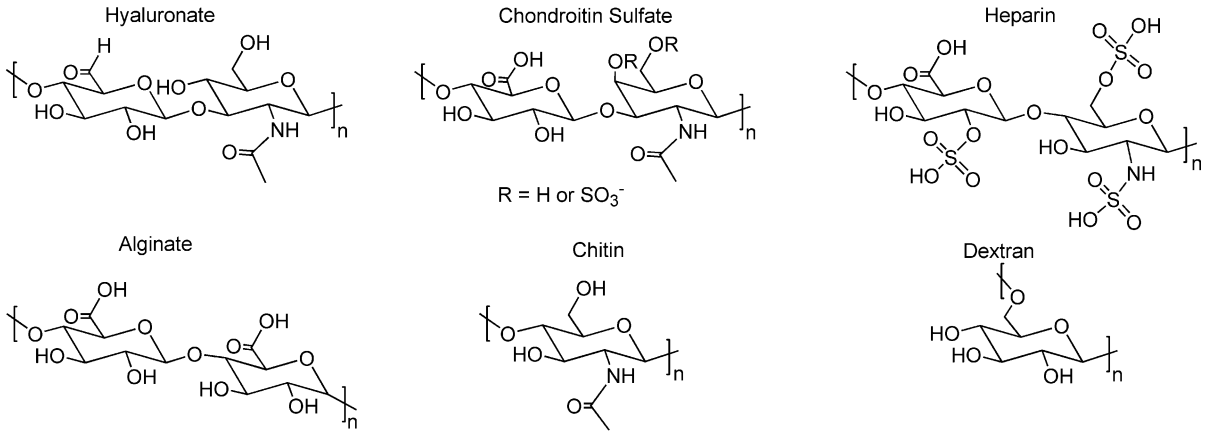
Chapter 1

General Introduction

DEXTRAN

Polymers such as proteins and polysaccharides, abundantly exist in nature and are major components of plants, animals and micro-organisms.[1] Polysaccharides are polymers composed of monosaccharide units connected through glycosidic bonds.[2, 3] The glycosidic bonds are formed by a condensation reaction between the anomeric carbon (C1) of one monosaccharide unit and an oxygen atom from a hydroxyl group on another monosaccharide unit. Since hydroxyl groups present on C2, C3, C4 and C6 carbons, the generated glycosidic bonds can be 1→2, 1→3, 1→4, or 1→6 linked providing linear to highly branched chains. Polysaccharides can be categorized into two groups: (1) mammalian polysaccharides such as hyaluronan, chondroitin sulfate, and heparin; (2) non-mammalian polysaccharides such as alginate, chitin, and dextran (Scheme 1).



Scheme 1. *Main structures of repeating units of some polysaccharides widely investigated in biomaterials applications. Top row: mammalian polysaccharides; bottom row: nonmammalian polysaccharides.*

Dextran is a neutral and hydrophilic polysaccharide consisting of an α-(1→6) linked D-glucose main chain with a relatively low level of α-(1→3) branched linkages.[4, 5] The molecular weight of dextran chains can vary from 1 to 2 000 kilodaltons (kDa). Although physiochemical properties of each dextran polymer depend on its particular structure (e.g. molecular weight and degrees of branching), the glycosidic bonds of dextran provide relatively high chain mobility and solubility, while the three pendant hydroxyl groups provide handles for its functionalization.[6-8] Moreover, dextran is biologically inert due to the α-(1→6) glycosidic bonds making it resistant to cleavage by most endogenous cellular glycosidases, but it is degradable by dextranase[9] which exists in mammalian (including human) tissues. Dextran is also non-immunogenic,[10-14] making it an attractive polymer for the design of novel biomaterials.

Dextran and dextran derivatives are already successfully commercialized in the biomedical field. Examples of dextrans used pharmaceutically include those of molecular weight 70 kDa

(Macrodex®) and 40 kDa (Rheomacrodex®) for plasma expansion.[15-17] Furthermore fluorescent dye-dextran conjugates (Molecular Probes®) are widely applied in numerous biological studies.[18] Dextran can also be crosslinked by epichlorohydrin to form hydrogel beads, which are used for separations of molecules based on differences in molecular weight (e.g. Sephadex®).[19] Dextran-based materials are also used in wound healing (e.g. Debrisan®).[20]

