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ORIGINAL CONTRIBUTION

Preparation and characterization of self-assembled nanoparticles based on glycol chitosan bearing adriamycin

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Abstract An anthracycline drug, adriamycin, was chemically conjugated onto the backbone of glycol chitosan via an acid-labile *cis*-aconityl linkage. The physicochemical characteristics of the glycol chitosan–adriamycin (GC–ADR) conjugates were investigated by dynamic light scattering, atomic force microscopy, and fluorescence spectroscopy. The GC–ADR conjugates were capable of forming nano-sized self-aggregates in an aqueous medium, when the adriamycin content in the conjugate was in the range of 2.0–5.0 wt.%. The self-aggregates were spherical in shape, and had mean diameters of 238–304 nm, depending on the adriamycin content. The critical aggregation concentrations of the conjugates, estimated by the fluorescence quenching method, were as low as $1.0–2.5\times10^{-2}$ mg/ml. The size of self-aggregates was not affected by the polymer concentration in the range from 50 to 2,000 μg/ml, and was maintained up to 8 days in phosphate-buffered saline (pH 7.4), indicating high colloidal stability. The release of adriamycin from self-aggregates was significantly dependent on the pH of the medium due to the *cis*-aconityl linkage; e.g., the amount of adriamycin released for 4 days was 7.3±0.3% at pH 7, whereas it was 29.3±1.9% at pH 4. The cell viability results demonstrated that free adriamycin shows more potent cytotoxicity than the conjugates, primarily attributed to the sustained release of adriamycin from self-aggregates. In conclusion, the self-aggregates, formed by GC–ADR conjugates, might be useful for the site-specific delivery of adriamycin in a sustained manner.

Keywords Glycol chitosan · Adriamycin · Self-aggregates · Sustained release · Cytotoxicity

Introduction

Adriamycin, exerting its cytotoxic activity against cancer cells by inhibiting the synthesis of nucleic acids, has been widely used in chemotherapy for various tumors [1–3]. The extended applications of adriamycin, however, have been limited due to its cytotoxicity to normal cells, causing serious side effects in the patients [4, 5]. In recent years, it has become apparent that the chemical conjugation of adriamycin to water-soluble macromolecules provides

opportunities to increase antitumor activity in vivo, compared to free adriamycin therapy [6–8]. Such macromolecular prodrugs have been demonstrated to decrease the toxicity of antitumor agents to normal tissues, to show prolonged longevity in the bloodstream, and to localize the cytotoxicity at tumor sites; this is primarily attributed to the shielding effect of hydrophilic macromolecules from rapid elimination by the reticuloendothelial system and renal excretion [6, 9, 10]. In addition, the unique characteristics of tumor tissues, such as hypervasculature, defective vascular architecture, and deficient lymphatic drainage systems, are believed to allow macromolecular prodrugs to be accumulated preferentially and to be retained more in tumor tissues than in normal tissues, which is called the "enhanced permeability and retention (EPR) effect" [6, 11, 12].

Polymeric amphiphiles capable of forming self-assembled nanoparticles have received increasing attention as drug carriers because they can imbibe a significant amount of hydrophobic drugs, release them in a sustained manner, and accumulate passively in tumor tissues, primarily due to the EPR effect [13–16]. A few representatives of polymeric amphiphiles, emerging as promising drug carriers, include amphiphilic block copolymers [14, 15, 17–19], hydrophobically modified water-soluble polymers [20, 21], and natural polysaccharides bearing hydrophobic twigs [22–27]. In recent years, our group also developed a novel type of polymeric amphiphile, composed of hydrophilic glycol chitosan and hydrophobic adriamycin [28]. This glycol chitosan–adriamycin (GC–ADR) conjugate self-associated in an aqueous medium to form compact nano-sized self-aggregates, to which a large amount of adriamycin could be encapsulated in as high as 38% of the loading content. When systemically administrated into tumor-bearing rats, the GC–ADR conjugate physically loaded with adriamycin was gradually accumulated in tumor tissues as a function of time, thus suppressing the tumor growth [28]. The detailed physicochemical properties of the GC–ADR conjugate, however, have not been fully understood yet. Therefore, in an attempt to address this question, we herein prepared three GC–ADR conjugates with different weight percentages of adriamycin. Thereafter, the conjugates were investigated with regard to the critical aggregation concentration (CAC), stability of self-aggregates in a physiological solution (pH 7.4), release behavior of adriamycin from self-aggregates, and in vitro cytotoxicity.

