Controlled drug release using ascorbate-responsive quercetin-conjugated alginate hydrogels

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Abstract In this paper, we report a new strategy for controlled cargo release using quercetin-conjugated alginate (Alg) hydrogels that has enhanced hydrogel stability and can release cargo molecules through on-demand metal cation reduction by a biological reducing agent, ascorbate. By conjugating hydrophobic quercetin to the Alg backbone, hydrogel stability was increased. The encapsulated cargos were released on demand through reduction of cross-linking Fe(III) ions by ascorbate, harnessing the differences in cross-linking capability between Fe(II) and Fe(III). We found that encapsulated fluorescein in quercetin-conjugated Alg hydrogels was released much slower than that in hydrogels without quercetin conjugation. In addition, the stability of the hydrogels and the release of encapsulated fluorescein were controlled by modulating oxidation state of Fe using ascorbate. We validated our strategy by demonstrating the enhanced cancer cell killing of the doxorubicin-encapsulated hydrogels upon treatment with ascorbate. We believe that the strategy would be used as an effective tool for the anticancer drug delivery that can be controlled by ascorbate as overcoming the drawback of Alg hydrogels.

Keywords Alginate · Biomaterials · Controlled release · Hydrogel · Quercetin

Introduction

Alginate (Alg) is a linear polysaccharide extracted from seaweed, chemically consisting of (1 → 4)-linked β-D-mannurionate and its C-5 epimer α-L-guluronate residues. Alg is a widely used natural polymer for preparing hydrogels, usually based on ionic interactions between carboxylic acids in Alg backbone and metal cations such as Ca$^{2+}$, Mg$^{2+}$, and Fe$^{3+}$ (Haug and Smidsrod 1965; Papa-georgiou et al. 2010). Due to the many excellent properties for bio-applications such as biocompatibility, non-toxicity, biodegradability, and suitability for chemical modifications (Lee and Mooney 2001, 2012), Alg hydrogels have been widely used in various fields encompassing pharmaceuticals (Wee and Gombotz 1998; Quong et al. 1998; Vandenberg et al. 2001; Wang et al. 2003; Iwasaki et al. 2004), food industry (Zhang et al. 2015; Waterhouse et al. 2014), and cosmetics industry (Liu et al. 2008). For example, the Alg hydrogels have been used in pharmaceuticals for injecting cells and drugs as a biocompatible carrier for sustained release, in dental implants, and as wound dressings (Ahmad and Khuller 2008; Matthew et al. 1995).

However, mechanical and biophysical instability of Alg hydrogels in physiological conditions, mainly stemming from interference in ionic interactions, leads to unavoidable rapid loss of metal cations and encapsulated cargos and therefore hampers in more effective usage of Alg hydrogels (Malafaya et al. 2007). As such, several tactics have been employed to improve hydrogel stability, mostly through introducing cross-linkers to the Alg backbone (Jeon et al. 2009; Lee et al. 2013). However, some cross-linking methods can affect cargo release because of unexpected conjugation of cargo molecules to the Alg backbones during cross-linking process. In addition,
improved stability of Alg hydrogels sometimes gives rise to inefficient controlled release of cargos. Therefore, a new approach has been necessitated, where enhanced stability of hydrogels can be compromised by external stimulus, resulting in controlled cargo release on demand (Swamy et al. 2013; Maciel et al. 2013). As a typical example, Maciel et al. reported glutathione (GSH)-responsive doxorubicin release using cysteine-conjugated Alg (Maciel et al. 2013). Cysteine played a role of cross-linker to make stable hydrogels with disulfide linkage, which was cleaved by GSH, a reducing agent in cells, inducing controlled release of encapsulated doxorubicin.

Here, we introduce an external stimulus-responsive Alg hydrogels for controlled cargo release. In our approach, mechanical property of Alg hydrogels was improved by incorporating hydrophobic moiety in Alg backbones (Bu et al. 2006; Yang and He 2012; Yang et al. 2007). Introducing hydrophobic moieties such as butyl and dodecyl chains has been known to improve mechanical property of Alg hydrogels for cargo delivery (Leonard et al. 2004a, b; Broderick et al. 2006). For on-demand controlled release of cargo, we devised a strategy to destabilize Alg hydrogels by reducing metal cations. Jin et al. reported an electrochemical protein release from iron–Alg thin films, where they used affinity differences of Fe$^{3+}$ and Fe$^{2+}$ toward carboxylate groups of Alg backbones (Jin et al. 2012). Fe$^{3+}$ has a stronger preference for coordinating to carboxylate than Fe$^{2+}$, and therefore, electrochemical reduction of Fe$^{3+}$ to Fe$^{2+}$ caused instability of protein-imbedded Alg complex resulting in the thin-film dissolution and the protein release. Taken together, we reported a new strategy for controlled cargo release using quercetin-conjugated Alg (QAlg) hydrogels that has enhanced hydrogel stability by incorporating hydrophobic quercetin to the backbone and can release cargo molecules through on-demand metal cation reduction by a biological reducing agent, ascorbate (Fig. 1).

