A Hyaluronic Acid-Chitosan-Gadolinium Nanosphere for Specific Tumor-Targeted MRI Contrast Agent

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A hyaluronic acid-chitosan-gadolinium (HA-CTS-Gd) nanosphere is prepared by HA modified CTS nanosphere and the complexation between Gd ions and functional groups in CTS and HA molecule. The nanosphere is used as a novel magnetic resonance imaging (MRI) contrast agent for targeting specific tumor. The results show that the HA-CTS-Gd nanosphere is stable in physiological environment due to the strong Gd chelating interaction between the abundant functional groups in HA and chitosan molecules, which leads to low toxicity. Moreover, the obtained nanosphere reveals a high CD44 targeting efficiency and relaxation efficiency in vitro, and it has a great agency for human colonic and mouse lymphatic tumor MRI in vivo. Thus, the novel MRI contrast agent can be taken up selectively by CD44 antigen and used as a potential MRI contrast agents in specific tumor.

Keywords chitosan, hyaluronic acid, contrast agent, magnetic resonance imaging

Introduction

At present, magnetic resonance imaging (MRI) has been a powerful tool among various clinical imaging diagnostic techniques because of its many advantages, such as no ionizing radiation, higher spatial resolution, infinite penetration depth, no ionizing radiation and precise three-dimensional positioning ability.[1] Furthermore, the specificity and sensitivity of MRI depend mainly on contrast agents. Specially, some functional contrast agents have been reported, which includes targeting, long circulation, hypotoxicity, and so on. Among them, the targeted contrast agents are paid more attentions because they could target to some special tissues and tumors, which helps them to accumulate in the special sites and increase intensity of MRI signals. For example, liver, blood pool and tumor targeting contrast agents have been developed and applied. However, as one of the most important tissue-targeting contrast agents, there are few reports about the lymphatic system specific contrast agents except the large molecule/gadolinium injection (Gd-DTPA),[2-4] protein/Gd-DPTA compound[5] and superparamagnetic iron oxide (SPIO).[6-9] These contrast agents could effectively gather in lymph node due to the phagocytosis of the macrophages in lymph vessel, leading to increase of noise ratio and signal intensity and better differing between the benign and malignant lymph nodes. For instance, Yan et al.[10] synthetized three dextran gadolinium complexes Dextran-DTPA-Gd as the potential MRI contrast agents in lymphatic system. Michel et al.[6] evaluated the MRI enhancement with SPIO for preoperative axillary lymph node staging in patients with breast cancer by using histopathologic findings as the standard of reference. However, these reported contrast agents are based on the mechanism of passive targeting, which brings that their imaging results could be affected by the quantity and function of macrophages in the lymph nodes and suffer finally wrong estimation by clinical doctors. Therefore, the development of active targeting contrast agents and their specific materials for the lymphatic system is still an interest and challenging task.

As we well known, the CD44 antigen is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration.[11] Specially, CD44 antigen is highly expressed at many tumor cells like epithelium, gastric tumor, ovarian tumor and colon tumor. Meanwhile, studies have also shown that the formation and metastasis of tumor in vivo is also associated with the CD44 receptor.[12] Thus, the research on the targeted diagnosis and treatment about tumor is popu-
larly responding to the CD44 receptor.\textsuperscript{12-14} Furthermore, it is well known that hyaluronic acid (HA) is widely used as a tumor targeting vector or targeting factor in the field of drug delivery.\textsuperscript{15,16} because it can specially bind to the CD44 and present good characters like bio-compatibility and biodegradability. Moreover, LYVE-1, a kind of high expression factor in the endothelial cells of adult lymphatic vessels, is also deemed to the first HA receptor to find and identify the lymphatic vessels.\textsuperscript{17,18} Therefore, HA can also be specifically recognized by LYVE-1, which is a very useful tool for the differential diagnosis of micro lymphatic vessels. In our preliminary report, pure HA molecule is used as a targeted matrix to lymphatic system.\textsuperscript{19} Furthermore, it is also reported that the endothelial specific expression of LYVE-1 in tumor tissues is expected to be used as a research area of lymphatic endothelial markers for tumor associated lymphatic endothelial cells.\textsuperscript{20} Therefore, HA has showed to be a highly promising specific targeting carrier for the tumor diagnosis and treatment.

