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Rapid communication

Insight into glycyrrhetinic acid: The role of the hydroxyl group on liver targeting

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ABSTRACT

Two kinds of glycyrrhetinic acid-modified chitosan/poly(ethylene glycol) nanoparticles (CTS/PEG-GA NPs) were prepared by an ionic gelation process in which the liver targeting ligand glycyrrhetinic acid (GA) was introduced into the nanoparticles at the C_{30}-carboxyl group (CTS/PEG-GA(c) NPs) or the C_3-hydroxyl group (CTS/PEG-GA(h) NPs). Their characteristics, especially their ability to target the liver, were compared. The results showed that both the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs are remarkably targeted to the liver. The accumulation in the liver is 51.3% and 56.5% of the injected dose for the CTS/PEG-GA(c)$_{4.60\%}$ NPs (the subscript number denotes the GA content as weight percent in nanoparticles) and the CTS/PEG-GA(h)$_{4.57\%}$ NPs at 3 h after injection, respectively. This is nearly 2.6–2.8 times higher than that obtained with the CTS/PEG NPs. According to our results, there is no significant difference between the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs in their ability to target the liver, when they were formed under identical conditions. This indicated that the C_3-hydroxyl group in GA has little influence on the targeting ability.

Licorice is one of the most widely used medicinal plants for the treatment of many pathologies. It has anti-hepatitis, anti-inflammatory and anti-hepatotoxic effects (Liu et al., 2007; Luk et al., 2007; Asl and Hosseinzadeh, 2008; Fiore et al., 2008; Makino et al., 2008; Beseda et al., 2010). The main chemical constituents of licorice are triterpene saponins, including glycyrrhizin (GL) and glycyrrhetinic acid (GA). GA is the hydrolysis product of GL. The distinct structural difference between GA and GL is the C_3 position, which is occupied by a hydroxyl in GA and a glycosyl in GL (Fig. 1A). In previous work, it has been shown that there are specific receptors for GA on hepatocyte membranes (Negishi et al., 1991). Recently, GA has been used as a ligand for liver targeting, and carriers modified with GA were shown to be more efficient for liver- or hepatocyte-targeted delivery (Mao et al., 2007; Huang et al., doi:10,1016/j.actbio.2010.04.021; Tian et al., 2010). Glycyrrhizin (GL), however, has a very low affinity for the GA receptor, and Negishi et al. (1991) speculated on the importance of the C_3-hydroxyl group for the interaction between GA and its receptor. As far as we know, no information is available about the influence of the C_3-hydroxyl group on liver targeting ability, and thus it is necessary to explore the significance of the C_3-hydroxyl group in the ability of GA to facilitate liver targeting. This is an important issue for the development of a GA-mediated liver-targeted system.

In this study, two kinds of GA-modified nanoparticles (GA-modified chitosan/poly(ethylene glycol) nanoparticles and CTS/PEG-GA NPs) were designed, in which GA was introduced at different sites: the C_3-hydroxyl group (CTS/PEG-GA(h) NPs) and the C_{30}-carboxyl group (CTS/PEG-GA(c) NPs). The main purpose of this work was to evaluate the function of the C_3-hydroxyl group in liver targeting. Thus, comparisons between the CTS/PEG-GA(h) NPs and the CTS/PEG-GA(c) NPs, especially the liver targeting ability, were carried out.