Due to their hydrophilicity, long shelf-life, relative stability, biocompatibility and biodegradable properties, dextrans have been studied as a starting material for drug delivery carriers. Cargo that was delivered in these studies included low molecular weight drugs, proteins/enzymes, and imaging agents by either direct attachment or through a linker since the 1980s.[11, 21-23] Degradation of linkages between dextran and the conjugated drug results in the delivery of therapies. Dextran conjugation was also used to overcome problems such as limited solubility of the drug, short plasma half-life high toxicity and/or undesired interactions with tissues. For example, the solubility of paclitaxel increased >2000 fold upon conjugation to aminated dextran (M_w = 70 000). Furthermore, modification of the dextran-paclitaxel conjugates with folate increased the uptake 2-3 folds in human oral carcinoma KB cells.[24] The *in vivo* biodistribution and circulation time can also be altered upon dextran conjugation. For example, prolonged circulation time and decreased uptake in kidney were observed for dextran-mouse epidermal growth factor (mEGF) conjugates, wherein mEGF was attached to aldehyde group functionalized dextran polymers *via* the formation of imine bonds which was further stabilized by reducing to secondary amide bonds with $NaCNBH_3$.[25, 26]

HYDROGELS

Hydrogels are a class of soft materials that possess a hydrophilic three-dimensional (3D) network hold together *via* chemical and/or physical bonds (i.e. crosslinks). Upon contact with water, the 3D-network swells and the (physically) entrapped molecules diffuse out of the network and the kinetics of release depend on both the properties of the encapsulated molecule as well as the physical characteristics of the hydrogel. The viscoelastic properties are similar to soft tissues and combined with the water-rich environment making these hydrogels an attractive biomaterial with potential applications in the fields of tissue engineering and regenerative medicine.[27, 28] These hydrogels can be synthesized from various precursors over a large molecular weight range. In this thesis the formation of hydrogels comprised of dextran covalently crosslinked by proteins is described.

In the last two decades, *in situ* forming hydrogels have been extensively investigated since they can be delivered to patients in a minimally invasive fashion.[29, 30] *In situ* forming hydrogels allow premixing of drug compounds or cells,[31] followed by injection of the formulation with its non-toxic gelation due to polymer crosslinking in the target tissues in a therapeutically relevant time

period. To this end, extensively applied chemoselective ligations for *in situ* gelation include Michael addition,[32] azide-alkyne Click reactions,[33, 34] Schiff base formation[35] and disulfide exchange.[36] Moreover, physical crosslinking methods with suitable gelation kinetics can also be used to form *in situ* hydrogels.[37] The physical crosslinks can consist of hydrogen bonds, hydrophobic interactions, π-π interactions or host-guest inclusion, to endow the hydrogels with dynamic, reversible and self-healing properties.[38]

Hydrogels have been studied as a biomaterial for the localized and sustained release of therapeutic agents in order to decrease the number of drug administrations, to prevent rapid drug degradation or to prolong therapeutic drug concentrations.[39] Typical drug release mechanisms applied in drug delivery systems include diffusion controlled drug release[40], degradation controlled drug release,[41] stimuli triggered drug release,[42] and affinity-based drug release. The affinity-based drug release mechanism uses non-covalent interactions between the therapeutic agents and hydrogel platform, such as electrostatic, hydrophilic-hydrophobic, hydrogen bonding, or van der Waals interactions, to control the release of the therapeutic.[43] These materials can improve drug loading, stability and release. Affinity-based carriers include specific host molecules such as cyclodextrin,[44-48] serum albumin[49-52] and heparin[53-55], or cavities formed within the solid polymer material by the molecular imprinting technique.[56, 57]

DEXTRAN GELS

Since the 1990s, hydrogels composed of dextran have been developed for macroscale therapeutics delivery and 3D cell culture.[58, 59] Chemically crosslinked dextran hydrogels can be prepared from a functionalized dextran precursor. For example, Hennink and co-workers prepared degradable dextran hydrogels by free radical polymerization of aqueous solutions of glycidyl methacrylated dextran or hydroxyethyl methacrylated dextran for the delivery of proteins.[9, 60-62] Liu et al. used UV-light exposure to initiate the crosslinking between dextran functionalized with both methacrylate and lysine groups and methacrylamide modified gelatin in presence of a photoinitiator and cells to obtain a 3D smooth muscle cell culture.[63]

To improve the biocompatibility of the gelation process and the obtained hydrogels, crosslinked dextran hydrogels can be prepared from a pair of polymeric precursors that can react with each other under physiological condition. Hiemstra et al. synthesized thiol modified dextrans and vinyl-sulfone modified dextrans to induce hydrogel formation *via* a Michael type addition reaction.[64] Wei et al. synthesized fulvene modified dextran and dichloromaleic-acid modified poly(ethylene glycol) to develop self-healing dextran hydrogel *via* reversible Diels–Alder reaction.[65] Supramolecular dextran hydrogels have also be developed *via* specific non-covalent interactions. For example, Huh et al. established a thermo-reversible hydrogel system with poly(ethylene glycol) grafted dextrans

and α-cyclodextrins which was crosslinked by the inclusion complex formation between poly(ethylene glycol) and α-cyclodextrins.[66] De Jong et al. established another thermo-reversible hydrogel system with L-oligo(lactic acid) grafted dextran and D-oligo(lactic acid) grafted dextran which was crosslinked by the stereocomplex formation between enantiomers.[67]