Materials and methods

Materials

Adriamycin (purity >98%), glycol chitosan ($M_n = 2.5 \times 10^5$, degree of deacetylation =88%), *cis*-aconitic anhydride, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimid (NHS), and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Dialysis membrane (molecular weight cutoff of 12,000 Da) was the product of Spectrum (Houston, TX, USA). Human hepatoblastoma cell line (HepG2) was obtained from the Korean Cell Line Bank. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, NY, USA). All other chemicals were analytical grade and used without further purification.

Conjugation of adriamycin to glycol chitosan

GC–ADR conjugates were prepared via two reaction steps: (1) chemical modification of adriamycin with *cis*-aconitic anhydride and (2) conjugation of *N-cis*-aconityl adriamycin to glycol chitosan. The overall synthetic route for GC–ADR conjugates is shown in Fig. 1.

N-cis-aconityl adryamycin was prepared as reported previously [28]. In brief, adriamycin (10 mg) was dissolved in 400 μl of pyridine, to which *cis*-aconitic anhydride (13.46 mg) in 1 ml of dioxane was slowly added. After being stirred overnight at 4°C, the solution was mixed with chloroform (5 ml) and 5% aqueous sodium bicarbonate solution (5 ml). The chloroform phase was decanted and the residual solution was extracted with ethyl acetate. The resulting solution was concentrated using a rotary evaporator and dried at room temperature under vacuum to obtain *N-cis*-aconityl adryamycin.

N-cis-aconityl adryamycin was chemically conjugated to the main backbone of glycol chitosan through formation of an amide bond, in the presence of EDC and NHS. Glycol chitosan (100 mg) was dissolved in distilled water (10 ml), followed by dilution with 10 ml of methanol. A predetermined amount of *N-cis*-aconityl adryamycin, dissolved in 1 ml of dimethylformamide, was added into the polymer solution. To activate carboxylic acid pertaining to *N-cis*-aconityl adryamycin, equal amounts (two equivalents/[−COOH]) of EDC and NHS were further added under gentle stirring. The resulting solution was stirred for 1 day at room temperature and was dialyzed against the excess amount of distilled water, followed by lyophilization to obtain the GC–ADR conjugate.

The adriamycin content in the GC–ADR conjugate was estimated by determining the absorbance at 481 nm with a UV/visible spectrophotometer (Lambda 18, Perkin Elmer, Wellesley, MA) and by comparing it to the standard curve obtained from the adriamycin solution [29]. The GC–ADR conjugates, synthesized in this study, were coded depending on the weight percentage of adriamycin; e.g., GC–ADR-5 indicates the glycol chitosan bearing 5 wt.% of adriamycin (Table 1).