**Fig. 1** Schematic diagram of controlled drug release from quercetin-conjugated alginate (QAlg) hydrogels by ascorbate. QAlg is prepared by coupling reaction between alginate and quercetin using EDC and DMAP. Stable drug-encapsulated QAlg hydrogels are prepared with Fe$^{3+}$ as a cross-linker. When Fe$^{3+}$ is reduced to Fe$^{2+}$ by ascorbate, hydrogel network is destabilized and encapsulated drugs are released.
Materials and methods

Materials

Quercetin dihydrate, Alg sodium salt, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), dialysis tube (MWCO 12400), fluorescein, FeCl₃, ascorbic acid, sodium citrate, reduced GSH, and 1,10-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO, USA). N,N-dimethylformamide (DMF) and iron(II) sulfate heptahydrate were from Daejung Chemicals and Metals Co., Ltd. (Siheung, Gyeonggi-do, Korea). Phosphate-buffered saline (PBS), RPMI-1640 medium, fetal bovine serum (FBS), and antibiotics (penicillin G and streptomycin) were obtained from WelGENE, Inc. (Seoul, Korea). Bovine serum albumin (BSA) was from SERVA (Heidelberg, Germany). Doxorubicin hydrochloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bio-rad protein assay kit based on Bradford assay was from Bio-rad ( Hercules, CA, USA). Dulbecco’s PBS (DPBS) was obtained from GE Healthcare Life Science (Logan, UT, USA). Trypan blue solution 0.4% was bought from Thermo Fisher Sci. (Carlsbad, CA, USA).

Methods

Synthesis of quercetin-conjugated alginate (QAlg)

Alg was dissolved in 10 mL distilled water at a concentration of 2% (w/v). Quercetin (500 μL, 100 mM in DMF) and DMAP (15 mg) were then added to the Alg solution, and EDC (200 μL, 20 mg/mL in distilled water) was added to the mixture four times every 1 h. After 5 h, the reaction mixture was dialyzed using distilled water for 3 days and subsequently lyophilized. Conjugation between quercetin and Alg was confirmed by nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FT-IR). 1H NMR spectra were recorded on a Bruker Advance III 500 (Karlsruhe, BW, Germany) at 500 MHz, and FT-IR spectra were recorded on a PerkinElmer Spectrum 100 (Waltham, MA, USA) at 4 cm⁻¹ resolution.

Formation of QAlg hydrogels

QAlg solution (500 μL, 2% w/v in PBS) was mixed with cargo molecules (100 μL, fluorescein, 0.2 mg/mL in PBS or doxorubicin hydrochloride, 2 mg/mL in PBS). The mixture solution was then added dropwise to FeCl₃ solution (10 mL, 0.2 M in distilled water) using a 26 G needle for formation of hydrogel beads. After 2 min, the resulting hydrogel was filtered and washed with PBS.

Fluorescein release profile of hydrogels

The hydrogels were incubated in PBS at room temperature, and the solution was collected at several time points during incubation for measuring the amount of released fluorescein using fluorescence intensity of fluorescein (λₑₓ = 494 nm and λₑᵣₓ = 521 nm). The residual fluorescein in hydrogels was measured after destructing hydrogel network. Ascorbate-responsive fluorescein release was performed using the identical protocol as described above except that the hydrogel was incubated in PBS in the presence of 3 mM ascorbate. Fluorescence intensities were measured by Gemini EM microplate reader (Sunnyvale, CA, USA).

Ferroin assay under various bio-reducing agents

1,10-Phenanthroline iron(III) chloride complex (3.7 mM) was prepared in distilled water. The complex was added to equal volume of a bio-reducing agent solution (19 mM in PBS) of sodium citrate, GSH, and ascorbate. As a positive control, 1,10-phenanthroline iron(II) sulfate complex (3.7 mM) was used. The absorbance of 1,10-phenanthroline iron(II) complex was measured at 508 nm using Shimadzu biospec1601 spectrophotometer (Kyoto, Japan).