Chitosan (CTS) is a natural polysaccharide derived from chitin, and has many unique physical, chemical and biological properties such as natural source, renew-able, good biological compatibility and biodegradable, non-toxic and low immunogenicity, and so on.\textsuperscript{21} At present, CTS has been widely used as the carrier material for low molecular weight drugs,\textsuperscript{22} gene\textsuperscript{23,24} and contrast agent.\textsuperscript{25,26} In addition, CTS contains a lot of free hydroxyl (OH) and amino (NH\textsubscript{2}), which can produce abundant derivatives to improve the properties through acylation, carboxylation, etherification, alkylation, esterification, aldehyde imidization, salt, chelating, grafting and crosslinking reaction.\textsuperscript{27} Thus, we prepare the HA modified CTS nanospheres through 1-(3-dimethylaminopropyl)-3-ethylcarboximidamide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) coupling chemistry with free amino in CTS and carboxyl group in HA molecule in the present experiment. Furthermore, a novel and stable contrast agent of HA-CTS-Gd nanospheres is obtained through a complexation between Gd ions and functional groups in CTS and HA molecule. The contrast agent is capable in CD44 antigen and highly targeted to specific system in vitro and in vivo.

**Experimental**

**Materials**

Chitosan, sodium tripolyphosphate (TPP), gadolinium chloride hexahydrate (GdCl\textsubscript{3}•6H\textsubscript{2}O), N-hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarboximidamide hydrochloride were analytical grade. All the chemicals above were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used without further purification. Hyaluronic acid was purchased from Bloomage Freda Biopharm Co., Ltd.

**Preparation of CTS nanospheres**

CTS nanospheres were prepared by ionic gelation according to the previous reports.\textsuperscript{28} 8 mg of CTS was dissolved in 4 mL of 1% acetic acid solution at room temperature. Furthermore, the pH value of the resultant solution was adjusted to 4 using 0.1 mol•L\textsuperscript{-1} NaOH solution after being filtered by 0.44 and 0.22 μm of filter paper in order. After that, 5 mg of sodium TPP was dissolved in 5 mL of double-distilled water at room temperature and the resultant solution was filtered as described above. Then, 1.5 mL of TPP solution was added to 4 mL of CTS solution under continuous stirring for 30 min. The resulting products were collected by centrifuging, washing with water three times and then lyophilized.

**Preparation of CTS-Gd nanospheres**

CTS-Gd nanosphere was prepared as following steps: 10 mg of CTS nanosphere was dissolved in 2 mL of PBS (pH = 7.4) and 5 mg of GdCl\textsubscript{3}•6H\textsubscript{2}O were added to the solution. The mixed solution was kept stirring overnight. The resulting products were obtained by centrifuging, washing with water three times and then lyophilized.

**Preparation of HA-CTS-Gd nanospheres**

HA-CTS nanosphere was prepared as following steps: Specially, 52 mg of HA was dissolved in 1 mL of double-distilled water, and activated by the addition of 12 mg EDC and 8 mg NHS orderly at room temperature. Then 8 mg of CTS nanosphere was dissolved in 4 mL of distilled water. Subsequently, both of above products were mixed and reacted for 1.5 h at room temperature. Finally, the HA-CTS products were obtained through further centrifuging, washing with deionized water and lyophilizing. Afterwards, 8 mg of HA-CTS nanosphere was dissolved in 2 mL of PBS (pH = 7.4) and 5 mg of GdCl\textsubscript{3}•6H\textsubscript{2}O were added to the solution. The mixed solution was kept stirring overnight. The resulting products were obtained by centrifuging, washing with water three times and then lyophilized.