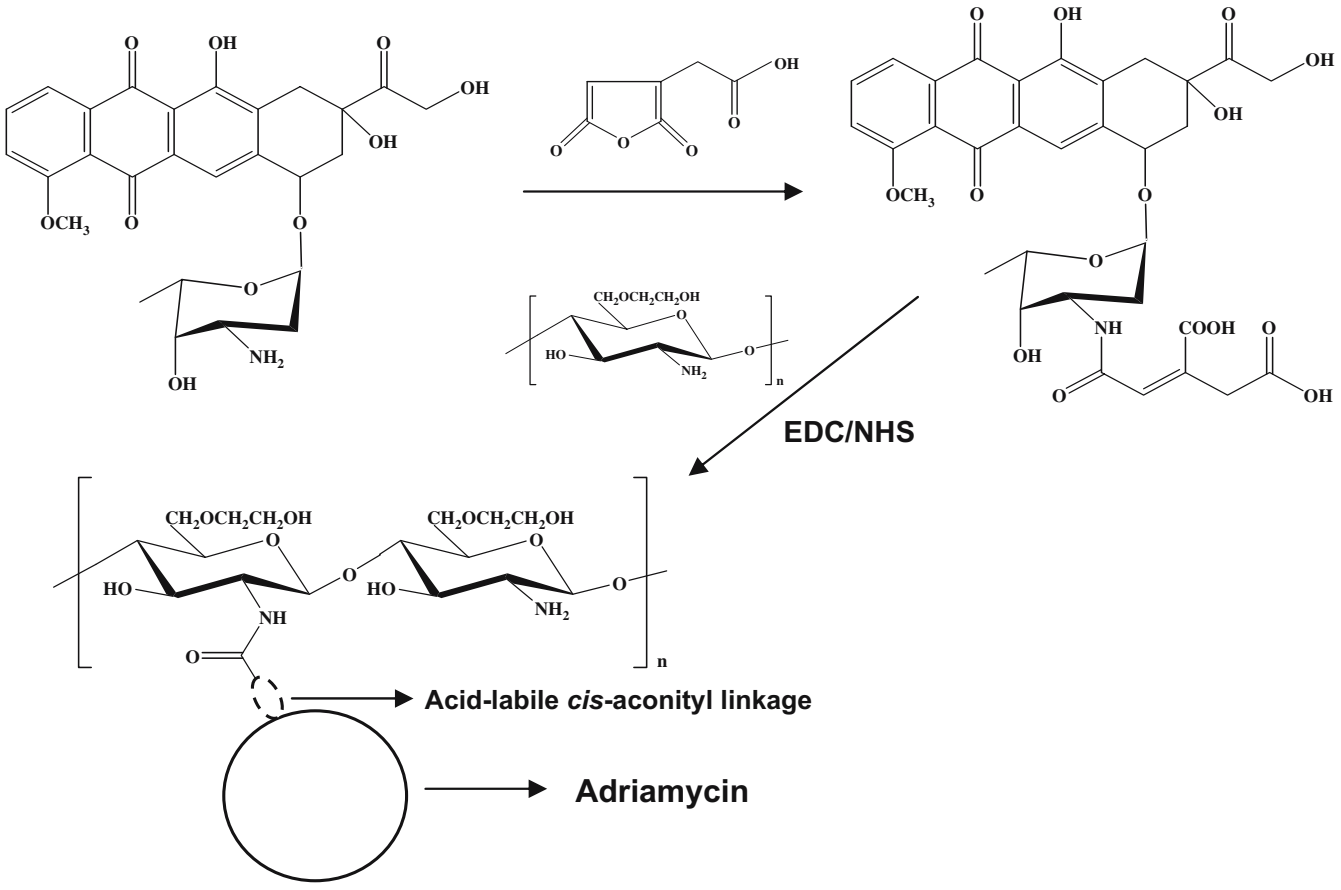

Fig. 1 Synthesis of glycol chitosan bearing adriamycin

Table 1 Composition and mean diameter of self-aggregates

Sample[a]	x[b] (%)	DS[c]	d[d] (nm)	CAC[e] ($\times 10^2$ mg/ml)	M_n[f]
GC	–	–	–	–	250,000
GC–ADR-3	3.1	1.0	304±38	2.5	258,000
GC–ADR-4	3.6	1.2	297±44	1.0	259,300
GC–ADR-5	5.0	1.6	238±40	1.0	263,200

[a]Glycol chitosan bearing adriamycin, in which the number indicates the weight percentage of adriamycin
[b]Weight percentage of adriamycin determined using a UV/visible spectrophotometer
[c]Degree of substitution, indicating the number of adriamycin particles per 100 sugar residues of glycol chitosan
[d]Mean diameter in PBS (pH 7.4), measured by dynamic light scattering (n=3). The polymer concentration was 0.5 mg/ml
[e]Critical aggregation concentration, determined by a fluorescence quenching method
[f]Number-average molecular weight, estimated from the weight percentage of adriamycin

Preparation of self-aggregates

The GC–ADR conjugate was suspended in phosphate-buffered saline (PBS) under gentle stirring at room temperature for 3 h. The solution was then sonicated five times using a probe-type sonifier (Sigma, Ultrasonic Processor, GEX-600) at 60 W for 2 min each. To keep the solution from being heated, the pulse was turned off for 1 s at intervals of 5 s. The solution of self-aggregates was passed through a 0.45-μm filter (Millipore, Billerica, MA, USA) and stored at 4°C, prior to use.