Chitosan (CTS, Mw = 50,000; DD (degree of deacetylation) > 95%) and diamine-poly(ethylene glycol) (PEG-$(NH_2)_2$, Mw = 3400) were used. Fig. 1B shows the synthesis of poly(ethylene glycol)-glycyrrhetinic acid (PEG-GA) from the carboxyl group (PEG-GA(c)) and the hydroxyl group (PEG-GA(h)). It was estimated that more than 96% of the amino groups of PEG-$(NH_2)_2$ reacted with GA or suc-GA. The CTS/PEG-GA NPs were formed by an ionic gelation process based on the interaction between the negative groups of penta-sodium tripolyphosphate (TPP) and the positively charged amino groups of CTS (Fig. 1C). Table 1 shows the particle size and the zeta potential of these nanoparticles. The CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs had similar sizes, zeta potentials and GA contents, when they were formed under identical conditions. The size increased, and the zeta potential decreased for the two kinds of nanoparticles, with increasing concentrations of PEG-GA. This was mainly because of the presence in the nanoparticles of PEG-GA, which could interact with CTS to form a semi-interpenetrating network through intermolecular hydrogen bonding between the electropositive amino hydrogen of CTS and the electronegative oxygen atom of polyethers (Yao et al., 1993; Calvo et al., 1997), leading to an enlargement of the size and reduction in the zeta potential of the nanoparticles. The morphology of the CTS/PEG-GA NPs is

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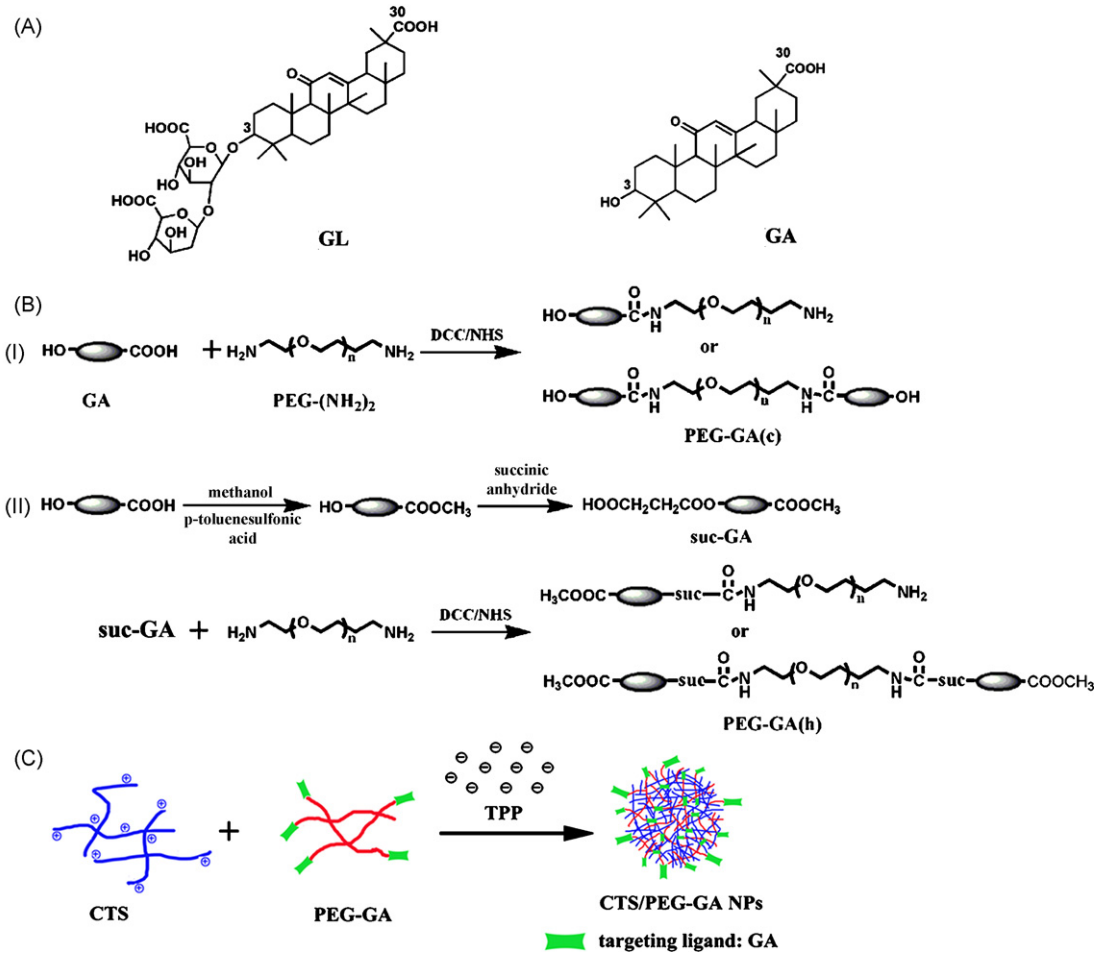

