Chapter 2. Experimental

2.1 Synopsis

This chapter outlines the materials and describes all experimental used throughout this thesis. All instrumentation used to characterise the results are described herein.

2.2 Materials

This section details all reagents and chemicals used in this study for the synthesis of Ca-Alg_2 gel beads, Ca-Alg_2/GO gel beads, Ca-Alg_2 dye or drug hydrogel and CPT/β-CD-g-Alg.

2.2.1 Chemicals and reagents

The chemicals and reagents, which used in this study, were listed in Table 2.1.

Table 2-1 Chemicals and reagents.

Chemical name	Manufacturer	Additional information
Milli-Q water		Resistivity is 18.2 MΩ cm. this water was used for all solution preparation and rinsing.
Acetic acid (HCl)	Scharlau, Australia	Dissolved in Milli-Q water to desired concentration
Acetone, AR grade	Ajax Finechem Pty Ltd, Australia	Used as purchased
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Australia	Used as purchased
β -cyclodextrin (β -CD)	Triway chemical Co. Ltd, China	Dissolved in Milli-Q water to desired concentration
Calcium chloride ($CaCl_2$), AR grade	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration

Copper sulphate ($CuSO_4.5H_2O$), AR grade	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
Camptothecin (CPT)	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
Dimethylsulphoxide (DMSO)	Sigma-Aldrich, Australia	Used as purchased
Dimethylsulphoxide, deuterated (d6-DMSO)	Cambridge isotope laboratories, Inc.	Used as purchased
Deuterium oxide (D_2O)	Sigma-Aldrich, Australia	Used as purchased
Ethanol, AR grade	Ajax Finechem Pty Ltd, Australia	Used as purchased
Graphene oxide (GO)	Graphene supermarket	Dissolved in Milli-Q water to desired concentration
2(hydroxymethyl) amino methane	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
Naphthalene	Sigma-Aldrich, Australia	Molten before used
Rose Bengal (RB)	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
Sodium alginate (Na-Alg)	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
Sodium hydroxide (NaOH), pellets	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration

Tetrabutylammonium hydroxide (TBAOH)	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration
ρ-Toluenesulfonic anhydride (Ts_2o)	Sigma-Aldrich, Australia	Used as purchased
Tris(2,2'bipyridyl) dichlororuthenium (II) hexahydrate (Rubpy)	Sigma-Aldrich, Australia	Dissolved in Milli-Q water to desired concentration

2.3 Polymer solution preparation

2.3.1 Sodium alginate (Na-Alg) solution

The Na-Alg solution was made according to the method described by Hassan et al.[20] and the 1 % wt/wt solution was prepared by dissolving Na-Alg (1.0 g) in Milli-Q water (100 mL). This dissolution was achieved by the stepwise addition of small amounts of Na-Alg powder into the Milli-Q water at room temperature while vigorously stirring at 5 rpm with an overhead stirrer (IKA Laborlechnik, model RW 20, USA), to give a clear solution (see Figure 2.1(a)).

2.3.2 Sodium alginate / graphene oxide (Na-Alg/GO) solution

Graphene oxide (GO) aqueous suspensions were prepared by mixing the dark brown powder of GO (0.02 g) into Milli-Q water (2 mL) using ultra-sonication treatment (Elma S 30 H Elmasonic sonicator, Germany) for 1 h. Na-Alg/GO was synthesised by the drop-wise addition of the GO solution into the aqueous Na-Alg solution (1 % wt/wt) under constant stirring at 5 rpm with a mechanical stirrer (IKA laborlechnik, model RW 20, USA) for 2 h at room temperature. A light brown translucent solution was formed (see Figure 2.1(b)).

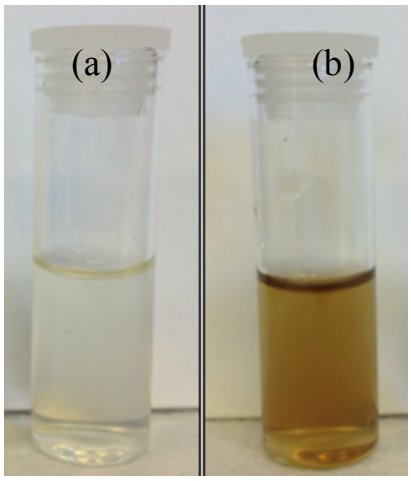

Figure 2-1 Images of aqueous solutions of (a) Na-Alg and (b) Na-Alg/GO.