Atomic force microscopy

The surface morphology of self-aggregates was observed using atomic force microscopy (AFM) (Park Scientific, Unnyvale, CA, USA), in which a silicon nitride tip on a cantilever with a spring constant of 0.12 N/m was used. The self-aggregate solution in distilled water (1 mg/ml) was used for the measurement, and the image was obtained under ambient condition by the noncontact mode.

Measurement of dynamic light scattering

For the determination of the particle size, dynamic light scattering (DLS) measurements were carried out using the helium ion laser system (Spectra Physics Laser Model 127-35, Mountain view, CA, USA), which was operated at 633 nm and 25±0.1°C. The scattered light was measured at an angle of 90° and was collected with a Brookhaven BI-9000AT autocorrelator (Holtsville, NY, USA). The hydrodynamic diameter of self-aggregates was calculated by the Stokes–Einstein equation. The polydispersity factor, represented as μ_2/Γ^2, was estimated from the cumulant method [30, 31], where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width.

Measurement of fluorescence spectroscopy

The CAC, the threshold concentration of self-aggregate formation by intra- or intermolecular association, was determined by the fluorescence quenching method. Potassium iodide (KI, 0.6 M), an adriamycin quencher, was dissolved in distilled water, and sodium thiosulfate ($Na_2S_2O_3$, 2×10^{-3} M) was added to prevent I_3^- formation. This quencher solution was mixed with the GC–ADR conjugate solution, by which polymer concentrations were in the range of 1.25–250 μg/ml in 0.3 M KI. The fluorescence spectra of the GC–ADR conjugates were obtained by using a ISS K2 Multifrequency Phase and Modulation Fluorometer (ISS, Champaign, IL, USA). The excitation and emission wavelengths were 471 and 595 nm, respectively.

Release studies

Self-aggregates, formed by the GC–ADR conjugate, were dispersed in physiological saline solution to give the concentration of 2 mg/ml. This solution (500 μl) was placed into the cellulose membrane tube. It was then immersed into 10 ml of citrate (pH 4.0) or phosphate (pH 7.0) buffer and was gently shaken at 37°C in a water bath at a frequency of 135 rpm. Periodically, the whole medium was replaced with fresh medium to maintain sink conditions. The amount of adriamycin released was determined using the fluorometer, based on the standard curve obtained from adriamycin in an incubation medium.

Evaluation of cytotoxicity

In vitro cytotoxicity of adriamycin and the GC–ADR conjugate was determined using HepG2 cells, grown in DMEM supplemented with 10% FBS. The cells were seeded on 96-well plates at a density of 1×10^3 cells/well and were exposed to different concentrations of adriamycin and the GC–ADR conjugate for 24 or 72 h. After being rinsed with culture media, the cells were treated for 4 h with 50 μl of MTT (0.5% w/v in PBS solution) and 200 μl of culture media, which allowed the production of formazan crystals. The quantity of formazan products, dissolved by adding dimethyl sulfoxide (200 μl) and Sorensen's glycin buffer (25 μl), was determined at 570 nm by using a microtiter plate reader (SOFTmax PRO, Molecular Device, CA, USA). Nontreated cells were used as a control (100%).