**Characterization**

The morphology of the samples was obtained by using a field-emission scanning electron microscope (FESEM, Hitachi, S-4800). The Fourier transformation infrared (FT-IR) spectra were recorded on a Nicolet 6700 FT-IR spectrometer. Size distributions and zeta potential of the sample were measured by dynamic light scattering (DLS), which was performed on a Malvern Zetasizer Nano ZS. Determination of the gadolinium concentration in a sample was performed by Varian ICP 710 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Analysis.

**Confocal imaging of cells**

Confocal imaging of cells was performed using a
Leica laser scanning confocal microscope. HCT 116 and HEK 293 cells (1 × 10⁶ cells/mL) were incubated with fluorescein isothiocyanate labelled nanospheres (CTS, HA-CTS-Gd) for 2 h for confocal imaging, and then fixed with 4% paraformaldehyde for 30 min and stained by DAPI for 8 min. All cells were washed twice with cell culture medium before confocal imaging. Imaging of FITC (fluorescein isothiocyanate) labelled nanospheres was carried out at 488 nm laser excitation, with its emission collected from 550 to 570 nm.

**Measurement of MRI signal**

All MRI scans were carried out with a 3.0 T whole body system (3.0 T Intera Achieva, Philips Medical Systems, Best, The Netherlands) with brain Crossed Coil. A dosage of 0.05—0.25 mmol·L⁻¹ Gd concentration of HA-CTS-Gd nanospheres was placed in a series of PE tubes for T₁-weighted MRI, using a standard spin echo sequence. The sequence parameters were TR/TE, 4.6/2.4 ms; flip angle, 12°; FOV, 26 cm; matrix, 320 × 256; effective slice thickness, 0.6 mm. The 3D MRIs were then reconstructed from the original data set at each time point using maximum-intensity projection (MIP).

**MRI in vivo**

The animal study was approved by the institutional review board for animal research. Female BALB/C nude mice (5 weeks of age, Slac, Shanghai, SPF) were used to prepare the tumor-xenografted mouse model. The HCT 116 and YAC-1 cells suspension (cells suspension 2 × 10⁶ cells) was subcutaneously injected into the backs of the mice. For interstitial MR lymphography, the nude mice were intravenously anesthetized with a solution containing 3% pentobarbital sodium (1 mL/kg, Nembutal, Sigma-Aldrich Montana American). During the process, the mice body temperature was kept at physiological level. HA-CTS-Gd (200 µL, 8 mg/mL) and CTS-Gd nanospheres (250 µL, 8 mg/mL) were injected into the veins around the mice’s heart. The injection sites for the contrast agents were carefully disinfected. After the injection, the injection sites were gently massaged for approximately 30 s to improve lymph drainage, and MR images were collected using small animal Crossed Coil.

All MRI was performed on a 3.0-T superconductive whole-body scanner (Signa HDxt GE Healthcare), using a dedicated six-channel phased array sensitivity encoding coil for optimized signal reception with the mice in the prostrate position. The conventional gradient system with the amplitude of 40 mT/m and a slew rate of 150 mT/m per millisecond. For interstitial MRI, a fast T₁-weighted 3D gradient-echo sequence (LAVA) in the coronal plane was acquired after gadopentetate dimeglumine injection. The sequence parameters were TR/TE, 4.6/2.4 ms; flip angle, 12°; FOV, 26 cm; matrix, 320 × 256; effective slice thickness, 0.6 mm. The 3D MRIs were then reconstructed from the original data set using maximum-intensity projection (MIP). During the acquisition, the examined mice remained in an unchanged position.