2.3.3 Na-Alg and Na-Alg/GO solutions storage

Both the Na-Alg and the Na-Alg/GO solutions were kept stored in a refrigerator for two weeks to prevent bacterial attack.

2.4 Ionotropic gel beads synthesis

2.4.1 Calcium alginate (Ca-Alg_2) ionotropic gel beads

Ca-Alg_2 beads were prepared using the dropping method. This process was performed by drop-wise addition of the aqueous Na-Alg solution (1 % wt/wt) (see Section 2.3.1) through a syringe pump (New Era-300 pump system, USA) at a flow rate of 3 mL min^{-1} into an aqueous coagulation bath of $CaCl_2$ (1.0 M, 150 mL) at room temperature. The bath was continuously stirred with a magnetic stirrer to prevent bead agglomeration. The speed of the stirrer was between maximum and minimum there was no other rotational speed. According to Hassan et al. [118] some precautions are needed in order to maintain a constant bead size and shape. Important factors included consistency in the height of the syringe tip from the electrolyte surfacesing the same syringe as a dropper and maintaining the drop rate throughout the bead preparation step.

The beads were then left in the coagulation bath for 24 h without any stirring in order

to obtain a complete formation of Ca-Alg_2 gel beads. The Ca-Alg_2 gel beads were then collected and washed three times with Milli-Q water (100 mL) to remove any excess $CaCl_2$. Figure 2.2(a) shows the resulting Ca-Alg_2 gel beads, which have a uniform spherical shape. The bead diameter was measured with a caliper for 10 Ca-Alg_2 gel beads and the average of the diameter was 0.46 ± 0.03 cm. In order to calculate the weight of the dried Ca-Alg_2 beads, the wet beads were dried in an oven at 100 °C to constant weight (approximately 2 days). The dried Ca-Alg_2 beads were characterised by Fourier transform-infrared (FT-IR) spectroscopy, thermogravimetric analysis (TGA) and focused ion beam/scanning electron microscopy (FIB/SEM) (see Chapter 3, Sections 3.4.1, 3.4.2, and 3.4.3, respectively).

2.4.2 Calcium alginate/graphene oxide (Ca-Alg_2/GO) ionotropic gel beads

Ca-Alg_2/GO beads were prepared using an aqueous solution of Na-Alg/GO (0.02 g GO in Na-Alg, 1 % wt/wt) (see Section 2.3.2) and a syringe pump (New Era-300 pump system, USA) at a flow rate of 3 mL min^{-1}. As the Na-Alg/GO beads formed they were dropped into an aqueous coagulation bath of $CaCl_2$ (1.0 M, 150 mL) at room temperature and the bath was continuously stirred with a magnetic stirrer to prevent bead agglomeration. The beads were then kept in the coagulation bath for 24 h without any stirring in order to obtain the complete formation of Ca-Alg_2/GO gel beads. Excess $CaCl_2$ was removed by filtration and the Ca-Alg_2/GO beads were washed three times with Milli-Q water (100 mL). Here, assume the complete exchange of the Na^+ with Ca^{2+} ions therefore the ratio of alginate to GO remains as 1:0.02. Figure 2.2(b) shows the resulting Ca-Alg_2/GO gel beads. The bead diameter was measured with a caliper for 10 Ca-Alg_2/GO gel beads and the average of the diameter was 0.46 ± 0.02 cm. In order to calculate the weight of the dried Ca-Alg_2/GO beads, the wet beads were dried in an oven at 100 °C to constant weight (approximately 2 days). The dried Ca-Alg_2/GO beads were characterised by FT-IR spectroscopy, TGA and FIB/SEM (see Chapter 3, Sections 3.4.1, 3.4.2, and 3.4.3, respectively).

