Cyclodextrin-grafted chitosan hydrogels for controlled drug delivery

Hiroyuki Kono*, Taku Teshirogi

Department of Science and Engineering for Materials, Tomakomai National College of Technology, Nishikioka 443, Tomakomai, Hokkaido 059 1275, Japan

* Corresponding author. Tel./fax: +81 144 67 8036.

E-mail address: kono@sem.tomakomai-ct.ac.jp (H. Kono).

ABSTRACT

A series of β -cyclodextrin-grafted carboxymethyl chitosan hydrogels (CD-*g*-CMCs) were prepared from carboxymethyl chitosan (CMC) and carboxymethyl β -chitosan (CMCD) using a water-soluble carbodiimide as a crosslinker in the presence of *N*-hydroxysuccinimide. Details of the hydrogel structures were determined via FTIR and solid-state NMR spectroscopic analyses. Increasing the feed ratio of CMCD to CMC in the reaction mixture led to an increase in CD grafting within the gel networks comprising CMC; this was confirmed by SEM observations and rheological analysis of the swollen hydrogels. The prepared CD-*g*-CMC hydrogels exhibited absorption properties toward acetylsalicylic acid (ASA, or Aspirin) due to the presence of CD in the structure; the amount of ASA absorbed into the hydrogels was enhanced with an increase in the amount of CD incorporated within the hydrogels. In addition, CD-*g*-CMC hydrogels provided a slower release of the entrapped ASA in comparison to the ASA release profile of a solely CMC-containing hydrogel. From these results, CD-*g*-CMC hydrogels have the potential to function as a biodegradable active material with controlled drug release ability.

Keywords: carboxymethyl chitosan, carboxymethyl cyclodextrin, hydrogel, acetylsalicylic acid, controlled drug release.

1. Introduction

Hydrogels are three-dimensional networks composed of hydrophilic polymers crosslinked through covalent bonds or held together via physical intermolecular interactions [1]. Over the past few decades, methods for administering drugs via hydrogels have gained increasing attention [2, 3], as regulating the rate of drug release with a controlled-release mechanism offers numerous advantages over conventional dosage regimens [4]. The use of polysaccharide-based hydrogels as a drug delivery carrier in biomedical and pharmaceutical applications has contributed to resolving relatively complicated biocompatibility problems owing to their non-toxicity, biodegradability, and biocompatibility [5, 6].

Chitosan is the partially or fully deacetylated form of one of the most abundant naturally occurring polymers, chitin, which is extracted from the exoskeletons of arthropods, crustaceans, and insects [7]. Chitosan is a cationic polymer that is composed of β -(1 $\rightarrow$ 4)-linked D-glucosamine (GlcN) residues, where specific GlcN residues within the polymer chain can be replaced by *N*-acetyl-D-glucosamine units. Chitosan is also well known as a promising biomaterial owing to its non-toxicity, antimicrobial properties, and biocompatibility [8, 9], and has been extensively studied as a carrier matrix candidate for drug delivery [6, 10, 11] and gene delivery [12] systems. In addition, chitosan is easily converted to gels [13], membranes [10, 11, 13], beads [14], nanoparticles [15] and scaffolds [16, 17]. Thus it can be adapted for a range of biomedical applications in tissue engineering, wound dressing, cancer drug delivery, and targeting in the area of nanobiotechnology [18–20]. Many approaches have been reported for the preparation of chitosan-based hydrogels, including those based on chemical [6, 21, 22] as well as physical [23–25] crosslinking methods. Although physical methods have the advantage of crosslink formation without the use of crosslinking agents, they exhibit a disadvantage in the lack of precise control over the quality of chemical properties (including degradation and dissolution) of the obtained gels [26]. On the other hand, chemical

crosslinking of hydrogels can be easily performed using small bifunctional molecules such as glutaraldehyde [27] and epichlorohydrin [28] as a crosslinker. The mechanical properties exhibited by chemically crosslinked hydrogels generally exceed those of physically crosslinked hydrogels for biomaterial applications [26]. However, these crosslinkers generally have associated toxicities, and a small amount of residual crosslinker in the obtained hydrogels can be considered as potentially harmful to human health [29].

β -Cyclodextrin (CD) is a cyclic oligosaccharide composed of seven glucopyranosyl units interacting via α -(1 $\rightarrow$ 4) linkages. CD possesses a hollow truncated cone structure in which the cavity at the center of the molecule is hydrophobic while the outer surface is hydrophilic [30]. This molecular structure is capable of forming inclusion complexes, so-called guest–host compounds, with aliphatic and, especially, aromatic molecules [30]. CD is frequently employed in pharmaceutical applications for numerous purposes including improving the bioavailability of drugs [31]. An elegant approach to functional biopolymers is represented by the synthesis of CD conjugates with biocompatible hydrophilic polymers, especially polysaccharides including chitosan; accordingly, many synthetic strategies for CD-grafted polysaccharides can be found in the literature [3, 32, 33]. However, because most of the methods reported require harmful reagents (such as the crosslinker species) for the preparation of polysaccharide/CD-based hydrogels, alternate methods avoiding harmful reagents are actively sought.

