Cyclodextrin-Functionalized Microgels and Injectable Hydrogels for the Delivery of Hydrophobic Drugs

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Abstract

The mechanical and chemical properties of hydrogels make them excellent vehicles to deliver drugs. However, current systems encounter difficulties with loading hydrophobic molecules into the aqueous gel network and the subsequent release of the drug from the gel matrix. Cyclodextrins (CDs) offer a potential solution to this drug delivery challenge. CDs have the unique property of possessing a hydrophilic exterior and a hydrophobic interior pocket which is capable of hydrophobic drug binding. CD molecules complexed with hydrophobic drugs have been demonstrated to significantly increase the bioavailability of those drugs in free solution. Thus, if these nanodomains are introduced into microgels or hydrogels, we anticipate that significantly higher hydrophobic drug loadings may be achieved together with improved controlled release of these drugs based on the properties of the hydrogel or microgel phase. We have fabricated *in situ* gellable and degradable hydrogels and microgels based on combinations of CDs and either functionalized carbohydrates (dextran) or thermosensitive synthetic polymers (poly(N-isopropylacrylamide), PNIPAM). To achieve this goal, we designed a series of microgels with grafted or immobilized CD groups and used multi-functional CD as a reactive crosslinker for making injectable bulk hydrogels.

β-Cyclodextrin (βCD) was modified with carboxymethyl groups and hydrazide groups to convert it into a functional molecule that can be incorporated into hydrogels and microgels. Both native and carboxymethylated βCDs exhibited similar levels of drug solubilizing ability, although at higher concentrations native βCD appears to be slightly more effective. Carboxymethylated βCD was more effective at solubilizing dexamethasone than hydrazide-functionalized βCD.

Higher functionalized βCD hydrazide derivatives displayed greater binding efficiency with dexamethasone than their less functionalized counterparts. Carboxymethylated βCD derivatives were found to bind the fluorescent probe 1,8- anilinonaphthalene sulfonate (1,8-ANS) with significantly higher affinity than unmodified or other functionalized βCD derivatives.

In hydrogel systems where βCD was covalently attached to the polymer network through cross-linking, a reduced release rate of dexamethasone was observed. This result was attributed to the immobilization of the βCD/drug complex within the hydrogel network. Furthermore, a step pattern was observed in the beginning of the release curve. Release was not controlled by the solubility of dexamethasone under these conditions but rather the absence of a concentration gradient of free dexamethasone between the gel phase and the saturated aqueous release phase.

In order to investigate the potential of hydrophobic drug delivery from microgels, a number of poly(N-isopropylacrylamide)-based microgel candidates were synthesized with different amounts and distributions of CDs via both chemical grafting and entrapment of linear CD-functionalized polymers. Results from FTIR, ^{1}H-NMR and 1,8-ANS fluorescence enhancement analyses indicate the successful modification of NIPAM based microgels with CD functional groups. NIPAM microgels functionalized with methacrylic acid (MAA) and thus, possessing a majority of functional groups at the particle core, exhibit the most ideal characteristics. Particle size measurements and electrophoretic mobility values indicated limited particle aggregation and both FTIR data and 1,8-ANS fluorescence enhancement data indicated that these microgels may possess the highest level of CD functionalization. On this basis, MAA microgels appear to be the most promising candidate for a hydrophobic drug delivery vehicle.

Acknowledgements

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Chapter 1 Literature Review

Drug Delivery

The effectiveness of a drug is not only determined by its ability to carry out its intended physiological action, but also by its tendency to remain localized at its target site at concentrations that will allow for a successful therapeutic intervention. Advances in synthetic chemistry and high throughput screening techniques have contributed to the development of drugs exhibiting increased efficacy; however, issues with the effective delivery of these compounds remain outstanding. Polymer-based delivery systems are considered the most important tools for addressing these problems as they can easily be adapted to fit the complex and dynamic nature of biological systems. Specific design principles can be applied to these systems in order to achieve long-term drug dosing through safe and non-invasive mechanisms. These systems offer advantages over traditional drug administration methods such as reduced side effects, lower doses, and improved patient compliance.

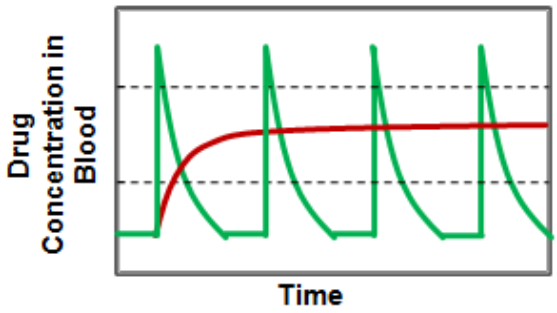

Figure 1.1 Schematic depicting both steady-state and fluctuating pharmacokinetic profiles

The most common route of drug administration is oral delivery, although absorption of the drug through this method is compromised by the tendency of drugs to be degraded in the gastrointestinal tract. (Saltzman 2001) Injections of pharmaceutical agents can allow for targeted delivery through intravenous, intramuscular or subcutaneous routes. However, the drug must exhibit significant levels of aqueous solubility in order to be administered in this manner. (Saltzman 2001) Current modes of drug administration experience difficulty in achieving steady-state drug concentrations, an ideal pharmacokinetic profile that results in clinical benefits. Sharp increases in local drug concentrations are usually observed, followed by a gradual reduction in the concentration until the next drug dosage occurs (Figure 1.1). Fluctuations in local concentrations of pharmaceutical agents can produce toxic effects at high levels or display an absence of therapeutic benefit at lower concentrations.

Polymer-based systems can be designed to generate specific drug delivery profiles. In these systems, release is controlled by the diffusion of the drug species through a polymer network. A concentration gradient is established between the polymer network containing a local drug reservoir and the external environment, creating a driving force for diffusion. The diffusivity of the drug molecule depends on the physicochemical properties of the drug (for example, level of hydrophobicity or hydrophilicity), the morphology of the polymer network, and the tendency of the drug to interact with the polymer chains. (Miro, Ungaro et al. 2011) Drug release may also be a consequence of the degradation of the polymer matrix or an effect of the swelling of the system over time. (Langer 1998)

Several types of polymer-based drug delivery systems have been reported. For example, a drug can be covalently attached to a polymer carrier through a degradable bond. Modification with a polymer such as polyethylene glycol can increase the half-life of a drug and bypass a

possible immunogenic response. (Srichana 2009) Biodegradable polymer networks are preferred drug delivery devices since the clearing of non-toxic degradation products circumvents issues with the removal of drug depleted devices. In these systems, a combination of diffusion and matrix degradation determine release rates. Mechanisms of degradation include surface or bulk erosion. Surface erosion results in the degradation of the exterior of the device with little water penetration into the core, while bulk erosion is characterized by water penetration throughout the structure and uniform degradation of the network. (Alvarez-Lorenzo, Moya-Ortega et al. 2011)

Hydrogels are one of the most investigated drug delivery platforms. These networks of cross-linked water soluble polymers typically exhibit low toxicity and high biocompatibility due to their high water content as well as their mechanical and chemical similarities to biological tissues.(Hoare and Kohane 2008) The polymer networks can be chemically cross-linked (for example, through UV photopolymerization) or physically cross-linked (through hydrogen bonding, electrostatic or hydrophobic interactions). Hydrogels can be modified in a number of ways to establish desired drug release and degradation profiles. For example, hydrolytically or enzymatically degradable cross-links can be introduced into the polymer network of a hydrogel. (Peppas, Hilt et al. 2006) Other properties, like cross-link density and swelling ability can also be adapted to fit specific delivery requirements. (Alvarez-Lorenzo, Moya-Ortega et al. 2011) A major disadvantage associated with hydrogels is the challenge in loading hydrophobic drugs inside an aqueous gel network. If a degree of drug loading is achieved, the gel network may collapse as a consequence of the increased internal hydrophobicity, reducing the average pore size of the network and impeding the diffusion limited release of the drug. Another major obstacle encountered in drug delivery applications of hydrogels is the fact that hydrogels are too elastic to

be easily injected, demanding that they first be synthesized outside of the body and then surgically implanted inside a patient. A less invasive alternative for introducing a hydrogel delivery vehicle inside the body would consist of an injectable linear polymer solution that forms a gel *in vivo*. (Hoare and Kohane 2008)

Similar to hydrogels, microgels are covalently cross-linked polymer networks that can swell in water. These spherical particles have diameters ranging from 50 nm to 5 μm and display properties that are intermediate between hydrogel and nanoparticle systems, including fast swelling kinetics, high surface area and increased bioavailability. On a macroscopic level, microgel systems behave like stable colloidal dispersions. (Pelton and Hoare 2011) Microgels can be synthesized from materials that exhibit unique responses to environmental stimuli, such as temperature, pH and even a specific wavelength of light. For example, microgels based on *N*-isopropylacrylamide or NIPAM display temperature responsive properties. (Pelton 2000) Above the lower critical solution temperature (LCST) of 32°C, a NIPAM microgel is insoluble and will shrink so as to collapse the polymer network. Below the LCST, a NIPAM microgel is soluble and, thus, the polymer network is swollen. These properties are conducive to both controlled drug loading and release. In the swollen state, the large average pore size within a NIPAM microgel facilitates drug uptake or (in the case of drug-loaded microgels) diffusion-limited release. In the shrunken state, a drug loaded NIPAM microgel can either exhibit a burst release or can trap the drug inside the matrix, depending on the physicochemical properties of the drug. (Pelton 2000) A significant drawback to microgels is the difficulty in achieving controlled and extended release of hydrophobic drugs (or, given the small diffusional path length in microgels, any type of drug). Our lab has previously shown that NIPAM-based microgels can bind large amounts of hydrophobic molecules, although drug release was prevented due to microgel condensation. (Hoare and Kohane 2008) Because the introduction of hydrophobic groups lowers the phase

transition temperature of the microgel, drug binding induces a local phase transition at the site of drug uptake, reducing the local pore size and thus the likelihood of release into an aqueous environment.

Hydrophobic Drug Delivery

Drugs that are classified as hydrophobic molecules can vary in their extent of aqueous dissolution and can range from sparingly soluble compounds to completely insoluble compounds. In general, a hydrophobic drug is considered insoluble if it exhibits a solubility in water of less than 0.1 mg/L.(Rabinow 2004) As described before, both microgels and hydrogels experience challenges with the loading and release of hydrophobic drugs. The introduction of hydrophobic binding sites to these polymer networks, such as grafts of aliphatic chains, is a possible solution. However, this modification would compromise the hydrophilicity of the polymer network and a collapse of the network would likely occur due to condensation effects.

Many strategies have been pursued in order to improve the administration of hydrophobic drugs. For example, salt derivatives of hydrophobic compounds are often synthesized as they exhibit increased aqueous solubility. (Stella and Nti-Addae 2007) However, chemical modification of a drug will likely affect its characteristic properties such as its mechanism of action and half-life. (Rabinow 2004) Increased solubility of a hydrophobic drug can also be achieved by pH adjustment, although this is impossible to accomplish under physiological conditions. As well, the introduction of organic co-solvents to aqueous suspensions of hydrophobic drugs has been proposed as a possible solution to address the limited solubility of these drugs. In most cases, excessive amounts of co-solvents would be required in order to attain reasonable levels of solubility, thus introducing high levels of toxicity to the system.(Rabinow

2004) Solubilization through lipid-based systems such as micelles and emulsions is another possibility. More specifically, nanosuspensions consisting of colloidal dispersions of hydrophobic drugs that are stabilized by surfactants have been developed. (Rabinow 2004) However, all of these systems contain components that will disturb the bilayer of a cell membrane by extracting important biological lipids such as cholesterol. Much research has been directed towards the development of liposome drug carriers. These vehicles can encapsulate large amounts of hydrophobic drugs within a vesicle, but they are marred by a low shelf life, difficulty in targeting specific tissues and rapid clearance by phagocytic cells of reticuloendothelial system (RES). (Langer 1998)

Polymer matrices composed of the synthetic, biodegradable polymers poly(lactic-*co*-glycolic acid) (PLGA) have also been suggested as possible vehicles for hydrophobic drug delivery. However, incorporating hydrophobic drugs within the PLGA matrix often results in the precipitation of the drug, inhibiting its release from the matrix. (Miro, Ungaro et al. 2011)

Based on the demonstrated challenges with other types of hydrophobic drug delivery vehicles as well as the issues with using existing hydrogels or microgels for hydrophobic drug delivery, it is evident that hydrogel and microgel platforms would be highly beneficial for the release of hydrophobic drugs if hydrophobic binding domains could be incorporated within the gel phase without reducing the overall hydrophilicity of the hydrogel. This can be accomplished through the incorporation of cyclodextrin, a molecule that exhibits both an (external) hydrophilic and an (internal) hydrophobic character.

Materials Used

Cyclodextrins

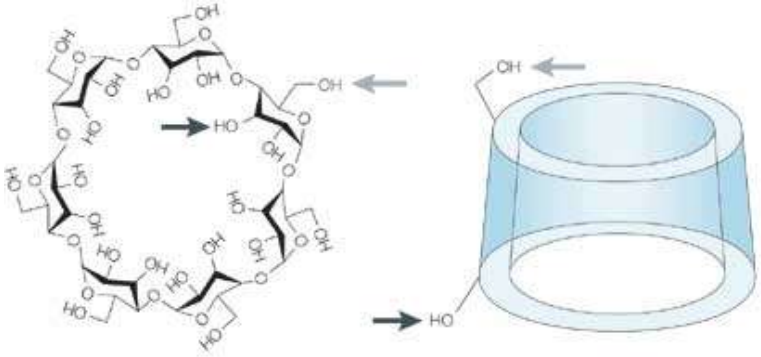

Figure 1.2 General structure of cyclodextrins. (Davis and Brewster 2004)

Cyclodextrins (CD) are cone-shaped molecules that have a hydrophilic exterior and a hydrophobic cavity (Figure 1.2). These molecules possess 1-2 nm pockets which can act as binding sites for hydrophobic drugs. CDs are synthesized through the enzymatic degradation of starch by amylase, originating from the microbe *Bacillus macerans.* (Brewster and Loftsson 2007) The hydrophobicity of the CD cavity is attributed to the presence of carbons and ethereal oxygens arising from the constituent glucose residues. Its polarity has been described to be similar to that of an ethanol/water solution.(Brewster and Loftsson 2007) There are three different types of CDs that can be stably produced: alpha (α), beta (β) and gamma (γ) CDs. These CDs vary in the number of constituent glucopyranoside units and thus, possess different cavity sizes. In order to establish a stable binding interaction, the CD cavity must be able to accommodate the guest molecule. For example, the cavity of αCD (composed of 6 glucopyranoside units) is too small to form an inclusion complex with most drug molecules. (Davis and Brewster 2004) βCD and γCD (composed of 7 and 8 glucopyranoside units, respectively) possess cavities that are large enough to accommodate most small molecule pharmaceutical agents. (Müller and Brauns 1985) βCD is

most often used as a pharmaceutical excipient because of it low cost and high availability. (Szente and Szejtli 1999)

Dextran

Dextran is a carbohydrate polymer that has a number of medical applications due to its generally high biocompatibility in various *in vivo* environments. (Cadée, van Luyn et al. 2000) For example, dextran has been used in surgical procedures to reduce tissue adhesion, as well as in drug conjugation techniques to improve drug half-life and targeting. (Srichana 2009) Dextran is synthesized by microbes such as *Leuconostoc, Streptococcus and Lactobacillis* through fermentation reactions. Once introduced into the body, dextrans are metabolized slowly by the enzyme dextranase, which is located within intra-abdominal organs. (Ito, Yeo et al. 2007)

NIPAM

Since the thermoresponsive behaviour of NIPAM was first described by Heskins and Guillet in 1968, it has been integrated in many polymeric systems.(Heskins and Guillet 1968) As described before, polymers that are based on NIPAM exhibit a phase transition temperature (also known as a lower critical solution temperature or LCST) of approximately 32°C. Below the LCST, the NIPAM polymer is soluble. When the temperature is raised above the LCST, the polymer will aggregate and form an insoluble mass. This temperature responsive behaviour can be rationalized through a thermodynamic explanation. When the temperature is below the LCST, the enthalpic gain generated by the hydrogen bonding of water molecules with the amide groups of the NIPAM molecule renders aqueous dissolution thermodynamically favourable. As the temperature is increased above the LCST, the entropic cost of water re-structuring around the hydrophobic isopropyl groups of NIPAM begins to effectively compete with the enthalpic gain of hydrogen bonding. This results in the aggregation of hydrophobic groups at the phase transition

temperature (at which the Gibbs free energy is equal to zero) in order to avoid the higher-level organization of water molecules around the NIPAM molecule (Pelton 2000).

Dexamethasone

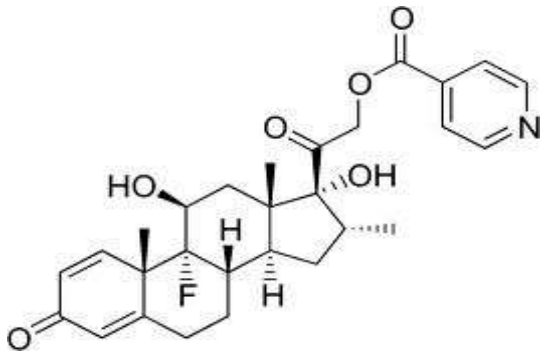

Figure 1.3 Structure of Dexamethasone

Dexamethasone is a corticosteroid that reduces inflammation, which can be physiologically manifested as swelling, increased body temperature, redness, and pain. It is used in the treatment of arthritis as well as various skin, blood, kidney, eye, thyroid, and intestinal disorders such as colitis. (MedlinePlus 2008) Dexamethasone can be a part of therapeutic interventions for ocular diseases such as macular edema. It is considered a hydrophobic drug as it displays an aqueous solubility of approximately 90 µg/mL in 10 mM PBS and about 113 µg/mL in water. (Loftsson, Hreinsdóttir et al. 2005) The relative hydrophobicity of a compound can be calculated by measuring the partition of the compound between an organic phase (typically octanol) and an aqueous phase. The partition coefficient (*P*) can be calculated by taking the ratio of the amount of the compound in the organic phase to the amount of the compound in the aqueous phase, followed by an application of a logarithm to the value.(Cerep 2010) The calculated log *P* of dexamethasone is 1.77. (Lu, Howard et al. 2008) In comparison to other

hydrophobic drugs like naproxen (log P=3.1), dexamethasone displays a relatively moderate level of hydrophobicity. (Loftsson, Hreinsdóttir et al. 2005)

Potential Applications of Research

The treatment of ocular diseases such as age-related macular degeneration, diabetic retinopathy and glaucoma require the delivery of dexamethasone to the posterior segment of the eye. (Loftsson, Sigurdsson et al. 2007) Conventional therapies consist of administering aqueous suspensions of these drugs topically at the front of the eye, although anatomic barriers formed by the sclera and conjunctiva as well as the secretion of tear fluid prevent therapeutic levels of drug from reaching the back of the eye. (Geroski and Edelhauser 2000) To address these issues, eye-drop formulations have incorporated a randomly-methylated CD derivative, which is able to increase the bioavailability of dexamethasone through its solubilizing efficacy and its ability to promote drug penetration through the lipophilic membrane. (Loftsson, Sigurdsson et al. 2007) Unlike other CD derivatives, randomly-methylated CDs display surfactant-like behaviour and can thus access the vitreous cavity of the eye by penetrating the lipophilic barrier of the sclera. (Másson, Loftsson et al. 1999) Following the administration of this eye-drop formulation, dexamethasone concentrations in the posterior segment of the eye were close to the estimated therapeutic concentrations. (Loftsson, Sigurdsson et al. 2007) However, a long-term solution would be preferable in the treatment of these inflammatory ocular diseases. This could be accomplished through a one-time injection of a device that delivers a controlled release of dexamethasone to the back of the eye, avoiding frequent drug dosages that lead to poor patient compliance. The potential transparency of hydrogel-based systems makes such systems of particular interest for ocular use. Thus, incorporation of CDs in hydrogel-based delivery systems, can potentially address the challenges that are unique to drug delivery to the back of the eye.

Objectives of Research

The objective of this work was to design a series of novel injectable hydrogel formulations containing high densities of CD sites with a particular focus on the delivery of dexamethasone. To achieve this goal, we designed a series of injectable βCD-containing hydrogels and microgels by grafting βCD to functionalized microgels, entrapping βCD-grafted linear polymer chains within microgels and using multi-functional βCD as a reactive cross-linker for making injectable bulk hydrogels. βCD was functionalized with carboxylic acid groups and hydrazide groups to convert it into a functional molecule amenable to incorporation into hydrogels and microgels. The complexation behaviour of these βCD derivatives was investigated. Dextran based hydrogels functionalized with βCD were synthesized. Drug release studies were carried out to determine the influence of the amount and organization of βCD within the hydrogel on the drug release profile. NIPAM based βCD-functionalized microgels were synthesized and various analytical tools were used to characterize the properties of these modified microgels.

Chapter 2 βCD Derivatives and their Complexation Behaviour

Introduction

In its native form, β-cyclodextrin (βCD) is not an ideal drug carrier. Properties such as its low aqueous solubility prevent its use as a pharmaceutical excipient in intravenous formulations. If a βCD/drug solution were to be administered intravenously, precipitation could occur either locally or at another site in the body, such as the kidney. (Davis and Brewster 2004; Brewster and Loftsson 2007). βCDs can also induce haemolysis by disturbing the lipid composition of the cell membrane. (Uekama, Hirayama et al. 1998) Unmodified βCDs prove to be best suited for oral drug administration, as they can be metabolized by the gastrointestinal system. (Brewster and Loftsson 2007)

Introducing new functional groups to βCD can increase its solubility, as well as its ability to form complexes with guest molecules. (Szejtli 1992) Poor solubility of the native form is attributed to an intramolecular hydrogen bonding network between the secondary hydroxyl groups in the molecule. Increased solubility can be achieved by modifying these hydroxyl groups with different chemical functionalities. (Davis and Brewster 2004) Modification of βCDs should be carried out with consideration of possible changes in structural dynamics of the molecule, as a larger size and higher number of substituents can hinder access to the CD binding cavity. However, increasing the number of substitutents can also have a positive effect on the solubility of the derivatized compound. Introducing different functionalities to βCD can impart advantageous properties to the molecule. For example, ionic groups can reduce the tendency of βCD to extract cholesterol and other important cellular membrane components. (Szejtli 1992; Uekama, Hirayama et al. 1998)

Because βCD has 21 possible hydroxyl groups that can be modified, an astronomical number of possible homologues and isomers of a derivative can be generated. In fact, the sheer

heterogeneity of these modified compounds accounts for the enhanced properties of functionalized βCDs. It has been demonstrated that selectively functionalizing βCD generates products with a tendency towards rapid crystallization whereas statistically modified products with low degrees of substitution give rise to an amorphous product with low toxicity. (Szente and Szejtli 1999)

There are a number of βCD derivatives that can be safely incorporated into pharmaceutical formulations, including hydroxypropylated βCD (HPβCD) and sulfobutylated βCD. (Brewster and Loftsson 2007) HPβCD is the most widely used and accepted CD-based pharmaceutical excipient due to its high aqueous solubility and minimal toxicity. The acute toxicity of native βCD in rats was reported to be 450 mg/kg, while its hydroxypropyl-methyl-ether counterpart had an LD_{50} greater than 2000 mg/kg. (Müller and Brauns 1985).