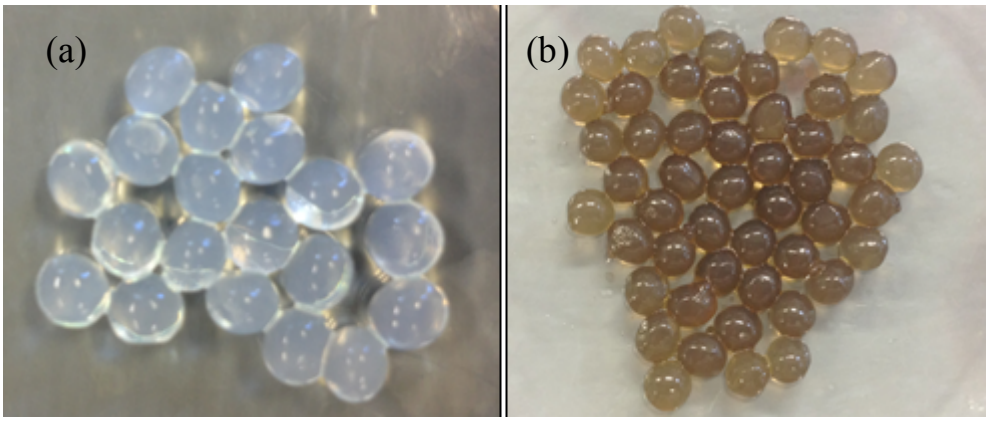

Figure 2-2 Images of (a) Ca-Alg_2 and (b) Ca-Alg_2/GO wet gel beads.

2.5 Batch Cu^{2+} ion adsorption experiments

The adsorption behaviour of Cu^{2+} ions by Ca-Alg_2 or Ca-Alg_2/GO beads were examined in batch mode under different experimental condition such as different initial Cu^{2+} concentration, different adsorbent dose (number of Ca-Alg_2 or Ca-Alg_2/GO beads), and different contact time between Ca-Alg_2 or Ca-Alg_2/GO beads and Cu^{2+} solution.

2.5.1 Preparation of electrolyte solutions

Aqueous copper sulfate solutions with different concentrations (2.5, 5.0, 7.5, 10.0, 25.0, and 40.0 mM) were prepared by dissolving the requisite amounts of the $CuSO_4.5H_2O$ in Milli-Q water.

2.5.2 Effect of initial copper concentration

The effect of initial copper concentration on the adsorption of Cu^{2+} ions by Ca-Alg_2 or Ca-Alg_2/GO beads was studied by adding a constant number (100) Ca-Alg_2 or Ca-Alg_2/GO wet beads into a beaker which contained 150 mL of each Cu^{2+} solution (2.5, 5.0, 7.5, 10.0, 25.0, and 40.0 mM). They were left at room temperature under

constant stirring with a magnetic stirrer for 90 min. Through preliminary experiments it was confirmed that 90 min was sufficient to attain equilibrium between the adsorbent (gel beads) and the Cu^{2+} ions and so this time was used for all experiments. The concentration of the Cu^{2+} ions remaining in each solution was determined using UV-Visible spectrophotometry (Varian Cary 50 scan spectrophotometer) at the maximum adsorption (810 nm) and the concentration calculated from a calibration curve (see Appendices Figure 1). The Cu^{2+} ion adsorption capacity at equilibrium (q_e) was then calculated using Equation 2.1 [119].

$$q_e = \left(\frac{C_0 - C_e}{W}\right) V$$

Equation 2.1.

Where C_0 is the initial concentration of Cu^{2+} ion (mg L^{-1}), C_e is the final concentration of Cu^{2+} ion (mg L^{-1}), V is the volume of Cu^{2+} ion solution (L) and W is the weight of the Ca-Alg_2 or Ca-Alg_2/GO dry beads (g).

2.5.3 Effect of adsorbent dose on Cu^{2+} ion adsorption

The effect of adsorbent dose on Cu^{2+} ion adsorption was studied by adding different numbers of Ca-Alg_2 or Ca-Alg_2/GO wet beads (100, 300, 500, 700, 900, and 1100) into different beakers containing a Cu^{2+} solution (10 mM, 150 mL). The mixture was stirred for 90 min at room temperature. The Cu^{2+} ion concentration was determined by the same procedure described in Section 2.5.2. Furthermore, adsorption percentage (%) was determined by the following Equation 2.2 [41].

$$Adsorption\ (\%) = \frac{C_0 - C_e}{C_0} \times 100\ \%$$

Equation 2.2

Where C_0 is the initial concentration of Cu^{2+} ion (mg L^{-1}), C_e is the final concentration of Cu^{2+} ion (mg L^{-1}).

