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ORIGINAL ARTICLE

Influence of histidine on the release of all-*trans* retinoic acid from self-assembled glycol chitosan nanoparticles

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Abstract

Objective: In this study, the influence of *N*-acetyl histidine (NAHis) on the all-*trans* retinoic acid (ATRA) release from the NAHis-conjugated self-assembled glycol chitosan (GC) nanoparticles was investigated. *Methods*: NAHis was conjugated to GC as a hydrophobic moiety to prepare the self-assembled nanoparticles, and ATRA was incorporated into the inner core of the NAHis–GC nanoparticles. The ATRA release from NAHis–GC nanoparticles was performed at 37°C in a phosphate-buffered saline buffer (pH 5.5 or 7.4) for 48 hours. *Results*: At a pH of 5.5, less than 20% (w/w) of total loading amount of ATRA was released from the nanoparticles after 48 hours. In contrast, two times greater amount of ATRA was released at a pH of 7.4. The ATRA release rate from the NAHis–GC nanoparticles was significantly slower at a pH of 5.5 than at a pH of 7.4. *Conclusion*: The release profiles of ATRA that was incorporated into the NAHis–GC nanoparticles were controlled by the NAHis content in the GC nanoparticles.

Key words: *All-*trans *retinoic acid; controlled release; glycol chitosan; histidine; self-assembled nanoparticles*

Introduction

All-*trans*-retinoic acid (ATRA) is effective against various types of cancer[1–3]. Additionally, ATRA prevents acne and dermatitis as well as skin aging[4]. However, the clinical use of ATRA is limited because of its poor aqueous solubility; its high instability in the presence of air, light, and heat; and its short half-life in blood[5,6]. Moreover, some side effects such as retinoid acute resistance, hypertriglyceridemia, and mucocutaneous dryness have been reported[7]. The development of new suitable formulations has been extensively considered to resolve these problems for clinical application of ATRA. Recently, liposomes, solid lipid nanoparticles (SLNs), microemulsions, micelles, and polymeric nanoparticles have all been investigated as potential carriers for ATRA delivery[8–11]. These formulations effectively improved the aqueous suspension ability and chemical stability and sustained the release of ATRA. In a previous study, SLNs were used for the topical delivery of ATRA[12]. The aqueous solubility and the stability of ATRA were improved through the use of the SLNs, and the ATRA release from the SLN was enhanced above the normal body temperature.

Hydrophobically modified glycol chitosan (GC) nanoparticles have been developed to improve the drug

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solubility, prolong the drug circulation time in blood, and reduce the drug toxicity[13–15]. A hydrophobically modified GC conjugate with a hydrophobic moiety, such as deoxycholic acid, lithocholic acid, and adriamycin, formed self-assembled nanoparticles in an aqueous medium[16]. The hydrophobic inner cores enabled various hydrophobic drugs to be efficiently incorporated into the nanoparticles with a sustained release profile. Recently, Park et al. demonstrated the use of *N*-acetyl histidine (NAHis)-conjugated GC self-assembled nanoparticles for intracytoplasmic drug delivery[16]. Paclitaxel that was incorporated into the NAHis–GC nanoparticles was used to deliver the drug into the cytosol of cancer cells. The nanoparticles were disassembled under acidic conditions because of the protonation of the imidazole groups in NAHis and the subsequent release of paclitaxel into the cytosol. This study indicated that NAHis–GC nanoparticles could possibly be promising vehicles for the intracytoplasmic delivery of drugs, proteins, and genes.

NAHis on GC can be used both as a hydrophobic moiety for self-aggregates and as a depot for ATRA, which forms a nano-complex with positively charged polymers, such as polyethyleneimine and chitosan[17]. ATRA was expected to stably incorporate into the NAHis–GC nanoparticles through ionic interactions with the amine groups of GC as well as the hydrophobic interactions with NAHis in a neutral medium. Moreover, NAHis on GC could exhibit additional ionic interactions with ATRA under slightly acidic conditions because its imidazol group is protonated in acidic conditions[16,18]. The NAHis–GC nanoparticles could serve as a good inner core for ATRA delivery.

In this study, NAHis–GC nanoparticles were examined as potential carriers for the controlled release of ATRA. The inner core of GC was hydrophobically modified with NAHis to influence the release of ATRA through ionic interactions caused by the protonation of the imidazole groups. Furthermore, the release of ATRA was controlled by the conjugation degree of NAHis on GC.

Materials and methods

Materials

ATRA, dimethylsulfoxide (DMSO), NAHis, *N*-(3-dimethyl aminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and hydroxysuccinimide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). GC (Mw 250 KDa, degree of deacetylation 88.7%) was obtained from Wako Chemical Co. (Osaka, Japan). All other chemicals were analytical grade and directly used without any further purification.