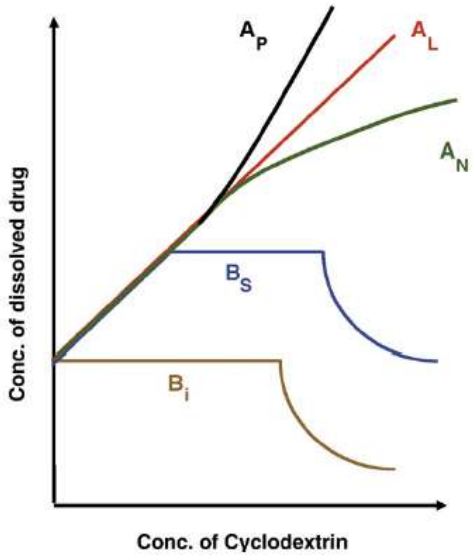

Figure 2.1 Different phase-solubility profiles. (Brewster and Loftsson 2007)

The complexing behaviour of CDs can be investigated by measuring a chemical or physical property of the guest molecule. For example, the stability constant of a CD/drug complex is

determined by constructing a Higuchi phase solubility diagram, which analyzes the CD concentration dependence of drug solubility (Figure 2.1) (Loftsson, Hreinsdóttir et al. 2005). Phase–solubility diagrams can be categorized as A or B systems. In A systems, the apparent solubility of the guest molecule increases as a function of CD concentration. More specifically, an A_L phase solubility profile indicates a linear relationship between CD concentration and solubilized drug. (Grant and Higuchi 1990). This describes the behaviour of most βCD derivatives and suggests a 1:1 binding stoichiometry between the CD host and guest drug. (Uekama, Hirayama et al. 1998) Under these solubilizing conditions, drug precipitation is highly unlikely if a solution containing this particular drug/CD complex were to be diluted. Following a dilution of the pre-formed complex, there will be a certain concentration of free drug according to the equilibrium constant that governs the complexation behaviour. The concentration of the free drug will not exceed the intrinsic solubility of the drug in an A_L type system. This will not be true for the case of non-linear drug/CD complexation relationships. (Rajewski and Stella 1996)

A_P solubility curves describe a system where the host molecule becomes more effective at solubilizing the drug at higher concentrations, while A_N curves describe a system where the host molecule becomes a less effective complexing agent at higher concentrations. (Grant and Higuchi 1990) In aqueous systems, native βCD complexation with guest molecules is illustrated by the Bs phase solubility curve, where at higher CD concentrations, the CD/drug complexes precipitate from solution. (Müller and Brauns 1985)

Another method of examining the complexation behaviour of modified CDs is to study guest molecules that can act as polarity probes. Anilinonapthalene sulfonates (ANS) (Figure 2.2) are fluorescent probes that exhibit different emission intensities based on the polarity of their local environment. Coumarins and nile red are other examples of polarity-sensitive fluorescent probes. (Wagner 2006) Upon excitation in a nonpolar environment, these probes are highly fluorescent;

while in a polar environment they become weakly fluorescent. Thus, when an ANS molecule binds inside a CD host, it exhibits an increased fluorescence. (Wagner and Fitzpatrick 2000)

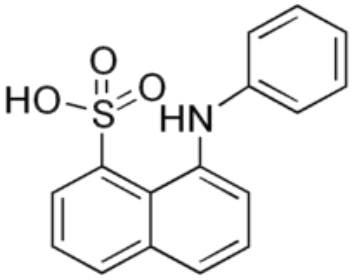

Figure 2.2 Structure of 1,8-ANS

In this study, the complexation behaviour of carboxymethyl cyclodextrin and adipic acid dihydrazide functionalized βCD were investigated with dexamethasone. For the purposes of this project, βCD was functionalized with carboxylic acid groups and hydrazide groups to convert it into a functional molecule amenable to incorporation into hydrogels and microgels. The ability of carboxymethylated βCD to form a complex with ANS was also studied. Carboxymethyl βCDs exhibit a high solubility in neutral and alkaline conditions, but a reduced solubility in acidic conditions. Because of this property, this derivative is thought to be well suited for drug delivery in the small intestine, as the release rate would be reduced under the acidic conditions of the stomach. (Stella, Rao et al. 1999) There have been many studies conducted on different βCD derivatives and their ability to solubilize a wide array of drugs (Arima, Adachi et al. 1990; Marques, Hadgraft et al. 1990; Kompantseva, Gavrilin et al. 1996). Of particular relevance to the current work, Brauns and Muller reported that carboxymethylated βCD (1.82 COOH/βCD) forms a complex with digitoxin and exhibits A_L type solubility behaviour. In comparison to dexamethasone, digitoxin possesses a similar log *P* (log *P*=1.26, while dexamethasone has a log *P* of 1.77), but a higher molecular weight (780.9 g/mol for digitoxin and 392.5 g/mol for dexamethasone). (Loftsson, Hreinsdóttir et al. 2005) The complexation efficiency between

digitoxin and carboxymethylated βCD was lower compared to its methylated counterparts, most likely due to the carboxymethyl group sterically blocking the CD cavity. (Müller and Brauns 1985) There have been no reported complexation studies on hydrazide-functionalized CD (βCD-Hzd), and no data on carboxymethylated βCD (βCD-COOH) induced solubilization of dexamethasone are available. Also, information on βCD-COOH based fluorescent enhancement of ANS probes is lacking.

Materials and Methods

Reagents: β-cyclodextrin (≥97% purity), adipic acid dihydrazide (ADH), N'-ethyl-N-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), chloroacetic acid (≥ 99.0% purity), dexamethasone (HPLC grade, ≥98.0% purity), 8-Anilino-1-naphthalenesulfonic acid ammonium salt (HPLC grade, ≥ 97.0% purity) were purchased from Sigma-Aldrich. Sodium hydroxide pellets were from EMD Chemicals, glacial acetic acid was from Caledon Laboratory Chemicals and all HCl and NaOH solutions were from Acculute Standards. All reagents were used without further purification.

Preparation and Characterization of βCD derivatives

Synthesis of Carboxymethyl Cyclodextrin

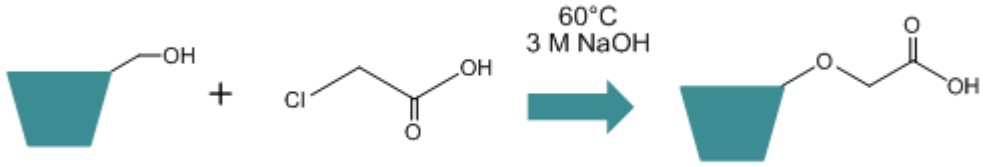

Figure 2.3 Schematic illustrating the carboxymethylation of βCD

Carboxymethyl CD was prepared based on the method described by Rivera(Rivera and University of Puerto Rico 2009). 5 g of βCD was dissolved in 42 mL of a 3 M solution of NaOH. 7.29 g of chloroacetic acid was added to the solution and the mixture was then stirred at room temperature until all components were dissolved. The solution was transferred to a water bath set at 70°C and the reaction was allowed to proceed for 90 min. The solution was cooled to room temperature and neutralized to pH 7.0 with glacial acetic acid. The reaction product was precipitated with methanol and collected through vacuum filtration. Due to the syrupy consistency of the product, it was stirred in acetone overnight. Following collection by vacuum filtration, the white powder was washed with acetone and dried in an oven at 60°C. To achieve a degree of substitution over 3, further carboxymethylations were performed by repeating the procedure described. (Huynh, Chaubet et al. 1998) The reaction time can also be varied in order to synthesize carboxymethylated products with degrees of substitution ranging from 1.4 to 2.6. The average number of carboxymethyl groups on each βCD molecule was determined through potentiometric titration. (See Appendix A1 for example calculation) Approximately 50 mg of product was dissolved in 50 mL of deionized water. The sample pH was adjusted to 3.4 with 0.1 M HCl (Acculute Standards). Samples were then titrated with 0.1 N NaOH (Acculute Standards), which was added at a rate defined by a delta pH/mV of 80 (Mandel PC Titrator). Carboxymethylated βCD products were synthesized with an average of 2.17 COOH per βCD molecule and 2.61 COOH per βCD molecule. Second and fourth reactions were performed on previous carboxymethylated products, generating βCD products with an average of 3.81 COOH per βCD molecule and 5.64 COOH per βCD molecule, respectively.

Synthesis of Hydrazide Functionalized Cyclodextrin

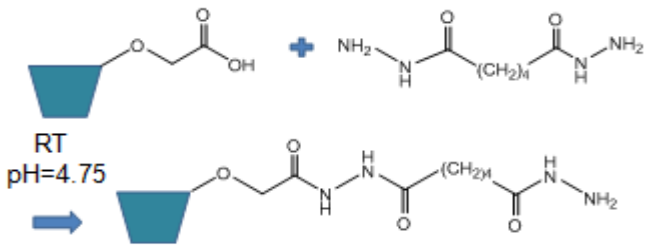

Figure 2.4 Schematic illustrating the hydrazide functionalization of βCD

Hyzdrazide groups were introduced by reacting 5 g of carboxymethylated βCD (3.81 COOH/ βCD and 5.64 COOH/ βCD) with 12.6 g of ADH in 120 mL of deionized water. The pH of the solution was adjusted to 4.75 with 1 M HCl and the reaction was started with the addition of 13.85 g of EDC. The pH was maintained at 4.75 until it stabilized, which took approximately 4 hours. The solution was neutralized to a pH of 7.0 with 1 M NaOH. Water was removed from the product under an aspirator vacuum in a rotary evaporator at 60°C. The product exhibited a syrupy consistency and was precipitated with a large excess of acetone, followed by stirring in acetone overnight. A white powder was generated and collected through vacuum filtration. The degree of substitution of the hydrazide-functionalized product was determined through potentiometric titration. The average number of hydrazide groups per βCD molecule was calculated by subtracting the number of unreacted carboxyl groups detected in the hydrazide-functionalized product from the number of carboxyl groups in the initial carboxymethylated product. Hydrazide-functionalized βCD products were synthesized with an average of 3.08 Hzd per βCD molecule and 5.07 Hzd per βCD molecule.

Dexamethasone Solubility Study

Sample Preparation

Solubility studies were performed according to the methods of Higuchi and Connors(Grant and Higuchi 1990). Excess amounts of dexamethasone were added to solutions containing increasing concentrations of βCD-COOH and βCD-Hzd and were shaken at room temperature for 3 to 7 days. Investigations were carried out in phosphate buffered saline (pH=7.4). In the case of native βCD and βCD-COOH, the drug solubilization study was carried out in 1 N NaOH. The undissolved drug was removed by passing the solutions through a 0.45 μm syringe filter (Pall Corp).

UV Spectrophotometry

Dexamethasone contents in 1 N NaOH solutions containing native βCD and βCD-COOH were determined by detecting the UV absorbance of the drug at 243 nm. (SpectraMax Plus384 Absorbance Microplate Reader).

HPLC

Quantitative determinations of dexamethasone in PBS solutions containing βCD-COOH and βCD-Hzd were performed on a high-performance liquid chromatographic (HPLC) system composed of a 2707 Autosampler, 2489 UV/Visible Detector and 1525 Binary HPLC Pump, all from Waters Corporation. Samples were analysed on a reversed-phase Atlantis C_{18} column (100 mm x 4.6 mm, Waters Corporation). The mobile phase consisted of 40% acetonitrile and 60% water. A flow rate of 1.0 mL/min resulted in retention times of approximately 3.7 min. The absorbance was measured at 263 nm.

Fluorescence Enhancement of ANS

Fluorescence

All solutions were freshly prepared using ultrapure deionized water (Millipore, Resistance >18 MΩ). Steady-state fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian). The excitation wavelength was set at 370 nm and emission scans were performed between 400 nm and 700 nm. Excitation and emission bandwidths of 10 nm were used and fluorescence measurements were made in a 1 cm^2 quartz cuvette at ambient temperature. In all solutions, the fluorescent probe concentration was 4.73×10^{-5} M, while the βCD concentration ranged from 3×10^{-4} M to 0.09 M.

Results

Carboxymethylation of Cyclodextrin

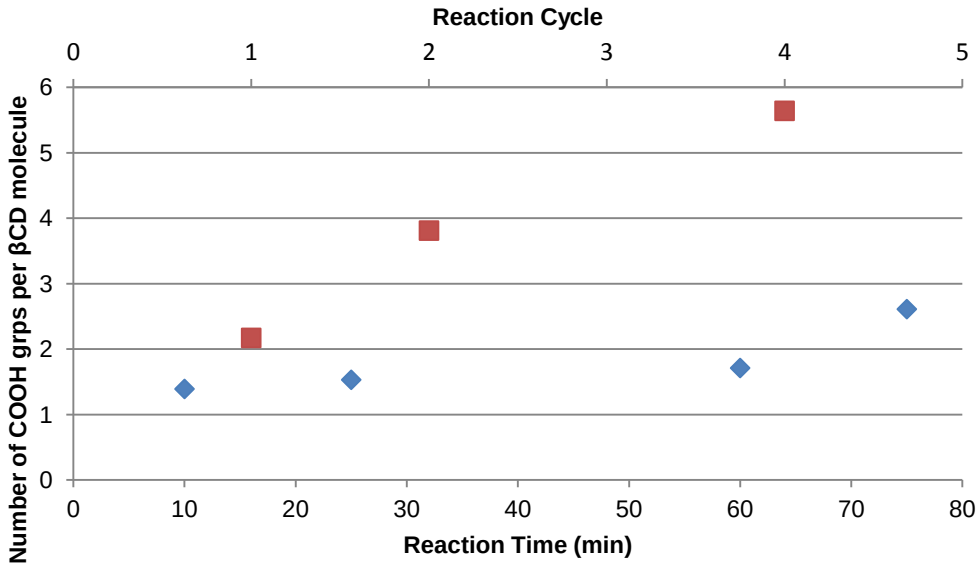

Figure 2.5 Effect of varying the reaction time (blue points, lower x-axis) and reaction cycle (red points, upper x-axis) on the number of carboxymethyl groups on βCD

Depending on the time over which the chloroacetic acid functionalization reaction was conducted, CDs with varying conversions of (mostly primary) alcohols to carboxylic acid groups could be produced. Figure 2.5 shows the average number of –COOH groups per CD chain present as a function of reaction time. By tuning the reaction time, CDs with as few as 1.39 –COOH/CD and as many as 2.61 –COOH/CD could be produced. Performing a secondary reaction could drive the degree of functionalization even higher, up to a maximum of 5.64 –COOH/CD over 4 reaction steps (total reaction time of 2 hours in each step). Thus, CDs useful for single site grafting (the lowest degree of functionalization) or as multi-valent crosslinkers (the higher degrees of functionalization) could be produced using the same reaction step but controlling reaction time. Figure 2.6 shows the titration curves of the carboxylic acid functionalized products generated

from 75 minute reactions. These derivatives were used in the drug solubility assays. Figures 2.7 and 2.8 show the titration curves of both hydrazide functionalized products that were also tested in the dexamethasone solubility assay. Note that hydrazides have very low pK_a values (2.5-3) and thus are not effectively titrated in Figures 2.7 and 2.8; as a result, the reduced titration volume observed is directly related to the degree of hydrazide functionalization.

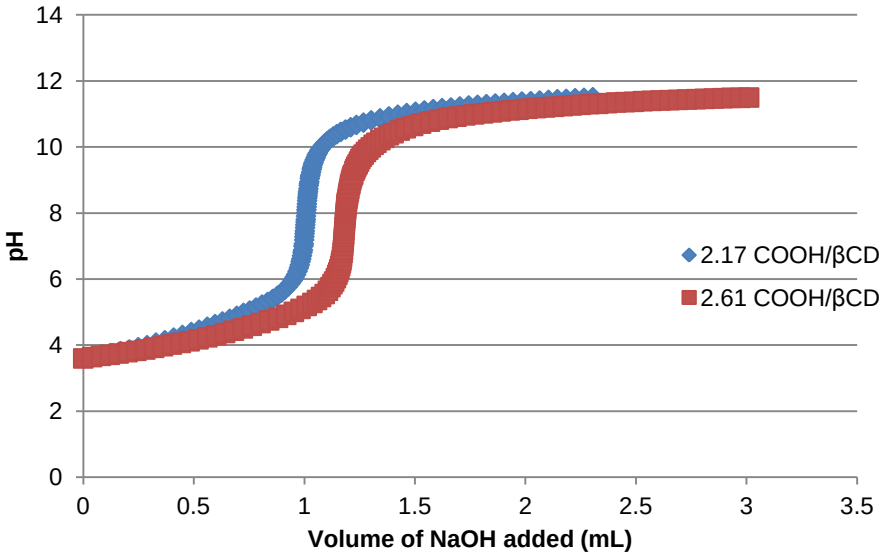

Figure 2.6 Titration curves of carboxymethylated βCD derivatives used in the dexamethasone solubility assays

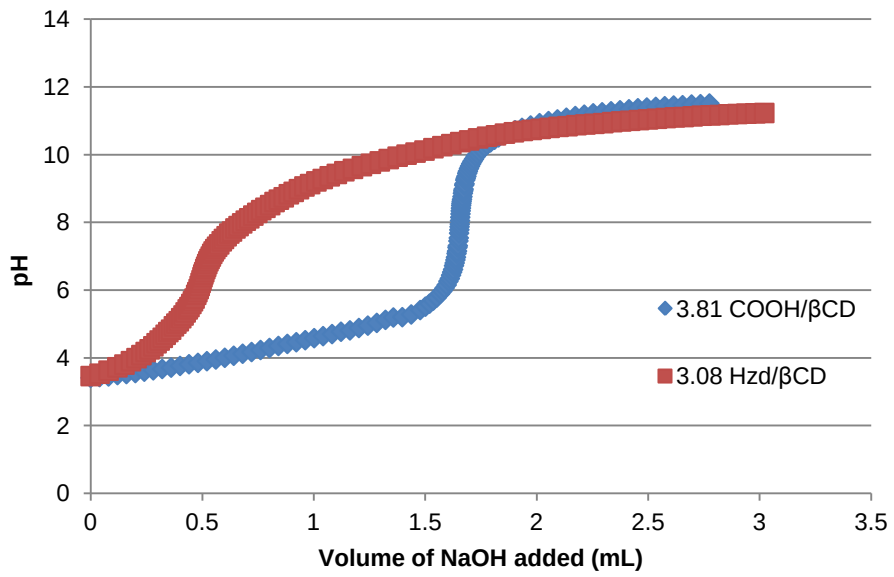

Figure 2.7 Titration curve of the hydrazide functionalized βCD (3.08 hydrazides/βCD) used in the dexamethasone solubility assay. The titration curve of the carboxymethylated βCD intermediate is also shown for comparison purposes.

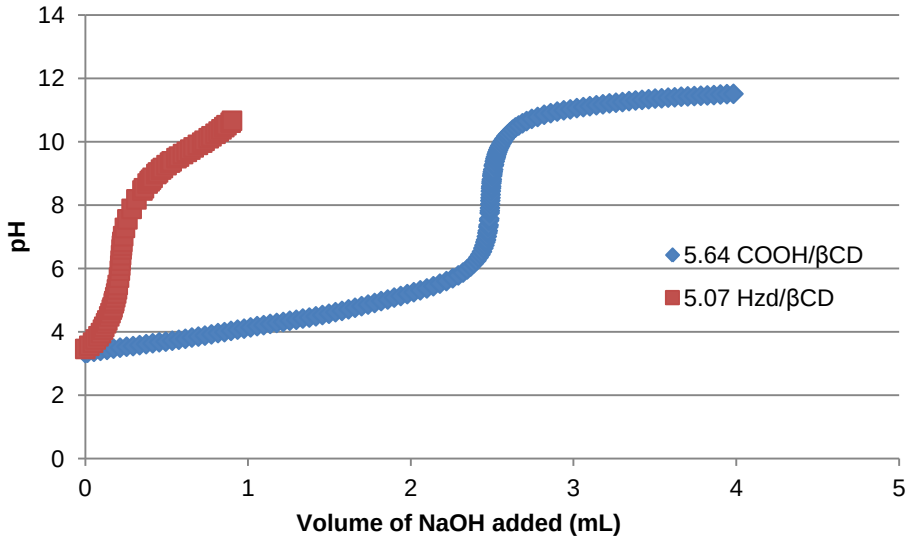

Figure 2.8 Titration curve of the hydrazide functionalized βCD (5.07 hydrazides/βCD) used in the dexamethasone solubility assay. The titration curve of the carboxymethylated βCD intermediate is also shown for comparison purposes

Dexamethasone Solubility Study

Comparison of Native βCD and CD-COOH

Figure 2.9 shows the phase-solubility diagram obtained for dexamethasone with native βCD and βCD-COOH (2.61 COOH/ βCD). Both complexes show A_p type solubility behaviour, where the curves deviate positively from linearity. If the diagram is extended beyond the solubility limit of both parent and βCD-COOH, the solubility of the drug is reduced, indicating that the complex has started to precipitate (Figure 2.10). The solubility limit of the βCD host was determined by noting the concentration of the derivative at which the maximum drug solubility occurs. The A_P type plots suggest that the drug solubility results from the formation of two types of complexes: D-CD, where one drug molecule (D) forms a complex with one CD and D-CD_2, where one drug molecule forms a complex with two CD molecules. (Grant and Higuchi 1990) Both βCDs exhibit similar levels of solubilizing ability, although at higher concentrations native βCD appears to be slightly more effective. This is likely attributable to the steric effects of the carboxymethyl substituent.(Müller and Brauns 1985)

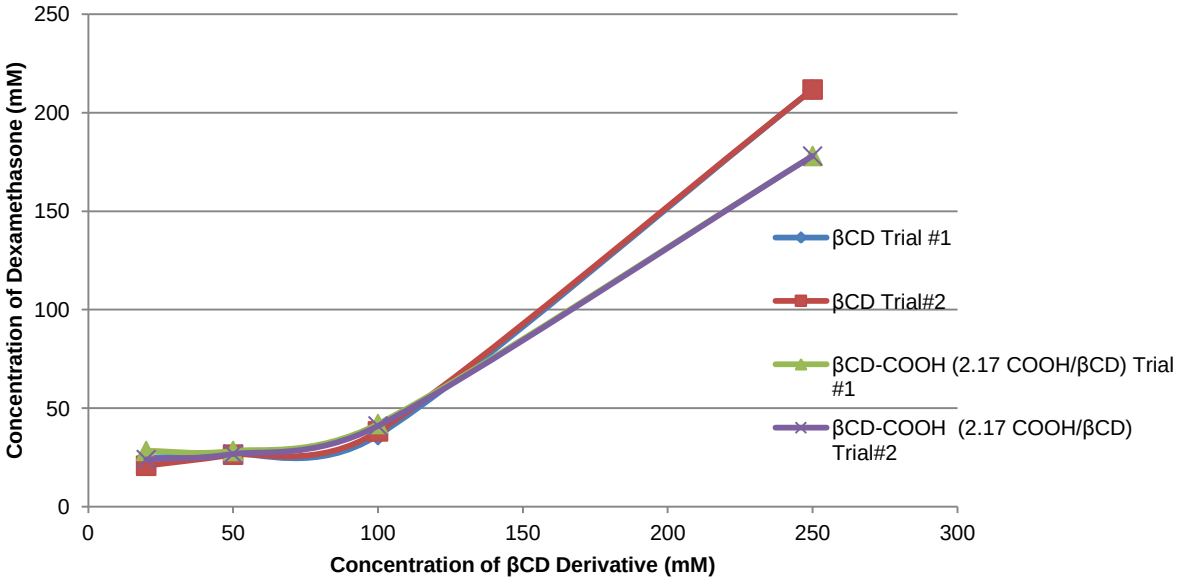

Figure 2.9 Phase-solubility diagram of dexamethasone-βCD and dexamethasone-βCD-COOH systems in 1 N NaOH at ambient temperature.