**Cell viability experiments**

HCT 116 cells (5 × 10⁵ cells per well) in log phase were incubated into 96-well flat-bottomed plates (Costar, Charlotte, NC). The cells were incubated for 24 h at 37 °C under 5% CO₂. The obtained HA-CTS-Gd nanospheres at concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL in DMEM (Gibco, USA) were added to the wells of the treatment group, respectively. The cells were incubated for 12 and 24 h at 37 °C under 5% CO₂. Then, the products were removed from the medium and 10% CCK/PBS solution was added to each well and incubated at 37 °C for 3 h. The amount of cell proliferation was measured by reading the optical density value at 450 nm using a plate reader. The following formula was used to calculate the viability of cell growth: Viability (%)=(mean of absorbance value of treatment group/mean absorbance value of control)×100%. The results were expressed as an average over five nominally identical measurement.

**Results and Discussion**

The HA modified CTS-Gd nanosphere (HA-CTS-Gd) was synthesized by conjugating activated HA with CTS through EDC/NHS coupling chemistry and complexing Gd ions. During the reaction, the synthesis of CTS nanosphere has been performed following ionic gelation and the conditions optimized to obtain nanospheres with narrow size distributions. In addition, the surface grafting of CTS nanosphere by HA results to a multifunctional platform for targeted delivery to special tumor cells and organism. Finally, the HA-CTS nanospheres and Gd ions could form stable complexes which prevent Gd ions from being released into solution, which entrusts MRI efficiency and safety of HA-CTS-Gd nanospheres. The whole procedure is schematically illustrated in Figure 1.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Schematic illustration of the formation of HA-CTS-Gd nanospheres.
The size, morphology and structure of the obtained products were characterized. As shown in Figures 2a and 2b, SEM images show that the sphere-like nanostructures of CTS and HA-CTS-Gd obtained from PBS solution are prepared. Closer observation reveals that the average size of HA-CTS-Gd nanospheres is larger than that of CTS nanospheres. Furthermore, the results of the DLS measurement also demonstrate that the average hydrodynamic size of the CTS and HA-CTS-Gd nanospheres in deionized water is 218 and 315 nm, respectively. Additionally, the zeta potential was also performed to characterize the process. The zeta potential values of CTS, HA-CTS and HA-CTS-Gd nanospheres are 40.5, −7.72 and 46.7, which suggests that the electronegative HA molecules are grafted and the electropositive Gd$^{3+}$ ions are chelated into CTS nanospheres successfully. Furthermore, FT-IR spectra the products were performed to prove that HA molecules have conjugated on CTS nanospheres. As shown in Figure 2d of the curve of CTS nanospheres, the typical peaks at 1635 and 1535 cm$^{-1}$ are attributed to characteristic stretching vibrations of C=O and C—N, respectively, where the band at 3200, 1070 and 1020 cm$^{-1}$ is the characteristic stretching and bending vibration of N—H, respectively. Compared with the curve of HA-CTS nanospheres, the intensity of 1635 cm$^{-1}$ has been improved obviously, which attributes to the addition of carboxyl group in HA molecule. Furthermore, the band at 3200 cm$^{-1}$ in the curve of HA-CTS-Gd nanospheres increases strongly, which implies the probable hydrogen bond interaction from Gd chelating between HA and chitosan molecules. Moreover, the amount of Gd ions is conducted by ICP-MS analysis. The result shows that 117.2 mg/g Gd ions can be chelated into HA-CTS nanospheres. Moreover, as shown in Figure 3, the Gd$^{3+}$ ions release after incubating the products in PBS solutions for 24 h through a leaching experiment, which shows that 0.7% Gd ions are released at 24 h. The result reveals that HA-CTS-Gd nanospheres are stable in physiological environment due to the strong chelating interaction from the abundant functional groups in HA and chitosan molecules. Moreover, as shown in Figure 3, the Gd$^{3+}$ ions release after incubating the products in PBS solutions for 24 h through a leaching experiment, which shows that 0.7% Gd ions are released at 24 h. The result reveals that HA-CTS-Gd nanospheres are stable in physiological environment due to the strong chelating interaction from the abundant functional groups in HA and chitosan molecules. Moreover, as shown in Figure 3, the Gd$^{3+}$ ions release after incubating the products in PBS solutions for 24 h through a leaching experiment, which shows that 0.7% Gd ions are released at 24 h. The result reveals that HA-CTS-Gd nanospheres are stable in physiological environment due to the strong chelating interaction from the abundant functional groups in HA and chitosan molecules.