2.6 Kinetic experiments

The influence of increasing contact time between the Ca-Alg_2 or Ca-Alg_2/GO wet beads and the Cu^{2+} ion solution was investigated by adding 100 Ca-Alg_2 or Ca-Alg_2/GO wet beads into Cu^{2+} ion solutions (15 mL) at concentrations of 5.0, 7.5 and 10.0 mM. The mixture was stirred for a designated period of time (5, 10, 20, 30, 45, 60, 90, and 120 min) at room temperature. The concentration of Cu^{2+} ions in the solution was determined using UV-visible spectrophotometry as per Section 2.5.2. The Cu^{2+} ion adsorption capacity at time t (q_t) in mg g^{-1}, was then calculated using Equation 2.3 [41].

$$q_t = \left(\frac{C_O - C_t}{W}\right) V$$

Equation 2.3

Where C_0 is the initial concentration of Cu^{2+} ion (mg L^{-1}), C_t is the concentration of Cu^{2+} ion (mg L^{-1}), at time t, V is the volume of Cu^{2+} ion solution (L) and W is the weight of the Ca-Alg_2 or Ca-Alg_2/GO dry beads (g).

2.7 Adsorbent characterisation techniques

The techniques used to characterise Ca-Alg_2 and Ca-Alg_2/GO gel beads are detailed in the following.

2.7.1 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy aids in the identification of various functional groups present in a compound by absorption of IR radiation at specific frequencies characteristic of the bond types present in the compound [120].

2.7.1.1 Sample preparation

FT-IR analyses were performed on KBr pellets. After the Ca-Alg_2 and Ca-Alg_2/GO wet gel beads were collected from the coagulation bath they were washed three times with Milli-Q water (100 mL) to remove the excess $CaCl_2$. The wet beads were dried

in an oven at 100 °C to constant weight (approximately 2 days). In order to make KBr discs, a mortar and pestle were first cleaned with ethanol, then the mixture of spectrophotometric grade oven dried KBr and the one dried bead was ground until a fine powder was obtained.

2.7.1.2 Instrumentation and data acquisition

Samples were analysed on a Perkin Elmer Spectrophotometer 400. Transmission spectra were recorded in the range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. Data were collected and exported into Microsoft Excel (2010) for re-plotting and analysis.

2.7.2 Thermogravimetric analysis (TGA)

TGA is an essential technique used for investigation of the pyrolysis and combustion behaviour of materials [121]. This technique measures the rate of change in the weight of the sample as a function of temperature [121]. TGA determines the thermal stability of the material [121].

2.7.2.1 Sample preparation

Approximately 10.3 mg of Ca-Alg_2 and 7.7 mg Ca-Alg_2/GO gel beads were prepared as described in Section 2.7.1.1, and used for TGA analysis. Each sample was placed in a platinum crucible and heated from 30 to 600 °C with a heating rate of 10°C min^{-1} under a nitrogen atmosphere at a flow rate of 50 mL min^{-1}.

2.7.2.2 Instrumentation and data acquisition

Samples were analysed on TA Instruments TGA 2950. All data were exported into Microsoft Excel (2010) for re-plotting.

2.7.3 Focused ion beam scanning electron microscopy (FIB/SEM)

SEM is an electron microscope, which allows visualization of a sample surface [122]. The electron beam is formed by the electron gun, which is condensed by the objective lens to a spot about 5-100 nm in diameter at the specimen plane [122]. SEM images are obtained by collecting the emitted electrons on a cathode ray tube [122].

FIB/SEM is a scanning electron microscopy equipped with a focused gallium ion beam, which allows three-dimensional imaging of a sample surface [123]. In this thesis FIB/SEM was used to probe the surface structure of fabricated Ca-Alg_2 and Ca-Alg_2/GO dry gel beads.

2.7.3.1 Sample preparation

FIB/SEM samples were prepared according to the method described by Hassan et al. [13]. The Ca-Alg_2 and Ca-Alg_2/GO wet gel beads were collected from the coagulation bath and washed three times with Milli-Q water (100 mL) to remove excess $CaCl_2$. One bead was completely dehydrated by shaking gently with absolute acetone several times. The excess acetone is removed using vacuum filtration then the bead was immersed in molten naphthalene (see Chapter 3, Section 3.4.3 for more details) for approximately 10 h. Finally, it was removed from the molten naphthalene and left in a desiccator to completely dry for approximately 6 days.