Fig. 1. (A) Chemical structures of glycyrrhizin (GL) and glycyrrhetinic acid (GA); (B) Synthesis of PEG-GA (I for PEG-GA(c) and II for PEG-GA(h)); (C) Preparation of the CTS/PEG-GA NPs.

displayed in Fig. 2. Nanoparticles were spherical in shape. Fig. 3 shows the FT-IR spectra of the CTS NPs (without adding PEG-GA) and the CTS/PEG-GA NPs. Compared with CTS NPs, the intensity at 1084 cm^{-1} in the spectrum of CTS/PEG-GA NPs, belonging to the C–O–C absorption, increased sharply. This is additional evidence for the existence of PEG-GA molecules in the nanoparticles. Meanwhile, the intensity of the 1084 cm^{-1} band increased as the concentration of PEG-GA increased, indicating the increase of PEG-GA content in the nanoparticles.

It was reported that nanoparticles with diameters larger than 200 nm are known to induce non-specific scavenging by monocytes and the reticuloendothelial system (Gabizon et al., 1990; Na et al., 2003). In this work, nanoparticles were prepared within the 200 nm size range, which would be suitable for the subsequent studies.

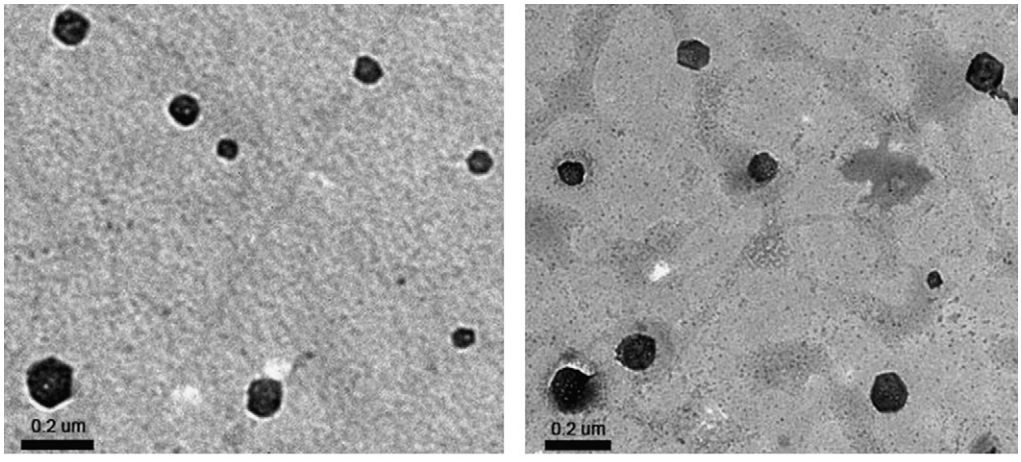

Fig. 2. TEM images of the CTS/PEG-GA NPs ((A) for CTS/PEG-GA(c) NPs and (B) for CTS/PEG-GA(h) NPs).

