structure. It is notable however that the toxicity of degradation products of PACA nanoparticle is reported, through the evaluation of data from clinical trials, not to induce any adverse effects of therapeutic significance (220). The molecular weight of native dextran and the degree of substitution with alkylglyceryl chains also appear to influence the cytotoxicity of the nanoparticulate formulations tested.
6. General Conclusions and suggestions for further work

The delivery of therapeutic agents to the brain is often limited by the BBB. Research findings have indicated that this barrier may be overcome by the use of nanoparticulate drug delivery vehicles which incorporate the therapeutic molecule that is not capable of crossing the BBB. To achieve this goal, it is essential that nanocarriers that do not alter the integrity of the BBB are developed.

This study designed and evaluated one such class of drug-carrying polymeric nanoparticles. To this end, dextran derivatives have been prepared through attachment of alkylglycerol of systematically varied chain length to the dextran core. These macromolecular structures were then combined with a widely used class of biocompatible moieties, the alkyl cyanoacrylates, to formulate nanoparticles. Alkylglycerols of systematically varied chain lengths were utilised to generate alkylglyceryl derivatives of two native dextrans with average molecular weights of 6 kDa and 100 kDa. These alkylglyceryl dextrans were characterised by MALDI-TOF MS, TGA, GPC, FTIR and NMR. The degree of substitution of each of the synthesised alkyl glyceryl dextrans, as determined using $^1$H-NMR spectroscopy, ranged between 30% and 200%. It was found that the longer the chain length of the progenitor alkylglycerol the lower the hydrophilicity of the alkylglyceryl dextran. GPC results demonstrated that the polydispersity index of alkylglyceryl dextrans was lower than that of the precursor dextran irrespective of average molecular weight (6 kDa or 100 kDa).

In the chemical attachment of alkylglycerol to dextran the carbon at position 2 was seen to be regioselectively favoured, as is consistent with its expected higher reactivity. The synthesised alkylglyceryl dextrans were readily amenable to formulation into nanoparticles through combination with alkyl cyanoacrylates (ethyl or butyl); the techniques of nanoprecipitation and emulsion polymerisation were both usefully employed for this purpose. Nanoparticles were characterised by DLS, NTA, elemental (CHN) analysis, NMR, FTIR, MALDI-TOF MS, SEM, DSC and TGA. Elemental analysis calculations determined that the ratio of alkylglyceryl dextran to alkyl cyanoacrylate in
General Conclusions

the formulated nanoparticles was in the respective, formulation-dependent, range of 3-19% and 80-95%; this was in agreement with NMR data.

The sizes of nanoparticles were of the order of 100-500 nm. Studies involving the systematic variation of the alkylglyceryl chain length indicated that there is no direct relationship between chain length and nanoparticle size. Notably, nanoparticles that had been prepared from alkylglyceryl dextran exhibited consistently higher negative zeta potentials than their congeners that had been formulated from native dextran. Autotitration experiments revealed that empty NPs exhibit decreasing zeta potentials with decreasing pH. The average sizes of nanoparticles appeared relatively stable across the pH range considered, while the sizes decreased with decrease in pH for some of the tested nanoparticles.

The nanoparticles were amenable to tagging with fluorophores and to loading with a range of model drugs (MIA, EGFP, Curcumin, Doxorubicin, Evans blue, TMRCA). Experiments involving nanoparticles that had been loaded with Curcumin demonstrated that the longer the alkylglyceryl chain length the smaller the size of nanoparticles.

Studies of the release of loaded Evans blue have shown the rapid release of this agent from the nanoparticulate matrix, presumably due to the incompatibility in the surface charges of the negatively charged Evans blue and the negative-zeta-potential nanoparticles. The release of Doxorubicin or that of Curcumin from their nanoparticulate host was notably slower than that observed with Evans blue.

Evaluated for cytotoxicity against bend3 cells, nanoparticles exhibited dose-dependent toxicity profiles: PBCA-Dex100G4 NPs were found to be more biocompatible than PECA-Dex100G4 NPs.

Integral to the future development of the technology towards clinical applications is an in vitro study that evaluates nanoparticles for their capability to permeate modelled BBB structures.
7. References

References

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References

References


8. Appendices

APPENDIX 1  \(^1\)H and 13C NMR spectra of butyl glycidyl ether
APPENDIX 2  \(^1\)H AND 13C NMR spectra of tetradecyl glycidyl ether
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APPENDIX 5  Snapshot of video measurement for PECA-NP loaded Rhodamine B and coated with Polysorbate 80
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APPENDIX 1

\(^1\text{H- and }^{13}\text{C-NMR spectra of butyl glycidyl ether}\)
APPENDIX 2

$^1$H- AND $^{13}$C-NMR spectra of tetradecyl glycidyl ether
APPENDIX 3

$^1$H- AND $^{13}$C-NMR spectra of hexadecyl glycidyl ether
APPENDIX 4

SEM image of PECA-Dex100G4
APPENDIX 5

Video snapshot of PECA-NP loaded Rhodamine B and coated with Polysorbate 80
APPENDIX 6

