Original Article
The construction of multifunctional nanoparticles system for dual-modal imaging and arteriosclerosis targeted therapy

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Abstract: Atherosclerosis is a major risk factor for the development of cardiovascular disease. Unfortunately, due to relatively low sensitivities and poor resolution, the results of surgical resection are often largely unsatisfactory. Moreover, many chemotherapeutic agents, such as curcumin (Cur), are restricted by the low blood-brain barrier (BBB) permeability. Recently, nanotechnology proposes new opportunities to overcome these treatment barriers. In this study, superparamagnetic iron oxide nanoparticles (SPIO) was prepared by the high-temperature solid-state method, and then loaded into amphiphilic polymer DSPE-PEG to form SDP nanoparticles by hydrogen bonding in oil phase. The curcumin was encapsulated in SDP nanoparticles by self-assembly. Finally, vascular cell adhesion molecule-1 (VCAM-1) and Cy5.5 were conjugated on into SDP/Cur nanoparticles by amidation reaction. The average particle size of the prepared multifunctional SDP-VCAM-1/Cur/Cy5.5 nanoparticles is 124.4 nm, which can provide the sustained release of Cur. Moreover, the nanoparticles are proved to have superparamagnetic properties and fluorescence properties. In vitro cell experiments show that nanoparticles have excellent biocompatibility, blood compatibility and macrophage targeting. These results show that SDP-VCAM-1/Cur/Cy5.5 nanoparticles can be used not only as dual imaging probe for magnetic resonance (MR) and fluorescence imaging, but also as carriers to deliver chemotherapeutic drugs to inflammatory tissue, thus providing a promising opportunity for the treatment, molecular imaging and targeted therapy in atherosclerosis due to their established specificity and safety.

Keywords: SPIO NPs, MRI, fluorescence imaging, VCAM-1, atherosclerosis

Introduction

With the development of society and the increase of life pressure, cardiovascular disorder has gradually become the most important factor threatening human health, in which atherosclerosis had extremely severe destructive [1, 2]. Atherosclerosis can cause local vascular lesions, which can lead to a series of complications, such as stroke, thrombus and even threaten our life [3]. In the early process of plaque formation, if the lesion area can be well diagnosed and treated, it can effectively reduce the damage of atherosclerosis to human beings [4]. Although certain therapeutic methods, including stent implantation, have been developed for atherosclerosis treatment, most of the patients are in the mid-late stage of the disease at the time of diagnosis [5, 6]. This is due to the wall thickening and lesion develops to the outside of the wall rather than the lumen in the early stage of the disease [7]. Thus, current diagnosis and treatment exhibit hysteresis. In early phase of plaque formation, the early diagnosis and treatment of atherosclerotic plaques by imaging will contribute to reduce the threat of atherosclerosis to human health.

Among the many imaging methods, magnetic resonance imaging (MRI) has become a powerful imaging technology recognized [8]. The imaging principle is the signal intensity, which is dependent on the relaxation of protons by an external magnetic field [9]. Currently, MRI is classified into different categories based on relaxation properties, i.e., longitudinal relaxation (T1) and transverse relaxation (T2) [10]. In general, the complex of gadolinium (Gd) is
selected as the contrast agent for T1 imaging, and iron oxide nanoparticles (Fe₃O₄) is often selected for T₂ imaging [11, 12]. The biological toxicity of Fe₃O₄ is much smaller than that of Gd, which has gradually become the mainstream contrast agent [13]. Superparamagnetic iron oxide nanoparticles (SPIOs) are excellent magnetic T₂ imaging contrast agents for medical use [14]. Its high magnetic capacity is mainly focused on two aspects: a) the size ranges of SPIOs is mostly between 10 and 30 nm owing to the strong quantum-confinement effect [15]; b) the surface effect (easy to agglomerate) was caused by particle boundary crystal structure defects [16]. Thus, many investigators generally attach a layer of oleamine or oleic acid on the surface of SPIOs to ensure the stability of SPIOs [17]. However, most of the biological environment is aqueous phase, and this kind of oil-soluble iron oxide often needs further modification before use. The common methods of modification include amphiphilic polymer micelles, bifunctional ligands, multi-dentate polymers, silicon shell coating and so on [18]. Previous studies have shown that when SPIOs are incorporated into DSPE-PEG micelles, SPIO nanoparticles could achieve longer circulation half-life, favorable biocompatibility and better surface modification [19].

