Multifunctional Lymph-Targeted ZnO:Gd@Hyaluronic Acid Nanospheres: Preparation and Application in Tumor Diagnose

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A novel multi-modal lymph-targeted contrast agent, ZnO:Gd@hyaluronic acid (HA) nanospheres, is prepared by Gd doped ZnO quantum dot, modified by silica shell and grafted by HA molecule. The physicochemical properties are characterized and the lymphatic targeting is evaluated *in vitro*. Moreover, the contrast agent is used for *in vivo* lymphoma magnetic resonance imaging (MRI). The results show that the ZnO:Gd@HA nanospheres with 19% of quantum yield and $5.8 \text{ mM}^{-1} \cdot \text{s}^{-1}$ of relaxivity could be used for *in vivo* lymphatic targeting studies. Furthermore, they are highly targeted to lymph system *in vitro* and *in vivo*, and highlight their MRI in lymph tumor. Thus, the novel MRI contrast agent can be taken up selectively by lymphatic system and used as a potential multi-modal contrast agent in lymphatic system.

Keywords multi-modal imaging, lymph-targeted, hyaluronic acid, quantum dot

Introduction

Recently, multi-modal imaging techniques have received considerable attention since they provide a wealth of accurate and detail information for biomedical applications. Among them, magnetic resonance imaging (MRI) is one of the most powerful techniques because of their noninvasive diagnosis, deep penetration into tissues and harmlessness. However, there are some drawbacks, including low sensitivity, spatial resolution, and so on.^[1] In contrast, fluorescence imaging offers much higher sensitivity and spatial resolution, but limited depth perception.^[2] Therefore, it is a promising approach to integrate the complementary techniques into one platform.

The preparation and application of multifunctional inorganic nanoparticles incorporating magnetic and fluorescent materials has become more and more popular.^[3] Some cadmium dichalcogenide nanocomposites have been focused because of their high quantum yield (QY) or relaxivity (r_1),^[4,5] but their disadvantages are the high toxicity due to the releasing of Cd^{2+} or/and Se^{2+} , which limits their biomedical applications.^[6,7] Therefore, several biocompatible nanomaterials have been designed to obtain multifunctional contrast agents.^[8-10] As a kind of versatile materials, ZnO was cheap and biocompatible.^[11] Various photoluminescent

ZnO nanoparticles have been investigated and demonstrated their promising biomedical applications.^[12] Furthermore, some multi-modal imaging nanoprobe based on ZnO have been developed.^[13,14] However, these nanoprobe rarely results from ZnO quantum dots with higher QY except there were few reports on Gd-doped ZnO nanoparticles. For example, Liu *et al.*^[15] fabricated Gd-doped ZnO quantum dots coated by silica. These biocompatible products exhibited both strong positive contrast effects with r_1 value of $16 \text{ mM}^{-1} \cdot \text{s}^{-1}$ and fluorescence intensity with QYs from 13.5% to 34%. Most importantly, they proved that Gd doping could enhance the fluorescence intensity of ZnO quantum dots when the Gd doping ratio is 0.08. Though the exact mechanism of this phenomenon is not clear, some researchers attributed this luminescence enhancement to less excited states and weak excited state absorption of Gd^{3+} , which led to emission intensity enhancement by effectively energy transfer.^[16]

As well known, tumor lymph metastasis is one of the most important reasons causing death. Compared to traditional lymph node biopsy for the diagnosis of metastatic lymph nodes, non-invasive imaging techniques play a great role. Thus, lymph system targeted contrast agent is attracting more and more interest.^[17-19] However, most of reported contrast agents for lymph metastasis are based on the mechanism of passive targeting,

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which brings that their imaging results could be affected by the number and function of macrophages in the lymph nodes and finally estimated wrongly by clinical doctors.^[20,21] Therefore, developing new targeted contrast agent for lymph metastasis is beneficial to the study and diagnosis of the tumor lymph node metastases, which could bring fresh prospect for the tumor treatment and vast their future development. Because of the interaction between hyaluronic acid (HA) and the CD44 receptor, HA is widely used as a tumor vector for the targeting procedure. CD44 is expressed in a variety of tumors such as breast, colon, intestinal, and brain.^[22,23] Furthermore, LYVE-1, as a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for HA, which can bind both soluble and immobilized HA. However, unlike CD44, the LYVE-1 molecule can colocalize with HA on the luminal face of the lymph vessel wall and is completely absent from blood vessels.^[24] Therefore, HA is a highly promising lymphatic targeting carrier.