Synthesis of NAHis–GC conjugates

The NAHis–GC conjugates were synthesized with slight modifications to a previously reported method[16]. First, 1 g of GC was dissolved in 50 mL of distilled water for 3 hours at room temperature. Then different amounts of NAHis (0.24 or 0.48 mmol) were added to the GC solution, along with EDC (0.72 or 1.44 mmol) and hydroxysuccinimide (0.72 or 1.44 mmol). The reaction mixture was stirred for 24 hours at room temperature and dialysed with a cellulose membrane (MWCO 12,000, Spectrum Laboratories, Rancho Dominguez, CA, USA) against distilled water for 5 days. The NAHis–GC conjugate powder was obtained after freeze-drying at –80°C with a chamber pressure of 5 mT for 20 hours. The NAHis–GC conjugates were suspended in DMSO (d-form) and the NAHis content was analyzed using ^{1}H-NMR spectroscopy with a 600-MHz NMR spectrometer (FT-NMR, JNM-ECA600; JEOL, Osaka, Japan) at 20°C.

Preparation of ATRA-loaded NAHis–GC nanoparticles

The ATRA-loaded NAHis–GC nanoparticles were prepared using a solvent-diffusion method[19]. The NAHis–GC conjugates (5 mg) and ATRA (0.5, 1, or 1.5 mg) were dissolved in 5 mL of DMSO, and the mixture solution was placed in a cellulose membrane (MWCO 12,000, Spectrum Laboratories). The solution was dialysed for 5 days against distilled water in the dark, and the final product was freeze-dried using the method described above.

Characterization of ATRA-loaded NAHis–GC nanoparticles

Transmittance electron microscopy (TEM) was carried out using a JEOL JEM-2000 FX II model (Osaka, Japan) at 80 kV to characterize the morphology. The nanoparticle suspensions were dropped onto a carbon film-coated copper gird and negatively stained with phosphotungstic acid. The size distribution of the ATRA-loaded NAHis–GC nanoparticles was determined using dynamic light scattering (DLS-7000, Otsuka Electronics Co. Ltd., Osaka, Japan). The ATRA-loaded NAHis–GC nanoparticles were filtered with a cellulose acetate syringe filter (0.8 μm pore-size; Milipore, Billerica, MA, USA) and diluted to an appropriate scattering intensity. Fourier-transform-infrared spectroscopy (FTIR) was performed using an FTIR 8000 instrument (Shimadzu Co. Ltd., Kyoto, Japan) to confirm the ATRA loading.

ATRA loading efficiency and release profiles

The ATRA-loaded NAHis–GC nanoparticles (each 1 mg) were dissolved in DMSO, and after the appropriate

dilution, the ATRA content was fluorometrically determined at 340 nm using a UV-spectrophotometer (UV-1200, Shimadzu Co. Ltd.) and calculated with the standard curve obtained from ATRA alone. The ATRA release from the ATRA-loaded NAHis–GC nanoparticles was measured in a phosphate buffer (0.1 M, pH 5.5 or 7.4) at 37°C. For these measurements, 1 mg of the ATRA-loaded NAHis–GC nanoparticles were suspended in 1 mL of distilled water. After sonication for 5 minutes, the nanoparticle suspensions were placed into a dialysis tube (MWCO 12,000, Spectrum Laboratories) and the ATRA release was determined using a UV spectrophotometer at 340 nm.

Results and discussion

Cationic polymers, such as chitosan, have been considered as good candidates for the formation of polyionic micelles because of the anionic properties of ATRA. Moreover, ATRA is readily incorporated into the hydrophobic inner cores of polymeric micelles because it is highly lipophilic. Recently, Jeong et al.[20] formed polyionic complex micelles composed of ATRA and poly(ethylene glycol)-grafted chitosan. In the study, the formation of the ATRA-loaded core-shell micelles was based on a polyion complex with chitosan. In this study, hydrophobically modified GC was suggested for ATRA delivery. NAHis was used as a hydrophobic moiety and was conjugated onto hydrophilic GC through the formation of an amide bond (Figure 1).

The NAHis content in the NAHis–GC conjugates was investigated using ^{1}H-NMR spectroscopy. In Table 1, the NAHis concentrations in NAHis–GC-Conj-1 and Conj-2 were 6.5 and 12.5 mol%, respectively.

The TEM images and size distribution results of the NAHis–GC-Conj-1 nanoparticles are presented in Figure 2. The nanoparticles showed a spherical shape

Table 1. NAHis contents in NAHis–GC conjugates.

