Research Article

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A novel CT contrast agent for intestinal-targeted imaging through rectal administration

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Abstract: In this study, a novel CT contrast agent used by rectal administration is developed for targeting intestinal imaging. Iopamidol, an iodinated contrast agent, is loaded in chitosan (CS) nanospheres modified by Anti-5-HT_{3R} (AH) antibody. The obtained AH-CS-I nanospheres (AH-CS-I Ns) would combine to 5-HT₃ receptors highly expressed on the gastrointestinal mucosal, enhancing the intestinal-targeting ability of the contrast agent. The AH-CS-I Ns were administered by the rectal route for intestinal CT imaging, and FITC-labeled AH-CS-I Ns were prepared for investigating the *in vivo* distribution of the contrast agent. As a result, obvious contrast enhancement could still be observed at 6 h post administration because of the poorly absorption of enteral AH-CS-I Ns. Unlike the intravascularly administered agents, AH-CS-I Ns would not accumulate in the kidney and induce adverse reactions. Therefore, this technology has potential applications in the examination of intestinal diseases and could reduce the side effect of commercial iopamidol.

Keywords: chitosan nanospheres, Anti-5-HT_{3R}, CT imaging, iopamidol, intestinal-targeted

1 Introduction

Colorectal cancer (CRC) is the fourth most common cancer in men and the third most common cancer in women worldwide (1–4). Due to its high incidence and mortality, developing diagnosis technology for detecting CRC is crucial (5–8). In addition to colonoscopy, X-ray-based computed tomography (CT) is a convenient technique for CRC diagnosis.

Contrast agents (CAs) are chemicals that are injected (or taken) into tissues or organs to enhance the performance of the CT-scan modality. At present, the most widely used CAs are iodinated contrast agents (ICAs). Among them, iopamidol is a type of nonionic and monomeric ICA with higher water solubility and lower osmolality when compared with high-osmolar ICAs. In clinical practices, intravenous administration is usually utilized to deliver iopamidol CAs; they would be quickly diluted in the circulating blood and distributed throughout extracellular fluid after the injection (9). Although the existing iopamidol CAs provide excellent CT imaging, they still could not be considered as targeted or molecularly specific CAs because the *in vivo* distribution of these CAs is non-specific for a particular disease. In addition, the elimination of iopamidol CAs is primarily renal, which may induce adverse reactions to the kidneys and increase the risk of contrast-induced nephropathy (CIN) (10). Hence, intravenous administration is not suitable to deliver CA for patients with abnormal renal function. Moreover, oral or rectal administration of iopamidol CAs would not result in the retention in the intestinal mucosa because these agents are not organ-specific, they cannot bind to aim tissue or protein effectively.

Recently, the design and use of nanomaterials for addressing medical issues continue to receive increasing interest; nanoscale particles can demonstrate new properties that can be exploited to design contrast agents for anatomical and functional imaging. Chitosan is a natural cationic polysaccharide with nontoxicity and high biocompatibility, which can easily be modified due to the presence of abundant reactive amino and hydroxyl

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groups on its molecular chain. Hence, this polysaccharide has many applications in drug or contrast agent delivery (11–14). Zhang et al. (15) prepared gadolinium-loaded chitosan nanoparticles (Gd-CSNPs) for magnetic resonance imaging (MRI). The obtained Gd-CS NPs exhibited high T1 relaxivity with no obvious cytotoxicity. HyoSook et al. (16) prepared an MRI contrast agent by coating superparamagnetic iron oxide (USPIO) with chitosan, the chitosan-coated USPIO shows no agglomeration and cytotoxicity, which is suitable for *in vivo* MRI imaging. Moreover, CS has certain antibacterial activity since it can act on the electronegative substances of microorganisms like proteins and cell membranes, which causes permeability changes and leakage of intracellular components; thus, the biological activity of microorganisms was destroyed (17–20).