Among the established crosslinking agents, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) is recognized as a virtually non-toxic reagent for the coupling of carboxylates and primary amines to generate the amide bond [17, 34, 35]; EDC initially reacts with a carboxyl group to form an amine-reactive *O*-acylisourea intermediate, which then reacts with a primary amine to form the amide bond. In the presence of *N*-hydroxysuccinimide (NHS), EDC first couples NHS to the carboxylate species, forming an NHS ester that is considerably more stable than the *O*-acylisourea intermediate, which allows for efficient conjugation to

primary amines. Indeed, NHS has been previously used to improve the efficiency of EDC coupling reactions [35]. This coupling method employing the dual EDC–NHS reagent has been used in diverse applications such as amide bond formation in peptide synthesis [36], attaching haptens to carrier proteins to generate immunogens [37], and labeling nucleic acids through their 5' phosphate groups [38]. Because there is no crosslinking moiety in the coupling product, the EDC–NHS coupling protocol has the significant advantage of biological safety over other commonly used crosslinkers.

In this study, we describe the preparation of smart polymeric hydrogels containing chitosan and cyclodextrin using the EDC–NHS coupling method, and examine their potential use as a carrier matrix for drug delivery systems, which includes an investigation of their drug loading and release behaviors. This new smart hydrogel is obtained using a simple two-step process, as shown in Fig. 1. The first step involves the preparation of carboxymethyl CD (CMCD) and carboxymethyl chitosan (CMC) from CD and chitosan, respectively. The second step is the crosslinking reaction between the carboxyl and primary amine moieties of neighboring CMC species to form a network structure of CMC chains; this is followed by the grafting of CMCD into the network via coupling of the CMC primary amine with the carboxyl group of CMCD. The conjugation reactions between CMC chains (crosslinking) and that between CMC and CD (CD-grafting to CMC) are performed simultaneously using EDC in the presence of NHS, thereby yielding a CD-*g*-CMC hydrogel. A series of CD-*g*-CMC hydrogels differing in the quantity of CD were prepared, and their swelling behaviors were investigated. Additionally, in order to examine the potential of the CD-*g*-CMC hydrogel as a suitable carrier matrix for drug delivery systems, drug adsorption/absorption and release behaviors of the hydrogels were characterized using acetylsalicylic acid (ASA, more commonly known as Aspirin) as a model drug.

(Please insert Fig. 1 here.)

2. EXPERIMENTAL

2.1. Materials

Chitosan (Mw; 4.6×10^4 , degree of deacetylation; 0.86) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Japan). The chitosan degree of deacetylation was determined via solid-state NMR analysis; details are presented below (Section 2.3.1 and Section 3.1). CD (purity 99.4%) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). EDC and NHS were purchased from Sigma-Aldrich Co., Ltd. (US). All solvents and other chemicals (analytical grade) were purchased from Kanto Chemicals Co., Inc. (Japan).

2.2. Preparation of CD-g-CMC hydrogel

2.2.1. Preparation of CMC

CMC was prepared according to a previously reported method [39]. In brief, chitosan (10 g, 57 mmol for the monomer unit) was added to a mixture of isopropyl alcohol (IPA; 80 mL) and aqueous 40% NaOH (w/v) solution (20 mL) and stirred using a Teflon impeller (300 rpm) at 25 °C for 1 h. To the suspension was added monochloroacetic acid (MCA; 15 g, 170 mmol) dissolved in IPA (20 mL) in five equal portions over a period of 20 min. The mixture was heated with stirring at 50 °C for a further 5 h. The reaction mixture was then neutralized using 4 M HCl solution. After removing the undissolved residue by filtration, the resulting CMC was precipitated by the addition of methanol. The precipitates were washed three times with methanol/water (1:1) and dried under vacuum at 40 °C.

2.2.2. Preparation of CMCD

CMCD was prepared according to a method reported in the literature [39]. Briefly, CD (22.8 g, 20 mmol) was completely dissolved in an aqueous 40% NaOH (w/v) solution (80 mL) containing MCA (3.8 g, 40 mM). The reaction mixture was then heated with stirring at 50 °C for 5 h. After neutralization with aqueous 4 M HCl solution, the obtained product was precipitated by addition of an excess amount of methanol. The precipitates were washed three times with methanol/water (1:1) and dried under vacuum at 40 °C.

(Please insert Table 1 here.)

2.2.3. Preparation of CD-*g*-CMC and CMC hydrogels

A series of CD-*g*-CMC hydrogels were prepared by varying the initial feed amounts of CMCD to CMC in the reaction mixture. A typical procedure for the preparation of a CD-*g*-CMC hydrogel is as follows: CMC (4.0 g, 17 mmol for the anhydroglucose unit (AGU)) and CMCD (4.0 g, 3.3 mmol) were completely dissolved in distilled water (100 mL) containing EDC (3.68 g, 19.2 mmol) and NHS (2.21 g, 19.2 mmol). The reaction was carried out at 25 °C for 24 h under continuous stirring with a Teflon impeller at 300 rpm. The obtained highly viscous solution was precipitated in methanol, and the resulting precipitate was then filtered. The obtained product was swollen with distilled water, and the resulting hydrogel was dialyzed against deionized water using dialysis tubing (cut-off, 2 kDa) for 3 d. The dialyzed hydrogel was precipitated with methanol, and then the precipitate was dried under reduced pressure at 40 °C. The resultant solid particles were cut and screened through a 20 mesh filter using a PLC-2M plastic cutting mill (Osaka Chemical Co., Japan) to obtain a white granular CD-*g*-CMC hydrogel (sample **1**). The other CD-*g*-CMC hydrogels (samples **2** and **3**) and CMC hydrogel were prepared using a method similar to that for preparation of sample **1**. The additive amounts of CMC and CMCD

are listed in Table 1. The CMC hydrogel was prepared without CMCD as a reference for samples 1–3. All hydrogels were stored in a desiccator under vacuum until ready for use.