However, previous MRI single-mode imaging system is often difficult to distinguish the imaging effect in the area with similar gray level due to the imaging mechanism. This requires that we select the regions that can specifically distinguish the gray levels of similar tissues in the choice of the second imaging mode to make up for the deficiency of MRI imaging [20]. Fluorescence imaging exhibits excellent performance, but it should be combined with the advantages of MRI imaging due to its poor penetration [21]. The preparation of a dual model probe with both MRI imaging and fluorescence imaging function has major research implications. The most commonly used fluorescent dyes for bio imaging are fluorescein, cyanine dyes, phthalocyanine dyes, rhodamine fluorescent dyes and so on [22]. Cyanine dyes are widely used in DNA sequencing, staining DNA, biomolecule labeling, in vivo imaging, photodynamic therapy and so on [23]. By conjugating a fluorescent dye to a monoclonal antibody, fluorescence imaging can penetrate several centimeters with near infrared light (NIR, 600 nm to 1000 nm wavelength), which is more suitable for small-animal fluorescence imaging in vivo. Among them, Cy5.5-NHS is more commonly used in Cy series cyanine dyes, which can directly connect with small peptides through chemically bind [24]. However, there are several major challenges for Cy5.5 in imaging applications such as the trend of rapid accumulation and degradation in aqueous solution, rapid removal from the body, prone to photo bleaching and lack of targeting [25]. These limitations can be overcome by introducing Cy5.5 into the nanoparticle (NP) delivery system [26]. Recently, a large number of polymer nanoparticles and inorganic nanoparticles have been introduced to solve the inherent problems such as Cy5.5 degradation, rapid blood clearance and in vivo imaging [27, 28].

The target molecule is also another important part of the nanoparticle targeting diagnosis system. Prior studies have shown that vascular cell adhesion molecule-1 (VCAM-1) is one of the most obvious signs of early atherosclerotic plaque, which is expressed by endothelial cells that form plaques [29]. The overexpression of VCAM-1 is found in only plaque-forming areas in aorta, and is considered to be a specific biomarker of area of aortic lesion formation [30], which makes targeting specificity the ideal choice for drug carriers to gather in atherosclerotic plaques [31]. VCAM-1 targeting has been used to potentiate the delivery of MRI and SPECT contrast agents for imaging at different formation stages of atherosclerosis plaque [32].

In this report, we synthesized and evaluated novel nanoparticles for the treatment and improvement of atherosclerosis, which integrates dual-modal imaging with MRI/fluorescent imaging and chemotherapy. As shown in Scheme 1, SPIO nanoparticles and curcumin (Cur) were coated on DSPE-PEG by double-solvent exchange method to obtain SDP/Cur nanoparticles, and then VCAM-1-targeted peptides and Cy5.5-NHS were grafted on DSPE-PEG by covalent bond to obtain SDP-VCAM-1/Cur/Cy5.5. Both SPIO NPs and Cy5.5 play a dual role in MRI/fluorescence bimodal imaging. The synthesis, size and spherical morphology of the polymer were confirmed by ¹H NMR and Fourier transform infrared (FTIR) spectroscopy.

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The fluorescence properties, magnetic properties and drug loading capacity of the nanoparticles were analyzed by fluorescence spectra, VSM and UV spectra, which has potential biological application. The drug release rate of nanoparticles on atherosclerotic plaque was obtained by simulating the microenvironment of atherosclerotic plaque. Finally, the cytotoxicity of nanoparticles was evaluated via CCK-8 assays and targeting of nanoparticles was demonstrated to the uptake in macrophages.