Results and discussion

Characterization of ADR-GC

To minimize side effects such as immunosuppression and cardiotoxicity, antitumor agents including adriamycin and daunomycin have been frequently conjugated to macromolecules [32–34]. In particular, when coupled by the acid-sensitive spacer, they are known to remain inactive at the physiological pH but to become active after cleavage under slightly acidic conditions, such as in the vicinity of tumor tissues [35, 36]. This strategy would also be effective to selectively release the drug to the intracellular regions after uptake of the conjugates, including endosomes and lysosomes [36]. One representative of acid-labile spacers is the *cis*-aconityl moiety, which has been successfully used for targeting of tumor cells [35–38]. In this study, to endow hydrophilic glycol chitosan with amphiphilic nature, adriamycin was covalently conjugated via an acid-cleavable linkage, a *cis*-aconityl bond, in the presence of EDC and NHS (Fig. 1). By varying the feed ratio of *cis*-aconityl adriamycin to glycol chitosan, various GC–ADR conjugates were produced. Of them, the conjugates with the adriamycin content, ranging from 2.0 to 5.0 wt.%, could self-associate to form nano-sized self-aggregates (Table 1); otherwise, the conjugates were fully soluble or precipitated in aqueous media, indicating a significant role of the balance between hydrophilicity and hydrophobicity in self-aggregation.

The mean diameters of self-aggregates, measured by DLS, were in the range of 238 to 304 nm, depending on the adriamycin content in the conjugate: the particle size of self-aggregates appeared to decrease with increasing the adriamycin content in the conjugate. Also, it was evident from the AFM experiment that self-aggregates formed by the GC–ADR conjugate are spherical in shape and have smooth surfaces (Fig. 2), in which the size of self-aggregates was smaller than that obtained by the DLS measurement because of the different conditions for sample preparation [26, 27]. For example, the DLS measurement was carried out using a self-aggregate solution in PBS (pH 7.4), whereas the AFM image was obtained using a dried sample, which had been dispersed in distilled water.

The fluorescence quenching technique has been extensively used to study the characteristics of micelles such as critical micelle concentration, aggregation number, and

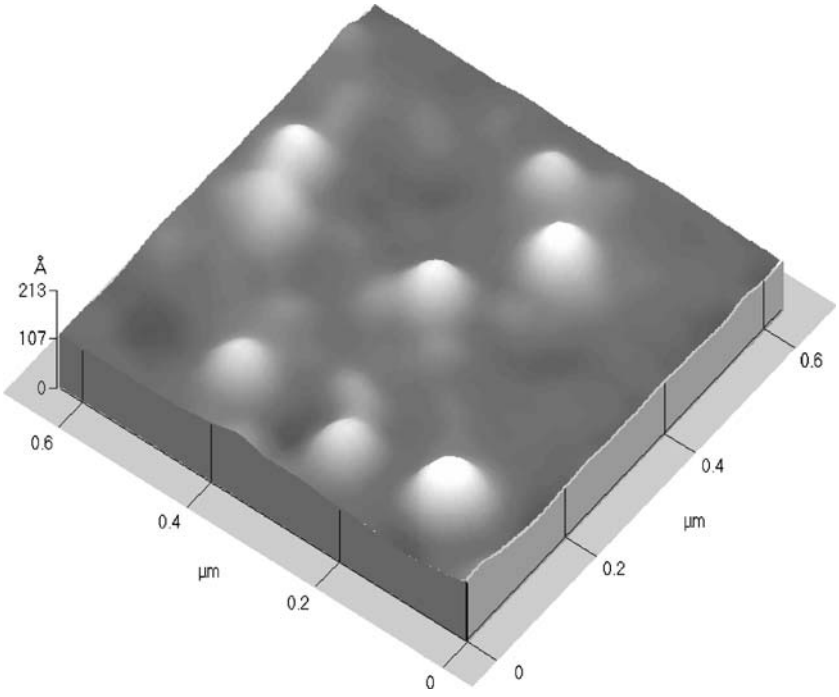

Fig. 2 AFM image of self-aggregates formed by GC–ADR-5. The sample was dissolved in distilled water at a concentration of 1 mg/ml. The AFM image was obtained under the ambient condition by the noncontact mode