In this paper, we develop a novel multi-modal contrast agent of ZnO:Gd@HA nanospheres based on Gd doped ZnO quantum dot, perform their magnetic resonance (MR) and fluorescence imaging *in vitro*, present evidence that they are highly targeted to lymph system *in vitro* and *in vivo*, and highlight their MRI in lymph tumor. Therefore, the contrast agent is capable of defining the multi-modal imaging about lymph tumor, which has great potential in biological area.

Experimental

Preparation of ZnO:Gd quantum dots

ZnO:Gd quantum dots were prepared according to the following processes with slight modifications.^[25] Methacrylic acid (MAA) was mixed with deionized water by $V(\text{MAA}) : V(\text{H}_2\text{O}) = 1 : 4$, which was heated to 60 °C and then reacted with appropriate amount of ZnO powder under stirring for 1 h to get zinc methacrylate $[\text{Zn}(\text{MAA})_2]$ aqueous solution. The unreacted ZnO powder was removed with filtration. The $\text{Zn}(\text{MAA})_2$ aqueous solution was dehydrated in a freeze drier at -80 °C to obtain anhydrous $\text{Zn}(\text{MAA})_2$ powder. Furthermore, 10 mmol of anhydrous $\text{Zn}(\text{MAA})_2$ powder and 0.8 mmol of $\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 100 mL of absolute ethanol, which was refluxed for 1 h at 80 °C. Finally, 153 mmol of $\text{LiOH} \cdot \text{H}_2\text{O}$ ethanol solution, a certain content of $\text{LiOH} \cdot \text{H}_2\text{O}$ dissolved in 100 mL of absolute ethanol by sonication, was added into the above mixture solution. After stirring for 24 h at room temperature, the as-prepared products were separated by centrifugation and then dispersed in 200 mL of ethanol or cyclohexane for further experiments.

Preparation of ZnO:Gd@silica nanoparticles

The ZnO:Gd@silica nanoparticles were prepared as follows: 7.5 mL cyclohexane of as-prepared ZnO:Gd nanoparticles, 1.77 mL Triton X-100, 1.8 mL *n*-hexanol,

48 μL deionized water and 240 μL ammonia aqueous solution (25 wt%) were mixed under stirring to form microemulsion system. After 30 min, 100 μL tetraethyl orthosilicate (TEOS) was added and reacted for 24 h, and then 50 μL γ -aminopropyltriethoxysilane (APS) was introduced. The reaction was continued for another 12 h at room temperature. The resulting ZnO:Gd@silica nanoparticles were collected by centrifugation.

Preparation of ZnO:Gd@HA nanospheres

HA was activated using *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC). Specially, 1.25 g of HA was dissolved in 200 mL double-distilled water. Subsequently, 0.23 g of EDC and 0.14 g of NHS were added into the solution and 0.1 g of ZnO:Gd@silica nanoparticles was dispersed in the solution. The mixed solution was stirred for 24 h at room temperature. Finally, the resulting products of ZnO:Gd@HA nanospheres were collected by centrifuging, washing with ethanol and water three times, and drying at 60 °C in sequence.

Characterization

Powder X-ray diffraction (XRD) patterns were recorded on a Rigaku D/MAX-2250V diffractometer using Cu-K α radiation (40 kV and 40 mA). The morphology of the samples was obtained using a high resolution transmission electron microscope (HRTEM, JEOL, JEM-2100F) and a field-emission scanning electron microscope (FESEM, Hitachi, S-4800), respectively. The Fourier transformation infrared (FT-IR) spectra were recorded on a Nicolet 6700 FT-IR spectrometer. The UV-vis spectra were investigated using a PerkinElmer Lambda 950 UV-vis spectrophotometer. Photoluminescence spectra were recorded using a Hitachi fluorescence spectrophotometer (F-7000).

Confocal imaging of cells

Confocal imaging of cells was performed using a Leica laser scanning confocal microscope. HCT116 cells (1×10^6 cells/mL) were incubated with ZnO:Gd@HA nanospheres for 2 h for confocal imaging, fixed with 4% paraformaldehyde for 30 min and stained by 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 8 min. All the cells were washed twice with PBS before confocal imaging. Imaging of ZnO:Gd@HA nanospheres was carried out at 405 nm laser excitation, with its emission collected from 500 to 550 nm.