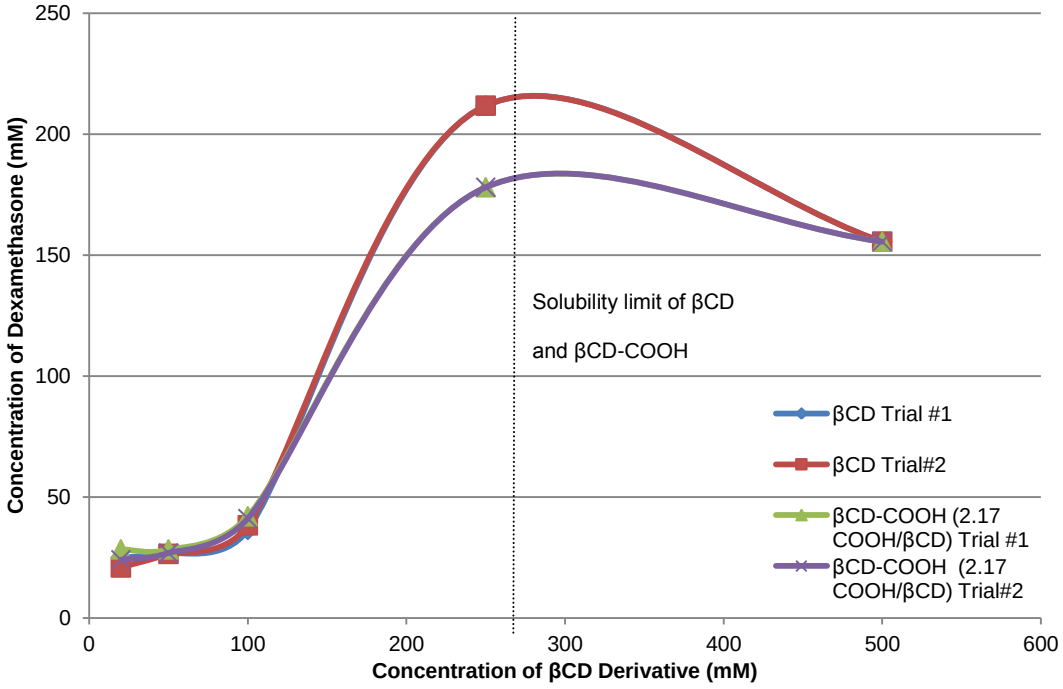

Figure 2.10 Phase-solubility diagram of dexamethasone-βCD and dexamethasone-βCD-COOH systems in 1 N NaOH at ambient temperature, extended beyond solubility limits of βCD and βCD-COOH

Comparison of βCD-COOH and CD-Hzd

The phase-solubility diagrams of βCD-COOH (2.61 COOH) and βCD-Hzd (3.07 Hzd/ βCD) are of the A_L type (Figure 2.11). Since the slopes of all three linear plots are less than one, the assumption of 1:1 drug/CD complexation is valid:

$$D + CD \overset{K_{1:1}}{\longleftrightarrow} D/CD$$

A more quantitative description of the complexation behaviours can be acquired by considering the values of the stability constant, $K_{1:1}$.

$$K_{1:1} = \frac{\text{Slope}}{S_o(1 - \text{Slope})}$$

$K_{1:1}$ is calculated using the slope of the phase-solubility curve and S_o, which is the solubility of the drug in the absence of βCD or the intrinsic solubility. Theoretically, the intrinsic solubility should be equal to the intercept of the phase-solubility plot (S_{int}), but this is hardly ever the case, especially for sparingly soluble drugs like dexamethasone. The S_o of poorly soluble drugs is difficult to determine accurately and the values tend to be much greater than $S_{int.}$, leading to inconsistent $K_{1:1}$ values. Loftsson and his collegues have found that the complexation efficiency (CE) is a more accurate way to compare and contrast complexation behaviours of βCDs. CE is defined as the ratio of complexed CD to free CD and it can be calculated in a manner that is independent of both S_o and S_{int}:

$$CE = S_o \cdot K_{1:1} = \frac{\left[\frac{D}{CD}\right]}{[CD]} = \frac{\text{Slope}}{(1 - \text{Slope})}$$

(Loftsson, Hreinsdóttir et al. 2007) Table 2.1 displays the calculated stability constants and complexation efficiencies of the βCD derivatives. The values indicate that carboxymethylated

βCD is more effective at solubilizing dexamethasone than hydrazide functionalized βCD. This was expected as the size and number of the hydrazide substituents will likely hinder drug binding. What is surprising is that a higher functionalized βCD-Hzd displays greater binding efficiency than its less functionalized counterpart. This could be due to the fact that greater functionalization leads to increased solubility of the derivative itself. The complexation efficiency calculated for carboxymethylated CD is comparable to that of hydroxylpropylated βCD (4.2 HP groups/ βCD) for dexamethasone. The CE value for HPβCD was determined to be 0.326 and a similar discrepancy between the two calculated stability constants was reported. (Loftsson, Hreinsdóttir et al. 2005)

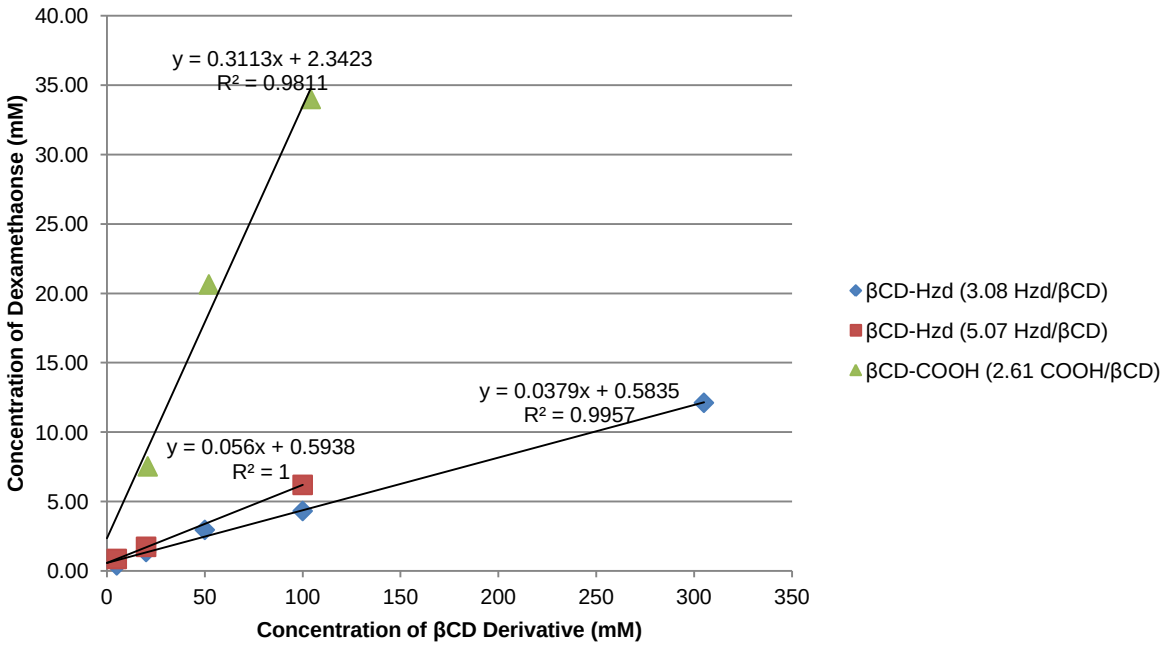

Figure 2.11 Phase-solubility diagram of dexamethasone-βCD-COOH and dexamethasone-βCD-Hzd systems in 10 mM PBS (pH=7.4) at ambient temperature

Table 2.1 Stability constants dependent on the intrinsic solubility (So) or intercept (Sint) of the phase-solubility plot and the complexation efficiency (CE) of varying derivatized βCDs measured in 10 mM PBS.

βCD Derivative	Slope	$K_{1:1}$ (M^{-1}) using S_o	$K_{1:1}$ (M^{-1}) using S_{int}	CE
βCD-COOH	0.3113	1109	193	0.452
βCDHzd (3.08)	0.0379	97	68	0.039
βCDHzd (5.07)	0.056	146	100	0.059

Fluorescence Enhancement of ANS

Upon incremental addition of βCD to the 1,8-ANS solution, there was an observed blue spectral shift from 525 nm to 500 nm. A 9-fold increase in fluorescence intensity was seen with the highest concentration of βCD tested (0.09 M) (Figure 2.12). The association constant (K) between βCD and ANS was calculated using the Benesi-Hildebrand method. A double reciprocal plot of ($C_{ANS}/F_{ANS\cdot\beta CD\text{-}COOH}$) vs ($1/C_{\beta CD\text{-}COOH}$) was constructed, where C_{ANS} is the concentration of 1,8-ANS in solution, $F_{ANS\cdot\beta CD\text{-}COOH}$ is the fluorescence intensity due to complexation and $C_{\beta CD\text{-}COOH}$ is the concentration of βCD. The association constant was determined from the ratio of the intercept to the slope of this graph. (Hoenigman and Evans 1996) In the double reciprocal plot for the βCD-COOH /ANS complex, higher levels of complexation are suggested by the two linear regions of the graph (Figure 2.13). The first linear portion holds information relating to K_2, which describes the 2:1 βCD/ANS complex; the latter linear portion contains K_1, describing the 1:1 βCD:/ANS complex. (Catena and Bright 1989) For this particular system, K_1 was determined to be 8571 M^{-1}, while K_2 was determined to be 200 M^{-1}. Native βCD complexes with 1,8-ANS in a 1:1 fashion, with an association constant of 70.4 M^{-1}. (Hoenigman and Evans 1996) Other βCD derivatives like HPβCD also exhibit a 1:1 binding stoichiometry and an association constant,

K=480 M^{-1}. (Wagner 2006). Thus, βCD-COOH derivatives bind ANS with significantly higher affinity than unmodified or other functionalized βCD derivatives.

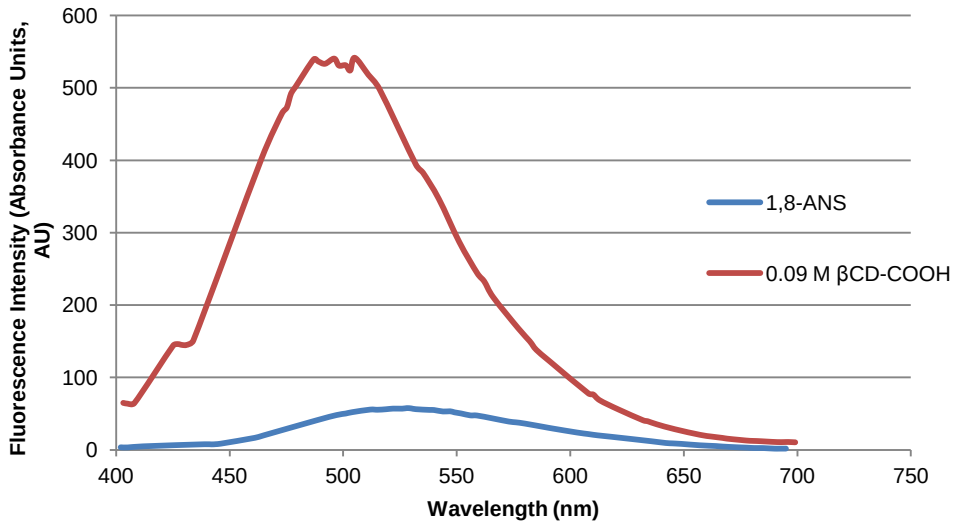

Figure 2.12 Relative fluorescence spectra of 1,8-ANS in a 10 mM phosphate buffer solution containing no βCD-COOH and 0.09 M βCD-COOH.

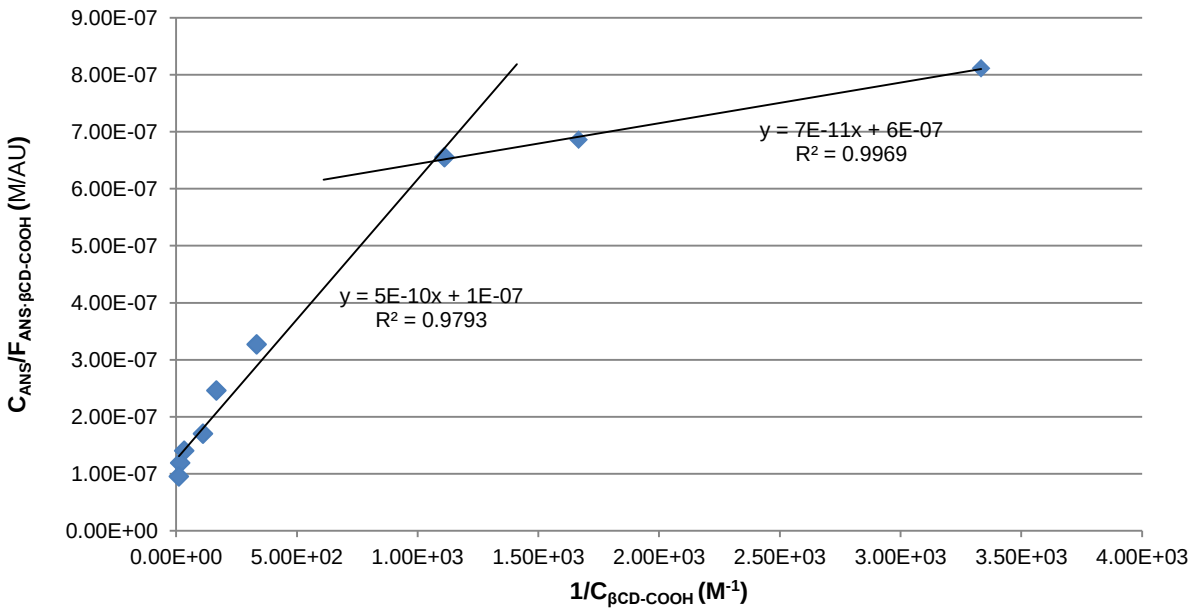

Figure 2.13 Double-reciprocal plot for the 1,8-ANS/βCD-COOH complex.

Discussion

It is difficult to acquire an appreciable comparison of the complexation behaviours of native and derivatized βCDs due to their solubility and stability differences in aqueous systems. βCD is poorly soluble at physiological pH while βCD –Hzd is hydrolytically labile at the 1 M NaOH solution used. (Sivakumaran, Maitland et al. 2011) For this reason, 1 M NaOH was chosen as the medium to study dexamethasone solubilization over a comparative CD concentration range. A quantitative understanding of drug complexation could not be obtained because of the lack of information regarding the intrinsic solubility (S_o) of dexamethasone under these conditions. In the A_p-type behaviour demonstrated by both parent βCD and βCD-COOH in NaOH, 1:1 and 1:2 D/CD complexes were formed. The construction of a ($[S_t] - [S_o]$)/[CD] vs [CD] plot would result in a linear correlation, where S_t is the solubility of the drug in the presence of CD. The binding constants can be derived from this linear plot, where the intercept represents

the product $K_{1:1}[S_o]$ and the slope represents the product of the two association constants, $K_{1:1}$ and $K_{1:2,}$ and $[S_o]$. (Savolainen, Järvinen et al. 1998) To determine the intrinsic solubility of dexamethasone in 1 M NaOH, a phase-solubility diagram of pure drug in 1 M NaOH would have to be generated. This experiment was attempted; however, the value calculated for the intrinsic solubility did not agree with the data collected from the solubility assay. This could have been due to a dilution error while preparing the samples for UV analysis.

The solubility relationships of carboxymethyl and hydrazide functionalized CDs with dexamethasone are considered first order with respect to CD. Complexation may be of higher order with respect to the drug, although the slope values of the A_L type solubility curves indicate that this is not likely. (Brewster and Loftsson 2007) The ability of cyclodextrin to solubilize hydrophobic components is not limited to inclusion-complex formation. It has been demonstrated that cyclodextrins can participate in the solubilization of hydrophobic molecules by forming micelles.(Loftsson, Hreinsdóttir et al. 2007) CD/drug complex formation is also strongly influenced by the composition of the solubilizing medium. It has been found that the presence of buffer salts and polymers can aid in complexation, suggesting that the calculated stability constants are also a reflection of the composition of the medium. (Brewster and Loftsson 2007)This could help explain why carboxymethylated CD displays two different complexation behaviours with dexamethasone in the two different solubilizing mediums tested (1 N NaOH and 10 mM PBS). However, the higher level of complexation observed in NaOH is mostly due to the improved solubility of the derivative under alkaline conditions.

Conclusions and Recommendations

Both native and carboxymethylated βCDs exhibit similar levels of drug solubilizing ability, although at higher concentrations native βCD appears to be slightly more effective. This is likely

attributable to the steric effects of the carboxymethyl substituent. Binding constants for native and carboxymethylated βCDs in 1 N NaOH will be determined once a value for the intrinsic solubility of dexamethasone under these conditions is confirmed. Carboxymethylated βCD is much more effective at solubilizing dexamethasone than the hydrazide functionalized βCD. In fact, the complexation efficiency associated with carboxymethylated βCD is 11.6 times higher than the complexation efficiency associated with hydrazide functionalized βCD. This result can be applied to cases where a CD derivative is to be physically incorporated in a polymer-based delivery system in order to increase the solubility of a given drug. An extension of this solubility assay would involve the investigation of the effect of the degree of carboxymethylation on CD complexation with dexamethasone. As well, this study showed that carboxymethylated βCD derivatives bind 1,8-ANS with significantly higher affinity than unmodified or other functionalized βCD derivatives.

Chapter 3 Injectable, *in-situ* gelling, cyclodextrin-dextran based hydrogels for the release of hydrophobic drugs

Introduction

The release of drugs from polymer-based systems can be adjusted to fit specific therapeutic needs. The polymer composition and network architecture of hydrogels can be altered in order to deliver drugs based on temporal control and on the physicochemical properties of the drug of interest. Accordingly, swellable and degradable hydrogels are well-suited for sustained drug release applications. However, achieving an extended delivery of drugs that exhibit extreme hydrophilic or hydrophobic character from these systems is a difficult task.

Both the loading and release of hydrophobic drugs is considered problematic. If drug loading is attempted following gel formation, the amount of drug incorporated is minimal and rarely reaches therapeutic levels. If a sufficient degree of loading is achieved, the absence of interactions between the hydrophobic drug and the hydrophilic matrix results in a rapid release profile.(Peng, Tomatsu et al. 2010) The tendency of hydrophobic molecules to form aggregates in aqueous environments will inhibit further release and may even impart a level of toxicity to the delivery vehicle.(Peng, Tomatsu et al. 2010) In the case of hydrophilic drug release from a hydrogel carrier, a similar burst release is observed. However, drug uptake in this system is considerable and occurs quickly. (Alvarez-Lorenzo, Moya-Ortega et al. 2011)

With an increasing number of drug candidates displaying a lipophilic nature, adapting hydrogels for the sustained delivery of hydrophobic drugs has attracted particular interest. (Davis and Brewster 2004) The unique properties of cyclodextrins (CDs) allow for the development of a hydrogel platform capable of delivering drugs with varying levels of aqueous solubility. Although it is evident that CDs can facilitate hydrophobic drug delivery by improving their loading within an aqueous environment, these molecules can also exert a finer level of control on the rate of drug

release. Factors that influence the rate of drug release include the solubility and diffusivity of the drug within the delivery matrix, as well as its initial distribution within the polymer network (Bibby, Davies et al. 2000) In addition to employing its influence on drug solubility, CDs can manipulate these features depending on whether they are physically incorporated or covalently attached to the polymer network. (Miro, Ungaro et al. 2011)

Mechanisms by which CDs modify drug release depend on the initial concentration of drug within the delivery matrix and on the difference between the diffusivities of free drug and drug complexed with CD. (Bibby, Davies et al. 2000) Under conditions where the drug concentration is below its solubility limit and thus, the addition of CD is not contributing to an increase in drug solubility, there is no change in the drug release rate if the free and complexed species display the same level of diffusivity. However, if free drug diffusivity is higher than that of its complexed counterpart, CD will reduce the concentration of diffusible species and consequently lower the concentration gradient in favour of a reduced release rate. These methods are applied to lower the release rates of hydrophilic drugs, whose diffusivities and solubilities are compromised by forming higher molecular weight complexes with CD. (Miro, Ungaro et al. 2011)

Upon the addition of CD to a hydrophobic drug carrier, the number of soluble drug molecules increases. If the complexed and free drugs demonstrate similar diffusivities, both species can diffuse out of the matrix and consequently, the release rate is increased. These conditions are illustrated by cases where CDs are physically incorporated within a hydrogel. However, if the drug diffusivity is much higher than its complexed form, the drug cannot diffuse out of the matrix as easily and thus, the release rate is reduced. (Miro, Ungaro et al. 2011)This

situation is demonstrated by cases where CD is covalently attached to the polymer network of hydrogels, either as a cross-linker or as a grafted substituent.

The diffusivity of the free and complexed drug forms also determines whether or not the degree of association between a drug molecule and CD influences the release rate. For cases where the complex is able to diffuse relatively freely, CD association is not considered a significant factor since complex formation is largely a diffusion controlled process. In fact, administering a solution containing CD/drug complexes results in an immediate dissociation of drug due to dilution effects. (Stella, Rao et al. 1999) When CDs are anchored to a polymer matrix, this dilution effect is not observed. In these cases, the affinity of the drug molecules for the CD cavity regulates the release of drugs from the polymer matrix. It is important to note that the covalent attachment of CDs to a polymer network does not compromise its complexation ability; in fact, grafting may even improve the proclivity of CD to complex, especially for larger molecules that exhibit higher levels of binding stoichiometry. (Alvarez-Lorenzo, Moya-Ortega et al. 2011) As well, it has been demonstrated that the addition of free water soluble polymers can increase the complexation efficiency of CDs (Loftsson and Friðriksdóttir 1998), effectively the case when CD is grafted to hydrogels. The incorporation of CDs within hydrogels can also increase the degree of water absorption into a hydrogel and consequently increase the release rate by accelerating the erosion of a degradable polymer matrix. (Bibby, Davies et al. 2000)

There are many published examples of CD-based hydrogels. (Rodriguez-Tenreiro, Alvarez-Lorenzo et al. 2007) (Sangalli, Zema et al. 2001; Woldum, Larsen et al. 2008) The simplest way of introducing CDs inside a hydrogel matrix is through the addition of CD in a drug loading solution. However, this is not an ideal approach as counterdiffusion of the CD/drug complex can

occur, resulting in subpar loading efficiencies and unpredictable release kinetics. (Hoare and Kohane 2008)

It is common for hydrogels to be synthesized from CD polymers generated using the cross-linking agent, epichlorohydrin. Temperature sensitive hydrogels were formed by grafting NIPAM to epichlorohydrin-cross-linked βCD polymers. Below the transition temperature of NIPAM, the hydrogel was swollen, allowing the fluorescent probe, 1,8-ANS, to form complexes with the CD cavities contained within the polymer network. Above the transition temperature, the hydrogel network collapsed, causing the NIPAM chains to sterically block the CD cavities and prevent complex formation. (Alvarez-Lorenzo, Moya-Ortega et al. 2011) Epichlorohydrin is considered highly toxic and thus, alternative synthesis schemes are being investigated. Cross-linking agents like diisocyanates have been used, for example, to form βCD bulk hydrogels designed for the extended release of antibiotics(Thatiparti, Shoffstall et al. 2010), although the toxicity profile of isocyanates remains problematic.

CD based hydrogels can also be synthesized by grafting CDs to an existing polymer network. For example, a reactive CD vinyl derivative was incorporated into hydrogels composed of polyvinylpyrrolidone cross-linked with polyethyleneglycol-dimethacrylate through UV-curing. These hydrogels were used to slow down the release rate of ibuprofenate, a water-soluble drug. (Nielsen, Madsen et al. 2009)

In other cases, physically-gelled hydrogel mixtures have been created by mixing water-soluble polymers, loaded drug, and other components that improve the structural integrity of the system, such as fillers and binders. For example, CD containing dry tablets were formed with hydroxypropyl methylcellulose and different ratios of the sparingly soluble drug sulphamethizole and the βCD derivative, hydroxyproyl βCD. Following hydration and subsequent physical gelation of the drug-loaded polymer solution, the release behaviour of the resulting hydrogel was

investigated. It was found that the cyclodextrin derivative enhanced drug solubility, thereby increasing the rate of drug release. (Pose-Vilarnovo, Rodríguez-Tenreiro et al. 2004). However, such formulations typically dissolve quickly *in vivo*, resulting in only short-term drug release over the course of minutes or hours.

A major obstacle hindering the translation of current hydrogel platforms to a clinical setting is the fact that most systems must be synthesized and then surgically implanted inside a patient. A less invasive alternative for introducing a delivery vehicle inside the body would consist of an injectable linear polymer solution that forms a gel *in vivo*. (Hoare and Kohane 2008). There are few examples of CD-based hydrogels that are injectable and *in situ* gelling, but two recent developments are worth mentioning. Kros and colleagues developed a covalently-crosslinking hydrogel system composed of maleimide functionalized dextran and thiol functionalized β-cyclodextrin (β-CDS). In this system, Dex-mal forms the polymer network while β-CDS acts as the cross-linker and host for the hydrophobic drug, all-trans retinoic acid (RA). The release rate of RA was controlled by its solubility in PBS and was sustained for a two week period. The release profile did not show an initial burst effect. (Peng, Tomatsu et al. 2010)

In 2007, Gref and colleagues developed a physically-crosslinking hydrogel consisting of a drug-loaded βCD polymer (synthesized with epichlorohydrin) and a dextran polymer grafted with alkyl side chains. Upon mixing the two polymer solutions, a gel formed immediately due to the inclusion of the alkyl side chains inside available CD cavities. The release of two hydrophobic drugs, benzophenone and tamoxifen, were investigated and a much longer release of the former was observed (sixteen days). (Daoud-Mahammed, Grossiord et al. 2008)

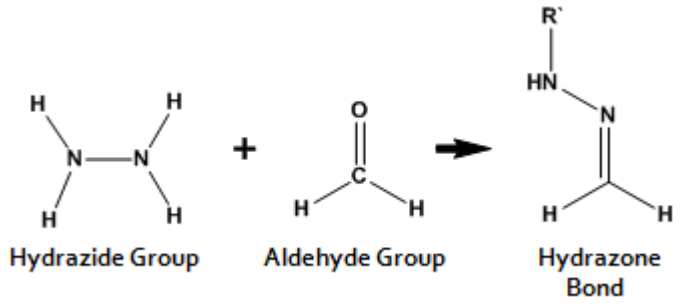

Figure 3.1 Reaction scheme for aldehyde-hydrazide chemistry.

In this study, a dexamethasone loaded dextran and cyclodextrin-based hydrogel was synthesized. Gelation of these materials occurs *in situ* and upon injection through the reaction of an aldehyde-functionalized dextran with a hydrazide-functionalized CD molecule (Figure 3.1). The dual role of CD as a cross-linker and host for the hydrophobic drug allows for the adjustment of hydrogel properties and release behavior by altering the CD/drug ratio. The hydrazone cross-links between the aldehyde and hydrazide-functionalized precursors allow for rapid hydrogel formation as well as offering the advantage of being slowly hydrolyzed at normal physiological pH. Through the addition of a hydrazide-functionalized dextran polymer, CDs were also physically entrapped within the hydrogel matrix. Two distinctly different release profiles were generated depending on the composition of the hydrogel formulated, demonstrating that CDs can help establish a hydrogel platform for hydrophobic drug delivery in addition to controlling the rate of drug release.

Materials and Methods

Reagents: Dextran from *Leuconstroc spp* [Mr- 500,000], sodium periodate (≥99.8% purity), β-cyclodextrin (≥97% purity), adipic acid dihydrazide (ADH), N'-ethyl-N-(3-dimethylaminopropyl)-carbodiimide (EDC), N-Hydroxysuccinimide (NHS), chloroacetic acid (≥ 99.0% purity) and dexamethasone (HPLC grade, ≥98.0% purity) were all purchased from Sigma-

Aldrich. Sodium hydroxide pellets were from EMD Chemicals USA, glacial acetic acid was from Caledon Laboratory Chemicals, ethylene glycol was from Fischer Scientific, and all HCl and NaOH solutions were from Acculute Standards. All reagents were used without further purification.