2.7.3.2 Instrumentation and data acquisition

Samples were sputter coated (Quorumtech sputter coater, K575X, Australia) with Pt (~3 nm thick). The images were obtained using Helios D433, Dual Beam FIB/SEM, FEI Co., USA, Adelaide microscopy).

2.8 Drug delivery

2.8.1 Fabrication of Ca-Alg_2 hydrogel membranes

Na-Alg solutions (2 % wt/wt) were made as described in Section 2.3.1. A Ca-Alg_2 cylindrical hydrogel was prepared by slowly pouring 2 mL of the Na-Alg solution into a test tube with a 1 cm diameter and height of 5 cm. $CaCl_2$ (1 M) solution was then added down the walls of the tube. The test-tube was allowed to stand for 2 h to achieve total formation of the Ca-Alg_2 cylindrical hydrogel. The Ca-Alg_2 hydrogel formed as shown in Figure 2.3 and was carefully removed and washed three times with MilliQ-water (50 mL) using a magnetic stirrer to remove the excess $CaCl_2$.

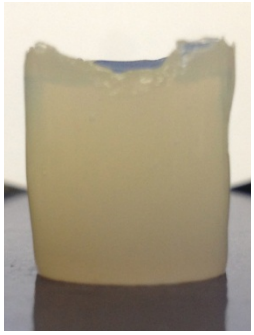

Figure 2-3 Images of Ca-Alg_2 cylindrical hydrogel.

2.8.2 Loading rose Bengal (RB) or Rubpy or camptothecin (CPT) into Ca-Alg_2 hydrogel membranes

Dye (RB or Rubpy) or drug (CPT) were loaded into Ca-Alg_2 hydrogel using an addition method, according to Stoppel et al. [124]. Na-Alg solutions (2 % wt/wt) were made as described in Section 2.3.1. RB or Rubpy or CPT solution (10 mg mL^{-1} of each dye in water or 10 mg mL^{-1} of CPT in DMSO) was added into 40 mL of Na-Alg (2 % wt/wt) solution under constant and gentle mechanical stirring (IKA laborlechnik, model RW 20, USA) for 2 h at room temperature. These solutions were then kept stored in a refrigerator to prevent bacterial attack. A Ca-Alg_2 dye or drug cylindrical hydrogel was prepared by slowly pouring 2 mL of the Na-Alg solution containing either the dye or drug into a test tube with a 1 cm diameter and height of 5 cm. $CaCl_2$ (1 M) solution was then added down the walls of the tube. The test-tube was allowed to stand for 2 h to achieve total formation of the Ca-Alg_2 dye or drug cylindrical hydrogel. The Ca-Alg_2 dye or drug hydrogel formed was carefully removed and washed three times with MilliQ-water (50 mL) using a magnetic stirrer to remove the excess $CaCl_2$.

The Ca-Alg_2 dye or drug cylindrical hydrogel was cut into individual discs, as shown in Figure 2.4(a, b, c and d) representing the pure Ca-Alg_2 hydrogel, the Ca-Alg_2 hydrogel loaded with RB, the Ca-Alg_2 hydrogel loaded with Rubpy and the Ca-Alg_2

hydrogel loaded with CPT, respectively. The disc thickness was measured with a caliper at different points and the average of the thickness was 0.74 ± 0.07 mm and the diameter was 1 cm.

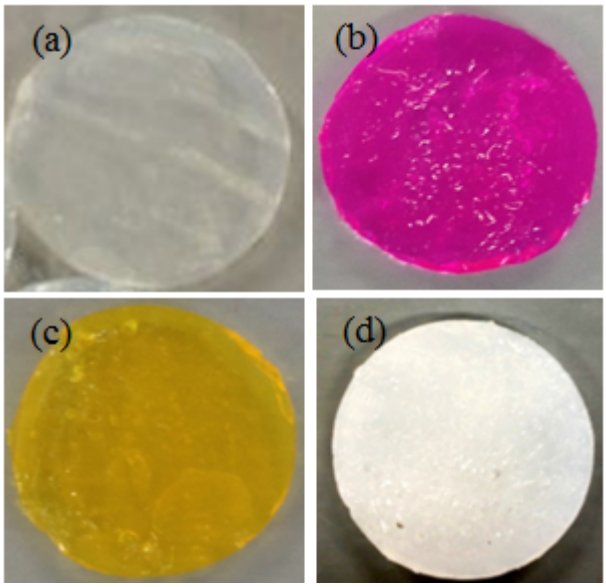

Figure 2-4 Optical images of (a) pure Ca-Alg_2 hydrogel, (b) Ca-Alg_2 hydrogel loaded with RB, (c) Ca-Alg_2 hydrogel loaded with Rubpy and (d) Ca-Alg_2 hydrogel loaded with CPT.