Hydroxy tryptamine (5-HT) is a sort of neurotransmitter that regulates intestinal motility and secretion (21,22). About 95% of 5-HT exists in the gastrointestinal tract, of which about 90% is synthesized and released by enterochromaffin cells (EC), and the remaining 10% exists in various nerve clusters in the intestinal tract. 5-HT_{3R} (5-HT₃ receptor) is one of the receptor subtypes of 5-HT which belongs to the ligand-gated ion channel family (23–25). In the intestinal tract, 5-HT_{3R} is widely expressed in the mucosal cell layer, the submucosa, and the nerve cells on the intermuscular plexus; hence, the 5-HT_{3R} is an ideal target for intestinal imaging (26–29). For example, Cheng et al. prepared a novel magnetic contrast agent based on 5-HT_{3R} for gastrointestinal mucosa-targeted imaging through oral administration (30).

In the present experiment, we report a novel CT contrast agent based on chitosan, iopamidol, and 5-HT_{3R} antibody. The preparation of iopamidol-loaded chitosan nanospheres and the CT imaging of intestinal mucosa were studied. The *in vivo* experiments showed that the CT contrast agent has good contrast capacity and would not cause kidney accumulation after rectal administration, indicating that the contrast agent may have potential applications in intestinal imaging in the future.

2 Materials and methods

2.1 Materials

Chitosan (M.W. <5,000) were purchased from Shanghai Ryon Biological Technology CO., Ltd. Glutaraldehyde solution (GA, 25%), ethylenediaminetetraacetic acid (EDTA), and ethanol were purchased from Sinopharm Chemical

Reagent Co., Ltd. Serotonin receptor 3 antibody (Anti-5-HT_{3R}) and fluorescein isothiocyanate (FITC) were purchased from Shanghai Fusheng Industrial Co., Ltd. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), ethylenediamine, and *N*-hydroxy succinimide (NHS) were purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD. Iopamidol 370 (Iopamiro) was obtained from Renji Hospital affiliated with Shanghai Jiaotong University. The CCK Kit was purchased from Beijing Fanbo Science & Technology Co., Ltd and was analytically pure. Fetal bovine serum (FBS), cyan-streptomycin, and culture medium (DMEM) were obtained from Grand Island Biological Company. Mouse colon cancer CT26 cell line was obtained from Shanghai Institute of Cell Biology. Female BALB/c mice (27 ± 2 g, 7 weeks old) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd, and all animal experiments were conducted in accordance with the guidelines approved by the Animal Protection and Use Committee of Shanghai Jiao Tong University. Water used in all the experiments was purified using a Milli-Q plus 185-water purification system (Millipore, Bedford, MA).

2.2 Preparation of chitosan nanospheres (CS Ns)

Chitosan nanospheres were prepared by the nonsolvent-aided counterion complexation method according to a previous report (31). About 50 mg of chitosan and 17 mg of EDTA were dissolved in 10 mL of deionized water. Then, ethanol was dropwise added under moderate stir until the clear solution became milky. The resulting CS Ns were obtained by being cross-linked by 30 µL of GA solution (25%) for 4 h at room temperature and purified by dialyzing against distilled water to remove the EDTA and ethanol.

2.3 Preparation of Anti-5-HT_{3R} antibody-modified chitosan nanospheres (AH-CS Ns)

Anti-5-HT_{3R} (AH) antibody was modified on the surface of CS Ns using EDC/NHS chemistry. Briefly, 100 µL Anti-5-HT_{3R} was dissolved in 10 mL deionized water, and then, 12 mg EDC and 8 mg NHS were added to activate the carboxyl group of the antibody at room temperature. About 8 mg of CS Ns were mixed with the above solution under moderate stir for 4 h at room temperature. The resulting

solution was washed three times by deionized water to remove uncoupled antibodies, which produces Anti-5-HT3R-modified chitosan nanospheres (AH-CS Ns).

2.4 Preparation of iopamidol-loaded chitosan nanospheres (AH-CS-I Ns)

Iopamidol-loaded chitosan nanospheres were prepared as follows: the obtained AH-CS Ns were added to an appropriate amount of iopamidol injection ($370 \text{ mg I}\cdot\text{mL}^{-1}$) and stirred for 4 h. The resulting Anti-5-HT3R-modified iopamidol nanospheres (AH-CS-I Ns) were obtained by being centrifuged at 7,000 rpm for 15 min and resuspended in distilled water. Specially, FITC-labeled AH-CS-I Ns were prepared by the addition of a certain amount of FITC power into an AH-CS-I Ns solution for fluorescent imaging.