2.3. Structural Analysis

2.3.1. FTIR spectroscopy

FTIR spectra were measured using a PerkinElmer Spectrum Two spectrometer (PerkinElmer Inc., US). FTIR spectra were recorded after grinding the sample into a powder and mixing well with KBr powder. The powder mixture was compressed into a transparent disk and scanned from 4000 to 400 cm^{-1} using an average of 16 scans with a resolution of 1 cm^{-1} .

2.3.2. NMR spectroscopy

All NMR spectra were recorded on a Bruker AVIII spectrometer (Germany; ^1H frequency of 500.13 MHz, ^{13}C frequency of 125.13 MHz) equipped with a two-channel 5 mm BBO probe incorporating a z-gradient coil. One-dimensional inverse-gated ^1H decoupled ^{13}C NMR spectra [30] were recorded using the default pulse program (Bruker Biospin). For the ^{13}C excitation pulse sequence, the flip angle (30°) pulse length, data acquisition time, and repetition time were set to 15 μs , 2.71 s, and 30 s, respectively. The obtained ^{13}C NMR chemical shifts were calibrated by assigning the methyl peak of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as 0 ppm.

Solid-state ^{13}C NMR spectra were recorded at room temperature on a Bruker Biospin AVIII 500 spectrometer with a 4 mm dual-tuned magic-angle spinning (MAS) probe at a frequency of 125.13 MHz. Dipolar-decoupled/MAS (DD/MAS) ^{13}C NMR experiments were performed on the hydrogel beads for quantitative analysis of the NMR spectra. ^{13}C -excitation pulse length (flip angle of 30°), data acquisition time, and repetition time were set to 1.5 μs , 20 ms, and 20 s, respectively. During the data-acquisition period, SPINAL-64 (small phase incremental

alternation with 64 steps) ^1H decoupling [42] was applied with a ^1H field strength of 100 kHz. The spectra were typically accumulated with 4096 scans to achieve an acceptable signal-to-noise ratio. ^{13}C NMR chemical shifts were calibrated based upon the carbonyl carbon resonance of D-glycine at 176.03 ppm, which was used as an external reference.

2.4. Swelling behavior

Water absorbencies of the CD-*g*-CMC and CMC hydrogels were determined by the following method [43, 44]: CD-*g*-CMC (100 mg) was swollen in a 20 mM citric acid–sodium citrate (100 mL; pH 4) buffer solution at 25 °C for 24 h. The swollen hydrogels at equilibrium were centrifuged at 15,000 rpm for 5 min, and the supernatant liquid solution was drained immediately. The weight of the swollen hydrogel (W_s) was measured, and the water absorbency was calculated using the following equation (Eq. (1)):

$$\text{Swelling index (\%)} = [(W_s - W_d)/W_d] \times 100 \quad (1)$$

where W_s and W_d are the weight of swollen and dried hydrogel, respectively. In addition, 20 mM NaH_2PO_4 – Na_2HPO_4 (pH 7) and 20 mM glycine–NaOH (pH 10) buffer systems were also used as an external solution for absorbency determinations.

2.5 Morphological observation

Scanning electron microscope (SEM) images of the dried and swollen hydrogels were obtained by use of a JEOL JSM-7500F scanning electron microscope (JEOL Ltd., Japan) operating at a potential of 1 kV. Cross-sectional structures of the swollen hydrogels were observed using samples prepared as follows: after being swollen in deionized water at 25 °C for 48 h, the hydrogels were carefully cut by a razor blade, frozen at -70 °C, and then freeze dried under vacuum until the water sublimed [46, 47]. The sample was then fixed on a specimen stub sputter-coated with platinum prior to observation.

2.6 Rheological properties

The rheological behavior of the swollen hydrogel was evaluated in triplicate using a Physica MCR 301 rheometer (Anton Paar GmbH, Austria) equipped with a Rheoplus 32 data analyzer and fitted with a Peltier temperature control. A 25 mm rough surface cone-plate measuring geometry was used to prevent sample slippage. The temperature was kept constant at 298 K for all measurements. The gap was set at 1.0 mm. Frequency-sweep tests were carried out to study the hydrogel's viscoelastic behavior. The storage (G') and loss (G'') moduli were evaluated as a function of angular frequency (ω) from 0.063 to 63 rad s^{-1} at constant strain ($\gamma = 0.01$).

2.7. Drug absorption and release studies

In the drug absorption experiments, ASA was dissolved in 20 mM phosphate-buffered saline (PBS) solution (7 mL; pH 7.4), followed by the addition of CD-g-CMC hydrogel (30 mg) to the ASA-containing buffer. Absorption experiments were then conducted in a thermostat-containing reciprocal shaker (120 rpm) at 25 °C for 24 h. At different time intervals, the mixture was filtered through a membrane filter (pore size: 0.45 μm); ASA concentration in the filtrate was determined at a wavelength of 234 nm [48] using an Epoch 96 well micro-volume spectrophotometer (BioTek Instruments, Inc., US). The amount of ASA absorbed in the hydrogels (q) was determined using Eq. (2):

$$q_t = V(C_0 - C_t) / W, \quad (2)$$

where q_t ($\mu\text{mol g}^{-1}$) is the ASA absorption capacity at contact time t , V is the volume of ASA solution (L), C_0 is the initial concentration of ASA ($\mu\text{mol L}^{-1}$), C_t is the concentration of ASA at contact time t ($\mu\text{mol L}^{-1}$), and W is the weight of hydrogel (g). The effects of contact time and BPA concentration on the ASA absorption capacities of each hydrogel were studied.