2.9 Release of RB or Rubpy or CPT from Ca-Alg_2 hydrogel membranes

The release profile of dye or drug from the Ca-Alg_2 hydrogel was studied, as described by Liu et al. [81]. The hydrogel discs were totally immersed in Tris buffer solution (0.02 M, 50 mL) with the pH modified to pH ~ 2.4 or to pH ~ 7.4 by the addition of HCl. The solution was stirred for a designated period of time (2, 4, 6, 8, 10, 13, 16, 19, 22, 25, 28, 43 and 103 min) at room temperature. Tris buffer solution (3 mL) at pH ~ 2.4 or to pH ~7.4 was removed at each time period and measured by UV-visible spectrophotometry (for RB and Rubpy) and fluorescence spectroscopy (for CPT) then return to the solution to maintain a constant volume. The wavelengths

of 550 and 285 nm were recorded for RB and Rubpy respectively, for pH ~ 2.4 and pH ~ 7.4. Whereas, the emission intensities at excitation 370 nm for CPT at 430 and 455 nm were recorded for pH ~ 2.4 and pH ~ 7.4, respectively.

The concentration of RB, Rubpy and CPT in Tris buffer solution was calculated from RB, Rubpy and CPT calibration curves, which were developed previously from known concentration of RB, Rubpy and CPT (Refer to Appendices Figure 2(a, b and c, respectively). The fractional amount (M_t/M_∞) of dye or drug released was calculated and the release mechanisms were analysed using Equation 4.2 and 4.4 (see Chapter 4, Section's 4.5 and 4.6 for more details).

2.9.1 Ultra-Violet-Visible (UV-Vis) spectrophotometry

UV-Vis spectrophotometry is a technique used to measure the molecule's absorbance. When the molecule absorbs ultraviolet or visible radiation, it undergoes to the change in its valence electron configuration [125]. RB has a strong absorbance band at 550 nm as a result of n-π* electronic transition and this band exploited to determine the concentration of RB in the Tris buffer solution (see Chapter 4, Section 4.4.1, Figure 4.1). Whereas, Rubpy has a strong absorbance band at 285 nm as a result of π-π* and n-π* electronic transition and this band exploited to determine the concentration of Rubpy in the Tris buffer solution (see Chapter 4, Section 4.4.1, Figures 4.2 and 4.3)

All samples were analysed on Varian Cary 50 UV-Vis Spectrophotometry, and a plastic cuvette (10 mm pathlength) were used for analysis. Absorbance spectra were recorded in the range of 200–800 nm. The baseline was corrected using Milli-Q water for all the samples.

2.9.2 Fluorescence spectrophotometry

Fluorescence spectrophotometry is a technique used to measure the intensity of photons emitted when a molecule in the lowest vibrational energy level of an excited

electronic state returns to a lower energy electronic state [125]. At an excitation wavelength of 370 nm CPT has two strong emission intensities at 432 nm (in acidic solution) and 448 nm (in alkaline solution) (see Chapter 4, Section 4.4.2, Figures 4.5 and 4.6, respectively) as a result of the opening of the lactone ring due to hydrolysis. Fluorescence emission spectra were recorded using Varian Cary Eclipse Fluorescence Spectrophotometry, and a plastic cuvette (10 mm pathlength) were used for analysis. Fluorescence emission data were recorded in the range of 400–600 nm. The baseline was corrected using Milli-Q water for all the samples.

2.10 Hydrogel disc characterisation techniques

2.10.1 Scanning electron microscopy (SEM)

2.10.1.1 Sample preparation

Three different discs were characterised with SEM spectroscopy, one disc was pure Ca-Alg_2 hydrogel, and another two disc were Ca-Alg_2 hydrogel loaded with CPT were immersed in pH ~ 2.4 and pH ~ 7.4 for 103 min. The three discs were frozen in the freezer then freeze-dried (Labconco) for 1 day.

2.10.1.2 Instrumentation and data acquisition

The three discs were sputter coated (Quorumtech sputter coater, K575X, Australia) with Pt (~3 nm thick). The images were obtained using Camscan MX2500 Scanning Electron Microscope.