Synthesis of Hydrazide Functionalized Cyclodextrin

Hyzdrazide groups were introduced by reacting 5 g of carboxymethylated βCD (3.81 COOH/ βCD) with 12.6 g of ADH in 120 mL of deionized water. The pH of the solution was adjusted to 4.75 with 1 M HCl and the reaction was started with the addition of 13.85 g of EDC. The pH was maintained at 4.75 until it stabilized, which took approximately 4 hours. The solution was neutralized to a pH of 7.0 with 1 M NaOH. Water was removed from the product under an aspirator vacuum in a rotary evaporator at 60°C. The product exhibited a syrupy consistency and was precipitated with a large excess of acetone, followed by stirring in acetone overnight. A white powder was generated and collected through vacuum filtration. The degree of substitution of the hydrazide-functionalized product was determined through potentiometric titration. The average number of hydrazide groups per βCD molecule was calculated by subtracting the number of unreacted carboxyl groups detected in the hydrazide-functionalized product from the number of carboxyl groups in the initial carboxymethylated product. Hydrazide-functionalized βCD products (βCD-Hzd) were synthesized with an average of 3.08 Hzd per βCD molecule .

Synthesis of Aldehyde Functionalized Dextran

10 mL of an 80 mg/mL aqueous solution of sodium periodate was slowly added to 1.5 g of dextran [Mr- 500,000] dissolved in 150mL of deionized water. The reaction was allowed to proceed for two hours and was stopped with the addition of 0.4 mL of ethylene glycol. The

modified polymer was dialyzed against water for six cycles and isolated through lyophilization. (3500 Da MWCO membrane, Spectrum Co)

Synthesis of Hydrazide Functionalized Dextran

Carboxymethylated dextran was synthesized according to the protocol described in Chapter 2, with an average functionality of 832.7 COOH/polymer chain. 1.5 g of the carboxymethylated product was reacted with 2.01 g of ADH in 150 mL of deionized water. The pH of the solution was adjusted to 4.75 with 1 M HCl and the reaction was started with the addition of 2.21 g of EDC. The pH was maintained at 4.75 until it stabilized, which took approximately 4 hours. The solution was neutralized to a pH of 7.0 with 1 M NaOH. The modified polymer was dialyzed against water for six cycles and isolated through lyophilization. (3500 Da MWCO membrane, Spectrum Co) The degree of substitution of the hydrazide-functionalized product was determined through potentiometric titration, which indicated an average of 601.3 hydrazide groups per dextran polymer chain. (Appendix A2)

Dexamethasone Loading

An excess of dexamethasone was added to 2 mL of a βCD-Hzd solution and was shaken for 3 to 7 days at ambient temperature. Drug uptake by βCD-Hzd was quantified using gravimetric methods. The drug suspension was diluted in 25 mL of water and centrifuged at 3000 rpm for 45 min. (Allegra® X-15R Benchtop Centrifuge, Beckman Coulter). The supernatant was removed and the pellet containing excess dexamethasone was dried and weighed. The amount of dexamethasone loaded into the βCD-Hzd solution was calculated by subtracting the amount of drug recovered after centrifugation from the amount of drug originally added to the solution. This value was related to the volume of βCD-Hzd solution added to the hydrogel disk (Appendix A3).

Dexamethasone loading in the control dextran hydrogel was achieved by suspending the hydrazide-functionalized dextran in a 90 µg/mL solution of dexamethasone in deionized water.

Preparation of Hydrogel

The hydrogel was formed using a double-barrel syringe (Medmix Systems: Switzerland). Each barrel contained the contents of the gel precursor solutions, composed of either a hydrazide phase or an aldehyde phase as described in Table 3.1. A 20-gauge needle was affixed to the mixer end of the syringe, allowing for ease of injection. Equal amounts of both phases were extruded through the mixing device and injected into silicone rubber molds (McMaster Carr: Illinois, USA). The dimensions of the mold resulted in a disk shaped hydrogel with an approximate volume of 0.226 mL.

Table 3.1 Dex-βCD hydrogel Compositions

Dex-βCD hydrogel	Hydrazide Phase	Aldehyde Phase
CD cross-linked gel (25 mg βCD-Hzd/gel)	110.6 mg/mL 4 wt% dextran-hydrazide polymer	8 wt% dextran-aldehyde polymer
CD cross-linked gel (15 mg βCD-Hzd/gel)	66.4 mg/mL 4 wt% dextran-hydrazide polymer	8 wt% dextran-aldehyde polymer
Physically entrapped CD (25 mg βCD-Hzd/gel)	110.6 mg/mL 8 wt% dextran-hydrazide polymer	8 wt% dextran-aldehyde polymer
Control Dextran gel	4 wt% dextran-hydrazide polymer	8 wt% dextran-aldehyde polymer

Drug Release Studies

Six hydrogels were used for each test sequence and were placed in cell culture inserts (8 um pore size, 2.5 cm diameter, Falcon) in a 12-well culture plate (Falcon). The inserts were perforated 20 times with a 20-gauge needle in order to ensure consistent flow of release medium into and out of the insert. A total of 2 mL of 10 mM PBS was introduced into each well. The release study was carried out in an orbital shaker at 37°C at 100 rpm (VWR Orbital Shaker). PBS release media was replaced every 30 min for the first 2 hours then every hour for the next five hours, followed by subsequent measurements over the course of 21 days. Quantitative determinations of dexamethasone in PBS release solutions were performed on a high-performance liquid chromatographic (HPLC) system composed of a 2707 Autosampler, 2489 UV/Visible Detector and 1525 Binary HPLC Pump, all from Waters Corporation. Samples were analysed on a reversed-phase Atlantis C_{18} column (100 mm x 4.6 mm, Waters Corporation). The mobile phase consisted of 40% acetonitrile and 60% water. A flow rate of 1.0 mL/min resulted in retention times of approximately 3.7 min. The absorbance was measured at 263 nm.

Results

Hydrazide Functionalized Cyclodextrin

Successful modification of βCD with hydrazide groups (βCD-Hzd) was confirmed through potentiometic titration. (Figure 3.2) βCD-Hzd derivatives were synthesized with an average of 3.08 Hzd per βCD molecule.

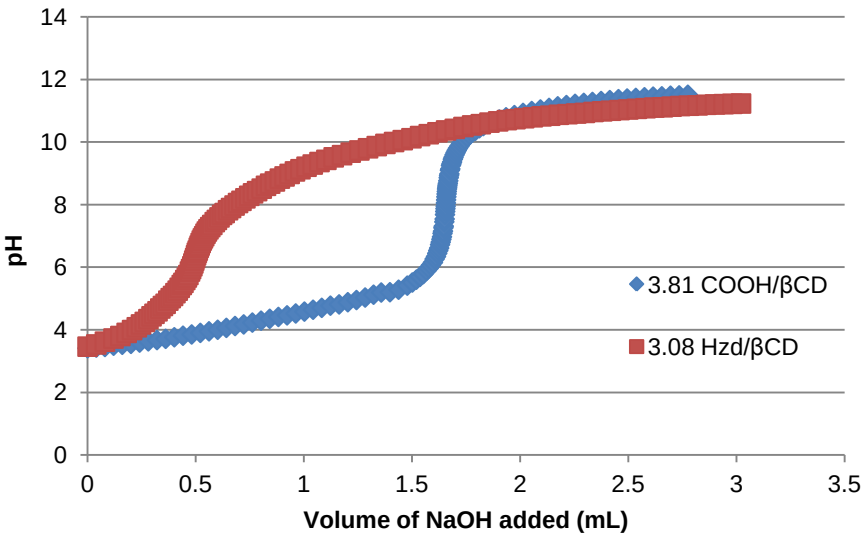

Figure 3.2 Titration curve of the hydrazide functionalized βCD (3.08 hydrazides/βCD) used in the hydrogel synthesis. The titration curve of the carboxymethylated βCD intermediate is also shown for comparison purposes.

Release Kinetics

Immobilization of CD in Hydrogel Network

Figure 3.3 shows the *in vitro* release curve of a hydrogel prepared by crosslinking aldehyde-dextran, hydrazide-dextran and hydrazide-βCD (Dex-βCD) containing 25 mg of βCD-Hzd cross-linker in each disk shaped gel. Hydrazide functionalized dextran polymer was added to these hydrogels at a concentration of 2 weight percent in order to improve their structural stability. However, hydrogels can be formed in the absence of this modified dextran polymer. The amount of dexamethasone loaded in each gel was determined to be 604.6 µg. During the 20 day sampling period of the kinetics study, 5.4% of the total drug was released from each gel, on average (Figure 3.4). The slow release of the drug can be attributed to the immobilization of the CD/drug complex within the hydrogel network. The general structure of this hydrogel network is

illustrated in Figure 3.5. It is interesting to note that a step pattern is observed in the beginning of the release curve. Following a 24 hour period, drug release is suspended due to the saturation of the release medium and release is only renewed when the medium is replaced with fresh PBS. Release is not controlled by the solubility of dexamethasone in PBS under these conditions, as the concentration of the "maximum release" drug solution (approximately 1 μg/mL) is significantly lower than the solubility of dexamethasone in PBS under the conditions of the release study (80 μg/mL). This result can be interpreted in terms of an absence of a concentration gradient of free dexamethasone between the gel phase and the saturated aqueous release phase; in other words, the number of drug molecules in the medium is the same as the number of diffusible drug species inside the gel. This effect is further illustrated by plotting cumulative drug release as a function of the sample number collected during the release study (Figure 3.6). The linear relationship between sample number and drug release indicates that there is a limit in release that occurs regardless of how much time has passed in the study. This limit is established as a result of the lack of mobile drug molecules inside the gel, which can be attributed to the fact that the CD/drug complexes are anchored to the polymer network of the gel. In comparison to the drug release observed from a solely dextran-based hydrogel (Figure 3.7), release achieved was extended and did not show an initial burst.

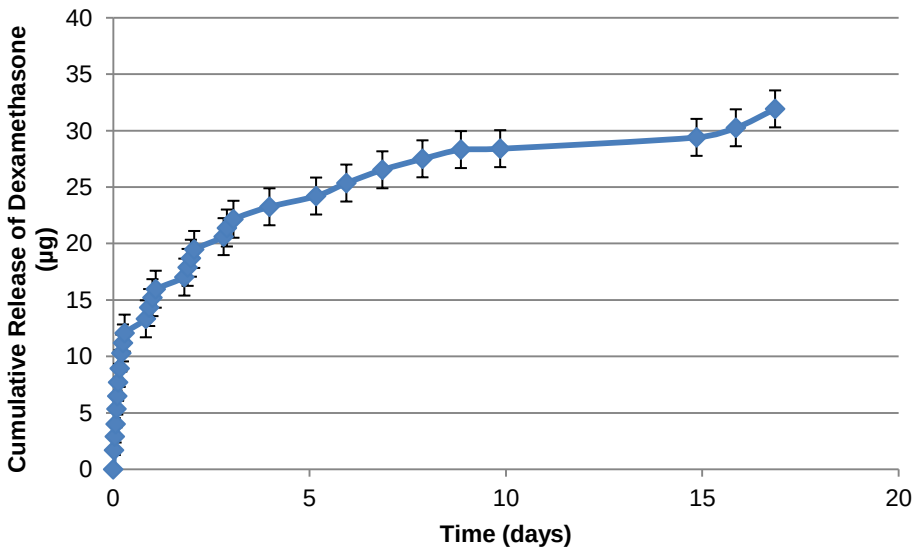

Figure 3.3 In vitro release of dexamethasone from a Dex-βCD gel into PBS buffer [25 mg βCD-Hzd, 2wt% Dex-Hzd] at 37^{0}C.

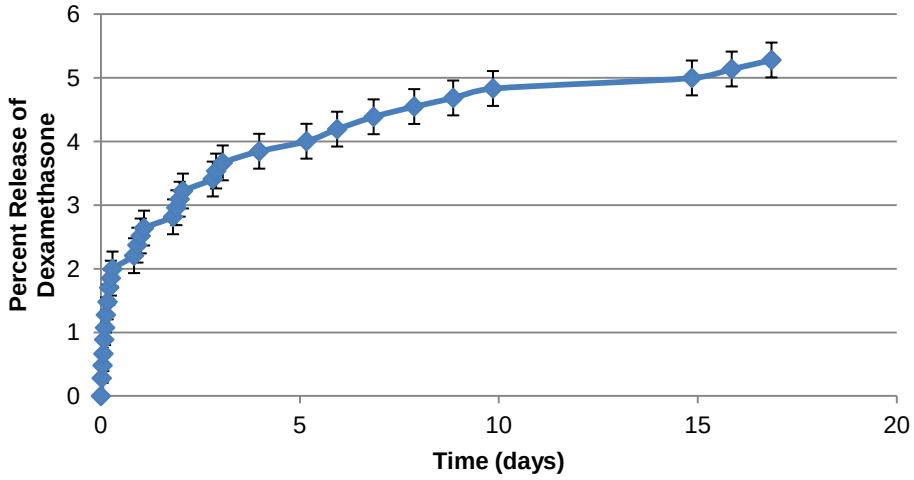

Figure 3.4 Percentage of total dexamethasone released from a Dex-βCD gel into PBS buffer [25 mg βCD-Hzd, 2wt% Dex-Hzd] at 37^{0}C.

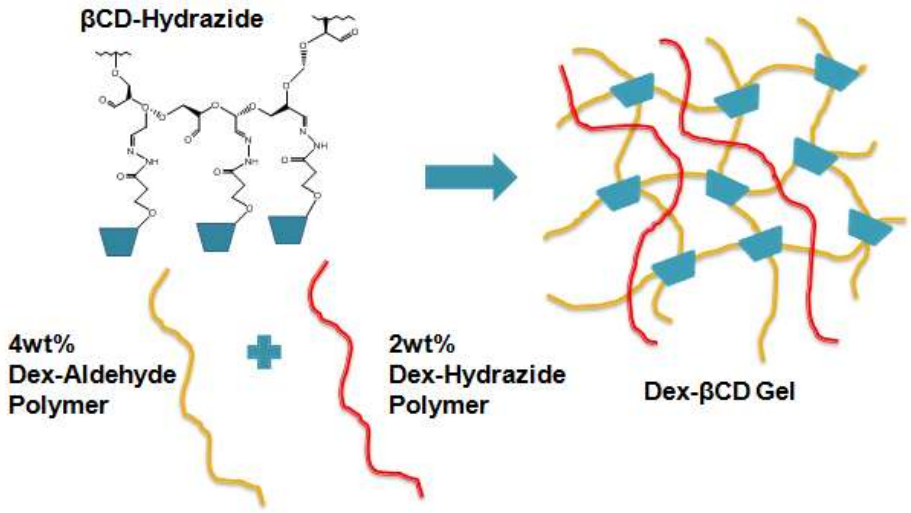

Figure 3.5 General structure of Dex-βCD gels cross-linked with βCD-Hzd, immobilizing CD/drug complexes.

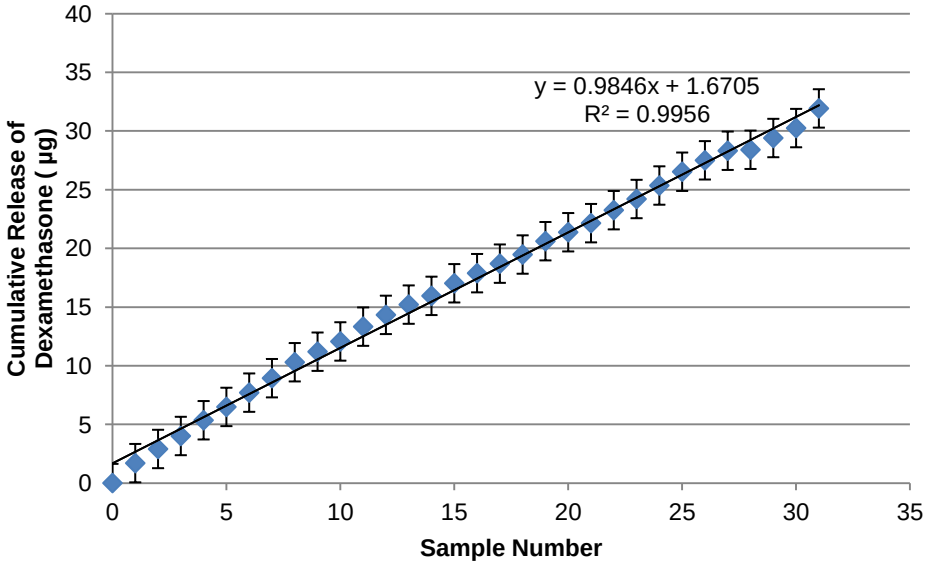

Figure 3.6 Cumulative dexamethasone release from a Dex-βCD gel [25 mg βCD-Hzd, 2wt% Dex-Hzd] as a function of the sample number collected during the release study.

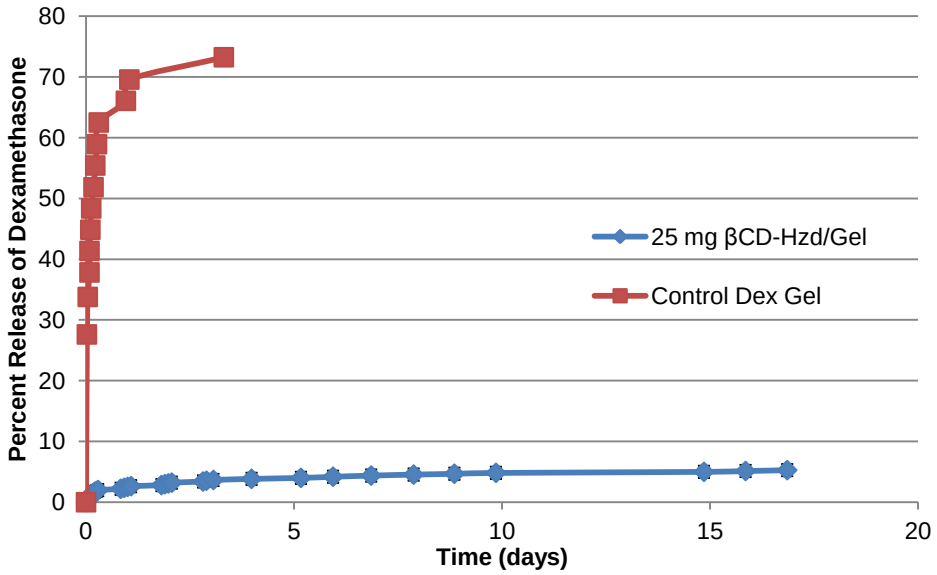

Figure 3.7 Comparison of percent dexamethasone release from a Dex-βCD gel and a control dextran gel [2wt% Dex-Ald, 2wt% Dex-Hzd] into PBS buffer at 37^{0}C.

Degree of Cross-linking

The degree of cross-linking can alter the swelling kinetics of the hydrogel and in turn, the drug release behaviour. The swelling response of hydrogel is enhanced when it has a lower cross-link density. For the case of hydrolytically degradable hydrogels, more swelling should enhance the release of drug through the accelerated erosion of the polymer matrix. (Saltzman 2001) Since CD functions as both the cross-linker and drug carrier, the amount of βCD in Dex-βCD gels was expected to influence the release characteristics. Figure 3.8 shows the release curves of Dex-βCD gels synthesized with different amounts of cross-linker. A lower cross-linking density was achieved by incorporating 15 mg of βCD-Hzd per gel, which was loaded with 508.5 µg of dexamethasone. During the 19 day sampling period, 5.8% of the total drug was released (Figure 3.9). In comparison to the gel synthesized with 25 mg of cross-linker, the release kinetics were very similar. A step release pattern is also observed in the release curves of both cross-linked

gels. The less cross-linked gel also exhibits a linear correlation between sample number and cumulative drug release, although a slight jump between samples 10 and 11 (representing a 24 hour sampling period) indicates that there are more diffusible drug species in this system. (Figure 3.10) The similar release kinetics of the two hydrogels indicates that when CD is immobilized, the release rate is gradual and primarily dictated by the complexation behaviour between CD and drug and not by the cross-linking degree or CD concentration.

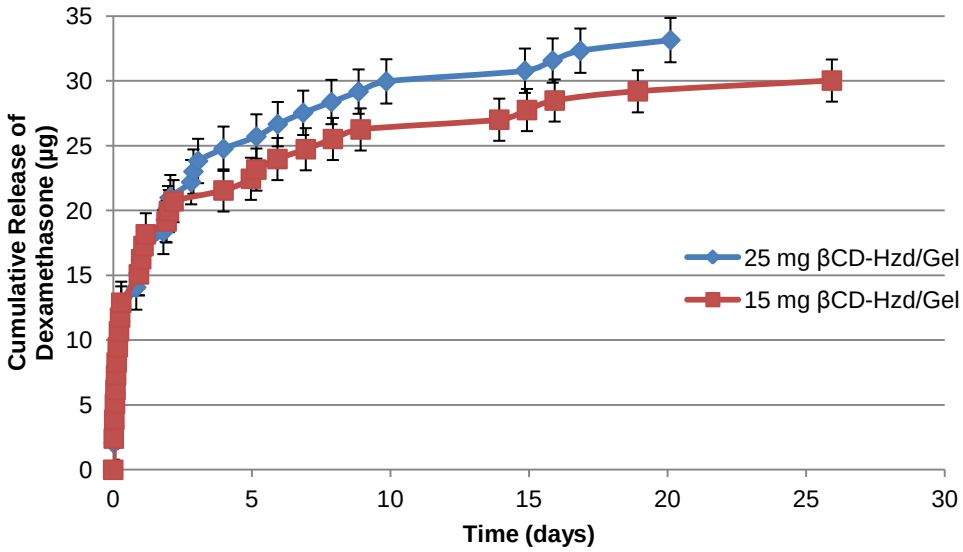

Figure 3.8 Comparison of dexamethasone release (into PBS) from Dex-βCD gels of different cross-link densities.

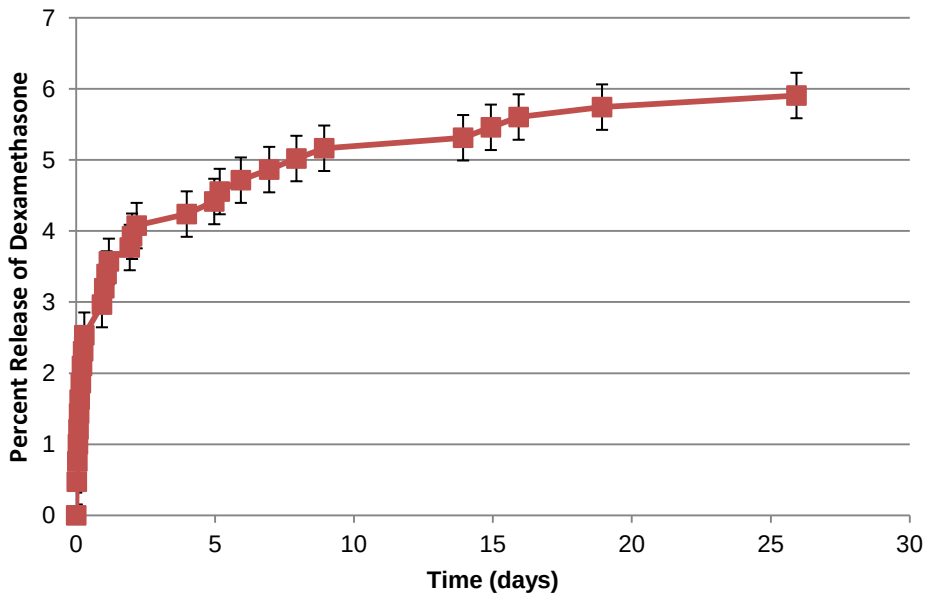

Figure 3.9 Percentage of total dexamethasone released from a Dex-βCD gel into PBS buffer [15 mg βCD-Hzd, 2wt% Dex-Hzd] at 37^{0}C

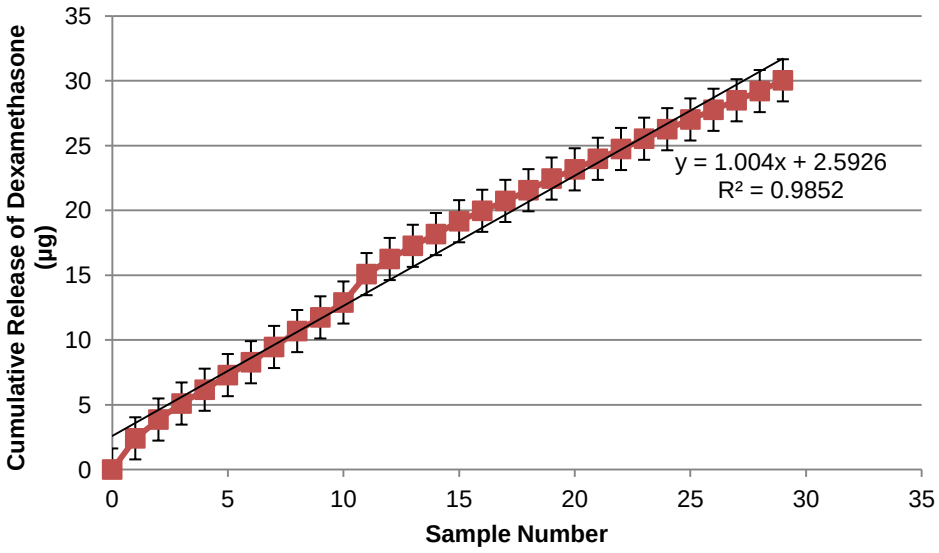

Figure 3.10 Cumulative dexamethasone release from a Dex-βCD gel [15 mg βCD-Hzd, 2wt% Dex-Hzd] as a function of the sample number collected during the release study.