Material and methods

Materials and reagents

Iron (III) acetylacetonate, manganese (III) acetylacetone, 1,2-hexadecanediol, oleic acid, oleylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). DSPE-PEG-NH$_2$ (purity 95%) was obtained from Aladdin Chemical Company (Shanghai, China). Curcumin (Cur, purity > 99%) was purchased from Shanghai Winherb Medical S&T Development Co., Ltd. (Shanghai, China). Vascular cell adhesion molecule 1 (VCAM-1) was purchased from Apeptide Bio-Technology Co., Ltd. (Shanghai, China). DMEM and FBS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trypsin-EDTA (0.25%), streptomycin and penicillin were purchased from Thermo Fisher Scientific. Cell Counting Kit 8 (CCK8) was purchased from Kaji Biological Technology Development Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) and DMEM/F12 cell culture mediums were purchased from Gibco. Unless otherwise noted, all other reagents were of analytical grade.

Preparation of the super-paramagnetic Fe$_3$O$_4$ nanoparticles (SPIO)

Superparamagnetic nanoparticles (SPION) were prepared by the high-temperature solid-state method as previously described [33]. Briefly, iron (III) acetylacetone (2 mmol), manganese (III) acetylacetone (2 mmol), 1,2-hexadecanediol (10 mmol), oleic acid (3 mmol) and oleylamine (3 mmol) were dissolved in 10 mL of benzyl ether in a reaction flask using a magnetic stirrer at 200°C for 2 h under argon. Following this, the reaction mixture was further heated to reflux (~300°C), stirred for 2 h. After cooling to room temperature, anhydrous ethanol (40 mL) was added and centrifuged (8000 rpm, 10 min) to obtain the brown-black complex. Precipitate was extracted and added into 10 mL of hexane, following add 0.05 mL oleic acid and 0.05 mL olemamide to remove undissolved impurities by centrifugation (8000 rpm,
Finally, the final product was collected by centrifugation at 8,000 rpm for 10 min, washed three times with absolute ethanol, and further dispersed in n-hexane.

**Synthesis of compounds**

**Preparation of SDP and SDP/Cur nanoparticles:** The SPIO@DSPE-PEG (SDP) nanoparticles were prepared with slight variations according to the method described [34]. SPIO NPs (10 mg, iron content) dissolved in 1 mL of tetrahydrofurane (THF) was mixed with DSPE-PEG-NH$_2$ (30 mg) dissolved in THF at room temperature for 15 min. Then, 20 mL ultrapure water was slowly and sequentially dropped into the mixed solutions under vigorous stirring, and performed with ultrasonication for 30 min. The obtained solution was dialyzed for 24 h in the distilled water with a dialysis bag (cut-off molecular weight, 3,500 Da). The whole preparation processes of SPIO@DSPE-PEG/Cur (SDP/Cur) nanoparticles were same as SDP except added 10 mg SDP and 5 mg Curcumin.

**Preparation of SDP-VCAM-1/Cur nanoparticles:** VCAM-1 (3 mg, 0.003 mmol) was dissolved in MES buffer (1 mL, 0.1 M). The carboxyl groups of VCAM-1 were activated with EDC solution (1.2 mg, 1 mg/mL) in MES buffer for 1 h to adjust its pH to 7.2, followed by the addition of the NHS solution (1 mg/mL, 25 μL) in PBS under stirring for 30 min to form active esters. Further, SDP/Cur (8 mg, 0.003 mmol) in PBS was added and stirring at room temperature for 4 h. The obtained solution was dialyzed for 4 h in the distilled water with a dialysis bag (cut-off molecular weight, 2,000 Da), lyophilized, and stored at 4°C. Approximately 4-16 mg samples were sealed in open ceramic and heated from 25 to 600°C at a heating rate of 10°C/min under nitrogen atmosphere. The fluorescent signals of nanoparticles were detected by a spectrofluorometer (678 nm excitation, 694 nm emission; Shimadzu RF530PC, Kyoto, Japan).