2.11 CPT/β-CD and CPT/β-CD-g-Alg inclusion complexes synthesis

2.11.1 Preparation of Camptothecin/β–cyclodextrin (CPT/β-CD) inclusion complex

An inclusion complex of β–CD and CPT was synthesised according to Tsai et al. [105]. β–CD (0.317 g) was dissolved in Milli-Q water (15 mL) at 70 °C in an oil bath for 30 min. CPT (0.1 g) in ethanol (4.7 mL) was slowly added to the β–CD solution with constant stirring for 6 h. The molar ratio for both compounds was 1:1. The solution was refrigerated overnight at 4 °C. The suspension was filtered by vaccum filtration and the precipitate was washed with ethanol to remove any unreacted CPT. The final precipitate was dried under vacuum for 4 h. The precipitate was characterised by ATR-FTIR, ^{1}H NMR and TGA spectroscopies (see Chapter 5, Sections's 5.5.1.1, 5.5.1.2 and 5.5.1.3, respectively).

2.11.2 Preparation of camptothecin / β–cyclodextrin-grafted-alginate (CPT/β-CD-g-Alg) inclusion complex

2.11.2.1 Preparation of β–cyclodextrin-grafted-alginate (β-CD-g-Alg)

*2.11.2.1.1 Preparation of mono-6-deoxy-6-(*p*-toluenesulfonyl) β–cyclodextrin (β-CD-6-OTs)*

β-CD-6-OTs was prepared following the procedure reported by Zhong et al. [126]. β–CD (5.75 g) and 2.45 g from *ρ*-Toluenesulfonic anhydride (Ts_2O) were dissolved in Milli-Q water (125 mL) and were stirred at room temperature for 2 h. A solution of NaOH (2.5 g in Milli-Q water 25 mL) was added with continuous stirring for 10 min then any un-adsorbed Ts_2O was removed via filtration. The filtrate was adjusted with NH_4Cl (13 g) to pH ~ 8 and refrigerated overnight at 4 °C. The solution was then added to acetone and the precipitate, which formed, was then filtered out under vacuum. The final white precipitate was dried under vacuum for 4 h. The precipitate was characterised by ATR-FTIR spectroscopy (see Chapter 5, Section 5.2.1).

2.11.2.1.2 Preparation of tetrabutylammonium-alginate (TBA-Alg)

TBA-Alg was synthesised as described by Pawar and Kevin [127]. Firstly, Na-Alginate (Na-Alg) was transformed to its acidic form (H-Alg). HCl (12 N, 2.5 mL) was added to ethanol (70 %, 50 mL) in a beaker. Na-Alg (2.5 g) was added to the mixture in an ice bath at 4 °C and stirred with a magnetic stirrer for 30 min. The mixture was filtered and the solid was washed with ethanol (70 %, 50 mL) and then with acetone (100 mL). The product was then dried in an oven at 37 °C for 24 h.

To prepare TBA-Alg, the H-Alg (1.64 g) was dispersed in Milli-Q water (225 mL). The mixture was stirred with a magnetic stirrer for 1 h, and then tetrabutylammonium hydroxide (TBAOH) (0.15 M) was added drop-wise with continuous stirring until a pH of 10 was obtained. The solution was frozen in the freezer then freeze-dried (Labconco) for 2 days. A final white cotton-like product was obtained. The precipitate was characterised by ATR-FTIR spectroscopy (see Chapter 5, Section 5.3.1).

2.11.2.1.3 Preparation of β–cyclodextrin-grafted-alginate (β-CD-g-Alg)

β-CD-g-Alg was prepared according to Zhang et al. [128]. TBA-Alg (0.158 g) and (6-OTs- β–CD) (0.5 g) were dissolved in a 1:1 molar ratio in dimethyl sulfoxide (20 mL) and were left at 70 °C in an oil bath for 24 h. Finally, the solution was precipitated in acetone and filtered under vacuum, then washed with cold deionised water. The final precipitate was dried in an oven at 40 °C for 30 min. The precipitate was characterised by ATR-FTIR and ^{1}H NMR spectroscopies (see Chapter 5, Section's 5.4.1 and 5.5.2.2.1, respectively).