Drug/CD Ratio

In previous studies of hydrogels supplemented with βCDs, it has been demonstrated that the [drug]/[βCD] molar ratios can influence release kinetics. For example, for the release of nicardipine hydrochloride (NIC) from a PEG monolithic device, it was found that the relative amount of NIC released during swelling increased as the ratio of [drug]/[βCD] was lowered. βCD was physically incorporated into these devices by equilibrium partitioning and the diffusivity of the complexed drug was greatly compromised in comparison to its free form. (Quaglia, Varricchio et al. 2001) Similarly, the mobility of dexamethasone is greatly reduced upon complexation with the βCD elements contained within the Dex-βCD gel scaffold. To assay the effect of [drug]/[βCD] ratio on the release kinetics, a Dex-βCD gel was synthesized with 15 mg of cross-linker per gel and loaded with 226 μg of dexamethasone. With a [drug]/[βCD] molar ratio of 0.0705, this gel exhibited a shortened release period of 6 days and released, on average, 12.8% of total contained drug (Figure 3.12). It was found that the release kinetics between the two gels loaded with different amounts of drug were similar, as the same mass of drug was released at each sampling point. (Figure 3.11 and Figure 3.13) The total duration of drug release from hydrogels loaded with less drug is likely to be shorter as there is a smaller amount of drug available in the reservoir. Again, the similar release kinetics of the two hydrogels indicates that when CD is immobilized, the release rate is gradual and primarily dictated by the complexation behaviour between CD and drug and not by the [drug]/[βCD] ratio.

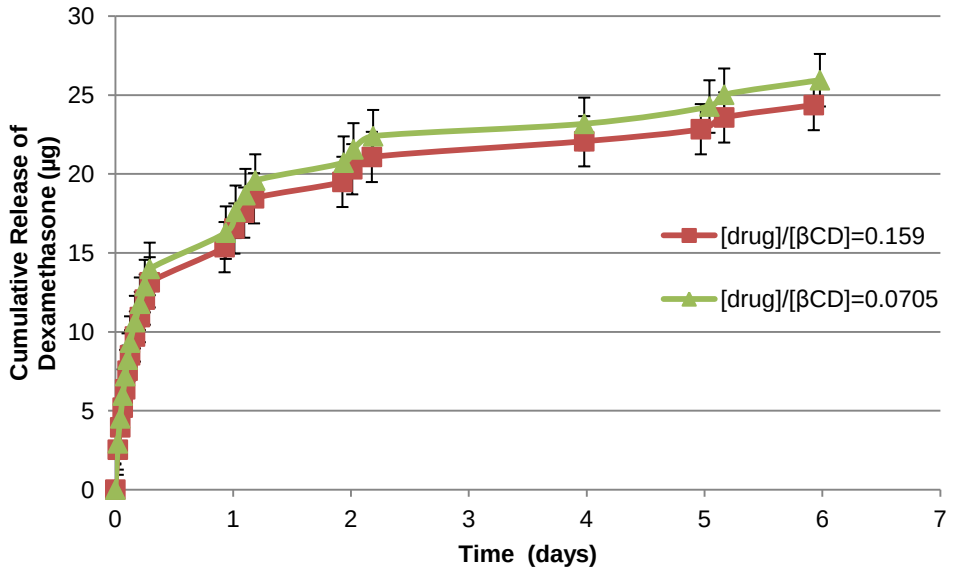

Figure 3.11 Comparison of dexamethasone release into PBS buffer from Dex-βCD gels synthesized with different [drug]/[βCD] molar ratios.

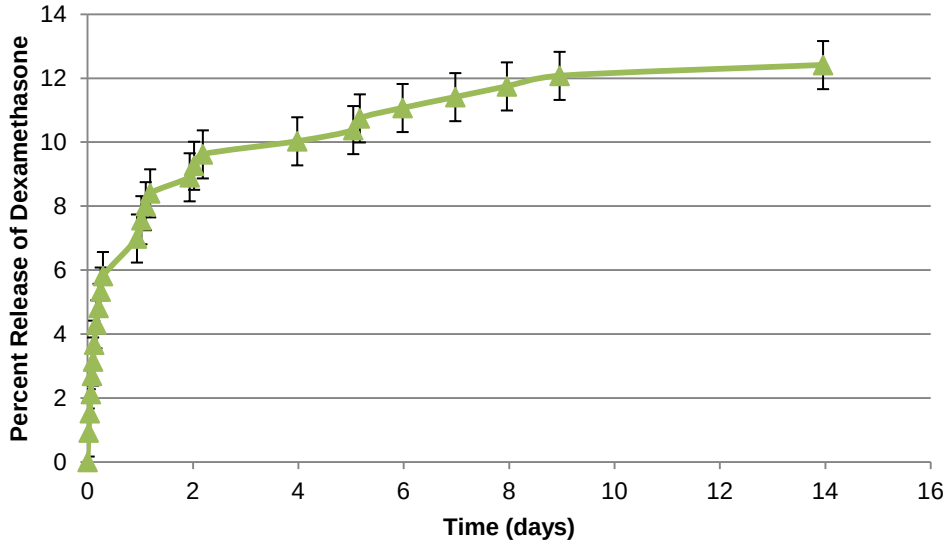

Figure 3.12 Percentage of total dexamethasone released from a Dex-βCD gel into PBS buffer [15 mg βCD-Hzd, 2wt% Dex-Hzd, [drug]/[βCD]=0.0705] at 37^{0}C

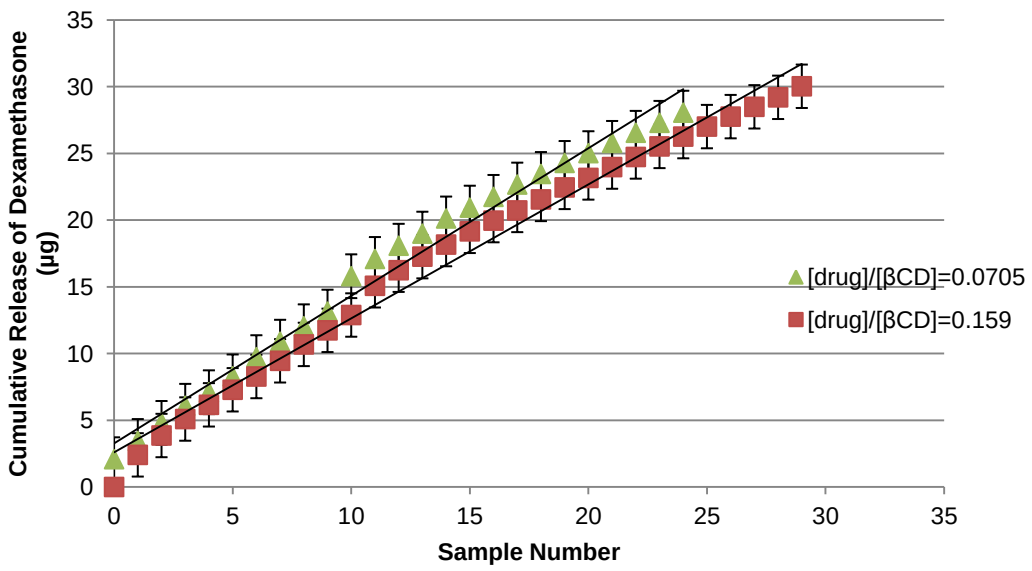

Figure 3.13 Cumulative dexamethasone release from a Dex-βCD gel [15 mg βCD-Hzd, 2wt% Dex-Hzd] as a function of the sample number collected during the release study.

CD Physically Incorporated in Hydrogel Network

The release of dexamethasone was significantly slowed down as a result of the covalent attachment of CDs to the hydrogel network. In order to increase the release rate from Dex-βCD gels, the CDs must be physically incorporated in the hydrogel matrix. By doubling the concentration of the Dex-Hzd polymer in the gel precursor solution, the hydrazide groups on the polymer will compete more effectively with those of the functionalized CD. The CD is less likely to cross-link the polymers and more likely to become physically entrapped in the hydrogel network (although a mixture of both chemically and physically bound CD groups is probable) as illustrated in Figure 3.14. In the entrapped CD hydrogel formulation, the ratio of the total number of hydrazides to the total number of aldehydes is approximately 1.3. In the CD cross-linked hydrogel formulation, the ratio of the total number of hydrazides to the total number of aldehydes

is approximately 1.2. Although the difference does not seem significant, the extra hydrazide groups available on the dextran polymer allow for greater polymer-polymer association. This is because polymer cross-linking is a cooperative process. The formation of one cross-link point brings other reactive groups together in close proximity, facilitating subsequent reactions. If these associations are occurring relatively quickly, a polymer network will be forming, creating a tortuous diffusion path for the βCD-Hzd molecules. Sterically blocking the diffusion of free βCD-Hzd molecules prevents them from accessing potential aldehyde reactive sites. By applying this synthetic strategy, gels were formed containing 25 mg of βCD-Hzd and loaded with 536.8 μg of dexamethasone. During the 20 day release study, 64% of the total contained drug was released from each gel (Figure 3.16). While there is some evidence for step-wise release between closely-spaced sampling points (attributable to the fraction of βCD molecules cross-linked to the hydrogel), when drug release is plotted as a function of the sample number collected during the release study, a linear relationship is not observed. Furthermore, the large increase in release observed between samples 10 and 11 represents a long, 24 hour period between the sampling steps (Figure 3.17). Together, these results indicate that the number of diffusible drug species is not limiting the release as observed with gels formed by βCD-Hzd cross-linking.

A particularly striking result is the 11-fold higher dexamethasone release observed in comparison to the gels composed of a βCD cross-linked structure (Figure 3.15). Because the CDs in both compositions were functionalized to the same degree, complexation behaviour between the drug and CD should be similar. Thus, differences in release kinetics are attributed to the higher mobility of the complexed drug when CD is physically entrapped in a hydrogel. In this system, the solubilizing effect of βCD is dominant, creating an influx of diffusible drug species and establishing a concentration gradient in favour of rapid, diffusion-controlled release.

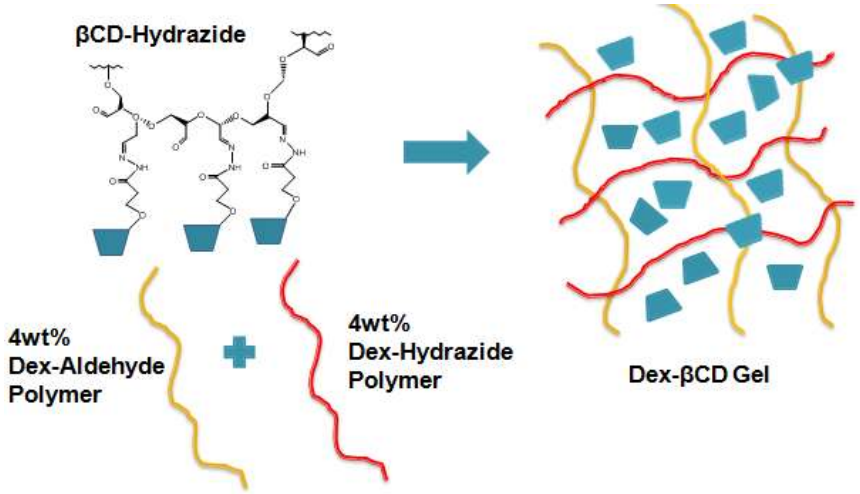

Figure 3.14 General structure of Dex-βCD gels with βCD-Hzd physically entrapped in the hydrogel matrix

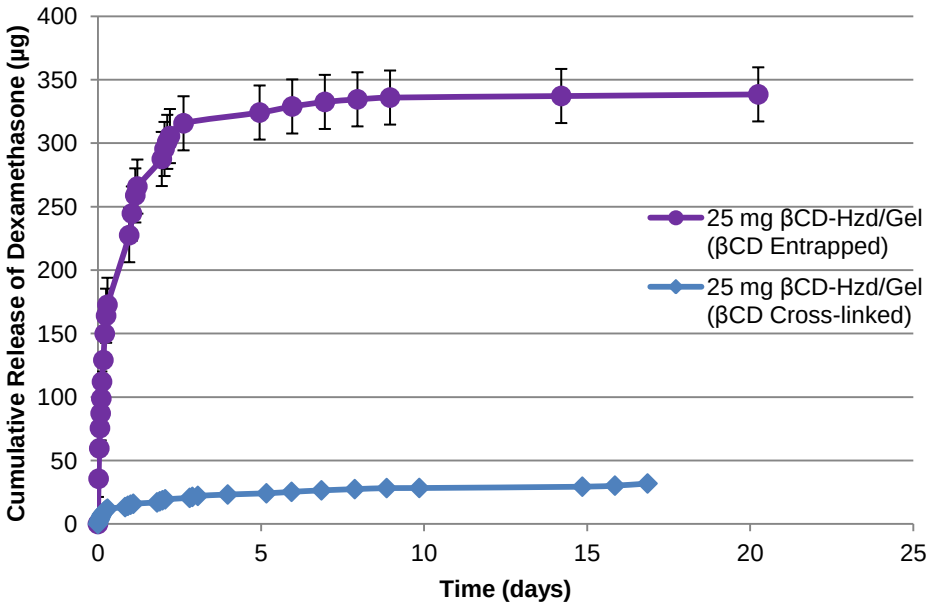

Figure 3.15 Comparison of dexamethasone release (into PBS) from Dex-βCD gels that are mostly cross-linked with βCD-Hzd or contain βCD-Hzd entrapped in the hydrogel matrix

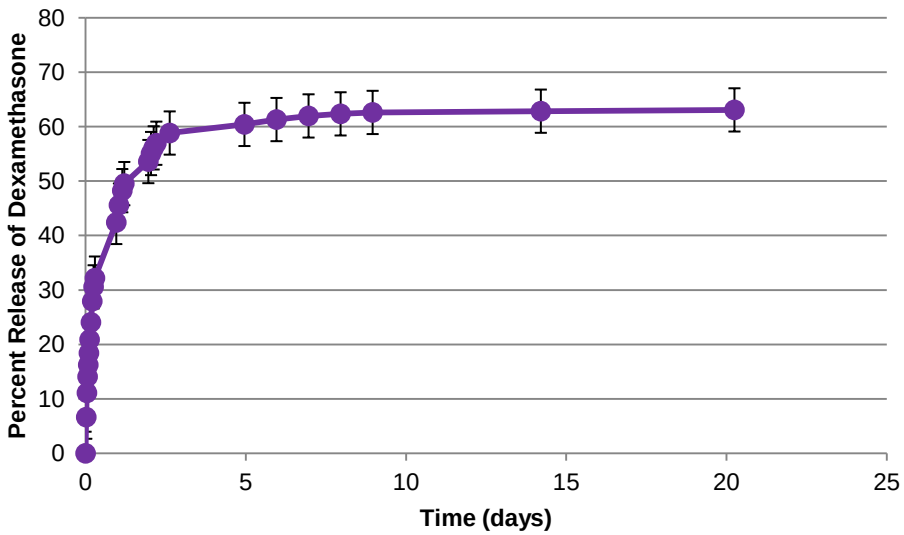

Figure 3.16 Percentage of total dexamethasone released from a Dex-βCD gel [25 mg βCD-Hzd, 4wt% Dex-Hzd] into PBS at 37^{0}C

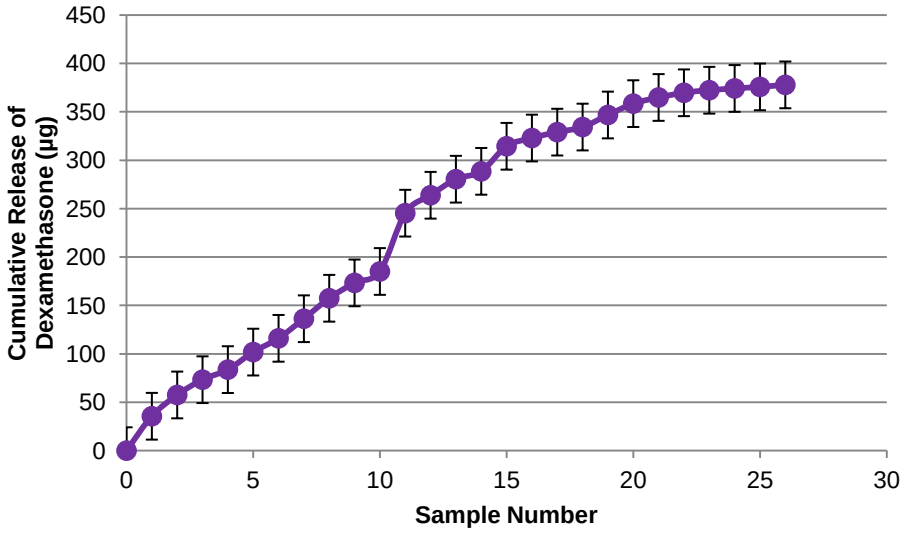

Figure 3.17 Cumulative dexamethasone release from a Dex-βCD gel [25 mg βCD-Hzd, 4wt% Dex-Hzd] as a function of the sample number collected during the release study

Swelling Characteristics

To interpret the effect of hydrogel swelling (regulating diffusion-driven release) on the overall release kinetics observed, Figure 3.18 shows the swelling responses of the varying hydrogels produced.

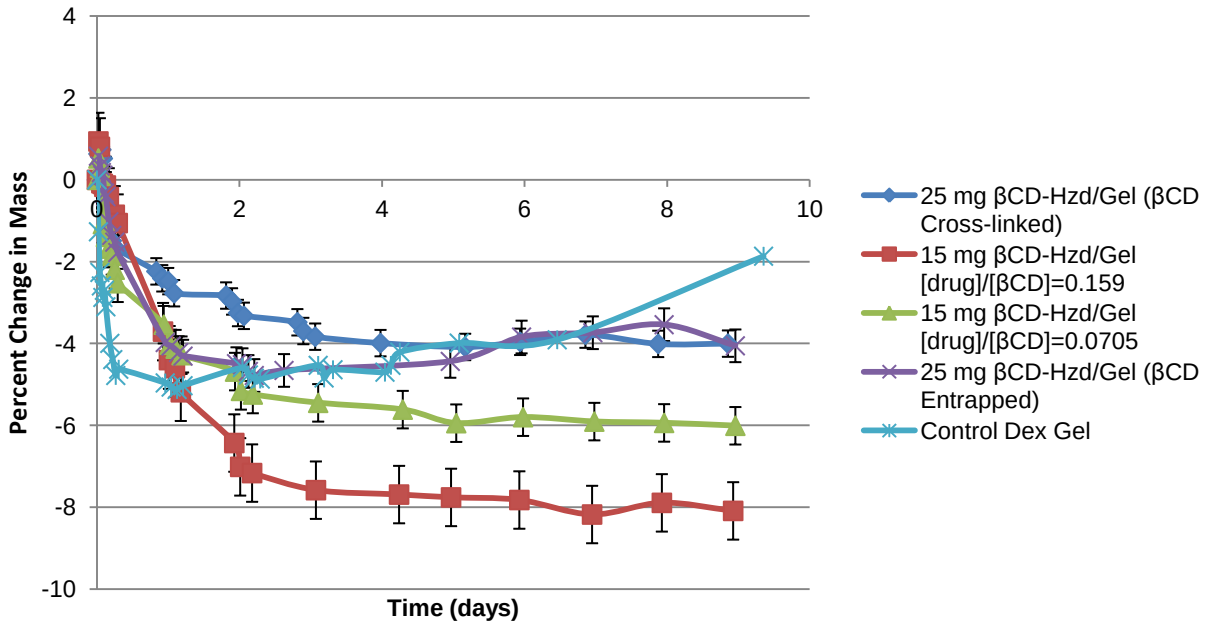

Figure 3.18 Swelling characteristics of Dex-βCD gels at 37^{0}C

Mass changes may be observed by two mechanisms: swelling (or deswelling) of the hydrogel due to elastic or mixing osmotic pressure contributions and/or degradation of the gel matrix over time. Since hydrazone bonds have lifetimes on the order of 3-4 months at physiological pH, osmotic pressure effects are likely driving the observed deswelling responses. (Sivakumaran, Maitland et al. 2011) Relative to their zero-strain state at *in situ* gelation, Dex/ βCD hydrogels tend to slightly deswell over time due to the mixing enthalpy of the crosslinked Dex/ βCD components, consistent with previous work.(Sivakumaran, Maitland et al. 2011) Generally, hydrogel swelling depends on the interaction between the polymer and the solvent, the molecular weight of the initial chains used to form the network and the molecular weight of polymer segments between the cross-links.

The presence of cross-linking always opposes gel swelling or shrinking as both conformational changes introduce strain to system. Hydrogel deswelling results from the compression of coiled polymers, reducing their conformational entropy. The more cross-linked a polymer network is, the more elastic it is, and thus, the more it will resist swelling or deswelling due to the reduction in conformational entropy. (Saltzman 2001). Indeed, gels with higher cross-link densities undergo less significant deswelling in comparison to their less cross-linked counterparts. Drug/CD molar ratios also seem to have an effect on the structural integrity of these gels. Gels with a higher drug/CD ratios deswell faster than those with a lower drug/CD ratio.

Previous studies have demonstrated that CD can act as a channelling agent and promote the diffusion of water inside polymer networks. (Bibby, Davies et al. 2000) It would be expected that gels with a lower drug/CD ratio would have more empty CD cavities, allowing for the increased absorption of water and consequently, an increased deswelling or degradation rate. However, this effect is not observed. Hydrogels containing CDs that are physically entrapped within the hydrated matrix display similar swelling kinetics as the control dextran gels.

Discussion

Approximately 30 µg of dexamethasone was released from all the gels in which CD was covalently attached to the polymer network, regardless of cross-linking density and drug/CD ratio. The step-wise pattern observed in the beginning of these release curves indicates that drug release is limited by the lack of diffusible drug species inside the gel. If the release medium was replaced more frequently so as to avoid this saturation effect, the cumulative drug release would be higher. However, given the extremely low percentage releases observed even at long sampling times (21 days), extremely long-term drug release is possible from such systems. Model studies simulating the potential uptake of dexamethasone by cell membranes in an *in vivo* environment over

extended release periods (2-3 months) will be conducted to investigate the feasibility of these materials for effective drug delivery *in vivo.*

In hydrogel systems where CD elements are immobilized, drug molecules diffuse through the polymer network by forming complexes with available CD cavities until they reach the surface of the hydrogel. The tendency for drug molecules to re-complex with a vacant CD element depends on the affinity of the drug for the CD derivative. The higher the association constant between the drug and CD, the slower the drug release will be through this series of complex-forming events. When there are fewer available CD cavities, the drug molecule must choose a diffusional path towards the hydrogel interface instead, increasing the rate of drug release. It is possible for drug molecules present in the release medium to diffuse back into the hydrogel and bind to vacant CD molecules in the polymer network.(Alvarez-Lorenzo, Moya-Ortega et al. 2011) Indeed, drug release in these systems is a dynamic process that is highly dependent on its surroundings, further necessitating future studies in cell-based release media to assess *in vivo* applicability.

In hydrogel systems where CD was physically entrapped within the matrix, an increased release rate was observed. Since the CD/drug complexes are mobile, the increased solubility of the drug afforded by the presence of CD could be translated to an increased level of drug release. Taken together, the observations from this study establish the role of CDs as tools for the controlled release of hydrophobic drugs.

Conclusions and Recommendations

In hydrogel systems where CD is covalently attached to the polymer network through cross-linking, a reduced release rate of dexamethasone was observed. This result was attributed to the immobilization of the CD/drug complex within the hydrogel network. In hydrogel systems where CD was physically entrapped within the matrix, an increased release rate was observed. Since the CD/drug complexes are mobile, the increased solubility of the drug afforded by the presence of CD could be translated to an increased level of drug release.

There are a number of different avenues that can be pursued based on the findings from the Dex-βCD hydrogel studies. The effect of the degree of cross-linking and the overall CD content can be further investigated by incorporating lower and higher hydrazide-functionalized βCD derivatives and examining any potential differences in release kinetics.