All supernatants were collected and the amount of free iopamidol was determined by UV spectrum according to the standard curve. The iopamidol loading content and encapsulation efficiency were calculated as:

$$\begin{aligned} \text{Iopamidol loading content (\%)} \\ = \frac{M_{\text{iopamidol in nanospheres}}}{M_{\text{nanospheres}}} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Encapsulation efficiency (\%)} \\ = \frac{M_{\text{total iopamidol}} - M_{\text{iopamidol in supernatant}}}{M_{\text{total iopamidol}}} \end{aligned} \quad (2)$$

2.5 Characterization

The morphology of the obtained products was observed using a field-emission scanning electron microscope (FESEM, Hitachi, S-4800). Transmission electron microscopy (TEM) images were recorded on a JEOL-2100F instrument using an accelerating voltage of 200 kV. The size distribution and zeta potential of the sample in PBS (pH = 7.4) were measured by dynamic light scattering (DLS), which was performed on a Malvern Zetasizer Nano ZS. FTIR spectra of the samples were recorded on a Nicolet iS50 FTIR.

2.6 Cell culture

CT26.WT mouse colon cancer cells were used to evaluate cell cytotoxicity of the CS and CS-I Ns. The RPMI 1640 solution and medium supplement with 10% FBS, $100 \text{ IU}\cdot\text{mL}^{-1}$ penicillin, and $100 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin were utilized as

the cell culture medium. Cells were cultivated at 37°C in a 5% CO_2 atmosphere.

2.7 Cell cytotoxicity

The cytotoxicity of CS and CS-I Ns was evaluated by a CCK-8 assay. CT26 cells were seeded into a 96-well plate (5×10^3 cells/well) and incubated at 37°C with 5% CO_2 for 24 h. Then, 100 μL of CS or CS-I Ns was put into culture medium for 24 h at a concentration of 25, 50, 100, and 200 ($\mu\text{g}\cdot\text{mL}^{-1}$), respectively. The culture medium without samples was put in the wells for control groups. Next, 10 μL of CCK-8 solution was added to each well, and the cells were further incubated for 4 h at 37°C . The absorption was recorded at 450 nm using a microplate reader (Bio-Rad, iMark/xMark, USA). The results are expressed as an average over five nominally identical measurements.

2.8 CT and fluorescence imaging

AH-CS-I Ns were delivered by rectal administration. Especially, 400 μL , $200 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ of FITC-labeled AH-CS-I Ns or AH-CS-I Ns was slowly injected into the anus of the mice with an enema tube. After 10 min, the mice were anesthetized by intravenous injection 100 μL of Zoletil 50, and then, *in vivo* CT imaging was performed by the Siemens CT SOMATOM Perspective imaging system. Similarly, *in vivo* fluorescence imaging was performed by IVIS Lumina LT Series III animal fluorescence imaging system. All mice fasted the day before imaging.

2.9 Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using a student's *t*-test. Differences were considered significant at a *P*-value of <0.05 .

3 Results and discussion

CS Ns were prepared by nonsolvent-aided counterion complexation method, after which Anti-5-HT3R modified

CS Ns (AH-CS Ns) were prepared through EDC/NHS coupling chemistry. Furthermore, iopamidol was absorbed into the AH-CS Ns for the synthesis of iopamidol-loaded AH-CS Ns (AH-CS-I Ns). During the reactions, some conditions were optimized to obtain sphere-like nanostructure with narrow size distributions. Finally, the CT contrast agent AH-CS-I Ns was applied for intestinal-targeted imaging by being inserted into the anus of the mice through the enema tube for rectal administration. The whole procedure is schematically illustrated in Figure 1.

The morphology, size distribution, zeta potential, UV, and FTIR spectra of the obtained samples were characterized. The SEM and TEM images of CS and CS-I Ns are shown in Figure 2. It can be seen that uniform CS Ns in sphere-shape were obtained on a large scale with an average size of about 100 nm, and these nanospheres all have smooth surface and clear boundary (Figure 2a). The morphology of CS-I Ns has not changed much compared to CS Ns (Figure 2b), suggesting that iopamidol was successfully encapsulated into the chitosan shells. TEM image of CS Ns further indicates that the CS Ns with core in dark and shell in bright are uniform nanospheres with a mean diameter of about 100 nm (Figure 2c), which implies that there is a hydrated layer on the surface of CS Ns. Figure 2d shows that the surface of nanospheres was coated with a layer of polymer after loading iopamidol, indicating that some iopamidol molecules may be absorbed on the surface of the chitosan carriers during

the drug-loading process, resulting in the increase of diameter.