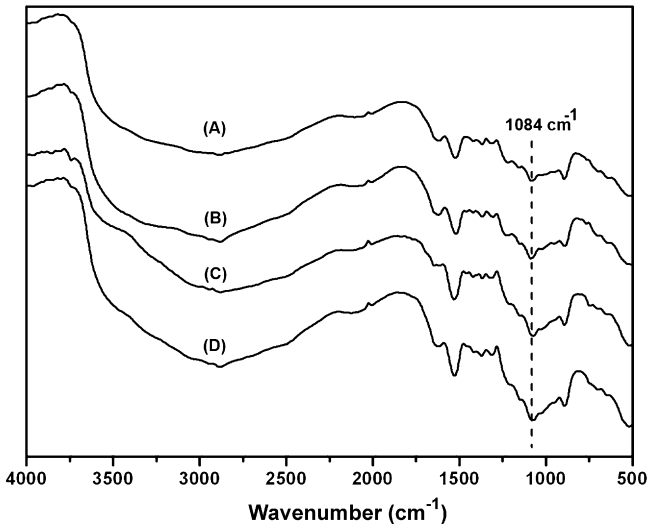

Fig. 3. FT-IR spectra of nanoparticles. (A) CTS NPs, (D) CTS/PEG-GA(c) NPs prepared at PEG-GA(c) concentration of 10.0 mg/mL and (B), (C) for the CTS/PEG-GA(h) NPs prepared at PEG-GA(h) concentrations of 5.0 mg/mL and10.0 mg/mL, respectively.

To ensure a sufficient liver targeting ability, CTS/PEG-GA NPs prepared at a PEG-GA concentration of 10.0 mg/mL were used, unless otherwise specified.

Nanoparticles were labeled with ^{99m}Tc, and single-photon emission computed tomography (SPECT) was used to visualize and quantify the biodistribution of nanoparticles *in vivo*. Fig. 4 shows the real-time images of the mice after tail vein injection with each kind of nanoparticles. Both the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs accumulated extensively in the liver at each time point. The radioactivity signal in the liver was much higher than that in the other organs; this allowed for a clear discrimination between the liver and other organs. Conversely, the CTS/PEG NPs localized mainly in the kidney, the bladder and the liver, and the level of radioactivity in the liver was followed by a gradual decline (Tian et al., 2010). Animals were sacrificed after the SPECT measurements, blood was drawn and organs (liver, heart, spleen, kidney, and lung) were excised. With the exception of the blood, the excised organs were carefully rinsed of the excess blood with physiological saline and wiped with filter papers. The radioactivity in each organ was counted in a γ-counter. For the blood, 0.2 mL blood was counted in the γ-counter, and the total blood volume was calculated as 7.2 mL per 100 g of rat. Fig. 5 shows the distribution of nanoparticles in each organ at 3 h after injection. The CTS/PEG NPs accumulated in the liver at a low level, and the percentage