Performing long-term release studies will determine the maximum drug release time, as well as answer questions regarding the unique release kinetics observed from the Dex-βCD hydrogels. For the case of the βCD cross-linked hydrogel, a long-term analysis will demonstrate whether the linearity observed between the sampling step and the cumulative drug release will persist. It is most likely that this behaviour is a result of a partitioning effect, where a certain percentage of dexamethasone remains complexed to CD at all times. Because the percentage of drug release observed is very low (below 6%), the total concentration of dexamethasone in the system has not changed significantly enough for the expected plateau in drug release to be observed as the sampling number increases. Performing long- term degradation studies and mechanical testing on these hydrogels will indicate how long these gels will remain intact, shedding light on their drug delivery potential.

It is also important to determine whether βCD is released from either type of hydrogel, along with free dexamethasone. βCD does not absorb at the same UV wavelength as dexamethasone, so it could not be detected through the applied HPLC protocol. βCD is usually detected through fluorescence spectroscopy, where the release medium can be incubated with a certain concentration of a polarity probe such as 1,8-ANS. If a level of fluorescence enhancement is observed, this would strongly suggest the presence of βCD. Since we have shown that carboxymethylated βCD demonstrates an 8-fold higher affinity for 1,8-ANS over dexamethasone (Chapter 2), it is expected that 1,8-ANS would displace any dexamethasone inside a βCD binding cavity, allowing for independent measurements of total dexamethasone release (via UV detection) and total βCD release (via fluorescence detection).

Another important factor to address is the observation of the gradual yellowing of the hydrogels during the first few days of the release study. Such yellowing may be related to the same mechanism by which paper yellows over time, given the chemical similarities between dextran and cellulose. However, while not absolutely required if the device is used outside the optical axis, maintaining the transparency of the hydrogel would be highly desirable for devices designed for ocular drug delivery. Monitoring a Dex-βCD hydrogel in the absence of light will help determine whether the modified dextran polymers possess any chemical cross-links that are photo-sensitive. If this is the case, other polymers possessing aldehyde or hydrazide functionalities (such as NIPAM copolymers) can be incorporated into the hydrogel and tested for transparency to confirm if carbohydrate redox reactions are the reason for hydrogel yellowing.

Chapter 4 Cyclodextrin Functionalized Microgels

Introduction

Altering the dimensions of a drug delivery vehicle can influence its release properties as well as its physicochemical responses to its local surroundings. In particular, systems in the submicron range can display unique behaviours that combine the advantages of both structures. The small size, high porosity and large surface area of microgels allow for fast responses to environmental fluctuations that are not afforded by systems at a macroscopic scale. (Kettel, Dierkes et al. 2011), (Liu, Fan et al. 2004) Microgels also demonstrate a higher bioavailability due to their small size, while maintaining their ability to swell in solvents (Ducheêne and Gref 2011). Disadvantages can also translate between these dimensional categories, a prime example being the difficulty in the loading and release of hydrophobic molecules.

Release profiles from cyclodextrin-based hydrogels have demonstrated that cyclodextrins (CDs) can promote hydrophobic drug delivery through their high solubilizing efficacy. (Duchêne, Ponchel et al. 1999) In the previous chapter, we have shown that altering the mobility of CDs in the hydrogel matrix proves to be a systematic way to enhance or inhibit drug release. Varying the distribution of CD groups in a microgel system should result in a similar level of control over release properties. However, more complexity is introduced on a microgel scale due to the sheer number of architectures that are possible in this system. Factors such as colloidal stability and particle size distributions are influenced by the arrangement of functional groups within a microgel matrix and must be considered when modifying current microgel platforms. It is interesting to note that CD drug binding may also be affected by environmental factors such as pH, temperature, and ionic strength**.** (Liu, Fan et al. 2004) In conjunction with the environmental responsiveness that characterizes microgel systems, CD-complexation provides a new tool for regulating drug release.

The literature covering CD based polymer systems indicates that similar strategies have been applied in the incorporation of CDs in both nanoparticles and microgels. Like bulk hydrogels, the simplest way of integrating CDs in particle based systems is through the equilibrium partitioning of drug/CD complexes into particle suspensions (Ducheêne and Gref 2011). In addition to improving drug dissolution, CDs can also function as steric stabilizers in the synthesis of polymer particles. Nanoparticles and microgels have been prepared from reactive CD monomers or from polymers that have been modified with CD functional groups. Lastly, a strictly nanoparticle based system has been established through the self-assembly of amphiphilic cyclodextrins. (Bilensoy and Hincal 2009)

Nanoparticles composed of poly(alkyl cyanoacrylates) can be synthesized in an aqueous medium in the absence of a radical initiator, as hydroxyl ions originating from the dissociation of water can start the polymerization process. HPβCDs can function as steric stabilizers, eliminating the requirement of surfactants in this particular nanoparticle synthesis. Further examination of these particles revealed that HPβCD was localized at the surface through polymer adsorption. Progesterone was added to the polymerization medium and its release kinetics were investigated. An initial burst release was observed, followed by a delayed release phase that levelled off after 30 hours. HPβCD itself dissociated from the nanoparticles after one hour, suggesting that the delayed release observed originates from progesterone molecules that have partitioned into the polymer matrix (Duchêne, Ponchel et al. 1999; Ducheêne and Gref 2011) Similar results were observed from saquinavir loaded poly(alkyl cyanoacrylate) nanoparticles. During the synthesis of these nanoparticles, the drug was present in the CD complex, as a diffusible species in the reaction medium as well as in the growing polymer particle. (Boudad, Legrand et al. 2001)

CD based microgels or nanoparticles are frequently synthesized through the copolymerization of vinyl- or (meth)acryloyl-modified CDs with other vinyl monomers such as 2-hydroxyethyl methacrylate (HEMA), N-isopropylacrylamide (NIPAm) , acrylic acid (AA), (meth)acrylated hyaluronic acid or poly(lactide)] (Lu, Yang et al. 2008) or N-vinylpyrrolidone (NVP). Pich et al. formed nanoparticles by copolymerizing vinylcaprolactam (VCL) with acrylate-modified CDs using a batch precipitation polymerization technique. In this particular system, CD was modified with multiple vinyl groups, allowing it to function as a cross-linking agent. FTIR analysis of the nanoparticles indicated that the incorporation efficiency of CD can be as high as 80.4%. The authors attribute this effect to an increase in the polymerization rate brought about by an increase in nucleation centres during the early stages of synthesis, presumably due to the presence of the reactive CD monomer. VCL-based microgels are temperature-sensitive and the incorporation of CD did not affect the transition temperature or characteristic swelling properties. (Kettel, Dierkes et al. 2011)

Liu et al. attempted to integrate CD functionality in NIPAM based microgels by creating a core-shell architecture. Core particles were synthesized through the copolymerization of NIPAM with acrylate derived CDs. These core particles were then added to a NIPAM polymerization reaction as seeds for core-shell particle formation, encapsulating the CD functional groups with a temperature-responsive polymer layer. There was a significant discrepancy between the amount of CD in the core particles and the amount contained in the monomer feed, suggesting that the CD monomer was not very reactive towards NIPAM. The drug release capability of these microgels was investigated using the model drug paeonol, a water soluble molecule that can form a complex with CD. It was expected that complexation with CD would reduce the rate of drug release. A slight delay in release was observed, although it was considered negligible. (Liu, Yu et al. 2009) A higher level of CD incorporation was achieved by

Lui et al. when they synthesized a microgel composed of an epichlorohydrin CD polymer associated with an interpenetrating network consisting of poly(methacrylic) acid. It was found that the release of methyl orange was independent of the phase-volume transition of these pH responsive microgels, suggesting that CD complexation behaviour can regulate the release of small molecules from a microgel platform. (Liu, Fan et al. 2004)

CD complexation is not limited to small molecules. Polymers modified with hydrophobic groups can thread through CD cavities, establishing a stable scaffold for a polymer network. Gref et al. developed a spontaneously forming nanogel system consisting of dextran grafted with alkyl side chains and a CD polymer. Although the alkyl chains formed inclusion complexes with CD cavities, some sites were reserved for drug binding. Benzophenone and tamoxifen were incorporated into nanogels by mixing the drug with either the CD polymer solution or both the precursor solutions. Drug loading for tamoxifen was found to be less efficient than benzophenone. As well, tamoxifen loaded suspensions were unstable and not further investigated. Burst release of benzophenone occurred in the first 15 minutes of monitoring and additional release was not detected after 24 hours. (Daoud-Mahammed, Couvreur et al. 2009)

Investigations of self-assembled arrangements of amphiphilic CDs have demonstrated their efficacy as drug delivery vehicles. Described as ‘skirt-shaped’ due to their morphology, these CDs are formed by modifying all of the primary or secondary hydroxyl groups with aliphatic chains varying in length from 3 to 14 carbons. Nanoparticles can spontaneously form when a solution of amphiphillic CDs in organic solvent is added to an aqueous phase that may contain surfactant. Although the organic solvent is later removed, its presence in the preparation of these nanostructures is considered a disadvantage to this potential delivery vehicle. The

surfactant characteristics of amphiphilic CDs are not only responsible for their ability to form micellar structures, but they also contribute to high drug loading efficiencies as the long aliphatic chains can also interact with hydrophobic molecules. The ester bond in these CD nanoparticles renders them biodegradable. Another advantage of preparing these nanoparticles is that they can be loaded with drug prior to their synthesis and can exhibit release patterns without an initial burst effect. (Bilensoy and Hincal 2009) Tamoxifen was loaded into amphiphilic βCD modified with aliphatic chains consisting of six carbons. A 65% drug loading efficiency was achieved and a controlled release profile was exhibited for 6 hours. (Duchêne, Ponchel et al. 1999)A significant disadvantage to these systems is the short release profiles observed, ranging from 2 to 96 hours.

When reviewing the literature examining CD-functionalized microgel systems, it is clear that sustained drug release has not been achieved from a majority of the vehicles that have been developed thus far. In order to investigate the potential of extended delivery, a number of microgel candidates were synthesized with different amounts and distributions of CDs. NIPAM forms the basis of these microgels due to its thermosensitive properties, which allow for the synthesis of stable and monodisperse particles. (Pelton 2000) Previous studies, as well as experience in our laboratory, have shown that vinyl CD derivatives exhibit limited reactivity towards NIPAM (Liu, Yu et al. 2009). Thus, a different synthetic strategy was pursued based on carboxylic acid containing NIPAM microgels. As a consequence of the different copolymerization kinetics between various carboxylic acid-containing monomers and NIPAM, carboxylic acids can be incorporated in different locations throughout a NIPAM microgel. When microgel synthesis is carried out with NIPAM and methacrylic acid, the carboxyl groups tend to be concentrated in the particle core. The addition of vinyl acetic acid results in the incorporation of carboxyl groups at the microgel surface, while acrylic acid functional groups tend to be

dispersed throughout the microgel matrix. (Pelton and Hoare 2011) (Hoare and Pelton 2006) In the synthetic strategy employed in this study, these microgels were conjugated with amine groups and then reacted with monocarboxymethylated CDs. Figure 4.1 illustrates the proposed structure of a VAA microgel modified through the application of this reaction scheme. In addition to using polyelectrolyte microgels as a foundation for CD-functionalized delivery vehicles, a CD entrapment strategy was also pursued. Linear NIPAM polymers were synthesized with βCD pendant groups and added to a microgel reaction, encapsulating CD functionalities within a NIPAM polymer shell (Figure 4.2).

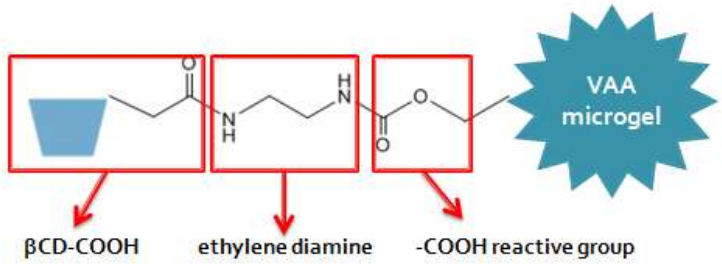

Figure 4.1 A schematic illustrating the βCD-functionalization of a VAA microgel

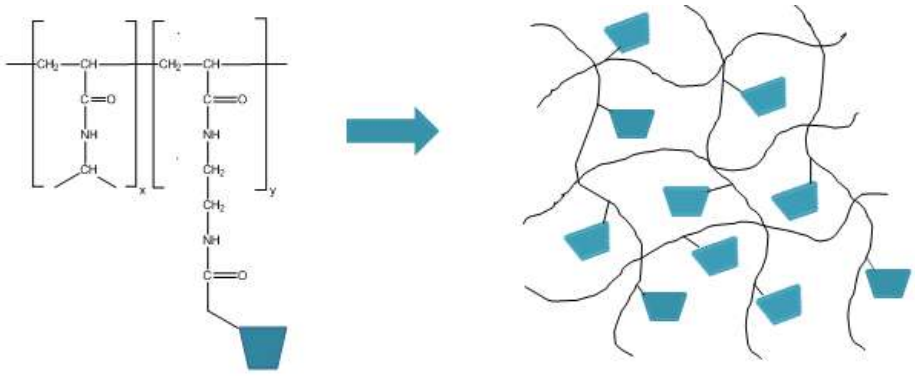

Figure 4.2 Entrapment of NIPAM copolymers with βCD pendants groups within a NIPAM based microgel

The arrangement of CD groups in the particle matrix should affect the colloidal stability, swelling properties and drug release kinetics of the microgels. Tools used to characterize properties that influence these microgel characteristics include dynamic light scattering and electrophoretic mobility measurements. Dynamic light scattering is a standard technique used for measuring particle size and polydispersity. Electrophoretic mobility values are related the surface charge density of microgels. FTIR can be used to detect the presence of CD and in some cases ^{1}H-NMR can also provide useful information regarding the CD content of microgels. Polarity probes like 1,8-anilinonaphthalene sulfonate (ANS) can also be used to detect CDs that are capable of complexing with guest molecules in a microgel system. Taken together, the structural and dynamic information regarding these particles will help define one of these candidates as a potential hydrophobic drug delivery vehicle for long-term release.

Materials and Methods

Reagents: N-Isopropylacrylamide (NIPAM, 99%, Acros Organics) was purified by recrystallization from a 60:40 toluene:hexane mixture. β-cyclodextrin (≥97% purity), N'-ethyl-N-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), chloroacetic acid (≥ 99.0% purity), 8-Anilino-1-naphthalenesulfonic acid ammonium salt (HPLC grade, ≥ 97.0% purity), 2-(N-morpholino)ethanesulfonic acid hydrate (MES, ≥99.5% purity), methacrylic acid (MAA, 99% purity), acrylic acid (AA, 99% purity), vinylacetic acid (VAA, 97% purity), *N*,*N*-methylene-bis-acrylamide (MBA, 99+%, Aldrich) and sodium dodecyl sulfate (SDS, 98% purity) were all purchased from Sigma-Aldrich. Ammonium persulfate (APS, 99% purity) was from BDH Chemicals, dimethyl 2,2'-azobis(2-methylpropionate) (AIBMe) was from Wako Chemicals, U.S.A and ethylenediamine (EDA, ≥99% purity) was from Acros Organics. Sodium hydroxide pellets were from EMD Chemicals USA, glacial acetic acid was from Caledon Laboratory

Chemicals and all HCl and NaOH solutions were from Acculute Standards. All other reagents were used without further purification.

Synthesis of Carboxylic Acid Functionalized NIPAM Microgels

To make the carboxylic acid functionalized microgels used in this study, 1.4 g of NIPAM and 0.10 g of MBA were dissolved in 150 mL of deionized water, along with the amounts of functional monomer given in Table 1.The amount of functional monomer was determined so that the final concentration of -COOH groups in VAA-1 and MAA-1 microgels were 6.5 mol%, while the concentration of –COOH groups in MAA-4 and AA-4 were 20mol%. A NIPAM microgel was also synthesized in the absence of carboxylic acid functionalized monomer as a control for CD grafting. The reactants were heated to 70 °C under a nitrogen purge and 200 rpm mixing. After 30 min of temperature stabilization, a solution of APS (0.1 g/10 mL water) was injected to initiate the polymerization. After 24 hours, the mixture was cooled to room temperature, dialyzed against water for six cycles and isolated through lyophilization. (12-14 kDa MWCO membrane, Spectrum Co).

Table 4.1 Polyelectrolyte microgel compositions

Microgel	Mass of Functional Monomer Added (g)	Functional Group Loading (mol%) (theory)	Functional Groups in Microgel (mol%) (measured)
VAA-1	0.3	6.5	6.1±0.4
MAA-1	0.068	6.5	5.9±0.3
MAA-4	0.272	20	19.9±0.7
AA-4	0.224	20	19.0±0.6

Synthesis of βCD Functionalized NIPAM Microgels

Carbodiimide chemistry was used to conjugate carboxymethylated βCD (βCD-COOH, 1.39 and 1.53 COOH/βCD) to amine-functionalized microgel intermediates (Appendix A4). Amine groups were introduced to the carboxylic acid functionalized microgels through a reaction with ethylenediamine (EDA). Briefly, 0.5 g of microgel was suspended in 60-100 mL of 0.1 MES buffer (pH=5.3) until a cloudy suspension was obtained. Amounts of EDA, NHS and EDC were added such that they were in a ten-fold molar excess of the amount of carboxylic acid groups available in the microgel for conjugation (to promote single-site functionalization over divalent crosslinking). The reaction was started with the addition of EDC dissolved in 5 mL of MES buffer and was allowed to proceed for 2 hours at ambient temperature. The modified microgel was dialyzed against water for six cycles and isolated through lyophilization. (12-14 kDa MWCO membrane, Spectrum Co) βCD-COOH was conjugated to the amine functionalized intermediate microgels in a similar fashion. Briefly, 0.3 g of microgel was suspended in 60-100 mL of 0.1M MES buffer until a precipitate-free suspension was obtained. Amounts of βCD-COOH, NHS and EDC were added such that they were in five-fold molar excess to the amount of reactive amine groups available in the microgel. ^{1}H-NMR analysis of VAA-CD indicated successful conjugation of βCD-COOH functional groups to the microgel (Bruker, 200MHz spectrometer). ^{1}H-NMR (D_2O): δ3.0= (0.29 H, H-2 and H-4 of one anhydroglucose unit), δ3.5= (0.55 H, H-5, H-3 and H-6 of one anhydroglucose unit).

Synthesis of NIPAM polymer with βCD pendant groups

A NIPAM copolymer containing 20 weight percent acrylic acid was synthesized by free-radical polymerization. Briefly, 4.5 g of NIPAM, 1.01 g acrylic acid and 0.0560g of the initiator, AIBMe, were dissolved in 20 mL of ethanol and heated to 50°C under a nitrogen purge and 200

rpm mixing. The reaction was carried out for 24 hours. The solvent was removed in a rotary-evaporator under vacuum at 50°C and the product was dissolved in 150 mL of deionized water. The polymer was dialyzed against water for six cycles and isolated through lyophilization. (12-14 kDa MWCO membrane, Spectrum Co) Carbodiimide chemistry was used to conjugate carboxymethylated βCD (βCD-COOH, 3.18 COOH/βCD) to an amine-functionalized polymer intermediate (Appendix A5). Amine groups were introduced to the carboxylic acid functionalized polymer through a reaction with ethylenediamine (EDA). Briefly, 2.0383 g of NIPAM-CoA polymer was dissolved in 60 mL of 0.1 M MES buffer (pH=5.3). Amounts of EDA, NHS and EDC were added such that they were in a ten-fold molar excess of the amount of carboxylic acid groups available in the polymer for conjugation. The reaction was started with the addition of EDC dissolved in 5 mL of MES buffer and was allowed to proceed for 2 hours at ambient temperature. The modified polymer was dialyzed against water for six cycles and isolated through lyophilization. (12-14 kDa MWCO membrane, Spectrum Co) βCD-COOH was conjugated to the amine functionalized intermediate polymer in a similar fashion. Briefly, 1.265 g of polymer was dissolved in 50 mL of 0.1M MES buffer. Amounts of βCD-COOH, NHS and EDC were added such that they were equal in molar amounts to the reactive amine groups available in the polymer. ^{1}H-NMR analysis indicated successful conjugation of βCD-COOH functional groups to the NIPAM polymer (Bruker, 200MHz spectrometer). ^{1}H-NMR (D_2O): δ3.0-3.5= (13 H, H-2, H-4, H-5, H-3 and H-6 of one anhydroglucose unit).

Synthesis of βCD Entrapped NIPAM microgel

The βCD-NIPAM polymer was entrapped within a NIPAM based polymer shell through a conventional microgel synthesis. Briefly, 0.4752 g of the βCD-NIPAM polymer, 1.4 g NIPAM, 0.1 g MBA and 0.05 g SDS were added to 150 mL of deionized water and heated to 70 °C under a nitrogen purge and 200 rpm mixing. After 30 min of temperature stabilization, a solution of APS

(0.1 g/10 mL water) was injected to initiate the polymerization. After 24 hours, the mixture was cooled to room temperature, dialyzed against water for six cycles and lyophilized. The amount of βCD-NIPAM polymer entrapped within the microgel was determined using gravimetric methods. Following the dialysis of the microgel reaction, free polymer should still be dissolved in the purified medium. The synthesized microgel was separated from the solution by conducting four cycles of ultracentrifugation (Beckman model L7-55, 1 hour at 50 000*g*). The unreacted βCD-NIPAM polymer was isolated by placing the supernatant under an aspirator vacuum in a rotary evaporator at 60°C. The amount of polymer incorporated in the microgel was determined to be 95.2 mg; 20% of the initial amount added to the synthesis.

Dynamic Light Scattering

Samples were prepared by suspending lyophilized microgel at a concentration of 2 mg/mL in 10 mM PBS. Dynamic light scattering measurements were performed on a Nano-S Zetasizer (Malvern Instruments Ltd). At least five replicates were conducted for each sample; the experimental uncertainties indicated represent the standard deviation of the replicates.

Electrophoretic Mobility

Samples were prepared by suspending lyophilized microgel at a concentration of 2 mg/mL in 10 mM PBS. Electrophoretic mobility values were measured using a ZetaPlus analyzer (Brookhaven Instruments Corporation) operating in phase analysis light scattering mode. A total of 10 runs (each comprised of 15 cycles) were conducted; the experimental uncertainties represent the standard error of the mean of the replicates.

FTIR

FTIR spectra were collected using a Thermo Nicolet IR300 spectrometer. Samples of lyophilized microgel and potassium bromide (KBr) were ground using a mortar and pestle and pressed into pellets. At least 64 scans were applied and data between 800 and 4000 cm^{-1} were recorded.

Fluorescence

Microgel samples were prepared at a concentration of 5mg/mL in 10 mM PBS with a 1,8-ANS concentration of $4.73x10^{-5}$ M. Samples were shaken in the dark for 45 minutes at ambient temperature, prior to fluorescence measurement. Steady-state fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian). The excitation wavelength was set at 370 nm and emission scans were performed between 400 nm and 700 nm. Excitation and emission bandwidths of 10 nm were used and fluorescence measurements were made in a 1 cm^2 quartz cuvette at ambient temperature.

Results

DLS

Since the carboxylic acid functionalized microgels were synthesized in the absence of surfactant, they were found to be generally larger than most conventional microgels, with (unmodified) diameters on the order of 1-2 microns in PBS. However, after CD grafting, DLS provides evidence of significant aggregation in many of the microgels, both in terms of large sizes and broad polydispersities measured. Interestingly, the stability of CD-grafted microgels was found to depend on both the number of graftable functional groups (i.e. the number of CDs that may be grafted to the microgel) and the distribution of those functional groups. MAA-1 CD retained high colloidal stability after CD functionalization, with the diameter exhibiting a slight decrease upon CD grafting; this particle size change is consistent with the consumption of carboxyl groups that

occurs through the grafting of βCD, reducing the charge density of the microgel and thus the Donnan equilibrium-induced swelling. However, despite having the same total number of functional groups, VAA-1 CD exhibited significant aggregation under the same conditions. Reactive functional groups (and thus CD grafting sites) are primarily located in the core of the microgel in MAA-1 CD while they are almost exclusively on the surface of VAA-1 CD. Thus, by consuming charged functional groups at the interface as opposed to the bulk, the colloidal stability is significantly reduced. This is likely a result of the conjugation protocol used to graft CD to the microgels. Ethylene diamine was used as an intermediate molecule to convert microgel-bound –COOH groups to $–NH_2$ groups that can facilitate carbodiimide-mediated attachment of the –COOH-functionalized CDs to the microgel. As a result, after the ethylene diamine conjugation step, microgels contain a mixture of cationic ($-NH_3^+$) and anionic (COO^- and initiator-derived $–SO_4^-$) functional groups in close proximity to wherever the original –COOH groups were located in the microgel. If this amphoteric polymer region is generated at or near the surface, salt bridges may form that can represent a significant a driving force for consuming interface and thus microgel aggregation.

Increasing the number of functional groups (MAA-4 CD) statistically introduces more reactive functional groups at or near the surface, leading to a loss of colloidal stability in the same manner as described above. However, MAA-4 CD (functional groups primarily in the core) yields significantly smaller aggregates than AA-4 CD (functional groups distributed throughout the bulk), further confirming the impact of charge localization on particle stability. Thus, to make a stable microgel for drug delivery, MAA-functionalized starting materials are most desirable, with the maximum MAA content at which colloidal stability may be fully maintained in PBS a subject of future study. However, for controlled release purposes in non-circulating drug release vehicles,

weakly bound microgel aggregates may also be useful as long as they remain easily injectable through a standard syringe.