Sample	X_S[a] (mmol)	X_C[b] (mol%)
NAHis–GC-Conj-1	0.24	6.5
NAHis–GC-Conj-2	0.48	12.5

[a]Amount of NAHis added for the synthesis of NAHis–GC conjugates.
[b]NAHis contents in the NAHis–GC conjugates determined by ^{1}H-NMR spectroscopy.

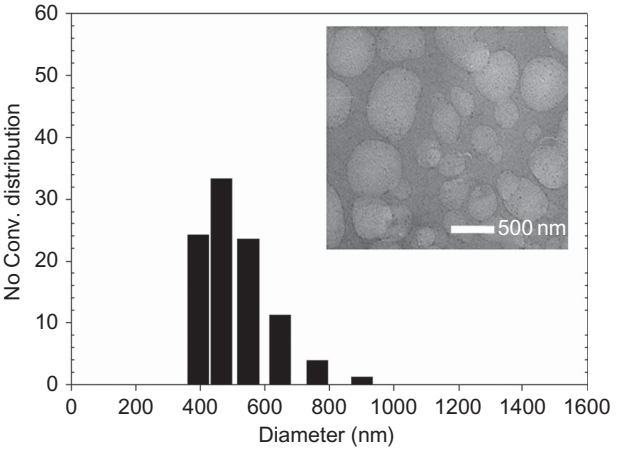

Figure 2. Size distribution of the NAHis–GC-Conj-1 nanoparticles. The inserted photograph is a TEM image of the nanoparticles with a scale bar of 500 nm.

with mean diameter of approximately 400 nm, as measured using dynamic light scattering. No difference in the morphology and mean diameter was observed for the NAHis–GC-Conj-2 nanoparticles compared to the NAHis–GC-Conj-1 nanoparticles.

NAHis–GC amphiphiles formed self-assembled nanoparticles in an aqueous medium. ATRA was loaded into the hydrophobic core of the NAHis–GC

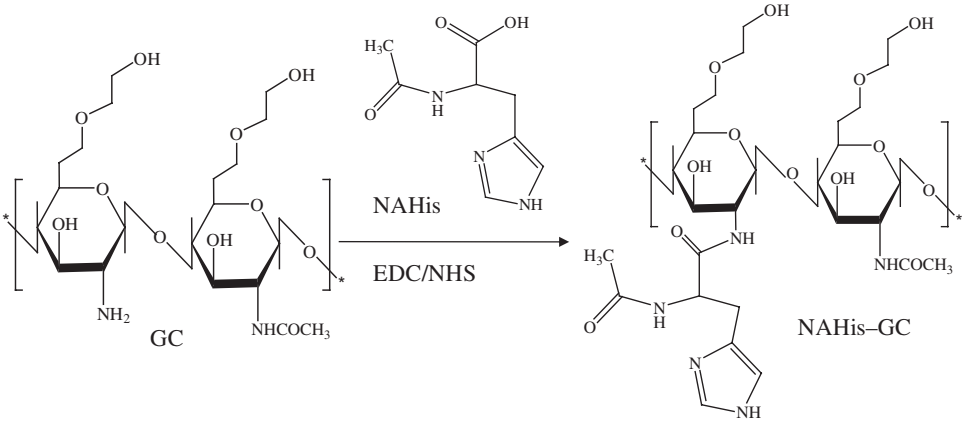

Figure 1. Schematic illustration of the synthesis of the NAHis–GC conjugates.

nanoparticles using a solvent diffusion method. ATRA was probably incorporated into the nanoparticles through ionic and hydrophobic interactions that were caused by its lipophilic and anionic properties. FTIR was carried out to confirm the existence of ATRA, and the spectrum results are shown in Figure 3. Figure 3b and d show the FTIR spectra of the NAHis-G- Conj-1 and Conj-2, respectively. The peaks at 3420 cm^{-1} (O–H stretch overlapped with N–H stretch), 1654 cm^{-1} (amide II band, C–O stretch of acetyl group), 1596 cm^{-1} (amide I band, N–H stretch), and 1057 cm^{-1} (C–O stretch) were observed for the NAHis–GC conjugates. Compared to the NAHis–GC conjugates, the peaks of the ATRA-loaded NAHis–GC nanoparticles exhibited some observably native ATRA peaks (Figure 3c and e). Moreover, the peaks at 1661 cm^{-1} (amide I band, C–O stretch) and 1576 cm^{-1} (amide II band, N–H stretch) notably increased because of the interactions between the carboxyl groups of ATRA and the amine groups of the NAHis–GC conjugates. These results indicated that ATRA was successfully loaded onto the NAHis–GC nanoparticles. The mean diameters and ATRA loading efficiencies of the ATRA-loaded NAHis–GC nanoparticles are presented in Table 2. The mean diameters of the nanoparticles with 0.5 and 1.0 mg of ATRA were similar. In contrast, the mean diameter of the nanoparticles