**In vitro release profile of curcumin**

Encapsulation efficiency (EE%) and drug-loading efficiency (DL%) of curcumin in SDP/Cur were determined. Firstly, a calibration curve was set with Cur at concentrations of 1.25, 1.56, 2.50, 3.12, 6.25, 10.00, 12.50, 20.00, 25.00, 40.00 μg/mL analyzed using a UV-Vis spectrophotometer (Shimadzu UV-3100PC, Kyoto, Japan) in the wavenumber range 300-
600 nm. The percentage of EE and DL were calculated according to the following formula:

\[
EE(\%) = \frac{\text{weight of the drug in nanoparticles}}{\text{weight of initially added drug}} \times 100%.
\]

\[
DL(\%) = \frac{\text{weight of the drug in nanoparticles}}{\text{weight of the nanoparticles}} \times 100%.
\]

The drug release study of nanoparticles was conducted using a dialysis bag method described by Xie et al. [35]. Briefly, SDP/Cur nanoparticles (20 mg) were placed in a dialysis bag (cut-off molecular weight, 3500 Da) and the tube was rotated in 200 mL of PBS solution at pH = 7.4 at 37°C. At the prescribed time points, 1 mL of the buffer solution was taken to analyze and replaced with the amount of the fresh PBS. The concentration of the released Cur was directly determined by UV-Vis spectrophotometer with the calibration curve of Cur. The cumulative amount of Cur in SPIO@DSPE-PEG/Cur nanoparticles was calculated according to the formula:

\[
\text{Cumulative amount released (\%)} = \frac{\text{weight of drug released}}{\text{weight of drug in nanoparticles}} \times 100%.
\]

**Biocompatibility evaluation**

Mouse peritoneal macrophages (RAW264.7) cells were cultivated for in vitro experimental studies. RAW264.7 cells was cultured in DMEM medium supplemented with 10% F12, 100 units/mL penicillin and 100 mg/mL streptomycin in 25 cm² cell culture flasks. All these cells were cultured in 5% carbon dioxide at 37°C. Cells were seeded on 24-well plates at 5 × 10⁴ cells per well and cultured until reaching confluence. Then, RAW264.7 cells were incubated in DMEM mediums containing SDP/Cy5.5, SDP/Cur/Cy5.5 and SDP-VCAM-1/Cur/Cy5.5 nanoparticles. At the prescribed time points, the medium was removed and 100 μL of fresh medium supplemented with 10 μL of CCK-8 solution was then added into each well. After incubation 2 h at 37°C, the absorbance (optical density, OD) was measured at a wavelength of 450 nm on a microplate reader. The cell viability was calculated according to the formula:

\[
\text{Cell viability (\%)} = \frac{A_s - A_n}{A_p - A_n} \times 100%.
\]

where \(A_s\), \(A_n\), and \(A_p\) are the absorbance of the sample, negative control and blank group, respectively. Control and blank group, respectively.

**Hemolysis test**

Blood was collected from SD rat and transferred to sodium citrate anticoagulant tubes at a 1:9 ratio. Then, red blood cells (RBC) were separated from plasma, and then were suspended in PBS to prepare a 16% (v/v) red blood cell suspension. Then the solution was mixed with 5 mL containing different concentrations of SDP, SDP/Cur/Cy5.5 and SDP-VCAM-1/Cur/Cy5.5 at 37°C for 6 h, then centrifuged (1000 rpm, 5 min) to sediment the red blood cell. Finally, the absorbance of supernatants was measured at a wavelength of 540 nm on a microplate reader. RBC which incubated with pure water and PBS were used as positive control and negative control, respectively. Hemolysis was calculated according to the formula:

\[
\text{Cell viability (\%)} = \frac{A_s - A_n}{A_p - A_n} \times 100%.
\]

where \(A_s\), \(A_n\) and \(A_p\) are the absorbance of the sample, the negative control, and positive controls, respectively.