Table 4.2 Particle sizes of βCD-NIPAM microgels. Asterisks indicate samples that display evidence of aggregation.

Base Microgel	Particle Size of Unmodified Microgel (diameter in nm)	Particle Size of CD-modified Microgel (diameter in nm)
VAA	245±13	77,690±10,590*
MAA-1	984±215	832±119
MAA-4	1001±77	33,050±14,040*
AA-4	2161±532	112,300±44,810*
NIPAM	1486±394	690±35

Electrophoretic mobility

Although the pKa of the carboxyl groups will vary in each of the unmodified microgels, most of the COOH groups will not be ionized at a lower pH value. Accordingly, the electrophoretic mobility values should increase as the pH is raised. As expected, more functionalized microgels such as MAA-4 and AA-4 exhibit larger negative mobility values at a pH of 10, when most of the COOH groups are ionized. Grafting of ethylene diamine to microgels should convert –COOH groups to $–NH_2$ groups (although retaining the anionic $–SO_4^-$ groups from APS initiator decomposition), reducing the measured mobility of the microgels. CD grafting will consume amine groups and introduce some additional –COOH groups, converting the mobility to a more negative value. However, given the presence of unreacted amine groups and grafting of CD residues with (on average) only 0.4-0.5 ungrafted –COOH groups/CD grafted (i.e. per original –COOH in the base microgel), the charge density and thus mobility of the CD-functionalized

microgels should be less negative than the original microgel. Indeed, MAA-1 CD (the most colloidally stable CD-functionalized microgel tested) displays lower mobility values than MAA-1 prior to CD grafting. However, this effect is not observed for the VAA-CD, AA-4 CD and CD entrapped microgels. This effect is likely attributable to the aggregation of these microgels observed in the DLS data. Aggregation can result in compaction of the particles via hydrophobic interactions, hydrogen bonding interactions, and (in this case, most likely) the formation of salt bridges between non-grafted NH_3^+ groups and either $–COO^-$ or $–SO_4^-$ groups. Compaction of the particles through any of these mechanisms upon aggregation would result in an increase in the effective charge density (charge/volume) within the microgels and a decrease in the electrophoretic softness of the microgel (related to the ease by which ions may diffuse through the microgel and introduce electro-osmotic effects on the mobility readings). Both these effects could result in the higher-than-expected mobility values observed as well as the inconsistent mobility trend with pH for the most highly aggregating microgel tested (AA-4 CD).

Table 4.3 Electrophoretic mobility values of βCD-NIPAM microgels

Microgel Type	Electrophoretic Mobility at pH=4 ($x10^{-8}$ m^2/Vs)	Electrophoretic Mobility at pH=7 ($x10^{-8}$ m^2/Vs)	Electrophoretic Mobility at pH=10 ($x10^{-8}$ m^2/Vs)
VAA	-0.884±0.33	-0.67±0.28	-0.77±0.27
VAA-CD	-0.712±0.05	-0.9±0.1	-1.11±0.1
MAA-1	-0.207±0.26	-0.421±0.164	-0.452±0.24
MAA-1 CD	-0.342±0.17	0.144±0.185	-0.215±0.23
MAA-4	-0.307±0.33	-0.941±0.08	-0.982±0.14
MAA-4 CD	-0.624±0.06	-0.931±0.14	-0.86±0.11
AA-4	-0.891±0.09	-1.6±0.07	-1.08±0.1
AA-4 CD	-0.84±0.08	-0.931±0.1	-1.28±0.15
CD Entrapped	-0.182±0.24	-0.304±0.12	-0.334±0.15
NIPAM	-0.392±0.06	-0.481±0.16	0.0711±0.229

FTIR

FTIR analysis can detect the presence of βCD functional groups in materials. Specifically, a band can be observed at 1032 cm^{-1}, originating from the C-O-C vibrations of the glucose units of βCD (Kettel, Dierkes et al. 2011). This band was seen when the MAA-1 CD, MAA-4 CD, and AA-4 CD samples were analyzed with FTIR (Figures 4.4, 4.5 and 4.6). By measuring the signal intensities (normalized to the baseline) of the amide C=O stretch at ~1650 cm^{-1} (from NIPAM residues and crosslinker) relative to the ether C-O-C stretch at ~1030 cm^{-1} (from CD residues) for both the modified and unmodified microgels, an estimate of the relative degrees of grafting between the different microgels can be achieved, as shown in Table 4.3.

Table 4.4 Peak intensity ratio for amide carbonyl stretch (from NIPAM amide group) relative to C-O carbohydrate ring stretch (from CD grafts) for CD-grafted microgels. Intensities were normalized to the baseline absorbance for each sample tested. Factor change noted in final column represents the ratio of relative peak intensities before and after CD grafting/entrapment.

Microgel	Peak Intensity Ratio (C=O stretch at ~1650 cm^{-1} / C-O stretch at ~1030 cm^{-1})		Factor Change on CD Grafting/ Entrapment
	Before CD Grafting/Entrapment	***After CD Grafting/Entrapment***	
MAA-1	20	3.5	5.7
MAA-4	20	0.7	28.5
AA-4	22	3.9	5.6
VAA-1	22	8.9	2.5
CD-entrapped	32	30	1.1

The intensity of the C-O-C peak was observed to be the highest in MAA-4 CD, as was the factor change in the amide:carbohydrate ring peak intensity ratio upon CD grafting; correspondingly, MAA-4 contained more functional groups than MAA-1 and exhibited better colloidal stability than AA-4, making the higher CD grafting observed in MAA-4 reasonable (Figure 4.5). Interestingly, the C-O peak intensity in the MAA-1 CD spectra was somewhat larger than that of AA-4 CD and both microgels exhibit the same increase in peak intensity ratio upon CD grafting; given the 4-fold higher –COOH content of AA-4 CD relative to MAA-1 CD, this result suggests that grafting is significantly more efficient in MAA-functionalized microgels than AA-functionalized microgels. We propose that the improved colloidal stability of MAA-functionalized microgels is primarily attributable for this observation. The characteristic band was visible but only at very low intensity the modified VAA microgel, indicating that low levels of βCD were incorporated through the applied conjugation strategy; correspondingly, the factor

change in peak intensity ratio was significantly smaller than in the MAA or AA-functionalized microgels. In contrast, the characteristic band at 1032 cm^{-1} was not observed in the CD entrapped microgel sample and essentially no change in the amide:carbohydrate ring peak intensity was observed relative to a NIPAM microgel prepared in the absence of the βCD-NIPAM polymer., This suggests that only small quantities of the βCD polymer were incorporated in the microgel, effectively below the lower limit of detection for FTIR analysis.

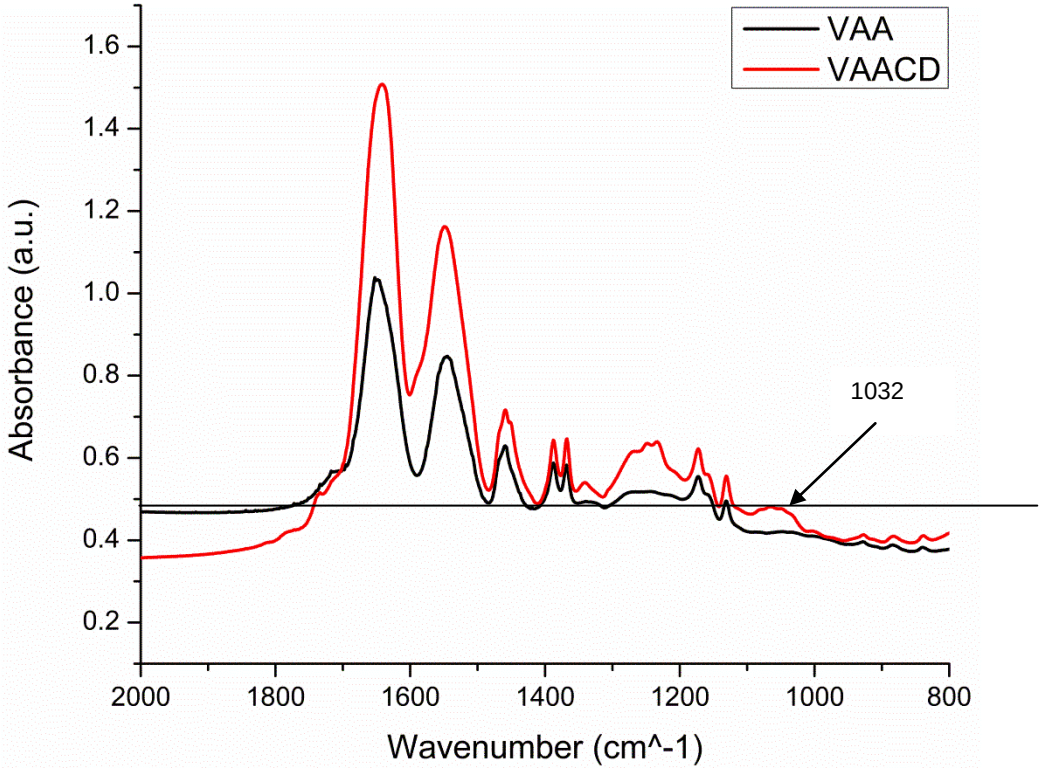

Figure 4.3 FTIR spectra of βCD modified and original VAA microgel samples

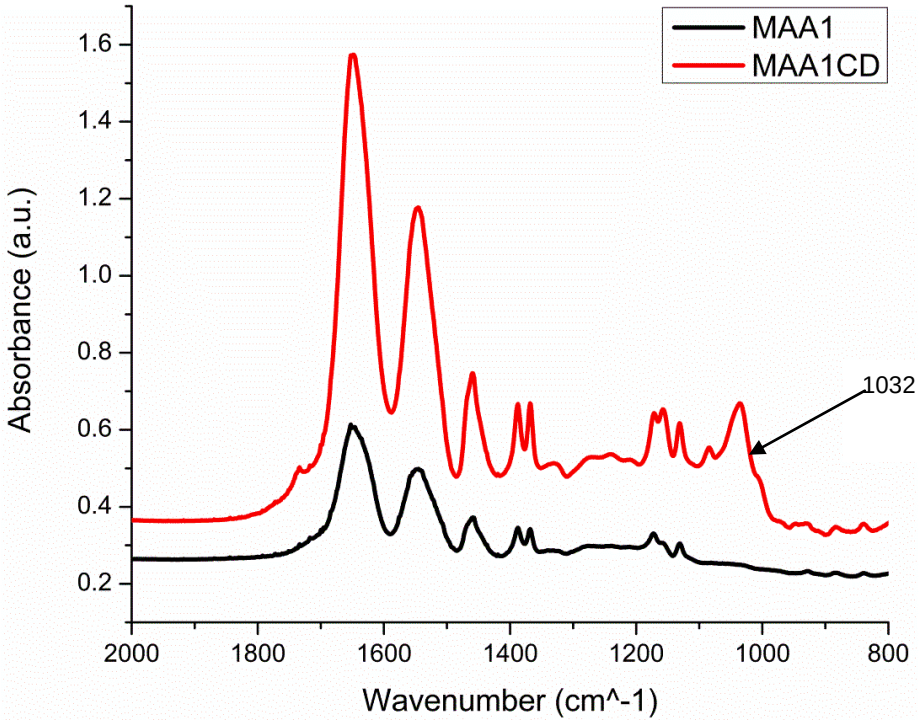

Figure 4.4 FTIR spectra of βCD modified and original MAA-1 microgel samples

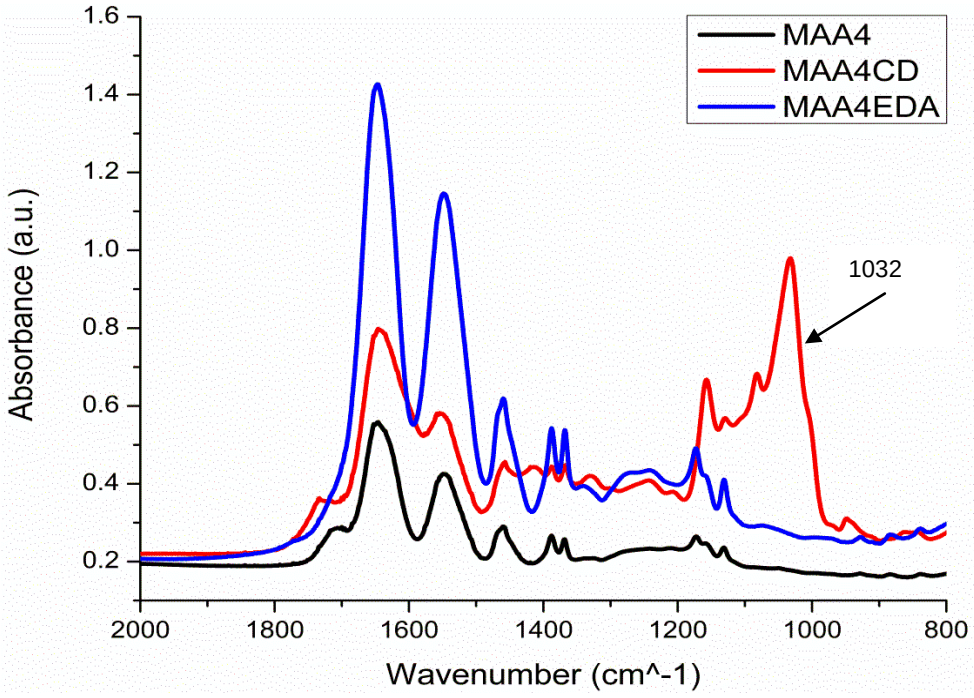

Figure 4.5 FTIR spectra of βCD modified and original MAA-4 microgel samples

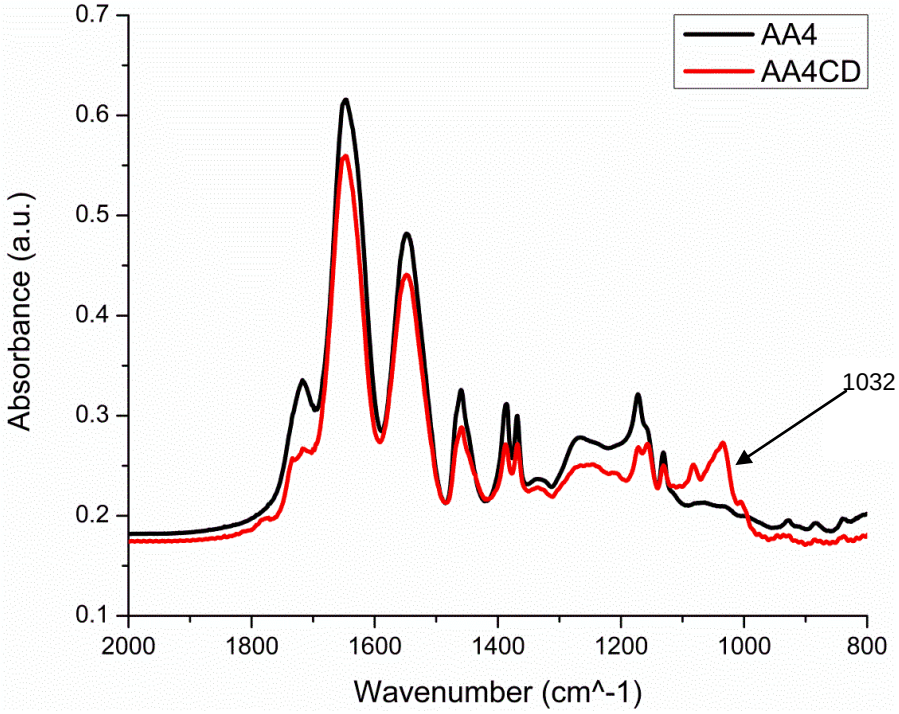

Figure 4.6 FTIR spectra of βCD modified and original AA-4 microgel samples

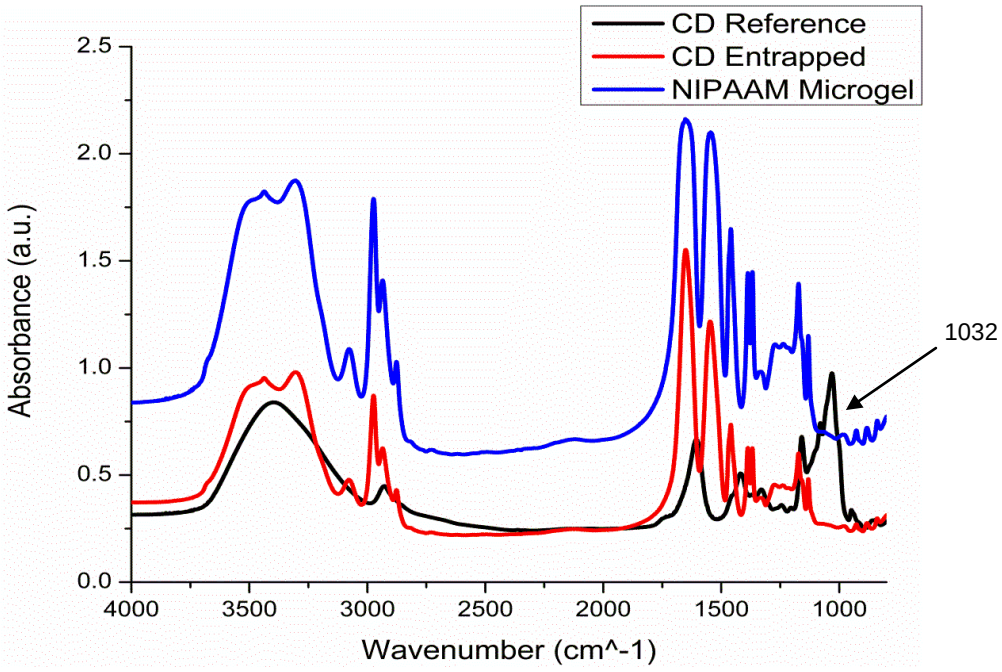

Figure 4.7 FTIR spectra of βCD entrapped microgel, βCD-COOH reactive unit and NIPAM microgel samples

^{1}H-NMR Analysis of VAA-CD and βCD NIPAM polymer

^{1}H-NMR analyses of the VAA-CD microgel and the βCD NIPAM polymer (entrapped inside the microgel) confirmed the successful modification of both materials with βCD functional groups. The peak found at a chemical shift of 3.5 ppm (designated as "1" in Figure 4.8) represents the H-5, H-3 and H-6 protons found in one anhydroglucose unit of the βCD molecule. The peak found at a chemical shift of 3.0 ppm (designated as "2" in Figure 4.8) corresponds to the H-2 and H-4 protons in one anhydroglucose unit.(Schneider, Hacket et al. 1998) The two peaks overlap in Figure 4.9, forming one peak that spans the chemical shift range between 3.0 ppm and 3.5 ppm. (designated as "3" in Figure 4.9) Quantification of the carbohydrate signal (in box 2, 2 x 7 = 14 protons/CD unit) relative to the NIPAM signal (the methyl signal at ~1.0ppm, 6 protons/NIPAM residue) suggests a relative CD content of 0.12 CD/NIPAM in VAA-CD. Taking into account the total number of proton corresponding to peaks 1 and 2, a relative CD content of 0.31 CD/NIPAM in the βCD NIPAM polymer was calculated. However, such calculations should be done with caution in microgel systems given that many NIPAM residues are incorporated in more densely crosslinked regions of the microgel and thus relax too slowly to be detected on the NMR time scale. Regardless, this result is qualitatively consistent with FT-IR of the VAA-CD microgel, in which a carbohydrate C-O stretch signal was detected. Note that solid state NMR is the preferred method for the analysis of microgels, as solution state NMR requires that the sample be completely soluble—a difficult requirement for larger and/or more crosslinked systems. For example, poor results were acquired for MAA-functionalized microgels, where the reactive functional groups (and thus grafted CD residues) are in the densely cross-linked microgel core. VAA-CD was chosen as a candidate for solution state NMR analysis as the relaxation of βCD protons incorporated at or near the surface (and are attached to weakly crosslinked "hairs" at the microgel surface) (Hoare, 2004) was easier to detect.

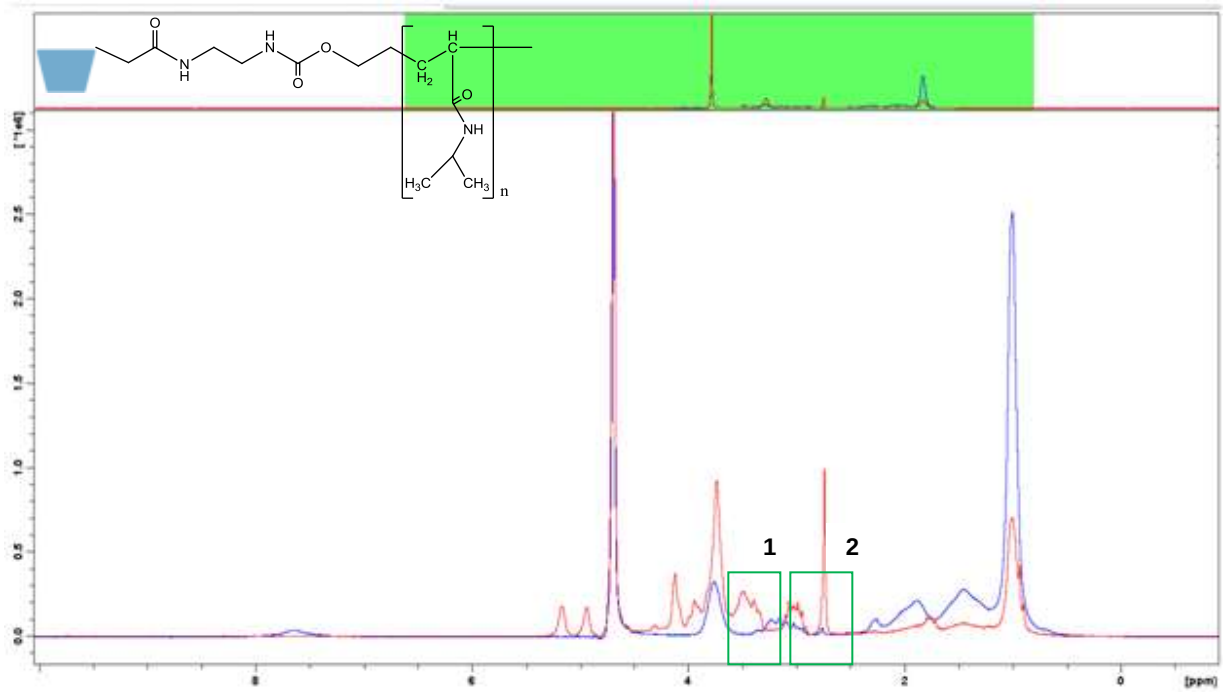

Figure 4.8 ^{1}H NMR of ethylene diamine-grafted VAA microgel (blue) and βCD-grafted VAA microgel (red) in D_2O

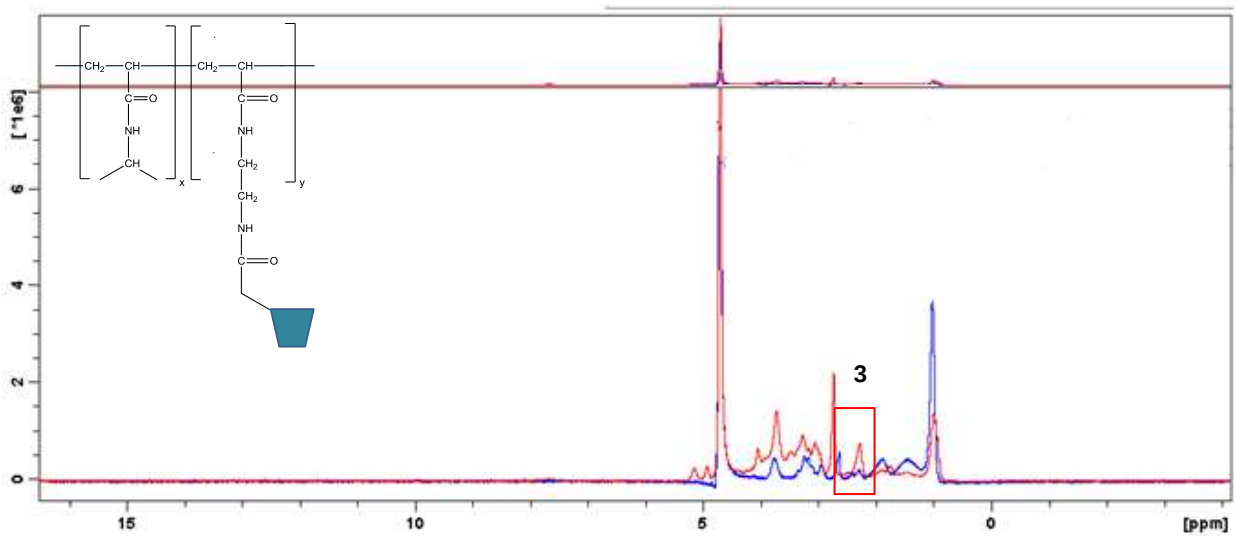

Figure 4.9 ^{1}H NMR of ethylene diamine-grafted NIPAM-co-AA polymer (blue) and βCD-grafted NIPAM polymer (red) in D_2O

Fluorescence Assay

In general, fluorescence enhancement of the 1,8-ANS signal is observed for all of the βCD functionalized microgels in comparison to their unmodified counterparts . Figs. 4.10-4.15 shows the fluorescence levels observed from all of the microgels, while Table 4.4 summarizes the fluorescence results in terms of both the absolute fluorescence at 530nm (a local maximum in the spectrum) and the ratio of fluorescences between CD-grafted and ungrafted microgels.