For drug-release experiments, the ASA-absorbed hydrogels were freeze-dried and the resulting hydrogel was used; a quantity of freeze-dried hydrogel (10 mg) was added to a polypropylene tube (15 mL capacity) containing 20 mM PBS solution (10 mL; pH 7.4). The tube was placed into the thermostat-containing reciprocal shaker (120 rpm) at 25 °C for 24 h. ASA release from the hydrogels was determined by applying the amounts of released and absorbed ASA to the following relationship (Eq. 3):

$$\text{ASA cumulative release (\%)} = (\text{amount of released ASA}) \times 100 / (\text{amount of absorbed ASA}) \quad (3)$$

(Please insert Fig. 2 here.)

3. Results and discussion

3.1. Characterization of CMCD and CMC

Quantitative ^{13}C NMR spectra of the obtained CMCs are shown in Fig. 2(A). Resonances located at 182.1 and 180.3 ppm were attributed to the carbonyl carbons of carboxymethyl-substituted amino and hydroxyl groups of chitosan, respectively; the resonance located at 180.0 ppm in the spectrum of chitosan could be assigned to the acetamide carbonyl carbon [49]. The degree of carboxymethyl substitution at the amino and hydroxyl groups of chitosan, denoted as DS_{NCM} and DS_{OCM} , could be determined using Eqs. (4) and (5), respectively:

$$\text{DS}_{\text{NCM}} = I_{\text{c}} / I_{\text{C1}} \quad (4)$$

$$\text{DS}_{\text{OCM}} = I_{\text{b}} / (I_{\text{C1}}) \quad (5)$$

where I_{b} and I_{c} are the integral values of carbonyl carbon resonances at 181.5 and 180.2 ppm, respectively, and I_{C1} corresponds to that of the C1 resonance at 106.2 ppm in (Fig. 2(A). By applying the I_{b} , I_{c} , and I_{C1} values to Eqs. (4) and (5), DS_{OCM} and DS_{NCM} were calculated as 0.65

and 0.21, respectively, indicating that the total degree of carboxymethyl substitution of CMC was 0.86.

Fig. 2(B) shows the quantitative ^{13}C NMR spectrum of CMCD. The degree of carboxymethyl group substitution (DS_{CMCD}) was determined as 0.14 using Eq. (6):

$$\text{DS}_{\text{CMCD}} = I_{\text{CM}} / I_{\text{C1}} \quad (6)$$

where I_{CM} and I_{C1} are the integral values of the carbonyl carbon (182 ppm) and C1 resonance of CMCD (107 ppm), respectively. The DS_{CMCD} value of 0.14 indicated that on average, one hydroxyl group was substituted for a carboxymethyl group per one CD molecule. CD-g-CMC hydrogels were next prepared using these CMC and CMCD.

3.2. Preparation of CD-g-CMC and CMC hydrogels

As shown in Fig. 1, the CD-g-CMC hydrogels were prepared from CMC and CMCD via EDC coupling in the presence of NHS. In this coupling reaction, two transformations occur simultaneously to form the CD-g-CMC hydrogel: an amidation crosslinking reaction between amino and carboxyl groups of neighboring CMCs to form the gel structure, and an amidation grafting reaction between the CMC amino and CMCD carboxyl groups. In this study, a series of three hydrogels (samples 1–3) and a CMC hydrogel as reference were prepared by varying the initial feed of CMCD to CMC, as summarized in Table 1. Among these samples, the prepared CMC hydrogel consisted solely of CMC (i.e., without added CMCD). During hydrogel preparation, the reaction mixtures became increasingly viscous soon after starting the reaction, and the mixture morphologies gradually changed from a solution to gel. After the reaction, the obtained transparent gels were purified, dialyzed against deionized water, and freeze-dried.

(Please insert Fig. 3 here.)