Thermograms of modified dextrans

Figure A6.1: TGA results of Dex6G8

Figure A6.2: TGA results of Dex6G12
Figure A6.3: TGA results of Dex6G14

Figure A6.4: TGA results of Dex6G16
Figure A6.5: TGA results of Dex100

Figure A6.6: TGA results of Dex100G4
Figure A6.7: TGA results of Dex100G8

Figure A6.8: TGA results of Dex100G12
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Figure A6.9: TGA results of Dex100G14

Figure A6.10: TGA results of Dex100G16
Appendices

APPENDIX 7

MALDI TOF spectra of modified dextrans

Figure A7.1 MALDI-TOF Spectrum of Dex100G4

Figure A7.2: MALDI TOF spectrum of Dex6G8
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Figure A7.3: MALDI TOF Spectrum of Dex100G8

Figure A7.4 MALDI TOF spectrum of Dex6G12
Figure A7.5 MALDI TOF spectrum of Dex100G12

Figure A7.6 MALDI TOF spectrum of Dex6G14
Figure A7.7 MALDI TOF spectrum of Dex100G14

Figure A7.8 MALDI TOF Spectrum of Dex6G16
Figure A7.9 MALDI TOF spectrum of Dex100G16
APPENDIX 8

MALDI TOF spectra of nanoparticles

Figure A8.1 MALDI TOF spectrum of PECADex100G4

Figure A8.2: MALDI TOF spectrum of PECADex6G8
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Figure A8.3: MALDI TOF spectrum of PECADex100G8

Figure A8.4: MALDI TOF spectrum of PECADex100G12
Figure A8.5: MALDI TOF spectrum of PECADex6G16

Figure A8.6: MALDI TOF spectrum of PECADex100G16
APPENDIX 9

Confocal microscopy image of PECA-Dex100G8 tagged with MIA in PBS
SEM image of PECA-Dex100G8 EGFP nanoparticles
Appendices

APPENDIX 11

Equations used to calculate the polymer mass composition from the results of elemental analysis

\[
\% H = \frac{(H_{atomic\ mass} \times X + H_{PECA\ m\ ratio}) + (H_{Dex\ m\ ratio} \times H_{atomic\ Mass}) + [DSd \times (H_{g\ m\ ratio} + 4) \times H_{atomic\ mass}]}{(molar\ mass\ of\ PECA \times X) + (molar\ mass\ of\ Dex) + (DSd \times molar\ mass\ of\ Alkyl\ Glycerol\ Chain)} \times 100
\]

\[
\% C = \frac{(C_{atomic\ mass} \times X + C_{PECA\ m\ ratio}) + (C_{Dex\ m\ ratio} \times C_{atomic\ Mass}) + [DSd \times (C_{g\ m\ ratio} + 2) \times C_{atomic\ mass}]}{(molar\ mass\ of\ PECA \times X) + (molar\ mass\ of\ Dex) + (DSd \times molar\ mass\ of\ Alkyl\ Glycerol\ Chain)} \times 100
\]

\[
\% N = \frac{(N_{atomic\ mass} \times X + N_{PECA\ m\ ratio})}{(molar\ mass\ of\ PECA \times X) + (molar\ mass\ of\ Dex) + (DSd \times molar\ mass\ of\ Alkyl\ Glycerol\ Chain)} \times 100
\]

\[
\% PECA = \frac{(m\ mass\ of\ PECA \times X)}{(molar\ mass\ of\ PECA \times X) + (molar\ mass\ of\ Dex) + (DSd \times molar\ mass\ of\ Alkyl\ Glycerol\ Chain)} \times 100
\]
APPENDIX 12

Calibration curves used for the determination of Curcumin

Calibration curve of Curcumin in DMSO

\[ y = 159.56x + 0.019 \]
\[ R^2 = 0.9964 \]

Curcumin calibration curve in PBS (0.1% Polysorbate 80)

\[ y = 97.601x + 6.5747 \]
\[ R^2 = 0.9997 \]
APPENDIX 13

SEM images of native dextran and Dex100G4

native dextran

Dex100G4
APPENDIX 14

Calibration curves for Doxorubicin

Calibration curve of Doxorubicin in DMSO

\[ y = 34296x + 10.862 \]
\[ R^2 = 0.9995 \]

Calibration curve of doxorubicin in PBS

\[ y = 9020.8x + 0.2966 \]
\[ R^2 = 0.9947 \]
APPENDIX 15

Calibration curve for BSA
APPENDIX 16

Calibration curve for Evans blue

Calibration curve of Evans blue in water

\[ y = 0.0254x + 0.0088 \]

\[ R^2 = 0.9999 \]