Besides macroscopic dextran hydrogels, various emulsification approaches were applied to obtain chemically crosslinked dextran microgels (also called as microspheres or microcapsules) and nanogels. Using a cyclohexane inverse miniemulsion, crosslinked nanogels with an averaged diameter below 100 nm were prepared starting from alkyne-dextran and azide-dextran.[68] To avoid the use of organic solvents, "water-in-water" emulsions were used to synthesize microgels. Aqueous mixtures of dextran and polymers such as polyethylene glycol (PEG) or poly(vinyl alcohol) (PVA) can form an aqueous two-phase system at elevated concentrations, which can be emulsified to obtain a water-in-water emulsion. De Geest et al. emulsified an aqueous solution containing dextran azide and dextran alkyne in an external aqueous PEG phase. $CuSO_4$ and sodium ascorbate were added afterward to obtain dextran microgels *via* Huisgen click reaction.[69] Similarly, Ghugare et al. prepared dextran microgels with an averaged diameter around 1000 nm using ultrasound assisted "water-in-water" emulsification. The emulsion contained dextran methacrylate, photoinitiator and PVA was crosslinked under UV-light afterwards.[70]

Additionally, charged dextrans, such as dextran sulfate, dextran phosphate and dextran amine, have been used to prepare polyelectrolyte complex. For example, positively or negatively charged colloids were obtained from non-stoichiometric polyelectrolyte complexes of dextran sulfate and chitosan.[71] Dextran sulfate and protamine were constructed to microcapsules for enzyme delivery by a layer-by-layer approach using a removable melamine formaldehyde template.[72] Complexes formed by cationized dextran amine and plasmid DNA enhanced gene expression *in vitro*, showing its potentials for gene delivery.[73]

GENERAL OUTLINE OF THE THESIS

In the present thesis, several dextran-based hydrogel systems are synthesized and characterized for biomedical applications. To prepare dextran polymers for crosslinking, the backbone is initially functionalized with maleimides or vinyl sulfones, which are able to form covalent bonds with nucleophilic thiols *via* a thiol-Michael addition reaction. These reactive groups (i.e. maleimides or vinyl sulfones) were coupled to the hydroxyl groups of dextran *via* an ester or an ether linkage. The stability of linkages strongly affected the properties and applications of the prepared hydrogel systems as described in the following chapters.

Human serum albumin (HSA), the most abundant protein in human plasma, strongly binds various

types of (hydrophobic) low molecular weight molecules. Inspired by this natural carrier, a covalently crosslinked dextran-albumin hydrogel system was developed in **Chapter 2** to explore the potential of HSA as a hydrophobic-drug cavity and crosslinker for affinity-based drug delivery. Native HSA was modified with 2-iminothiolane (2-IT) to introduce thiol groups at the lysine residues. Circular dichroism spectroscopy revealed that the conformation of thiolated HSA (sHSA) is mostly maintained showing that sHSA was capable of simultaneously being a drug carrier and the crosslinker in the hydrogel system. The hydroxyl groups of dextran were modified with maleimide groups through the formation of ester linkages resulting in functionalized dextran (i.e. Dex-Mal). Next, sHSA was added to Dex-Mal resulting in gelation of the mixture under physiological conditions in a relatively fast manner. The resulting dextran-albumin (**Dex-Mal-sHSA**) hydrogel was characterized by oscillatory rheology experiments. *In vitro* release profiles of three hydrophobic drugs (i.e., ibuprofen, paclitaxel and dexamethasone) from the dextran-albumin hydrogel were recorded, showing high drug loading efficiency and demonstrating sustained release of the corresponding guest molecule.

To improve the stability and to gain control over the properties of the abovementioned dextran-albumin hydrogel system (Dex-Mal-sHSA), two new affinity-based drug delivery hydrogel systems were developed in **Chapter 3**. Firstly, a fraction of the dextran hydroxyl groups were modified with vinyl sulfone. The effect of reaction temperature, reaction time and dextran molecular weight was studied to control degree of vinyl sulfone modification of dextran (i.e. Dex-VS). In comparison to the Dex-Mal polymer in Dex-Mal-sHSA hydrogels used in Chapter 2, the obtained vinyl sulfone functionalized dextran polymers (Dex-VS) showed to be more stable against hydrolysis. Next, a new dextran-albumin hydrogel system (**Dex(VS)-sHSA**) with improved stability was developed by reacting Dex-VS with sHSA. However, the vinyl sulfone groups show relatively slower reaction kinetics towards thiol groups than the maleimide groups. To accelerate the gelation process while maintaining the concentration of albumin constant, a third macromolecular precursor, poly(ethylene glycol) dithiol (PEG-DT), was introduced into the dextran-albumin hydrogel. By balancing the number of reactive vinyl sulfone groups from Dex-VS and thiol groups from both sHSA and PEG-DT for the thiol-Michael addition reaction, the covalently dual-crosslinked **Dex(VS)-sHSA-PEG** hydrogel system was prepared where sHSA acts both as a drug carrier and crosslinker. Three Dex-sHSA-PEG hydrogels with different weight concentrations were studied by oscillatory rheology experiments and swelling tests and compared to the corresponding Dex(VS)-sHSA and Dex-VS-PEG hydrogels. By introducing the PEG crosslinker the gelation kinetics and the mechanical properties of the resulting three-component Dex-sHSA-PEG hydrogels could be more easily controlled in comparison to the other two dextran-albumin hydrogels. The capacity of the network to release a drug molecule, Doxorubicin, in a sustained manner was validated through *in vitro* studies using MCF-7 breast cancer cells.