microviscosity of micellar interiors [26, 39–41]. It has been demonstrated that the fluorescence of free adriamycin exposed to an aqueous phase is readily quenched by a water-soluble quencher (iodide ion), whereas that of adriamycin in a micellar solution is not significantly affected because the access of the quencher to the hydrophobic cores of micelles is limited [39]. Therefore, the self-aggregation behavior of the GC–ADR conjugates at various concentrations in an aqueous phase was estimated by the fluorescence quenching technique, in which KI was used as a quencher of adriamycin. Figure 3 shows the fluorescence intensity ratio (F_1/F_0) of adriamycin in GC–ADR-5, a representative conjugate, in the absence and presence of 0.3 M KI. No significant changes in the intensity ratio were detected at low concentration ranges of the GC–ADR conjugate because the fluorescence of adriamycin was effectively quenched. As the concentration increased, however, the intensity ratio increased markedly, reflecting attenuation of adriamycin quenching by iodide ion due to the formation of self-aggregates. The CACs of the GC–ADR conjugates were determined from the crossover point at the low concentration ranges and are listed in Table 1. The

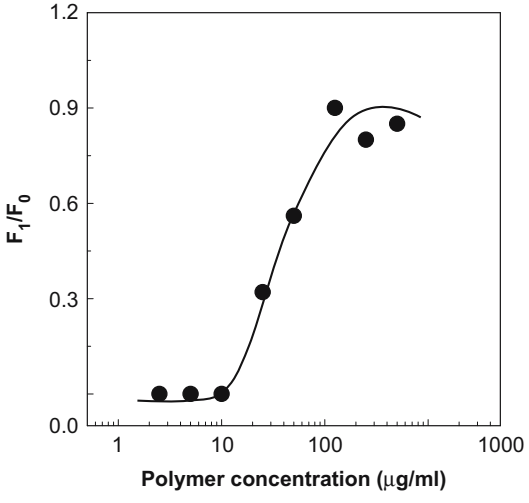

Fig. 3 Plot of the fluorescence quenching of adriamycin in GC–ADR-5 solutions by iodide ions. The fluorescence intensity of adriamycin was determined in the absence (F_0) and the presence (F_1) of iodide ions

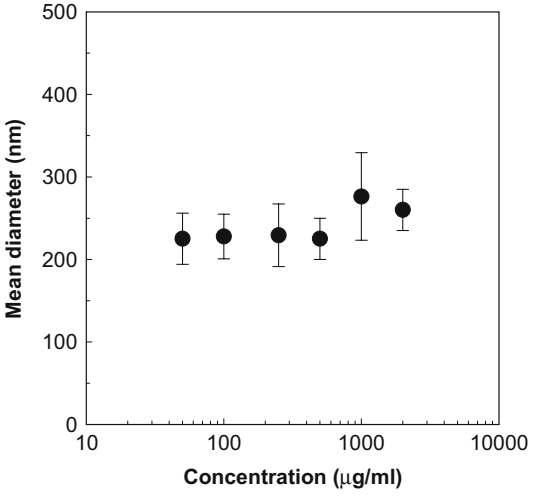

Fig. 4 Mean diameter of self-aggregates, formed by GC–ADR-5, in PBS (pH 7.4) as a function of polymer concentration. The *error bar* is for standard deviation (n=3)

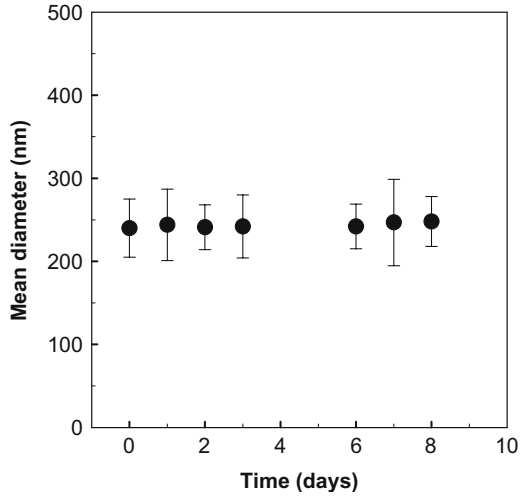

Fig. 5 Colloidal stability of self-aggregates in PBS (pH 7.4). The GC–ADR-5 solution (0.5 mg/ml) was stored at 37°C up to 8 days. The *error bar* is for standard deviation (n=3)