Measurement of MRI signal

All MRI signals 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. Different mass concentrations of ZnO:Gd@HA nanospheres were placed in a series of PE tubes for T_1 -weighted MR imaging, using a standard spin echo

Figure 1 Schematic illustration of the formation of ZnO:Gd@HA nanospheres.

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*

Animal experiments were approved by local governmental authorities. Male Specific Pathogen Free nude mice, aging 4–5 weeks and weighing 18–20 g, were supplied by Department of Experimental Animals, Shanghai Jiaotong University, and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Shanghai Jiaotong University.

YAC-1 (mouse lymphoma cells) tumor bearing a group of nude mice was established by subcutaneous injection of 2×10^6 cells suspended in 200 μL PBS. When the diameter of tumor was around 8–10 mm, the group of nude mice was subcutaneously injected in tumor region by ZnO:Gd@HA nanospheres (0.2 mL, 0.2 mg/mL), and MR images were collected using small animal Crossed Coil. All MR imaging 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 in the supine position. The conventional gradient system with an amplitude of 40 mT/m and a slew rate of 150 mT/m per millisecond. For interstitial MR imaging, a fast T_1 -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.

Cell viability experiments

HCT116 cells (5×10^3 cells per well) in log phase were incubated into 96-well flat-bottomed plates. The cells were incubated for 24 h at 37 °C under 5% CO_2 .

The obtained ZnO:Gd@HA nanospheres at concentrations of 0, 50, 100, 200, 400 and 500 $\mu\text{g/mL}$ in PBS were added to the wells of the treatment group, respectively. The cells were incubated for 12 h at 37 °C under 5% CO_2 . Then, sample was removed from the medium and 10% CCK/PBS solution was added to each well and incubated at 37 °C for 4 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: $\text{Viability}(\%) = (\text{mean of absorbance value of treatment group} / \text{mean absorbance value of control}) \times 100\%$. The results were expressed as an average over five nominally identical measurement.

Results and Discussion

The HA conjugated ZnO:Gd@HA nanospheres are synthesized by conjugating activated HA with surface-aminolated ZnO:Gd@silica nanoparticles through EDC/NHS coupling chemistry. The whole procedure is schematically illustrated in Figure 1. During the reaction, ZnO:Gd quantum dots are prepared (step a) and encapsulated in surface-aminolated amino silica shell (step b). Then, the surface-aminolated ZnO:Gd@silica nanoparticles are grafted by the EDC/NHS-activated HA (step c). Thus, the multifunctional ZnO:Gd@HA nanospheres could be obtained.

The XRD pattern of the as synthesized products is shown in Figure 2. All of diffraction peaks could be indexed to ZnO with hexagonal wurtzite structure (P6₃mc space group and JCPDS No. 80-0075). Furthermore, the peaks, assigned to crystalline Gd, Gd oxide, Zn-Gd alloys, or Zn-Gd-O particles in the XRD, could not be found, which reveals that Gd-doping do not change the wurtzite structure of ZnO. However, different to the standard data, the relative intensity of the (002) plane decreases distinctly, implying that the Gd-doping could distort the host lattice.^[16] When ZnO:Gd was coated by silica shells and further surface was conjugated by HA, the peaks are similar to those of ZnO:Gd products except to the decreasing intensity and an new amorphous peak at about 20° due to the formation of amorphous species. The above results reveal successful preparation

Figure 2 The XRD pattern of obtained ZnO:Gd and ZnO:Gd@HA products.

of ZnO:Gd and their modify by HA.

The morphology of the obtained ZnO:Gd products was characterized. Typical TEM image is shown in Figure 3a. It is clear that the nanoparticles are essentially mono-dispersed. HRTEM image reveals the successful preparation of ZnO:Gd quantum dots with the size about 4 nm (Figure 3b). Furthermore, the corresponding particle core size histogram is shown in Figure 3c. The result demonstrates that the majority of particle size is 3.8 nm (the standard deviation was around 7.8%) for ZnO:Gd quantum dots. The result was further proved by UV-vis absorption spectra. As shown in Figure 3d, the ZnO:Gd quantum dots shows a broad absorption band at 325–350 nm. Additionally, their size is evaluated according to Meulenkamp's experiential formula as following: $1240/\lambda_{1/2} = a + b/D^2 - c/D$.^[26] Herein, $\lambda_{1/2}$ represents the wavelength where the absorption is half of that at the absorption peak or shoulder; D represents the average size of nanoparticles; a , b , and c are parameters when D is within the range 25–65 Å. According to the calculation, the particle size is about 4.0 nm, which was in accordance with the result of HRTEM.