Cyclodextrins are able to form a reversible host-guest complex with suitable hydrophobic molecules. In **Chapter 4** the potential of cyclodextrin-modified dextran based drug carriers in a zebrafish embryo toxicity assay was explored. Zebrafish embryos have become a promising model organism for developmental and reproductive toxicity screening studies.[17, 19, 74] However, ineffective uptake of certain compounds by zebrafish embryos was reported raising concerns of the predictive capacity of zebrafish embryo based toxicity assays.[75] It was reported that zebrafish embryos show a weaker than expected sensitivity for neurotoxic compounds with respect to acute toxicity.[76] Here, a previously reported β-cyclodextrin decorated dextran-poly(ethylene glycol) **Dex-CD/PEG** drug carrier system[45] was used to enhance the uptake of the model drug valproate in the zebrafish embryo toxicity assay. The polymeric drug carrier was synthesized by crosslinking maleimide modified dextran with poly(ethylene glycol) dithiol, wherein cyclodextrins were conjugated to the dextran backbone to ensure binding of valproate through the formation of a non-covalent host-guest complex. The uptake of fluorescent labelled Dex-CD/PEG drug carriers into the gastrointestinal tract of zebrafish embryos was observed by fluorescent microscopy. Comparison of zebrafish embryos exposed to valproate or valproate loaded Dex-CD/PEG carrier showed that the Dex-CD/PEG carrier improves the valproate uptake *via* the gastrointestinal tract. Correlations between the composition of Dex-CD/PEG carriers and the viability of 4-dpf zebrafish embryos after 48-hour exposure to valproate loaded Dex-CD/PEG carrier were also studied to investigate the role of Dex-CD/PEG carriers in the toxicity assay.

In **Chapter 5**, the capacity of hydrogels to guide the formation of giant unilamellar vesicles is explored. Giant unilamellar vesicles (GUVs) are widely used cell membrane model systems for biophysical measurements.[77-79] Recently, it was reported that GUVs can be prepared through hydration of a lipid film on a hydrogel consisting of agarose, which was more straightforward and rapid to generate GUVs in solutions of physiologic ionic strength.[80] However, using agarose for the hydrogel film results in contamination of the vesicles due to its dissolution during the hydration process. To address this issue, the chemically crosslinked polymer system **Dex-Mal-PEG** was covalently anchored to a glass slide as a substrate for GUV formation. Using this method, polymer and additive-free GUVs can be prepared rapidly in high yield under physiological ionic strength conditions. Moreover, by varying physiochemical properties of Dex-Mal-PEG hydrogels through controlling the molar ratio of the maleimide and thiol groups, the molecular weight of PEG-DT and the degrees of substitution of Dex-Mal and PEG-DT, the effect of the network on the yield and size distribution of the prepared GUVs was investigated.

In **Chapter 6**, an attempt was made to direct the assembly of dextran polymers *via* specific coiled-coil interactions. The coiled-coil motif is one of the basic folding motifs in natural proteins, which is a left-handed superhelix formed through the winding of two or more right-handed α-helical peptides around each other.[81, 82] Peptide **E** (amino acid sequence: $(EIAALEK)_3$) and peptide **K** (amino acid sequence: $(KIAALKE)_3$), able to assemble into heterodimeric coiled coils, were grafted

to dextran-vinyl sulfone polymers to obtain multivalent peptide-dextran conjugates. Two pairs of dextran-peptide **E** and dextran-peptide **K** bioconjugates were synthesized, wherein the dextran polymer was attached at either the N- or C-terminus of the peptides. The effect of conjugation on peptide conformation and the interaction between complementary dextran-peptide conjugates was studied by circular dichroism spectroscopy, fluorescence spectroscopy and dynamic light scattering measurements.

Finally in **Chapter 7**, the findings and conclusions of this thesis are summarized and possibilities for future research are presented.

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