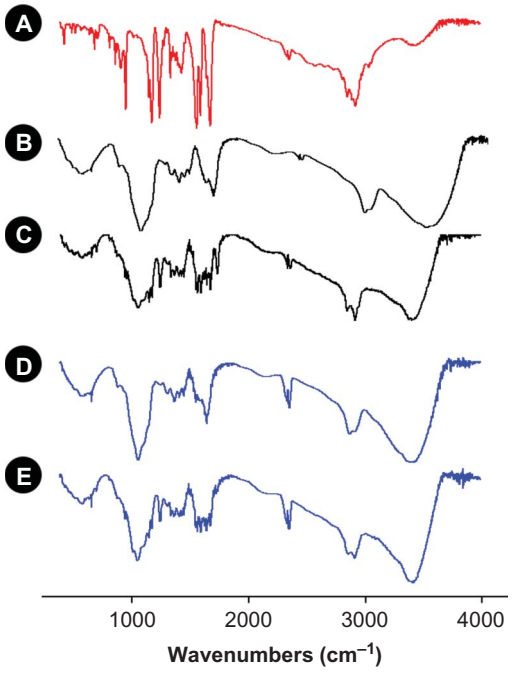

Figure 3. FTIR spectra of the (a) ATRA, (b, d) NAHis–GC conjugates, and (c, e) ATRA-loaded NAHis–GC nanoparticles. (b) NAHis–GC-Conj-1. (c) The nanoparticles prepared with NAHis–GC-Conj-1 and ATRA (1 mg). (d) NAHis–GC-Conj-2. (e) The nanoparticles prepared with NAHis–GC-Conj-2 and ATRA (1 mg).

Table 2. Mean diameters and ATRA loading efficiencies of ATRA-loaded NAHis–GC nanoparticles.

Sample	ATRA (mg)	Mean diameter[a] (nm)	ATRA loading efficiency[b] (%)
NAHis–GC-Conj-1	0.5	461 ± 70.5	64.7 ± 0.5
	1.0	508 ± 114	82.6 ± 0.3
	1.5	388 ± 79.7	85.3 ± 0.6
NAHis–GC-Conj-2	0.5	422 ± 74.0	64.3 ± 0.4
	1.0	412 ± 37.9	88.6 ± 0.6
	1.5	339 ± 80.2	89.9 ± 0.3

[a]Mean diameters of ATRA-loaded NAHis–GC nanoparticles were measured by dynamic light scattering ($n = 3$).
[b]ATRA loading efficiencies (%, w/w) were determined using the following formulation (n = 3): Loading efficiency (%) = (Residual amount of ATRA in NAHis–GC nanoparticles/feeding amount of ATRA) × 100.

slightly decreased when 1.5 mg of ATRA was incorporated into the nanoparticles because of the ionic and hydrophobic interactions between ATRA and the NAHis–GC conjugates. The ATRA loading efficiencies of the NAHis–GC nanoparticles were more than 80% (w/w) of feeding amounts for all the samples except for the nanoparticles loaded with 0.5 mg ATRA. The release studies of ATRA from the NAHis–GC nanoparticles were performed at 37°C in phosphate-buffered saline for 48 hours. Buffers with a pH of 5.5 and 7.5 were used to investigate the effect of the imidazole groups on the ATRA release. Figure 4 shows the ATRA release rate from the NAHis–GC nanoparticles. The ATRA release rate was slower at a pH of 5.5 than at a pH of 7.4. At a pH of 7.4, the rate of ATRA release from the NAHis–GC-Conj-2 nanoparticles was slower than the NAHis–GC-Conj-1 nanoparticles. Interestingly, less than 20% (w/w) of ATRA was released from the NAHis–GC nanoparticles at a pH of 5.5. The imidazole group in histidine is known to be protonated in a slightly acidic medium. In a previous paper, Park et al.[16] reported that the drug release from histidylated self-assembled nanoparticles is pH-dependent and correlated to the protonation of the imidazole groups of histidine. In a slightly acidic medium, the imidazole group was protonated, and the hydrophilic and hydrophobic balance of the self-assembled nanoparticles collapsed. Then the drug was released into the nanoparticles. On the other hand, in this study, the protonation of imidazole groups was expected to enable an interaction with the carboxyl groups of ATRA in an acidic medium. The slow ATRA release at a pH of 5.5 was possibly caused by the protonation of the imidazole groups (Figure 5) and the strong ionic and hydrophobic interactions between the NAHis–GC conjugates and ATRA. A previous report showed that ATRA was continuously released from polyethylene glycol-grafted chitosan micelles for 1 month[20]. Therefore, the ATRA