Figure 3 (a) TEM image, (b) HRTEM image, (c) particle core size histogram, and (d) UV-Vis spectra of obtained ZnO:Gd quantum dots.

Furthermore, the ZnO:Gd quantum dots were coated by silica shell (ZnO:Gd@silica) and further surface conjugated by HA molecule (ZnO:Gd@HA). As shown in Figure 4a, SEM image shows the obtained products are sphere-like structure. The inserted statistical size analysis of SEM image in Figure 4a reveals that the average diameter of ZnO:Gd@HA nanospheres is 200–300 nm (the standard deviation was around 4.6%). Moreover, HRTEM image in Figure 4b indicates these nanospheres contain many nanoparticles inside (highlighted by circles) and thin silica shell outside, implying the successful silica coating. The result implies that the ZnO:Gd@HA nanospheres are built up by primary ZnO:Gd quantum dots. Furthermore, FT-IR spectra of ZnO:Gd@HA nanospheres and HA molecule are shown in Figure 4c. As shown in curve of HA, the typical peaks such as 1610, 1560 and 1040 cm^{-1} are monitored. Among them, the bands at 1610 and 1560 cm^{-1} are attributed to characteristic stretching vibrations of C=O and C–N, respectively, where the band at 1040 cm^{-1} is the characteristic bending vibration of N–H. Compared to curves of HA and ZnO:Gd@HA nanospheres, the main characteristic peaks of HA molecule are found in the spectra of ZnO:Gd@HA nanospheres, such as 1610 and 1040 cm^{-1} . Additionally, some typical peaks about vibrations of C–N coming from APS and HA molecules are shown in 1490–1570 cm^{-1} . Moreover, the characteristic peaks of the Si–O–Si symmetrical stretching at about 1100 cm^{-1} , the Si–C bending at about 950 cm^{-1} and the Si–C stretching at about 1250 cm^{-1} , are the proof of the silica shells. The results further indicate the silica shell and HA molecule successfully encapsulate some primary ZnO:Gd nanoparticles to produce ZnO:Gd@HA nanospheres. Moreover, UV-vis absorption spectrum is performed to evaluate optical property. As shown in Figure 4d, the ZnO:Gd@HA nanospheres show a broad absorption band at 350–400 nm, which is red-shifted by about 40 nm. The reason could lie in the assembly of nanoparticles and surface conjugation of

Figure 4 (a) SEM, (b) HRTEM, (c) FT-IR spectra, and (d) UV-vis spectra of obtained ZnO:Gd@HA nanospheres. The inset is the particle core size histogram of ZnO:Gd@HA nanospheres.

HA molecules.

The luminescence property of ZnO:Gd@HA nanospheres was investigated to evaluate its multifunctional properties. Figure 5 indicates the room temperature photoluminescent spectra of ZnO:Gd@HA nanospheres. The sample reveals an emission with a maximum centered at 530 nm due to the near-band-edge emission, which is the green emission component proved by the inserted photoluminescent photograph. The QY of obtained ZnO:Gd@HA nanospheres are measured according to a reported literature,^[27] in which the rhodamine 6G ethanol solution (QY=95%) was chose as a standard. Thus, the QY of ZnO:Gd@HA nanospheres is measured and calculated to be 19%.

Figure 5 The photoluminescence spectra of ZnO:Gd@HA nanospheres and rhodamine 6G ethanol solution as the standard. The inset is photoluminescent photograph of ZnO:Gd@HA nanospheres.

The magnetic resonance property of ZnO:Gd@HA nanospheres is further investigated. T_1 -weighted MR images of ZnO:Gd@HA nanospheres in H_2O are measured using a 3T MRI system. With increase of the concentration, the ZnO:Gd@HA nanospheres could accelerate longitudinal relaxation of water protons and exert bright contrast (Figure 6a). The resulting r_1 value is evaluated from a plot of the T_1 relaxation rate ($1/T_1$) versus the Gd^{3+} ion concentration, and determined to be $5.8 \text{ mM}^{-1} \cdot \text{s}^{-1}$, which is larger than that of Magnevist ($r_1 = 4.5 \text{ mM}^{-1} \cdot \text{s}^{-1}$).^[28] Therefore, these paramagnetic properties can provide ZnO:Gd@HA nanospheres as a good MRI probe.