CAC values of the GC–ADR conjugates were in the range of 1.0 to 2.5×10^{-2} mg/ml, which were lower than those of low-molecular-weight surfactants (e.g., 1.0 mg/ml for deoxycholic acid and 2.3 mg/ml for sodium dodecyl sulfate in water) but were similar to those of other polymeric amphiphiles [19–21, 42, 43]. Compared to glycol chitoans bearing 5β-cholanic acid ($4.7–21.9\times10^{-2}$ mg/ml), which were developed in our group as carriers for peptide delivery [26, 27], GC–ADR conjugates showed lower CAC values, indicating that the hydrophobic anthracycline drug (adriamycin) allows the conjugates to form self-aggregates at low concentrations.

The mean diameter of self-aggregates, formed by GC–ADR-5, was measured at different polymer concentrations in a PBS solution (pH 7.4), as shown in Fig. 4. It was likely that the size of self-aggregates is scarcely affected by the polymer concentration in the range of 50 to 2,000 μg/ml. This implies that the interparticle interaction between self-aggregates is almost negligible, which is critical for their use as drug carriers because they should be exposed to extremely diluted conditions whenever administrated in the body. To evaluate the stability of self-aggregates, the change in their size was determined at 37°C up to 8 days (Fig. 5). Self-aggregates maintained their size for the period of time applied in this study, indicating the high colloidal stability of self-aggregates in an aqueous medium.

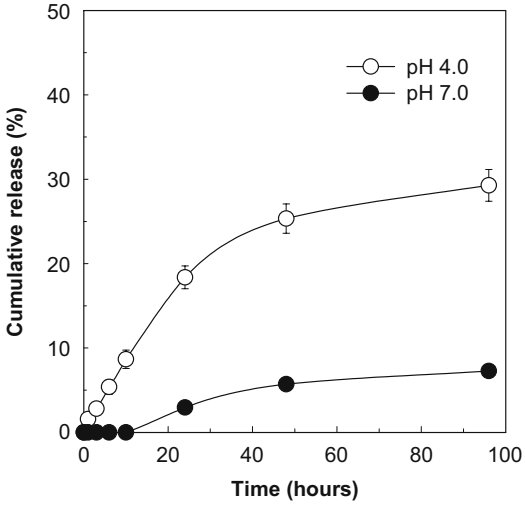

Fig. 6 Release profiles of adriamycin from self-aggregates of GC–ADR-5 as a function of time. The self-aggregates were exposed at 37°C to different milieus, such as citrate (pH 4.0) and phosphate (pH 7.0) buffers. The *error bar* represents standard deviation (n=3)

In vitro release profile of adriamycin

Because adriamycin is conjugated into the backbone of glycol chitosan via the acid-labile *cis*-aconityl linkage, it