3.3. Structural analysis of hydrogels

3.3.1. FTIR spectroscopic analysis

Fig. 3 shows the FTIR spectra of CD-*g*-CMC hydrogels, CMC hydrogel, and CMC and CMCD starting materials. These spectra displayed common absorption bands at 2930, 1591, and 1406 cm^{-1} , which can be assigned to the asymmetric stretching vibration of aliphatic CH_2 as well as C=O asymmetric and symmetric stretching vibrations of the carboxylate group, respectively [50]. Except for these absorption bands, all CD-*g*-CMC samples showed the typical amide I band with a strong intensity at 1640 cm^{-1} [50], indicating that successful amidation crosslinking had occurred between the CMC molecules. In addition, both the CD-*g*-CMC samples and CMCD showed bands at 894 cm^{-1} due to the vibration of the α -(1 $\rightarrow$ 4) glucopyranose ring [51], indicating that CD was grafted to CMC through the amide bond between the amino group of CMC and carboxymethyl group of CMCD. The intensity of the band at 894 cm^{-1} of the CD-*g*-CMC samples was enhanced by an increase in the feed amounts of CMCD during hydrogel preparation, indicating that a higher feed ratio of CMCD prompted the grafting of CMCS to CMC. This was also confirmed by a comparison of absorption band intensities at 2930 and 2875 cm^{-1} ; the absorption band at 2930 cm^{-1} assigned to the asymmetric stretching vibration of aliphatic CH_2 was attributed to the carboxymethyl CH_2 group and C6 of CMC and/or C6' of CMCD, while the band at 2875 cm^{-1} could be assigned to the symmetric CH_3 vibration of the acetamide group in CMC [52]. Thus, the bands at 2875 cm^{-1} could not be observed in the CMCD spectrum. In the FTIR spectrum of CD-*g*-CMC, the intensity of the bands located at 2875 cm^{-1} obviously increased with an increase in the feed amounts of CMCD during CD-*g*-CMC preparation, indicating that the grafting of CMCD onto CMCS was prompted by an increase in the CMCD feed amount in the reaction mixture. In order to obtain more detailed and clear information about the molecular structure of CD-*g*-CMC and CMC hydrogels, solid-state ^{13}C NMR measurements were next performed.

(Please insert Fig. 4 here.)

3.3.2 NMR spectroscopic analysis

Fig. 4 shows the solid-state ^{13}C NMR spectra of CD-*g*-CMC and CMC hydrogels and also provides a ^{13}C resonance assignment. In these spectra, carbon atoms of the AGU in CMCD are designated by prime notation; accordingly, C1', C4' and C6' resonances appeared in the regions encompassing 110–90 ppm, 88–80 ppm, and 67–58 ppm, respectively, while C2', C3', C5', and methylene carbon resonances overlapped in the region of 80–67 ppm [52]. Most CMC ^{13}C resonances overlapped those of CMCD; chemical shifts for C1, C4, and C6 appeared in the region of 110–90 ppm, 88–80 ppm, and 67–58 ppm, respectively, and C3, C5, and methylene carbons were located in the 80–67 ppm region. C2 and the acetamide CH₃ group of CMC appeared in the region of 58–52 ppm, and 24–20 ppm, respectively [52]. Carbonyl carbons of CMC and CMCD appeared in the range 182–170 ppm with two peaks at 178 ppm and 172 ppm; the resonance at 178 ppm was assigned to the free carboxylate carbonyl carbon, while that at 172 ppm was attributed to the overlap of the amide carbonyl carbons and acetamide groups [50].

To estimate the amounts of CMCD grafted to CMC in the CD-*g*-CMC hydrogel, the integration values of the carbonyl carbons at 182–170 ppm (I_{CO}) were determined when the sum of C1 and C1' integrals at 108–93 ppm of each CD-*g*-CMC were set to 1 (Fig. S1). A gradual increase in I_{CO} was observed with an increase in the feed amount of CMCD to CMC during CD-*g*-CMC preparation. From the I_{CO} value for each sample, the average number of grafted CMCD per one monomer unit of CMC, defined as the graft degree of CMCD (n_{CD}), could be determined using Eq. (7):

$$n_{\text{CD}} = (0.16 + 0.86 \cdot I_{\text{CO}}) / 7 (I_{\text{CO}} - 0.14) \quad (7)$$

where the constants 0.14, 0.16, 0.86, and 7 correspond to the DS_{CMCD} of CMCD starting material, acetylation degree of CMC amino groups, total degree of CMC carboxymethyl group substitution ($DS_{NCM} + DS_{OCM}$), and number of AGUs in one CMCD molecule, respectively. Therefore, the average number of CMC monomer per one CMCD molecule could be calculated using Eq. (8):

$$(\text{average number of CMC monomer of per one CMCD molecule}) = 1 / n_{CD} \quad (8)$$

These structural parameters for each CD-*g*-CMC are also summarized in Table 1. From this data, it was clearly revealed that n_{CD} increased with an increase in the feed ratio of CMCD to CMCs during CD-*g*-CMC preparation.

(Please insert Fig. 5 here.)

3.4 Swelling behavior

The dried CD-*g*-CMC samples consist of white particles and absorb water readily, forming transparent hydrogels upon soaking. Fig. 5 shows the SEM images of the dried and swollen CD-*g*-CMC hydrogel (sample 3). The surface morphology of the dried hydrogel is smooth, while a porous structure could be observed on the surface and in the cross-section of the CD-*g*-CMC hydrogel. This suggests that the porous structure helps retain water inside the gel, resulting in water absorbency.

(Please insert Fig. 6 here.)