To evaluate the targeting specificity of HA-CTS-Gd nanosphere to CD44, cellular uptake efficiency of HA-CTS nanosphere was observed by confocal laser scanning microscopy. Based on previous studies, receptor-mediated endocytosis, especially an interaction between HA and the CD44 receptor, was identified as the principal cellular uptake mechanism of HA-based nanosphere. Therefore, some cells with wide expression

![Figure 2](image1.png)  
**Figure 2** SEM image of (a) CTS and (b) HA-CTS-Gd nanospheres, (c) DLS data of CTS and HA-CTS-Gd nanospheres and (d) FT-IR spectra of CTS, HA-CTS and HA-CTS-Gd nanospheres.

![Figure 3](image2.png)  
**Figure 3** The release of gadolinium from HA-CTS-Gd nanospheres in PBS solution.

of CD44 were always used to simulate the interaction of lymph system and HA-based nanosphere. Thus, in present experiment, HCT 116 cells were used as high CD44 receptor-expressing cells, while HEK 293 cells were regarded as control groups because of the low expression of endogenous ligand receptor, and FITC (fluorescein isothiocyanate), an near-infrared fluorescence dye, was used for the detection of cellular uptake and distribution of the nanospheres. As shown in Figure 4, HCT 116 and HEK 293 cells with blue fluorescence were dyed with DAPI and green FITC-labeled nanospheres. In HCT 116 cells group, HA-CTS nanospheres embrace the cells largely and distribute around them tightly (Figure 4f), which is different to the result of CTS nanospheres (Figure 4c), a general cellular uptake to nanospheres. The result shows that HA-CTS nanospheres have a strong affinity of HCT 116 cells. Furthermore, the addition of Gd in HA-CTS nanospheres has few influence on targeting specificity (Figure 4i). However, all of CTS and HA-CTS nanospheres in HEK 293 cells group reveal poor ability of phagocytosis on cells (Figures 4c', 4f' and 4i'). The above results imply
that the developed HA-CTS-Gd nanospheres are potential in high targeting efficiency to the CD44 receptor over-expressed tumor cells.

The MRI property of HA-CTS-Gd nanosphere was further investigated to evaluate their diagnose function. MRI signals of HA-CTS-Gd nanosphere were measured in vitro. Different mass concentrations of HA-CTS-Gd nanospheres in the centrifuge tubes, as well as pure water for the background signal, were measured for their $T_1$ relaxation time by a 3T MR imaging scanner. $T_1$-weighted maps in Figure 5a show that the $T_1$-weighted MRI signal intensity is continuously enhanced, resulting in brighter images with increasing the mass. Furthermore, as shown in Figure 5b, the longitudinal proton relaxation rate as a function of Gd ion concentrations led to an $r_1$ relaxivity of 9.2 mM$^{-1}$s$^{-1}$, which was much larger than that of Magnevist ($r_1 = 4.5$ mM$^{-1}$s$^{-1}$).

Because of the excellent MRI in vitro and targeting specificity, we further explore the potential to use the HA-CTS-Gd nanospheres for diagnosis of a xenografted tumor model. Human colonic tumor, prepared by the injection of HCT 116 cells suspension into the backs of the mice from the vein around the mice’s heart, was used as the model for MRI. As shown in Figures 6a—6d, before and after 10, 20 and 60 min post injection, the nude mice are imaged by MR. Compared to Figure 6a without any injection, we can clearly see that the signal of tumor region increases obviously in a typical $T_1$-weighted MRI at 10 min after injection (Figure 6b).