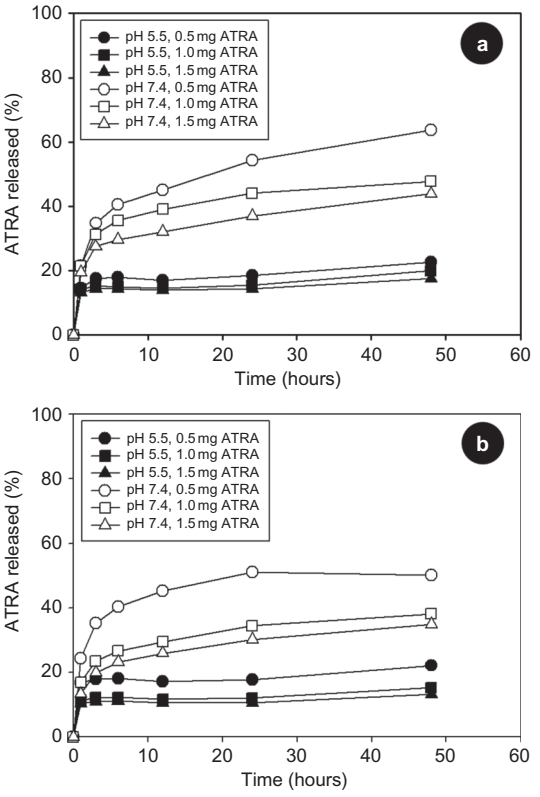

Figure 4. Release profiles of ATRA from the NAHis–GC nanoparticles in 7.4 PBS buffer at a pH of 5.5 and 37°C ($n = 3$). (a) and (b) The nanoparticles prepared with NAHis–GC-Conj-1 and Conj-2, respectively. The quantitative data were expressed as means, which were compared using an independent samples t-test.

release from the NAHis–GC nanoparticles could possibly be controlled by changing the histidine content in the conjugates.

Conclusion

In this study, NAHis–GC nanoparticles were used for the controlled release of ATRA. The NAHis–GC conjugates formed self-assembled nanoparticles with core-shell structures. ATRA was successfully loaded into the core of the NAHis–GC nanoparticles. The release rate of ATRA was significantly slowed with increasing histidine content in the NAHis–GC conjugates. Therefore, the ATRA release from the NAHis–GC nanoparticles was controlled by the histidine conjugation.

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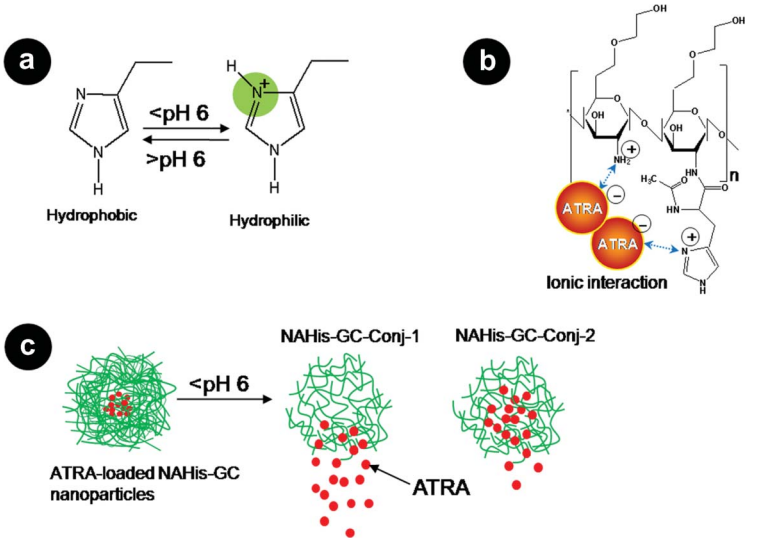

Figure 5. Schematic representation of the ATRA release from the NAHis–GC nanoparticles. (a) The protonation of the imidazole groups of NAHis; (b) ionic interaction between negatively charged ATRA, the protonated imidazole groups, and GC in a slightly acidic medium; and (c) the NAHis influence on the ATRA release from the GC nanoparticles. The rate of ATRA release from the NAHis–GC nanoparticles was slower at pH < 6 with increasing NAHis content in the GC nanoparticles.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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