Fig. 6 shows the absorbency of the CD-*g*-CMC and CMC hydrogels swollen in 20 mM buffer solutions at pH 4, 7, and 10 for 24 h. The absorbencies of all hydrogels showed similar trends; the absorbency at pH 4 was low, while absorbency at pH 7 was almost equal to that at

pH 10, indicating that ionic groups in the hydrogels take an important factor to absorb water inside the gel. According to previous reports, the pK_a of the chitosan amino group is ca. 6.3 [53, 54], and that of the carboxymethyl group in CMC is 4.2 [54]. Therefore, the free amino groups of the hydrogels are protonated to form NH_3^+ at pH 4, whereas the carboxylic group is considered to exist as the acid and carboxylate anion in the almost same population. In contrast, at pH 7 and 10, the free amino and carboxyl groups exist as the neutral NH_2 and anionic COO^- species, respectively. In this study, the degree of deacetylation of the starting chitosan was 0.86, and the DS_{NCM} of CMC was 0.21, indicating that the free amino groups in CMC were present in a ratio of 0.65. On the other hand, the free carboxylic groups in the CMC were present in a ratio of 0.86 ($DS_{NCM} + DS_{OCM} = 0.86$). These results indicate that the CMC prepared in this study was an anion-rich amphoteric chitosan material. During the simultaneous crosslinking reaction of CMC and grafting reaction between CMC and CMCD, a portion of the free amino groups were consumed to form the carboxymethyl groups of CMC and CMCD; thus, all the obtained hydrogels were also anion-rich amphoteric hydrogels. In the buffer solutions at pH 7 and 10, therefore, an electrostatic repulsion between the hydrogel carboxylate anions would occur, which would serve to enhance its absorbency. In the case of the pH 4 buffer solution, most of the ammonium cation was considered to be neutralized by the carboxylic anions; accordingly, electrostatic repulsion between the ammonium cations and carboxylate anions could not occur, thereby lowering the extent of water swelling.

The most notable difference in absorbency between the CMC hydrogel and CD-*g*-CMC hydrogels was observed at pH 7 and 10; absorbencies of the CMC hydrogel at pH 7 and 10 were 42.1, and 42.8 g/g-polymer, respectively, while those of CD-*g*-CMC hydrogels were lower, in range of 12–20 g/g-polymer. In addition, the CD-*g*-CMC hydrogel absorbency was gradually decreased with an increase in the n_{CD} value of each sample. As described above, the dominant charged species in the CD-*g*-CMC hydrogels at pH 7 and 10 is the deprotonated

carboxyl group found in the CMC moiety. The increase in the extent of CMCD grafting is accompanied by a decrease in the amount of CMC per unit mass of CD-g-CMC, thereby lowering the absorbency of CD-g-CMC at pH 7 and 10.

(Please insert Fig. 7 here.)

3.5 Rheological properties

The G' and G'' of the CD-g-CMC and CMC hydrogels as a function of ω are shown in Fig. 7. This figure shows that the value of G' is always higher than that of G'' and that there were no cross-over points for any of the hydrogels, indicating that the hydrogels had a characteristic gel structure [46, 47, 55]. The value of G' for the CMC hydrogel was the highest among all the samples over the entire frequency range, and the G' value of CD-g-CMC hydrogels decreased with an increase in the extent of CMCD grafting. In addition, on going from 0.063 rad s^{-1} to 63 rad s^{-1} , we observed a 2.9, 3.6, 3.3, and 5.7 fold increase in G'' for the CMC hydrogel, CD-g-CMC hydrogel sample 1, sample 2, and sample 3, respectively. These results indicate that the addition of a large amount of CMCD to the reaction mixture to produce CD-g-CMC hydrogels caused a significant decrease in the moduli because of a decreased network structure composed of CMC molecules [46, 47].

3.6 Drug absorption capacity

Among pharmaceutical drugs, ASA is well known for its ability to undergo complexation with CD [56, 57]. Encapsulation of ASA with CD has been performed by physical mixing [56], precipitation [58], a solid dispersion/co-evaporated dispersion method [59], etc., to enhance ASA solubility, reduce irritation in the human body, and prevent its premature degradation via ester cleavage in aqueous solution. In addition, the ASA-CD complex structure in solution has

been previously proposed based on NMR spectroscopic analysis [60, 61]. In this study, to confirm the encapsulation abilities of the CD-*g*-CMC hydrogel, its absorption behavior toward ASA was characterized in order to elucidate its potential as a drug delivery system carrier.

(Please insert Fig. 8 here.)

Fig. 8 shows the absorption of ASA in the CD-*g*-CMC and CMC hydrogels as a function of time. Elevated absorption rates were observed at the beginning of the experiment, after which saturation was gradually achieved over the course of 12 h, finally reaching a plateau. The amount of ASA absorbed by the CD-*g*-CMC hydrogels at the saturated time was strongly depended on the n_{CD} of the hydrogels; the amount of absorbed ASA after 24 h was 6.3, 15.7, and 23.9 $\mu\text{mol/g-polymer}$ for CD-*g*-CMC hydrogels of samples **1**, **2**, and **3**, respectively. The CMC hydrogel also absorbed ASA; after 24 h, this amount was substantially lower (only 2.4 $\mu\text{mol/g-polymer}$) than that of the CD-*g*-CMC hydrogels. These results indicated that the ASA molecules were not only absorbed in the hydrophobic CD cavity in the CD-*g*-CMC hydrogels, but were also adsorbed on the hydrogel surface or absorbed in the hydrogel interior, although the amount of ASA absorbed in the CD cavity was higher than that found on the gel surface or interior.

(Please insert Fig. 9 & Table 2 here.)