2.11.2.2 Preparation of camptothecin/β–cyclodextrin-grafted-alginate (CPT/β-CD-g-Alg) inclusion complex

An inclusion complex of β–CD-g-Alg and CPT was synthesised according to Tsai et al. [105]. β–CD-g-Alg (0.063 g) was dissolved in Milli-Q water (15 mL) at 70 °C in an oil bath for 30 min. CPT (0.015 g) was slowly added to the β–CD-g-Alg solution with constant stirring for 6 h. The molar ratio for both compounds was 1:1. The solution was refrigerated overnight at 4 °C. The suspension was filtered by vaccum

filtration and the precipitate was washed with ethanol to remove any unreacted CPT. The final precipitate was dried at room temperature. The precipitate was characterised by ATR-FT-IR, ^{1}H NMR and TGA spectroscopies (see Chapter 5, Section's 5.5.2.1, 5.5.2.2.2 and 5.5.2.3, respectively).

2.12 CPT release experiment

In vitro release profiles of CPT from CPT/β-CD and CPT/β-CD-g-Alg were studied by a dialysis method described by Zeng et al. [116]. Free CPT (1.0 mg) was dissolved in a mixture containing Tris buffer solution (1 mL) (pH ~ 7.4, 20 mM) and DMSO at 9:1 v/v. CPT/β-CD or CPT/β-CD-g-Alg (10 mg) was dispersed in 1 mL Tris buffer solution (pH 7.4, 20 mM). Each sample was loaded into a dialysis membrane (MWCO: 12,000-14,000) and the dialysis membrane was then immersed in Tris buffer solution (100 mL) at 37 °C with constant stirring. The permeate solution (3 mL) outside the dialysis membrane was withdrawn and measured at a wavelength of 370 nm by UV-visible spectrophotometry to determine the concentration of CPT then returned back to the solution to maintain a constant volume. The drug release experiments were performed in duplicate. The concentration of CPT in Tris buffer solution was calculated from CPT calibration curve, which was developed previously from known concentration of CPT (Refer to Appendices Figure 3). The fractional amount (M_t/M_∞) of CPT released and the diffusion coefficient was calculated using Equation 6.3 (see Chapter 6, Section's 6.4 and 6.5 for more details).

2.13 Inclusion complexes characterisation techniques

The techniques used to characterise CPT/β-CD and CPT/β-CD-g-Alg are detailed in the following.

2.13.1 Fourier transform infrared spectroscopy

2.13.1.1 Instrumentation and data acquisition

Samples were analysed on a Perkin Elmer Spectrophotometer 400, Transmission spectra were recorded in the range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. An air

background was used as a blank for all spectra collected. Data were collected and exported into Microsoft Excel (2010) for re-plotting and analysis.

2.13.2 ^{1}H nuclear magnetic resonance spectroscopy

Proton solution NMR spectroscopy is a powerful technique to identify a molecules structure by studying the magnetic properties of nuclei [120]. NMR spectroscopy was used in this work to prove the fabrication and characterise the inclusion complexes [129].

2.13.2.1 Instrumentation and sample preparation

^{1}H NMR spectra was recorded using a Bruker Avance NMR spectrometer 400 MH_Z. Approximately 5 mg of β-CD or CPT/β-CD was dissolved in 1 mL D_2O and approximately 5 mg of β-CD-g-Alg or CPT/β-CD-g-Alg was dissolved in 1 mL d6-DMSO and every solution placed in an NMR tube, which was pre-cleaned with acetone and was dried in an oven for 15 minutes. Chemical shifts (δ) were recorded in ppm and referenced to the peak D_2O and d6-DMSO (4.7 and 2.5 ppm, respectively).

2.13.2.2 Data acquisition

Data were collected and exported into ACD / NMR Software for analysis.

2.13.3 Thermogravimetric analysis (TGA)

2.13.3.1 Sample preparation

Solid samples from Na-Alg, β-CD, CPT, CPT/β-CD and CPT/β-CD-g-Alg (0.5-13 mg) were used for TGA analysis. Each sample was placed in a platinum crucible and heated from 30 to 600 °C with a heating rate of 10 °C min^{-1} under a nitrogen atmosphere at a flow rate of 20 mL min^{-1}.

2.13.3.2 Instrumentation and data acquisition

Samples were analysed using Simultaneous Thermal Analyser (Perkin Elmer STA 8000). All data were exported into Microsoft Excel (2010) for re-plotting.