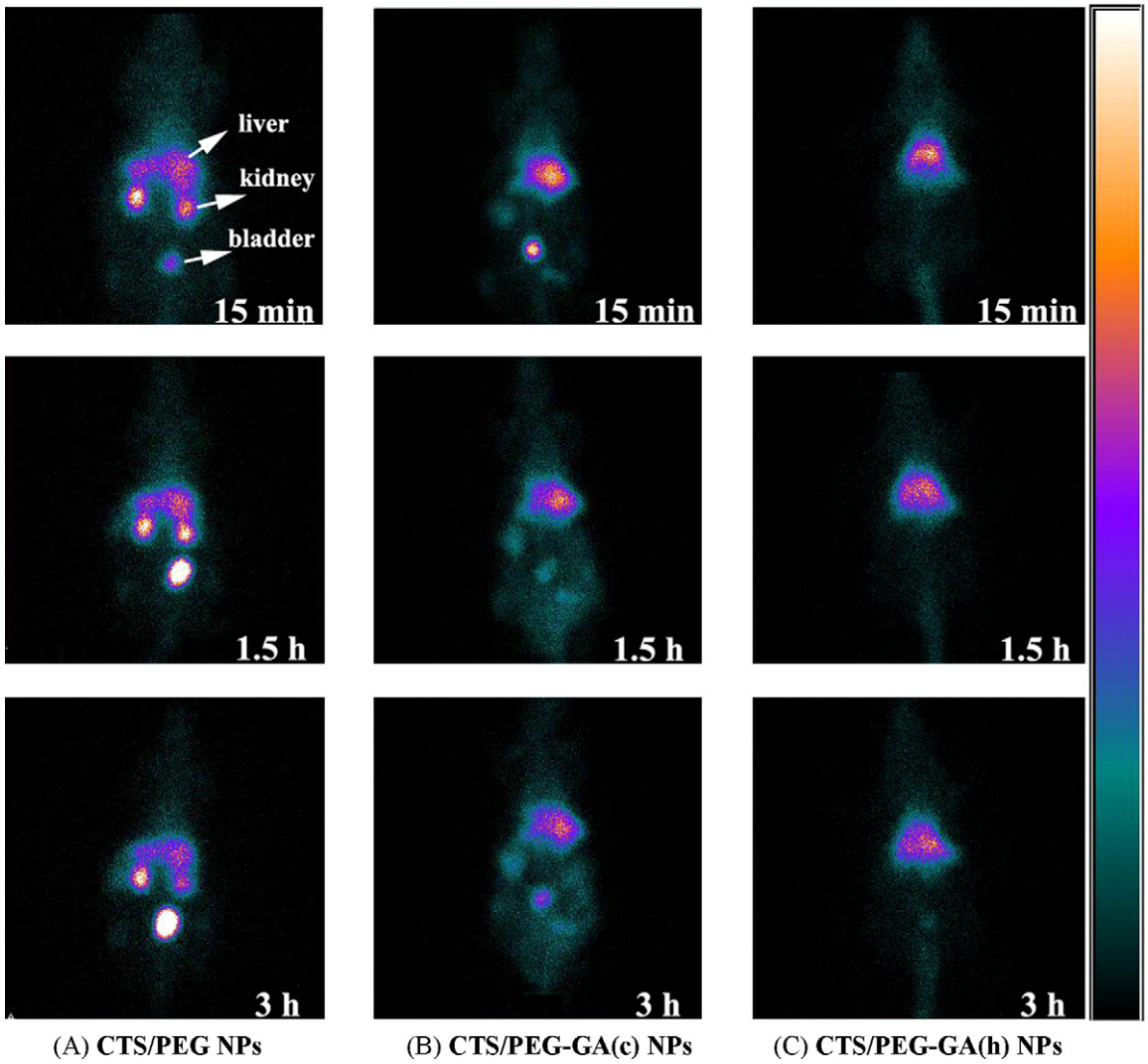

Fig. 4. Images of the mice injected with CTS/PEG NPs (A), CTS/PEG-GA(c) NPs (B) and CTS/PEG-GA(h) NPs (C) at 15 min, 1.5 h and 3 h after injection (observed by SPECT).

Table 1
The particle size, zeta potential and GA content of the nanoparticles.[a]

PEG-GA concentration (mg/mL)	CTS/PEG-GA(c) NPs			CTS/PEG-GA(h) NPs		
	Diameter (nm)	Zeta potential (mV)	GA content[c] (wt%)	Diameter (nm)	Zeta potential (mV)	GA content (wt%)
0	171.6 ± 5.1	36.55 ± 0.97	\	\	\	\
5	188.8 ± 9.2	29.22 ± 1.38	2.51%	186.1 ± 5.3	28.40 ± 2.44	2.39%
10	195.6 ± 4.9	18.42 ± 0.43	4.60%	190.5 ± 6.7	21.60 ± 1.22	4.57%
10[b]	190.5 ± 7.6	22.55 ± 1.45	\	\	\	\

[a] Nanoparticles were prepared with concentrations of CTS and TPP at 2.0 mg/mL and 1.0 mg/mL, respectively; the volume ratio of the CTS solution to the TPP solution was 5:2. The results are given as mean ± SD; $n = 3$.
[b] Without conjugation with GA.
[c] Single batch.