Fig. 9(A) shows the effects of the initial ASA concentration ($[\text{ASA}]_{\text{init}}$) on its absorption into the CD-*g*-CMC hydrogel after 24 h. In our previous study of the absorption of bisphenol A in CD-containing hydrogels prepared from the copolymerization of sodium carboxymethyl cellulose and CD [52], it was revealed that the absorption behavior of bisphenol A toward the

hydrogels could be well fitted to the Langmuir adsorption isotherm equation [62]. In addition, a water-insoluble CD polymer prepared by the polymerization of CD with 1,2,3,4-butanetetracarboxylic dianhydride as a crosslinker showed a similar Langmuir-type behavior toward bisphenol A [51]. Therefore, using the data shown in Fig. 9(A), it was confirmed that the Langmuir model could be applied to the absorption of ASA by the CD-*g*-CMC hydrogel. In the Langmuir absorption model, the maximum ASA absorption capacity (q_m) of the CD-*g*-CMC hydrogel and equilibrium binding constant (K_b) of the absorption system can be described by Eq. (9):

$$1/q = K_d / \{q_m [ASA]_{eq}\} + 1/q_m \quad (9)$$

where q is the equilibrium absorption capacity of ASA absorbed in the CD-*g*-CMC hydrogel, q_m is the maximum absorption capacity of the CD-*g*-CMC, $[ASA]_{eq}$ is the ASA equilibrium concentration in solution, and K_d is the dissociation constant of the absorption system. Fig. 9(B) shows the plot of the equilibrium ASA concentration, ($[ASA]_{eq}$) vs. q , after an absorption time of 24 h. Double reciprocal plots of q and $[ASA]_{eq}$ were next constructed for CD-*g*-CMC (Fig. 9(C)). The absorption isotherms of ASA for the CD-*g*-CMC hydrogel were confirmed to be typical of Langmuir-type behavior, which can be described by Eq. (9). Based on Fig. 9(C), q_m of the CMC hydrogel and CD-*g*-CMC hydrogels (samples 1–3) were 7.32, 17.8, 25.5, and 40.8 $\mu\text{mol g}^{-1}$, with dissociation constants K_d of 55.5, 49.5, 21.2, and 15.0 $\mu\text{mol L}^{-1}$, respectively (Table 2). Therefore, the equilibrium binding constant K_b could be determined using Eq. (10):

$$K_b = 1 / K_d \quad (10)$$

As summarized in Table 2, the values of K_b for CMC and CD-*g*-CMC hydrogels (1–3) were 1.80×10^{-2} , 2.02×10^{-2} , 4.72×10^{-2} , and $6.65 \times 10^{-2} \mu\text{mol}^{-1} \text{L}$, respectively. This data indicates that a stable inclusion complex was formed between the ASA and CD sites of CD-*g*-CMC, and that the inclusion complex between the CMC hydrogel and ASA was less stable as a result of the increase in K_b with an increase in n_{CD} of the hydrogel.

(Please insert Fig. 10 here.)

3.7 ASA release behavior

In vitro ASA release from the ASA-absorbed CD-*g*-CMC and CMC hydrogels was investigated by monitoring the amounts of released ASA from a hydrogel–PBS mixture at 37 °C as a function of time. The ASA release profiles are depicted in Fig. 10; as evidenced, the CD-*g*-CMC hydrogels presented a fast release into the medium up to a release time of 2 h, at which point 45.4, 53.7, and 59.0% ASA remained within CD-*g*-CMC hydrogel samples 1–3, respectively. After this initial fast release profile, CD-*g*-CMC hydrogels showed a slower and steadier ASA release into the medium. In contrast to these results, ASA-absorbed CMC hydrogel showed a more rapid release of ASA; most of the drug (86.0%) was released to the medium within 2 h. A number of different factors are thought to be involved in the complex formation of CD-*g*-CMC hydrogels with ASA: hydrophobic–hydrophobic (van der Waals) interactions between the hydrophobic moiety of the ASA molecules and hydrophobic CD cavity in CD-*g*-CMC hydrogels, hydrogen bonding between the polar functional groups contained in both ASA and CD-*g*-CMC hydrogels, and hydrogen bonding between the polar moieties of ASA and high-energy water molecules released from the hydrogel CD cavity during complex formation [63, 64]. The ASA release rate from CD-*g*-CMC during release experiments was quite fast during the initial 2 h time period. Such an abrupt release was attributed to the remaining free ASA on the hydrogel surface and interior and/or ASA weakly interacting with CD-*g*-CMC hydrogels through hydrogen bonding [63, 64]. After this initial burst, ASA was released in a sustained manner; the release rate was observed to decrease for all CD-*g*-CMC hydrogel samples with increased time. This slow release of ASA from CD-*g*-CMC hydrogels was ascribed to host–guest complex formation between ASA and the CD cavity in the CD-*g*-

CMC hydrogel. In the case of ASA-absorbed CMC hydrogels, such a sustained and slow ASA release was observed because of a lack of host–guest complexation between ASA and CMC hydrogel. The unique drug release profiles observed for the present CD-*g*-CMC hydrogel systems indicate their utility in controlled drug delivery applications, in this example, yielding an anti-inflammatory effect at wound sites to potentially effect long-term healing.

4. Conclusion

A novel type of CD-*g*-CMC hydrogel having the capability for controlled drug release could be prepared from CMCD and CMC by the reaction with the zero-bond crosslinker of EDC in the presence of NHS. Because the dual EDC–NHS reagent allows for the simultaneous carboxyl-to-amine crosslinking between CMC molecules as well as grafting between CMC and CMCD, no crosslinking agents were incorporated or remained in the molecular structure of the obtained CD-*g*-CMC hydrogels; this synthetic method is advantageous for yielding biocompatible hydrogels for use in pharmaceutical applications. The CD-*g*-CMC hydrogels showed excellent drug absorption/adsorption abilities, with the ASA-absorbed CD-*g*-CMC hydrogels exhibiting slow and sustainable ASA release. The observed ASA release profiles could be attributed to the formation of a host–guest complex between ASA and the hydrophobic cavity of CD in the hydrogels; this valuable property might be enable the use of CD-*g*-CMC hydrogels as carriers controlled drug delivery systems.