The size distribution of the samples was measured by DLS; the average hydrodynamic sizes of the CS, CS-I, and AH-CS-I Ns were 180, 245, and 251 nm, respectively (Figure 3a). The results reveal that the iopamidol-loading process leads to the increase of particle size, but the antibody modification has little influence on the size of CS-I Ns. Additionally, the polydispersity index (DPI) of CS, CS-I, and AH-CS-I Ns was 0.044, 0.036, and 0.154, respectively, indicating the narrow size distribution and excellent water dispersity of the samples. It is worth noting that the average hydrodynamic size of CS and CS-I Ns was larger than their dimension shown in SEM or TEM images because the chitosan molecules are prone to bond with H_2O molecules, resulting in the size increase which is accordant with the TEM result. Furthermore, zeta potential of CS, CS-I, and AH-CS-I Ns was 6.9, 16.7, and 25.7 mV, respectively, which suggests that both the iopamidol-loading and antibody modification changed the surface charge density of nanospheres which made AH-CS-I Ns dispersed in PBS (pH = 7.4) more steadily (Figure 3b). Iopamidol loading content and encapsulation efficiency of CS-I Ns were measured by UV spectrophotometry, the iopamidol-loading content for CS-I Ns is determined to be 15%, and encapsulation efficiency is determined to be 54%. Figure 3c is FTIR spectra of the samples. In spectra of CS, the wide peak at the range of $3,200\text{--}3,600\text{ cm}^{-1}$ was

Figure 1: Schematic illustration of the formation of AH-CS-I Ns preparation and its application in intestinal-targeted CT imaging.

Figure 2: The morphology of the obtained nanospheres: (a) SEM image of CS Ns, (b) SEM image of CS-I Ns, (c) TEM image of CS Ns, and (d) TEM image of CS-I Ns.

Figure 3: Characterization of obtained nanospheres. Size distribution (a) and zeta potential (b) of CS Ns, CS-I Ns, and AH-CS-I Ns. (c) FTIR spectra of CS and CS Ns.

related to OH and NH groups, the peak at $2,900\text{ cm}^{-1}$ was related to stretching vibration of C–H. After addition of glutaraldehyde to form CS Ns, a new peak at $1,615\text{ cm}^{-1}$ was observed, which was related to the formation of amide group because of the Schiff base reaction of glutaraldehyde and amine groups of CS.

The EDC/NHS coupling chemistry was used to conjugate the 5-HT3R antibody to the surface of CS Ns; the mechanism of this method is shown in Figure 4. First, the carboxyl groups in the antibody are exposed to EDC/NHS, and reactive NHS esters are formed, when a primary amine group on the surface of CS Ns comes in contact with the ester, a covalent bond is formed. These results

suggest that AH-CS-I Ns with a positive potential value and stable structure were prepared, and the positive charge of chitosan molecules and Anti-5-HT3R antibody modification strongly contribute to its intestinal-targeted properties for use in intestinal imaging.

To examine the feasibility of the obtained CS and CS-I Ns for use in biomedical applications, their cytotoxicity was investigated. As shown in Figure 5, the cell viability was measured by the CCK-8 assay. The cell viabilities of all the groups decreased slightly with increasing concentrations of CS and CS-I Ns but were still higher than 90% when the dose of CS-I Ns arrived at $200\text{ }\mu\text{g}\cdot\text{mL}^{-1}$. The results proved that the iopamidol-loaded chitosan

Figure 4: The mechanism of antibody-conjugation on the surface of CS Ns.

Figure 5: The *in vitro* cytotoxicity of CS and CS-I Ns.

nanospheres had no obvious cytotoxicity and were suitable for potential CT imaging applications.

To investigate the *in vivo* bio-distribution of AH-CS-I Ns after the drug administration, FITC-labeled AH-CS-I Ns were prepared as a Fluorescence probe and delivered to the intestinal tract of the mice. The mice were scanned by IVIS Lumina LT Series III animal fluorescence imaging system at 90 min post the rectal administration, and fluorescent signal was tested under the excitation at 465–490 nm and recorded at 500–550 nm. Compared to the control group, a red and channel-like region was found at the bottom-left of the abdomen (Figure 6), suggesting that FITC-labeled AH-CS-I Ns mainly accumulated in the colon region at 90 min post injection.