of the nanoparticles in the liver was only 19.9% of the injected dose. In contrast, animals treated with GA-modified nanoparticles (CTS/PEG-GA NPs) showed a significantly higher accumulation in the liver than in other tissues. The accumulation in the liver was 51.3% and 56.5% for the CTS/PEG-GA(c)$_{4.60\%}$ NPs (the subscript number denotes the GA content in the nanoparticles as the wt%) and the CTS/PEG-GA(h)$_{4.57\%}$ NPs, respectively. This was about 2.6 – 2.8-fold higher than that for the CTS/PEG NPs. It has already been shown that there are receptors for GA on the cellular membranes of hepatocytes (Negishi et al., 1991). Therefore, nanoparticles modified with GA could be recognized, and be transferred into hepatocytes. This enhances their ability to target the liver.

It can also be seen that there was no significant difference between the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs in their liver targeting ability, when they were formed under identical conditions. This indicated that the C_3-hydroxyl in GA has little influence on the targeting ability. Indeed, Lin et al. (2008) showed that GL-modified nanoparticles were more efficient in hepatocyte-targeted delivery. In this work, GA was conjugated with PEG. Both the PEG-GA(c) and the PEG-GA(h) have similar molecular weights and steric hindrances, and the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs showed equivalent targeting ability when they were formed under the same conditions. Therefore, considering that differences between GA and GL in terms of structure, the low affinity of GL to the GA receptor observed in previous work (Negishi et al., 1991) may be the result of the relatively high steric hindrance conferred by the glycosyl at the C_3 position. Moreover, nanoparticles had an increasing liver targeting ability as the GA content in the nanoparticles increased. The liver accumulation was 19.9%, 44.3% and 56.5% for the CTS/PEG-GA(h) NPs with a GA content of 0, 2.39% and 4.57% (wt%) in the nanoparticles at 3 h after injection, respectively (Fig. 5). This indicated that the higher the GA content, the more readily the nanoparticles are recognized by the GA receptor and taken up by the liver. However, taking into consideration the possibility that GA molecules existed both on the surface and inside the nanoparticles, the density and orientation of GA on the surfaces of nanoparticles, which are important factors in the recognition events between the ligand and the receptor, are not clearly defined. Therefore, designing a clearer and understandable system will be the primary focus of our future studies to fully understand the GA-mediated liver-targeted system.

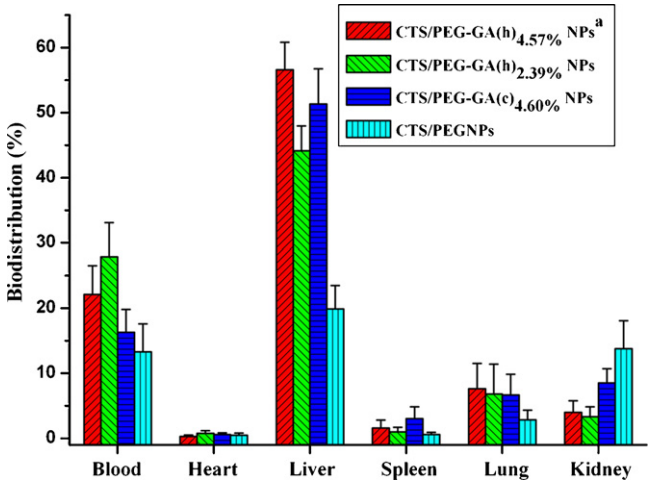

Fig. 5. Tissue distribution of ^{99m}Tc radiolabeled nanoparticles at 3 h after injection ($n = 3$). [a] The subscript number denotes the GA content in the nanoparticles as the wt%.

In conclusion, two kinds of GA-modified nanoparticles (CTS/PEG-GA(c) NPs and CTS/PEG-GA(h) NPs) were prepared, and they showed a remarkable ability to target the liver. There was no significant difference between the CTS/PEG-GA(c) NPs and the CTS/PEG-GA(h) NPs on their targeting ability when they were formed under the same conditions, indicating that the C_3-hydroxyl group in GA has little influence on the targeting ability.

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