Cytotoxicity assay of doxorubicin-encapsulated QAlg hydrogels against MCF-7 cells

MCF-7 cells were cultured in RPMI-1640 containing 10% (v/v) FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified 5% CO₂ atmosphere. Cells (ca. 50,000) were plated in a six-well plate and incubated for 24 h. The media was removed, and the cells were incubated with 5 mL cell culture media containing 1 mL PBS as a control, 1 mL doxorubicin (3 μg/mL), or 1 mL extracts from the hydrogels for additional 24 h. Cytotoxicity of the cells was verified through trypan blue exclusion test. The cell was detached from the plate using trypsin–EDTA solution, and the cell suspension was centrifuged (100 × g, 5 min). The cell pellet was resuspended in 0.1 mL DPBS and was mixed with 0.4% trypan blue solution in an equal volume ratio for 3 min at room temperature. The number of viable and non-viable cells in 10 μL of the mixture was counted using a hematocytometer.

Measurement of albumin-binding capacity of hydrogels

Hydrogels without cargo were prepared with a cross-linking agent Ca²⁺ or Fe³⁺. BSA (5 mL, 20 mg/mL in PBS) was added to the hydrogels and incubated for 1 h. After removing unbound BSA by filtration, the hydrogel network
was destroyed, and the bound BSA to the hydrogels was quantified using standard Bradford assay at 590 nm.

**Results and discussion**

QAlg was synthesized by ester coupling chemistry between the carboxylate of Alg backbones and quercetin catechol group using a carbodiimide coupling reagent. The conjugation of quercetin to Alg backbone was verified with \(^1\)H NMR and FT-IR which showed peaks of the aromatic ring protons in quercetin at around 8 ppm and peaks of the ester bond at 1653 and 1233 cm\(^{-1}\), respectively, resulting from quercetin conjugation (Figs. S1, S2). First, we prepared QAlg hydrogels encapsulating cargo molecules and verified enhanced hydrogel stability by measuring cargo release rates using fluorescein as a model drug. QAlg or Alg solution (2 % w/v in PBS) was mixed with fluorescein (0.2 mg/mL in PBS) at a ratio of 5:1 (v/v). The mixture was then added dropwise to \(\text{FeCl}_3\) solution (0.2 M in distilled water), and the resulting hydrogels were filtered and washed with PBS. For investigating fluorescein release profile, the hydrogels were incubated in PBS at room temperature, and the solution was collected at several time points during incubation, and fluorescence intensity of fluorescein (\(\lambda_{\text{Ex}} = 494 \ \text{nm} \) and \(\lambda_{\text{Em}} = 521 \ \text{nm}\)) was measured. Fluorescein in Alg hydrogels was released rapidly leading to 90 % cumulated release in 3 h and complete release after 4 h, whereas QAlg hydrogels showed slower release of fluorescein and afforded only \(\sim 35\ \%\) cumulated release of fluorescein after 4 h (Fig. 2A, diamond and square). This result indicates that QAlg hydrogels had the better mechanical stability than Alg hydrogels and that incorporating quercetin to Alg backbone enhanced hydrogel stability as expected. Next, ascorbate-responsive fluorescein release from QAlg hydrogels was performed in PBS in the presence of 3 mM ascorbate which is known for the concentration without cytotoxicity to normal cells (Padatty et al. 2006). After 30 min, 42.5 % of fluorescein was released with ascorbate treatment, which was four times faster release than that without ascorbate treatment (11 %) indicating ascorbate can accelerate cargo release from QAlg hydrogels (Fig. 2A, triangle and square). We also investigated the cargo loading capacity of hydrogels by destructing hydrogel network.

Figure 2B shows the relative amount of fluorescein loaded in Alg hydrogels and QAlg hydrogels indicating that the QAlg hydrogels contained six times more fluorescein than Alg hydrogels. The more loading capacity of QAlg hydrogels can be attributed that quercetin conjugation to carboxylate groups of Alg backbone decreased anionic charge and increased hydrophobicity of hydrogels so that fluorescein loading in QAlg hydrogels was preferred.