Table 4.5 Absolute fluorescence and % fluorescent intensity change upon CD grafting/entrapment for microgels tested

Microgel	Fluorescence Intensity at 530 nm (emission)		% Intensity Change on CD Grafting/ Entrapment
	Before CD Grafting/Entrapment	***After CD Grafting/Entrapment***	
MAA-1	89	128	+45
MAA-4	14	138	+880
AA-4	31	62	+200
VAA-1	60	108	+70
CD-entrapped	57	163	+290

The different microgels, even prior to CD grafting, all facilitate significantly different 1,8-ANS fluorescences, suggesting that each (unmodified) microgel has significantly different hydrophobic domain character due to differences in both the degree of functionalization and the location of the functional groups. The enhancement in fluorescence signal in a CD-functionalized microgel relative to the bare microgel also changes significantly in each of the samples tested. The % fluorescence enhancement results are largely consistent with the IR results, in which MAA-4 CD exhibited by far the highest CD signal, followed by AA-4 CD and VAA-CD. However, MAA-1 CD had a significantly higher CD peak in IR than would be anticipated based on the fluorescence probe result; furthermore,, the CD entrapped microgel exhibits the highest level of absolute fluorescence and a high fluorescence enhancement (~290% relative to a NIPAM microgel prepared in the absence of the βCD NIPAM polymer) even though IR analysis indicated that it contained the lowest amount of βCD groups. There are a number of possible explanations for these observations. Firstly, 1,8-ANS is a polarity probe and the increase in fluorescence observed

can result from the binding of the molecule to any hydrophobic region, not just the cavity of βCD. Given the different distributions of functional groups within these microgels, different levels of local hydrophobicity might also be expected. In particular, for the entrapped microgel, the presence of NIPAM-rich CD-grafted chains entrapped inside a non-functionalized NIPAM microgel should result in the formation of more local hydrophobic nanostructure, even at ambient temperature, that can also effectively provide a hydrophobic environment for 1,8-ANS, independent of the presence of CD. Similarly, the fact that the fluorescence observed for the unmodified MAA-1 microgel is only slightly lower than the level detected from the βCD functionalized microgel (Figure 4.11) indicates the presence of hydrophobic regions within this particular functionalized NIPAM microgel; such a morphology would be consistent with previous literature on such microgels, in which the outer shell of the microgel is primarily NIPAM with functional groups localized in the microgel core. As well, the βCD cavities within a microgel may be sterically hindered by nearby polymer substituents (as indicated in Chapter 2), preventing the binding of the fluorescent probe.

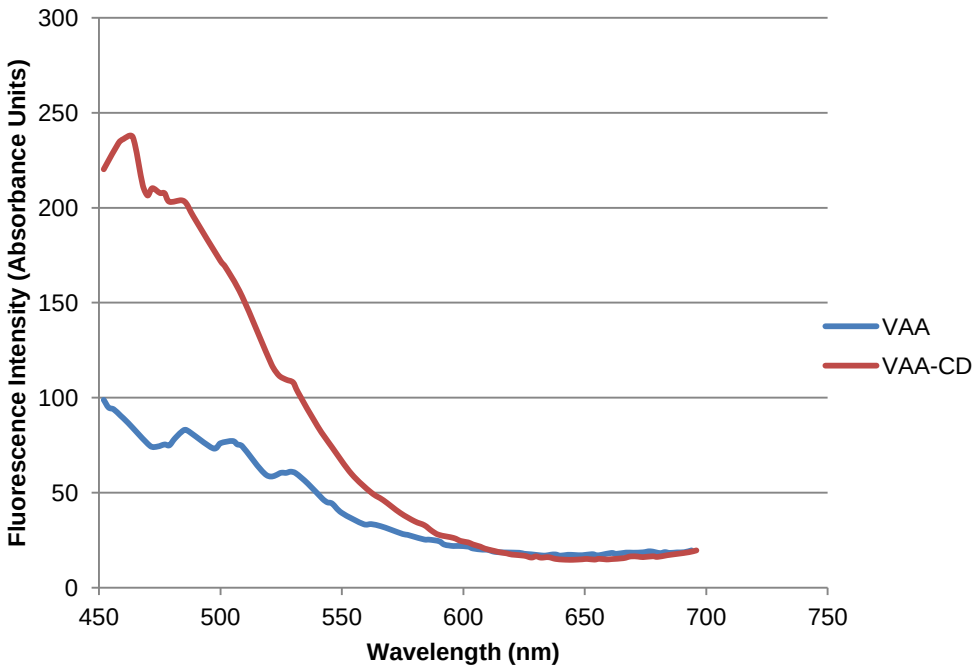

Figure 4.10 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD modified and original VAA microgel samples

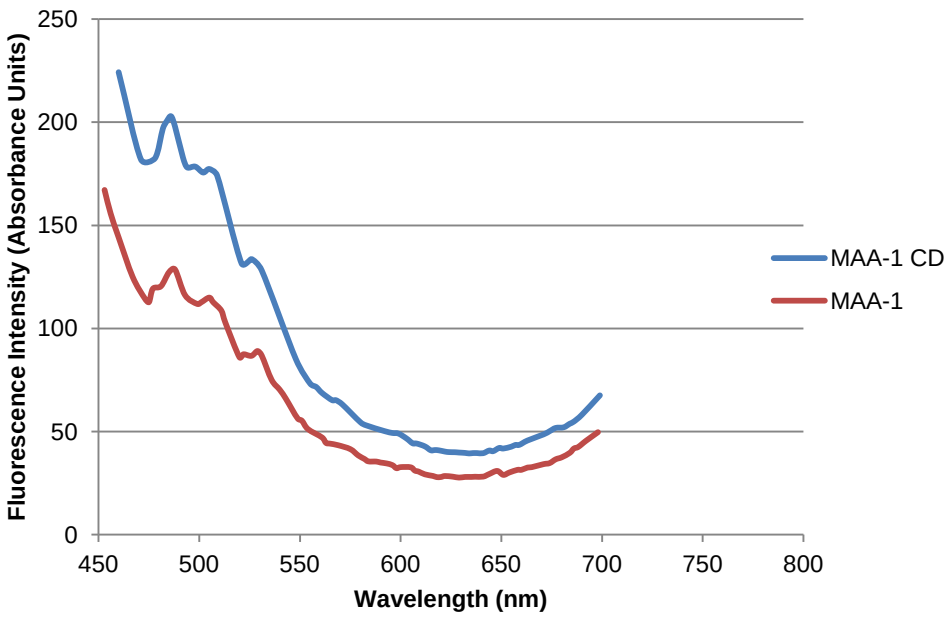

Figure 4.11 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD modified and original MAA-1 microgel samples

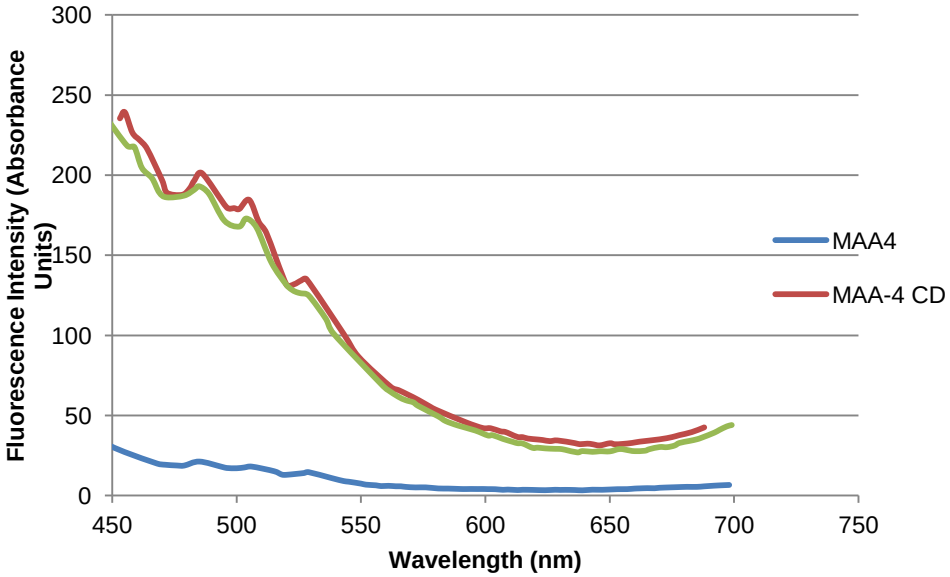

Figure 4.12 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD modified and original MAA-4 microgel samples

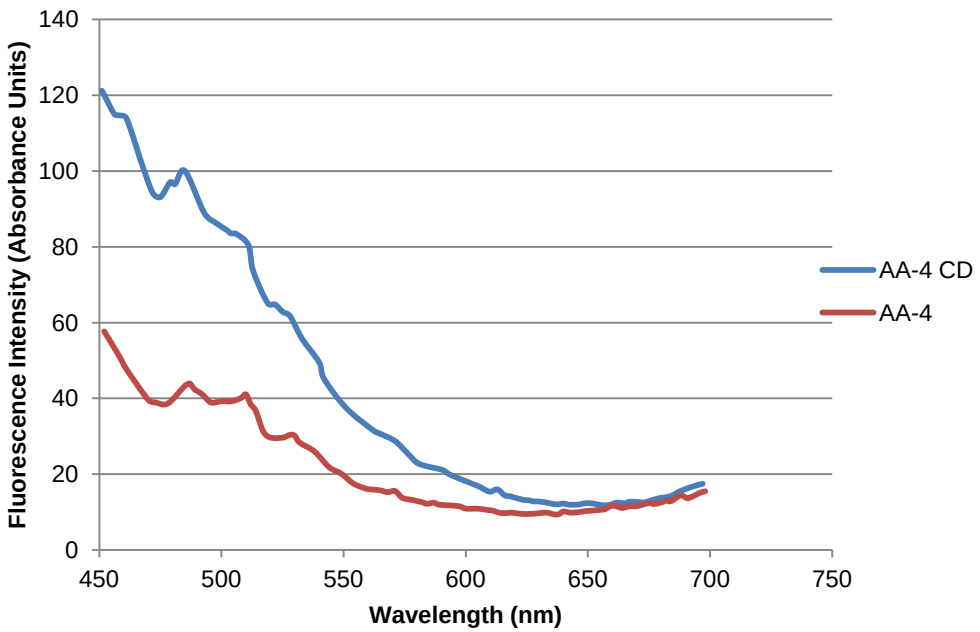

Figure 4.13 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD modified and original AA-4 microgel samples

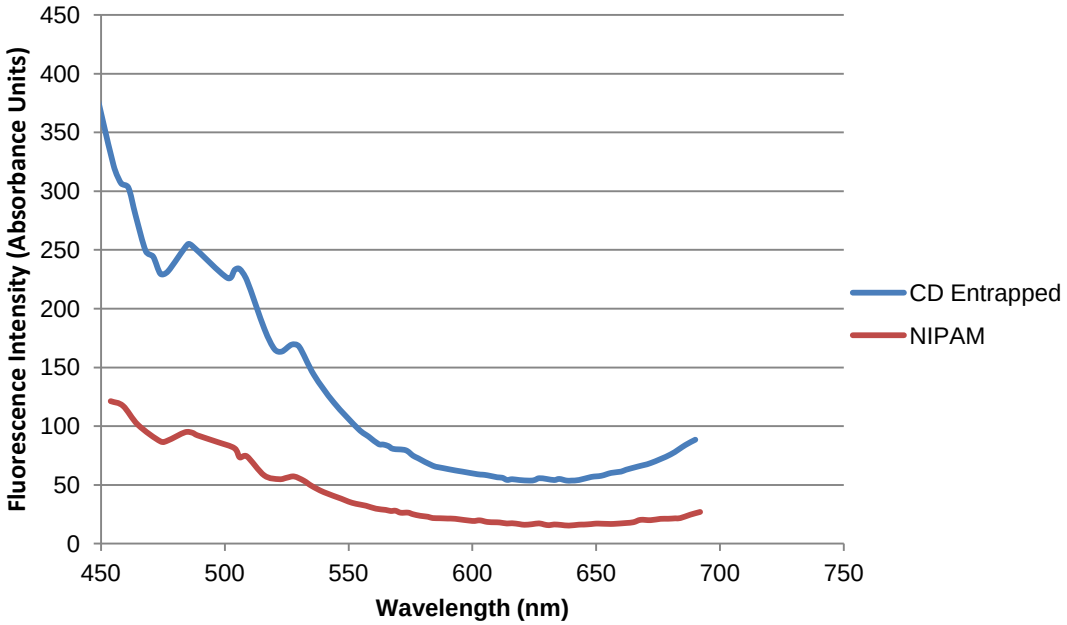

Figure 4.14 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD modified and original VAA microgel samples

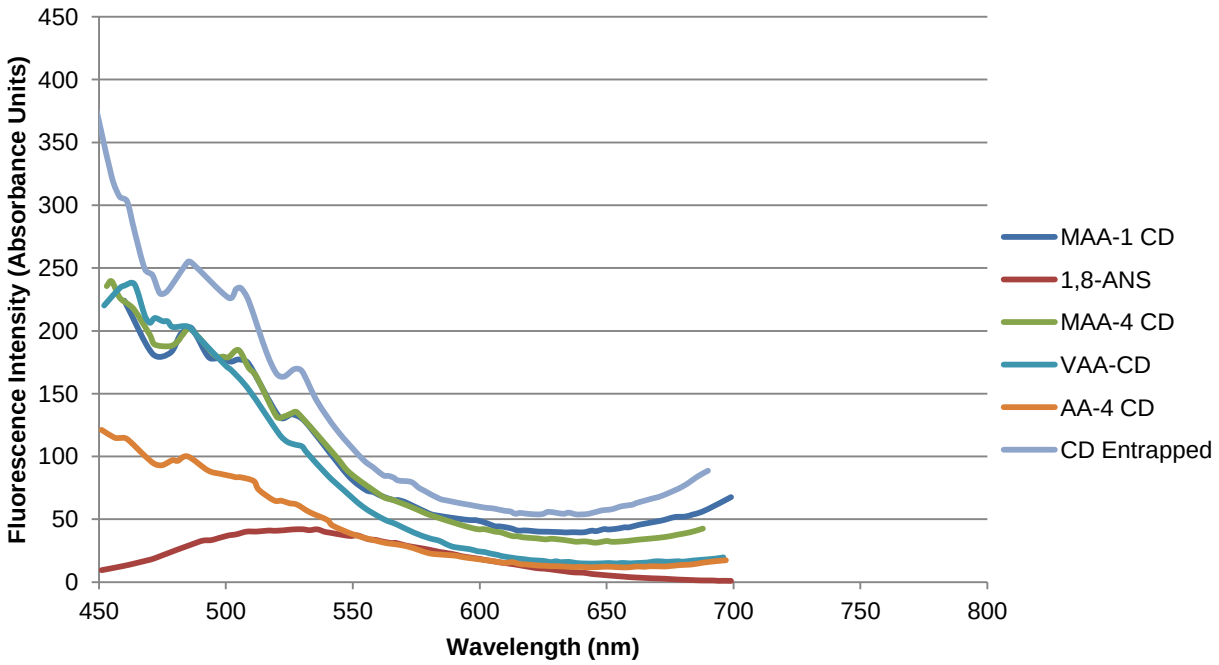

Figure 4.15 Relative fluorescence spectra of 1,8-ANS in 10 mM phosphate buffer solutions containing βCD -NIPAM microgel samples

Discussion

The results from the different analytical methods applied to the microgels confirm the successful modification of NIPAM based microgels with βCD functional groups. From the analysis, it appears that the MAA-4 CD microgel exhibits the most ideal characteristics of a microgel. While particle size measurements indicate limited particle aggregation upon re-suspension, MAA-4 CD can be immediately redispersed in water from its lyophilized state and both FTIR data and the 1,8-ANS fluorescence enhancement data indicate that MAA-4 CD may possess the highest level of βCD functionalization. On this basis, MAA-4 CD appears to be the most promising candidate for a hydrophobic drug delivery vehicle. The drug delivery potential for all of the synthesized microgels will be investigated in future work.

Conclusions and Recommendations

The results from the different analytical methods applied to the microgels confirm the successful modification of NIPAM based microgels with βCD functional groups. It appears that the MAA-4 CD microgel exhibits the most ideal characteristics of a microgel. Particle size measurements indicate limited particle aggregation upon re-suspension and both FTIR data and the 1,8-ANS fluorescence enhancement data indicate that MAA-4 CD may possess the highest level of βCD functionalization. On this basis, MAA-4 CD appears to be the most promising candidate for a hydrophobic drug delivery vehicle.

The dexamethasone loading capacity of each of these microgels will be investigated as well as their drug release properties. Different reaction schemes generating microgels with increased colloidal stability will also be pursued.

Chapter 5 Conclusions

In this thesis we have shown the following:

Chapter 1

- Both native and carboxymethylated βCDs exhibit similar levels of drug solubilizing ability, although at higher concentrations native βCD appears to be slightly more effective. This is likely attributable to the steric effects of the carboxymethyl substituent.
- Carboxymethylated βCD is more effective at solubilizing dexamethasone than hydrazide functionalized βCD.
- Carboxymethylated βCD derivatives bind ANS with significantly higher affinity than unmodified or other functionalized βCD derivatives.

Chapter 2

- In hydrogel systems where CD is covalently attached to the polymer network through cross-linking, a reduced release rate of dexamethasone was observed. This result was attributed to the immobilization of the CD/drug complex within the hydrogel network
- In hydrogel systems where CD was physically entrapped within the matrix, an increased release rate was observed. Since the CD/drug complexes are mobile, the increased solubility of the drug afforded by the presence of CD could be translated to an increased level of drug release.

Chapter 3

- The results from the different analytical methods applied to the microgels confirm the successful modification of NIPAM based microgels with βCD functional groups.
- It appears that the MAA-4 CD microgel exhibits the most ideal characteristics of a microgel.
- Particle size measurements indicate limited particle aggregation upon re-suspension and both FTIR data and the 1,8-ANS fluorescence enhancement data indicate that MAA-4 CD may possess the highest level of βCD functionalization. On this basis, MAA-4 CD appears to be the most promising candidate for a hydrophobic drug delivery vehicle.

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Appendices

Appendix A1:

Example Calculation of average COOH functionality:

After subtracting a blank titration (salt solution) from the sample titration curve, it was determined that 0.88 mL of 0.1 M NaOH was added to 51.2 mg of the carboxymethylated product:

$$\frac{8.8x10^{-5}\ moles\ of\ NaOH\ added}{0.0512\ g}$$

$=1.72x10^{-3}\ moles/g$

M_W of anhydroglucose unit: 162 g/mol

M_w of substituted carboxymethyl group: 59.05 g/mol

Degree of substitution (per anhydroglucose unit):

$$DS = \frac{162\frac{g}{mol}x\ 1.72x10^{-3}\ \frac{mol}{g}}{\left[1-\left(59.05\ \frac{g}{mol}x\ 1.72x10^{-3}\ mol\right)\right]}$$

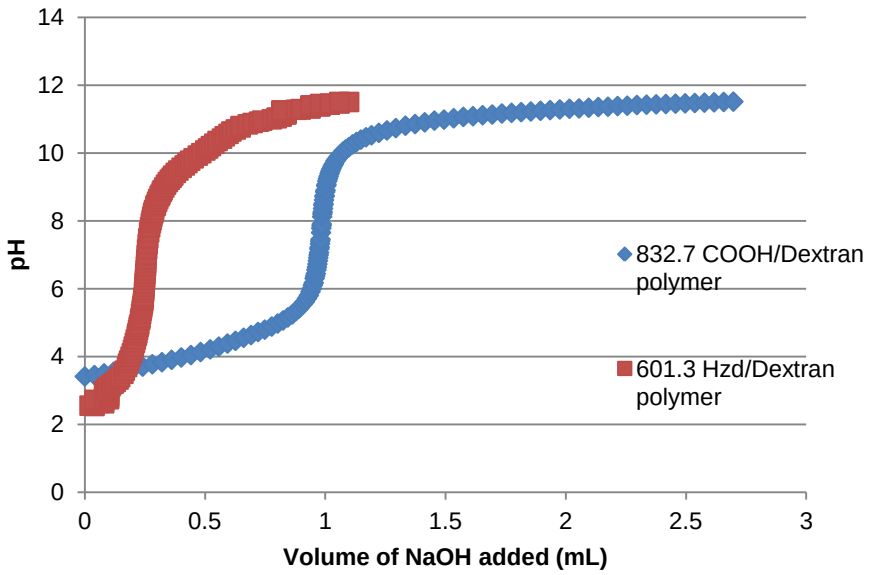
$=0.202$

Since βCD has 7 anhydroglucose units:

2.17 COOH groups/ βCD

Appendix A2:

Titration curve of the hydrazide functionalized dextran used in the synthesis of Dex-βCD hydrogels. The titration curve of the carboxymethylated dextran intermediate is also shown for comparison purposes.



Appendix A3:

The amount of dexamethasone loaded into the βCD-Hzd solution was calculated by subtracting the amount of drug recovered after centrifugation from the amount of drug originally added to the solution. This value was related to the volume of βCD-Hzd solution added to the hydrogel disk:

The volume of the hydrogel disk is approximately 0.226 mL. Since equal amounts of both the hydrazide and aldehyde phases are extruded through the double-barrel syringe, approximately 0.113 mL of the βCD-Hzd solution is added to the hydrogel volume.

Amount of dexamethasone loaded into CD cross-linked gel (25 mg βCD-Hzd/gel):

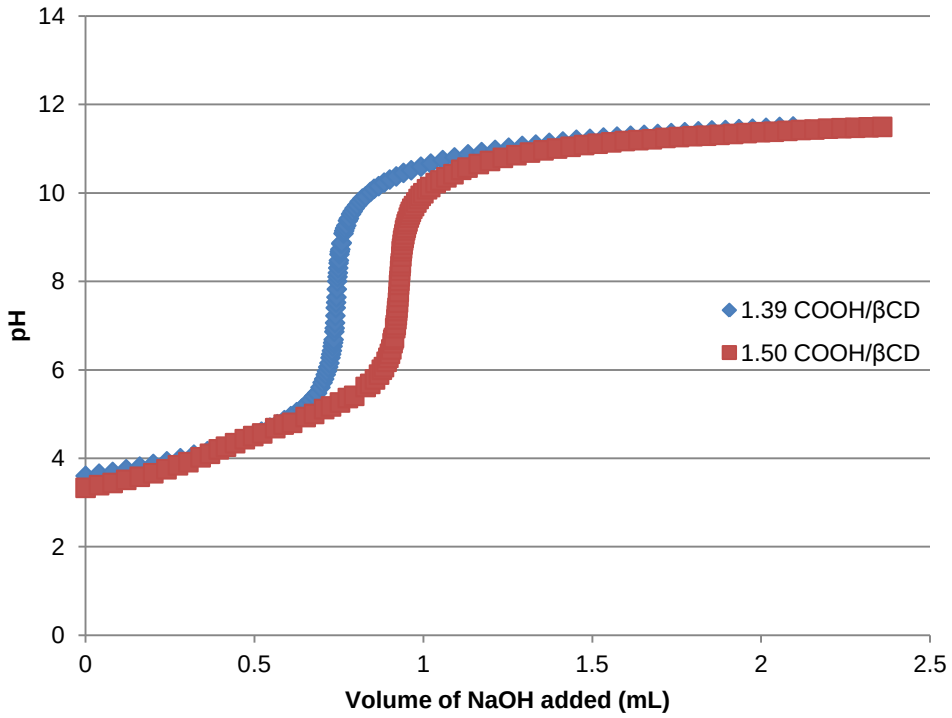
Amount of dexamethasone loaded into 2 mL of 110.6 mg/mL solution of βCD-Hzd: 10.7 mg

$$\frac{10.7\ mg\ dexamethasone}{2\ mL\ \beta CD - Hzd\ soluiton} = \frac{x\ mg\ dexamethasone}{0.113\ mL\ \beta CD - Hzd\ solution}$$

$$x = 604.6\ \mu g$$

Appendix A4:

Titration curve of the carboxymethylated βCD used in the synthesis of βCD functionalized microgels.



Appendix A5:

Titration curve of the carboxymethylated βCD used in the synthesis of βCD NIPAM polymer, entrapped within a NIPAM based microgel.