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Figure Captions

Fig. 1. Scheme for CD-*g*-CMC hydrogel synthesis from chitosan and CD, and a schematic illustration of the structure of the CD-*g*-CMC hydrogel. In this figure, although carboxymethylation at the chitosan C6 hydroxyl group is illustrated, carboxymethylation also can occur at other hydroxyl positions of chitosan as well as CD, which have been omitted from this figure.

Fig. 2. Quantitative ^{13}C NMR spectra of CMC (A) and CMCD (B) in D_2O . Resonance assignments are also indicated.

Fig. 3. FTIR spectra of CMC, CMCD, CMC hydrogel, and CD-*g*-CMC hydrogels.

Fig. 4. Solid-state DD/MAS ^{13}C NMR spectra of the CMC hydrogel and CD-*g*-CMC hydrogels. These spectra were roughly normalized by the methyl carbon peak intensity at 22 ppm.

Fig. 5. SEM images of the CD-*g*-CMC hydrogel (sample 3): (A) and (B) surface of the dried hydrogel; (C) surface and (D) cross-section of the swollen hydrogel.

Fig. 6. Water absorbency of the CMC hydrogel and CD-*g*-CMC hydrogels immersed in 20 mM buffer solutions (pH 4, 7, and 10) after 24 h.

Fig. 7. Viscoelastic behavior of the CMC and CD-*g*-CMC hydrogels swollen in pure water (weight was 20 times its dry weight). These measurements were performed at 298 K.

Fig. 8. Time dependency of ASA absorption in CMC hydrogel and CD-*g*-CMC hydrogels. ASA absorption experiments were performed under the following conditions: hydrogel content (dried): 30 mg; initial ASA concentration: 500 $\mu\text{mol L}^{-1}$; buffer: 20 mM PBS solution, pH 7.4; temperature: 25 °C; volume of ASA solution: 7 mL.

Fig. 9. (A) Effect of initial ASA concentration on ASA absorption (q) by CMC hydrogel and CD-*g*-CMC hydrogels. ASA absorption experiments were performed under the following conditions: hydrogel content (dried): 30 mg; buffer: 20 mM PBS solution, pH 7.4; temperature: 25 °C; volume of ASA solution: 7 mL; contact time: 24 h. (B) Relationship between equilibrium ASA concentration ($[\text{ASA}]_{\text{eq}}$) and ASA absorption (q) in hydrogels. (C) Linear representation of Langmuir-type absorption of ASA for CMC hydrogel and CD-*g*-CMC hydrogel beads.

Fig. 10. ASA release profile from ASA-absorbed CMC hydrogel and CD-*g*-CMC hydrogels in 20 mM PBS solution, pH 7.4.

Table 1. Reaction conditions, yields, and structural parameters of CMC and CD-g-CMC hydrogels.

Sample No.	Initial additive amounts / g		Yields / g ^a	<i>I</i> _{CO} ^b	Structural parameters	
	CMC	CMCD			<i>n</i> _{CD} ^c	<i>n</i> _{CD} ^{-1 d}
1	4.0 (17 mmol)	4.0 (3.3 mmol)	4.3	0.92	1.83 × 10 ⁻²	54.6
2	4.0 (17 mmol)	8.0 (6.6 mmol)	4.6	0.86	4.20 × 10 ⁻³	23.8
3	4.0 (17 mmol)	12.0 (9.9 mmol)	4.9	0.71	7.77 × 10 ⁻³	12.9
CMC hydrogel	4.0 (17 mmol)	0 (0 mmol)	3.2	0.67		

^a Yield for each entry is of the dried hydrogel obtained from the reaction of CMC and CMCD.
^b Values of *I*_{CO} were determined from the integration of the carbonyl carbon region (183–172 ppm) in each solid-state ¹³C DD/MAS NMR spectrum (Fig. S1) when the integral value of the C1 signal (110–90 ppm) was set to 1.
^c Average number of grafted CMCD molecules per one AGU of CMC in each hydrogel.
^d Average number of AGU of CMC per one CMCD molecule grafted with CMC in each hydrogel.

29 **Table 2.** Maximum adsorption capacity (q_m), dissociation constant (K_d), and equilibrium
30 binding constant (K_b) for the ASA absorption by CD-*g*-CMC hydrogels and CMC hydrogel.^a

Sample No.	$q_m / \mu\text{mol g}^{-1}$	$K_d / \mu\text{mol L}^{-1}$	$K_b / \mu\text{mol}^{-1} \text{L}$
1	17.8	49.5	2.02×10^{-2}
2	25.5	21.2	4.72×10^{-2}
3	40.8	15.0	6.65×10^{-2}
CMC hydrogel	7.32	55.5	1.80×10^{-2}

31 ^a Determined by the Langmuir-type plot of the ASA adsorption systems shown in Fig. 9(C).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Graphical abstract for “Cyclodextrin-grafted chitosan hydrogels for controlled drug delivery”