Closer observation reveals that some subtle structures in tumor region are also found clearly. Moreover, the brightness of tumor region hardly decreases at 20 and 60 min post injection, which reveals the long residence time in tumor due to the specific binding between...
HA-CTS-Gd nanospheres and their receptor. However, during the MRI process, the signal of mice body shows almost no change. The results imply that HA-CTS-Gd nanospheres could strongly target to the human colonic tumor because of the high CD44 expression in the original HCT 116 cells. To further prove the above opinion, a control experiment was performed. The result of a control group of CTS-Gd nanospheres with the same Gd content shows the signal intensity of the tumor region and body increase synchronously at 10 and 20 min post injection although CTS-Gd nanospheres could enhance the signal of the tumor region due to possible the charge effect between the negative cells and the positive nanospheres. Additionally, the subtle structures in tumor region are difficult to be found. And the signal decays at 30 min post injection (Figures 6e—6g). Moreover, we monitored the MRI signals at the special tumor sites at $T_1$-weighted MRI images (labeled by arrows). As shown in Figures 6i and 6j, the signal intensity of the injection of HA-CTS-Gd nanospheres still increased at the time of 60 min, while that of the injection of CTS-Gd nanospheres decreased obviously at the time of 30 min. The above results reveal that CTS-Gd nanospheres as a MRI contract agent are difficult to distinguish the human colonic tumor and the body tissue, which suggests that there is no specificity of CTS-Gd nanospheres to human colonic tumor. Therefore, the vein injection of HA-CTS-Gd nanospheres leads to a quite uniform and specific distribution of the particles within the human colonic tumor region, allowing for effective and long-periodic MRI of the whole tumor.

Moreover, we further explore the potential usage of the HA-CTS-Gd nanospheres for diagnosis of a lymphatic xenografted tumor model. YAC-1 (mouse lymphoma cells) tumor was used as model because LYVE-1, as a new homologue of the CD44 glycoprotein,\textsuperscript{[19]} is a lymph-specific receptor for HA. As shown in Figure 7, similar to the MRI of human colonic tumor by HA-CTS-Gd nanospheres (Figures 6a—6d), the same phenomenon has been found except that the brightness of tumor region slightly decreases at 60 min post injection. Furthermore, the signal intensity of the special tumor sites (labeled by arrows) decreased slightly at the time of 60 min after the injection of HA-CTS-Gd nanospheres (Figure 7e). Therefore, these results show that the obtained HA-CTS-Gd nanospheres have a great potential to be used as a contrast agent for in vivo MRI of lymphatic tumor.

The cytotoxicity was investigated to examine the feasibility of the obtained products for biomedical application. As shown in Figure 8, using the viability of untreated cells for controlled, the effect of varying concentrations (0—1.0 mg/mL) of HA-CTS-Gd nanospheres on the viability of HCT 116 cells after exposure for 12 h was investigated. It is an interesting phenomenon that when the cell was incubated in low concentration for 24 h, its viability even exceeded cells in controlled. Moreover, 85% cell viabilities are still maintained even up to a relatively high dose of 1.0 mg/mL at 48 h. Therefore, the results demonstrate that the obtained HA-CTS-Gd nanospheres have a low cytotoxicity.

Figure 7 $T_1$-weighted MRI in vivo of mouse lymphatic tumor by HA-CTS-Gd nanospheres at different scanning time: (a) 0, (b) 10, (c) 20 and (d) 60 min. The time-enhancement curves of MRI signals at the tumor sites in vivo after the injection of (e) HA-CTS-Gd nanospheres.

Conclusions

In conclusion, HA-CTS-Gd nanospheres were prepared successfully and used as MRI contrast agent for tumor MRI. The characterization in vitro indicates that the obtained nanospheres possess stable physicochemical property and could target to cells which own high expression of CD44. Moreover, the obtained products have good biocompatibility because its low cell cytotoxicity. Furthermore, the nanospheres show outstanding $T_1$-weighted MRI signal which is better than those traditional contrast agents. In vivo experiment shows that the nanosphere is a great MRI agency for
human colonic and mouse lymphatic tumor. Therefore, we believe that the obtained product is a good MR contrast agent candidate for lymphatic system.

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