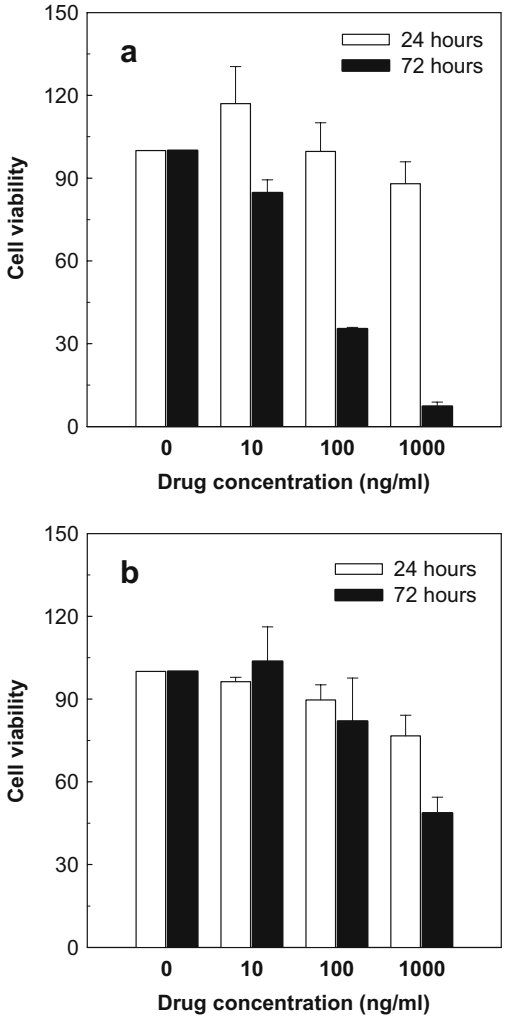

Fig. 7 Cytotoxicity of **a** adriamycin and **b** GC–ADR-5 against HepG2 cells at various drug concentrations. The *error bar* is for standard deviation (n=5)

can be expected that the release rate of adriamycin from self-aggregates depends on the pH of the medium. Figure 6 shows the release behavior of adriamycin from self-aggregates at different pHs. It was evident that the release rate of adriamycin from self-aggregates is much higher at low pHs; e.g., the amount of adriamycin released for 4 days was 7.3±0.3% at pH 7, whereas 29.3±1.9% was released at pH 4. At pH 4, adriamycin was released from self-aggregates in a biphasic fashion, characterized by a fast release phase on the initial day followed by a slower release phase on the remaining days. On the contrary, at pH 7, a negligible amount of adriamycin was released for 9 h, followed by a slow release over the remaining period of time. The slow release of adriamycin at pH 7 is consistent with the results reported in other groups who investigated the anthracycline drug conjugates with the *cis*-aconityl bond and confirmed no release or below 10% release of the drugs after 24 h at pH 7 [44–46]. It is of interest to note that the release rate of adriamycin significantly decreased after 1 day in an acidic condition (pH 4), in which free adriamycin may be continuously generated due to the cleavage of the *cis*-aconityl linkage. One possible reason is that cleaved adriamycin from the self-aggregates can be entrapped physically in the inner core of self-aggregates by hydrophobic interaction, which may induce the sustained release of adriamycin [46].

In vitro cytotoxicity of self-aggregates

The in vitro cytotoxicity of adriamycin and self-aggregates, formed by GC–ADR-5, was tested against HepG2 cells by using MTT assay. Figure 7 shows cell viability results, measured after 24 and 72 h at various drug concentrations in the range from 10 to 1,000 ng/ml. At 24 h, both adriamycin and self-aggregates appeared to exhibit slightly lower cell viability at higher concentrations, whereas there were no noticeable differences between adriamycin and self-aggregates. However, at 72 h, cell viability significantly decreased, depending on the concentrations of adriamycin and self-aggregates. It is important to emphasize that adriamycin exhibited more potent cytotoxicity than self-aggregates, as judged by cell viability results at 72 h; e.g., at the drug concentration of 1,000 ng/ml, cell viability was 48.7±5.8% for self-aggregates but was only 7.3±1.5% for adriamycin. Considering the release profile of adriamycin from self-aggregates shown in Fig. 6, it can be suggested that the sustained release of adriamycin significantly affects the cytotoxicity of self-aggregates. Similar observations were reported by other groups who tested the in vitro cytotoxicity of nanoparticles, releasing adriamycin in a sustained manner [25, 47]. Because the self-aggregates have been demonstrated to gradually accumulate in the tumor tissues for long periods of time, at least up to 8 days [28], the sustained release of adriamycin from self-aggregates may allow the drug to be delivered selectively into the tumor tissues.

Conclusion

This study explored the formation of self-aggregates, on the basis of glycol chitosan partially modified with adriamycin, in an aqueous solution. The physicochemical properties of self-aggregates were investigated by the measurements of DLS and fluorescence spectroscopy. The self-aggregates were spherical in shape, their mean diameters were in the range of 238 to 304 nm depending on the weight percentage of the conjugated adriamycin, and they were stable in an aqueous solution without interparticle interaction for a long period of time. Adriamycin, conjugated into the backbone of glycol chitosan, was released out of self-aggregates in a sustained manner, thus showing lower cytotoxicity than adriamycin.

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