Next, we investigated whether ascorbate can reduce \(\text{Fe}^{3+}\) ion and \(\text{Fe}^{2+}\) inducing hydrogel instability and accelerate cargo release. This experiment is significant because stability of QAlg hydrogels against biological reducing agents other than ascorbate should be secured for ascorbate-responsive cargo release. We performed ferroin assay for identifying the oxidation state of iron ion, which selectively gives absorbance at 508 nm for \(\text{Fe}^{2+}\) ion. We examined three biological reducing agents, citrate, GSH, and ascorbate. \(\text{Fe}^{2+}\) in PBS and \(\text{Fe}^{3+}\) in PBS without reducing agent were used as a positive control and a negative control, respectively. Figure S3 clearly shows distinct absorbance at 508 nm for the solution incubated with ascorbate, suggesting that only ascorbate has enough reducing power for the reduction of \(\text{Fe}^{3+}\) to \(\text{Fe}^{2+}\). This result also implies that accelerated fluorescein release by ascorbate resulted from reduction of \(\text{Fe}^{3+}\) to \(\text{Fe}^{2+}\) and subsequent destabilization of the hydrogels.

Fidelity of our strategy was further verified by observing cancer cell killing effect. We prepared doxorubicin-encapsulated hydrogels QAlg and investigated...
doxorubicin release profiles using the identical approach as described above except for using 2 mg/mL doxorubicin for cargo loading. Figure 3A shows the released amount of doxorubicin from the hydrogels after 30 min. Similar to the result of fluorescein release from the hydrogels, doxorubicin released faster from the Alg hydrogels than from the QAlg hydrogels, and ascorbate treatment induced rapid drug release. To examine the anticancer activity of doxorubicin-encapsulated hydrogels, the in vitro cytotoxicity was evaluated against MCF-7 cells (a breast cancer cell line) using trypan blue exclusion test. PBS was used as a control, and doxorubicin was used at the same concentration (3 μg/mL) of the released doxorubicin concentration from QAlg hydrogels with ascorbate. We observed that doxorubicin-encapsulated QAlg hydrogels showed better cancer cell killing effect with the treatment of ascorbate than without the ascorbate treatment (Fig. 3B). The enhanced anticancer ability of the QAlg hydrogels with ascorbate compared with doxorubicin was also observed, although same amount of doxorubicin to the released doxorubicin from QAlg hydrogels with ascorbate was used. We presumed that this enhanced ability might be attributed to the synergetic effect of iron ion and ascorbate in using doxorubicin. Ascorbate and iron ion are used as adjuvants of doxorubicin because of its synergetic effect in inducing apoptosis by way of lipid peroxidation and DNA degradation (Kurbacher et al. 1996; Quiles et al. 2002).

For the practical use of our system, circulation time in blood stream might be considered. It has been known that binding of plasma protein such as albumin increases circulation time in blood, and quercetin is known to have a decent affinity toward BSA. We compared the BSA binding capacity of the hydrogels that were prepared with two metal cation cross-linkers, Fe$^{3+}$ and Ca$^{2+}$ without cargo loading. The hydrogels were incubated with BSA (20 mg/mL in PBS) for 1 h, and unbound BSA was removed by filtration. The hydrogel network was then destroyed, and the bound BSA to the hydrogels was quantified using standard Bradford assay. As expected, QAlg hydrogels showed better BSA binding capability than Alg hydrogels (Fig. S4). Interestingly, BSA bound 2.5 times more to Fe$^{3+}$ cross-linked QAlg hydrogels than to the hydrogels without quercetin conjugation.

In summary, we reported quercetin-conjugated Alg hydrogels for controlled cargo release. By incorporating hydrophobic quercetin moiety into Alg backbone, hydrogel stability enhanced, leading to decreased loss of loaded cargos. Upon an ascorbate treatment, cargo molecules were released on demand through compromising hydrogel stability by reduction of cross-linking metal cations. Using quercetin as a hydrophobic moiety and ascorbate as a reducing agent is advantageous. Quercetin is a well-known polyphenolic flavonoid compound with low toxicity found abundantly in many common foods, showing various biological activities such as apoptosis induction, angiogenesis inhibition, and anti-proliferative action in many kinds of cancer cells (Nijveldt et al. 2001; Hyun et al. 2010). Quercetin also acts as a multidrug resistant protein inhibitor that prevents efflux of drugs such as doxorubicin from cancer cells (Cooray et al. 2004). In addition, quercetin is known to bind to form a complex with human plasma proteins such as albumins leading to increased circulation time of QAlg (Zsila et al. 2003). Furthermore, ascorbate can reduce potential lethal cardiotoxicity of doxorubicin which is the major side effect in using doxorubicin (Viswanatha Swamy et al. 2011).

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