Furthermore, confocal laser scanning microscopy was used to evaluate the fluorescent imaging of and cellular uptake efficiency of the ZnO:Gd@HA nanospheres. As shown in Figure 7, green fluorescence of the ZnO:Gd@HA nanospheres excited by 405 nm is shown, which is similar to their results of photoluminescent spectra. Moreover, HCT116 and HEK293 cells with blue fluorescence are dyed with DAPI. In HCT116 cells group, ZnO:Gd@HA nanospheres embrace the cells largely and distribute around them tightly (Figure 6c). However, In HEK293 cells group, ZnO:Gd@HA nano-

Figure 6 (a) T_1 -weighted maps of various Gd^{3+} ion concentrations in ZnO:Gd@HA nanospheres and (b) the linear relationship between relaxation rates ($1/T_1$) and Gd^{3+} ion concentrations in ZnO:Gd@HA nanospheres.

spheres reveals poor ability of phagocytosis on cells. As we known, because of the interaction between HA and the CD44 receptor, HA is widely used as a tumor vector for the targeting procedure because CD44 is expressed in a variety of tumors such as breast, colon, intestinal, and brain. Thus, in present experiment, HCT116 cells are used as high CD44 receptor-expressing cells, while HEK293 cells are regarded as control groups because of the low expression of endogenous ligand receptor. The above results imply that the CD44 receptor overexpressed target ability of the obtained HA-based nanoparticles is achieved, which is potential in high targeting efficiency to some kinds of tumors.

Figure 7 Laser scanning confocal microscopy images of HCT116 and HEK293 cells incubated with ZnO:Gd@HA nanospheres. All images are taken under the identical instrumental conditions and presented at the same intensity scale.

Because of the excellent MRI *in vitro* and targeting specificity, we further explore the potential to use the

ZnO:Gd@HA nanospheres for diagnosis of a xenografted tumor model. YAC-1 (mouse lymphoma cells) tumor was used as model because LYVE-1, as a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for HA. Thus, a group of nude mice with about 120 mm³ tumor volume were intratumorally injected with 0.2 mL, 0.2 mg/mL ZnO:Gd@HA nanospheres. Before and after 5, 15 and 25 min post injection, the nude mice were imaged by MR. As shown in Figure 8, compared to before injection, we can clearly see that the signal of tumor region increases obviously in a typical *T*₁-weighted MRI at 5 and 15 min after injection. Closer observation reveals that some subtle structures in tumor region are also found. However, the brightness of tumor region decreases slightly at 25 min post injection, which could contribute to the metabolism of ZnO:Gd@HA nanospheres. Thus, the intratumoral injection of the ZnO:Gd@HA nanospheres leads to a quite uniform distribution of the particles within the tumor region, allowing for effective and long-periodic MR imaging of the whole tumor, which could come from the high targeting efficiency to the kinds of tumor. Therefore, these result shows that the obtained ZnO:Gd@HA nanospheres have a great potential to be used as a contrast agent for *in vivo* tumor MR imaging.

Figure 8 MR images *in vivo* at different scanning time after post injection. (a) 0 min, (b) 5 min, (c) 15 min and (d) 25 min.

To examine the feasibility of the obtained ZnO:Gd@HA nanospheres for use in biomedical applications, their cytotoxicity was investigated. As shown in Figure 9, the effect of different ZnO:Gd@HA nanospheres on cells proliferation was assessed with HCT116 cells by CCK-8 assay. Using the viability of untreated cells as control, the cellular viabilities decrease with an increasing the concentrations of ZnO:Gd@HA nanospheres, and about 80% cell viabilities are maintained even up to

a relatively high dose of 500 µg/mL after exposure for 12 h. In other words, upon incubating with the ZnO:Gd@HA nanospheres of different concentrations, less than 20% of cells died after 12 h. Therefore, the results demonstrate that the obtained products have a low cytotoxicity.

Figure 9 The CCK-8 assay results of the obtained ZnO:Gd@HA nanospheres.

Conclusions

ZnO:Gd@HA nanospheres are successfully prepared and used as a novel lymphatic system specific multi-modal contrast agent. *In vitro* characterizations reveal that the ZnO:Gd@HA nanospheres possess suitable and stable physicochemical properties, high quantum yield and relaxation effectiveness and low cytotoxicity. Furthermore, the contrast agent shows an excellent *T*₁-weight signal compared to traditional MRI contrast agents, and significantly higher selectivity to some kinds of lymphoma. Therefore, we believe that the as-synthesized contrast agent is a potential candidate for diagnosis and therapy of lymphatic system.

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