Furthermore, the mouse was sacrificed, and *ex vivo* fluorescence images were obtained for the major organs such as heart, liver, spleen, lung, kidneys, and intestine at 6 h post rectal administration (Figure 7). It can be seen that a strong fluorescence signal was recorded in the

Figure 6: Fluorescent images of mice at 90 min post rectal administration with FITC-labeled AH-CS-I Ns (a) and PBS (b).

Figure 7: *Ex vivo* fluorescence images of major organs dissected from mice at 6 h post rectal administration.

intestine and also some weak signals were founded in the liver and lung of the mouse, suggesting that the uptake of AH-CS-I Ns by gut is limited and most nanospheres could be accumulated in the intestine, but still a little nanospheres could be absorbed into the circulating blood and sent to the liver. However, there is no evidence that the AH-CS-I Ns would be eliminated by kidneys.

To evaluate the contrast capacity of the AH-CS-I Ns, the contrast agents were delivered by rectal administration. In addition, the mice without rectal administration of AH-CS-I Ns were set as a control group. Based on the result of Figure 5, the bottom-left of the mice is selected as an interesting region. Figure 8 shows CT images of mice at different time points after rectal administration

Figure 8: *In vivo* CT images of mice treated with AH-CS-I Ns at different times points.

of AH-CS-I Ns. For the control group, CT images could only display limited areas or organs clearly, which have a greater difference in density from adjacent tissues, such as bone areas, but it is hard to observe the detail of soft tissues, such as heart, lung, liver, intestinal tract, and other organs. However, after rectal administration of CT contrast agent AH-CS-I Ns, the brightness of the target region is increased at high degrees when compared with the control group. As shown in Figure 8, after using the CT contrast agent, the brightness of the target regions of the mice increased significantly, and the detailed intestinal cavity can be identified easily with time elapsing. The results may relate to the contrast agents modified by the Anti-5-HT₃R antibody, which binds to the 5-HT₃ receptor expressed on the surface of the intestinal mucosa (31). Besides, the intestinal mucosa is often negatively charged; so, the positively charged chitosan carriers could easily be absorbed. Thus, with the metabolism of CT contrast agent inside the intestinal tract, the intestinal tract can be imaged more clearly, which leads to the visible structure of the intestinal cavity. Additionally, the above results show that the CT contrast agent AH-CS-I Ns were still effective at 6 h post the rectal administration, because the absorption of diagnostic enteral iodinated contrast agents is limited. Unlike administered contrast agents intravascularly, the AH-CS-I Ns would not be eliminated by kidneys; instead, it would be accumulated in the intestinal tract for a long time due to the antibody on the surface of agents and the excretion of the agent is through the fecal route.

It is worth noting that delivering contrast by enteral administration has few known adverse reactions. Therefore, the CT contrast agent is helpful for the analysis and diagnosis of intestinal diseases.

4 Conclusion

In summary, Anti-5-HT₃R modified CS Ns were prepared through EDC/NHS coupling chemistry, and then, iopamidol was loaded into the AH-CS Ns for the synthesis of the CT contrast agent AH-CS-I Ns. The morphology, size distribution, zeta potential, UV-Vis, and FTIR spectra of the samples were characterized; the results show that AH-CS-I Ns have uniform morphology with 100 nm in dimension. Anti-5-HT₃R modification can strongly contribute to their intestinal-targeted properties. Furthermore, cell cytotoxicity assays showed that the cell viability was still higher than 90% when the dose of CS-I Ns reached 200 µg·mL⁻¹, which means that AH-CS-I Ns have no obvious

effect on cellular viability. Additionally, FITC-labeled AH-CS-I Ns were also prepared for fluorescence imaging system to investigate its *in vivo* distribution through rectal administration. Finally, CT contrast capacity of AH-CS-I Ns was evaluated, in which an obvious contrast enhancement was observed compared with control group. Thus, the obtained CT contrast agent could actively target to the intestinal mucosa and has certain potential application for intestinal imaging in the future.

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