**Coagulation studies**

Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) are basic tests to record the time taken for the activation of blood clotting. Briefly, blood samples were collected and immediately centrifuged (3000 rpm, 20 min) to obtain the serum. At the prescribed time points, 5 mL of serum was allowed to mix with sample solution, then incubated at 30°C for 10 min. Finally, the PT or APTT reagents (100 μL) were added, and APTT and PT values of the samples were determined full-automatic blood coagulation analyzer (Sysmex Corporation, Kobe, Japan).

**Cellular uptake of nanoparticles**

RAW264.7 cells were cultured in confocal cell dishes at a density of 2 × 10⁵ cells per well until they became completely adherent. Then the culture medium was exchanged by fresh medium, and the cells were treated with SDP/Cy5.5 and SDP-VCAM-1/Cy5.5 nanoparticles. Follow-
Incubation for 4 h and 12 h, the cultured cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde for 20 min. Then, cells were washed twice using PBS, followed nucleus staining with DAPI for 15 min. Intracellular localization was observed by confocal laser scanning microscopy.

**Statistical analysis**

All the data were presented as mean ± standard deviation (SD), and the statistical comparisons between groups were analyzed via one-way ANOVA statistical analysis by GraphPad Prism 7 software. The values of * (P < 0.05), ** (P < 0.01), and *** (P < 0.001) were determined to be statistically significant.

**Results and discussion**

**Synthesis and characterization**

Scheme 1 shows a schematic diagram of the preparation of Cy5.5 labeled and VCAM-1 targeted DSPE-PEG and magnetic nanoparticles loaded with curcumin micelles (SDP-VCAM-1/Cur/Cy5.5). Firstly, Superparamagnetic iron oxide nanoparticles (SPIO) was prepared by the high-temperature solid-state method and then loaded into amphiphilic polymer DSPE-PEG by hydrogen bonding in oil phase. Secondly, curcumin (Cur) was assembled on SDP micelles by self-assembly method. Through the amidation reaction between the carboxyl groups on VCAM-1 peptide and the amino groups on SDP using EDC and NHS, the VCAM-1 loaded magnetic nanomicelles (SDP-VCAM-1/Cur) were obtained. Finally, the SDP-VCAM-1/Cur/Cy5.5 nanomicelles were prepared by the covalent-coupling reaction between the amino groups on SDP/Cur-VCAM and NHS on Cy5.5 hydroxysuccinimide ester.

The FTIR spectra of SPIO nanoparticles is shown in Figure 1A. The two peaks at 1570 cm\(^{-1}\) may be attributed to the C = O symmetric stretching vibration and the C = N asymmetric stretching vibration, which is related to inorganic carboxylate. The two peaks at 2920 cm\(^{-1}\) and 2850 cm\(^{-1}\) indicate the C-H telescopic vibration in oleic acid molecules. VCAM-1 was connected to DSPE-PEG through the amidation reaction between the carboxyl of VCAM-1 and the amine of DSPE-PEG as shown in Figure 1B. Characteristic absorption peaks of DSPE-PEG/VCAM-1 at 1527 cm\(^{-1}\) of N-H, 1438 cm\(^{-1}\) of C-N and 1664 cm\(^{-1}\) of C = O indicated the formation of amide bond. Meanwhile, characteristic absorption peaks of DSPE-PEG/Cy5.5 at 1676 cm\(^{-1}\) of C = O indicated the for-
mation of amide bond. Cy5.5 was connected to DSPE-PEG via the amidation reaction between the NHS of Cy5.5 and the amine of DSPE-PEG. As is shown in Figure 1C, chemical shift at 3.67 ppm presented the -CH\(_2\)- of PEG chain, and chemical shifts at 2.68 ppm was the characteristic peaks of VCAM-1. VCAM-1 and Cy5.5 were grafted to DSPE-PEG via amide reaction and characteristic \( ^1 \)H-NMR spectra was displayed. By mass spectrometry, we confirmed that VCAM-1 is covalently conjugated to SDP nanoparticles (Figure 1D-F).

XRD results further confirmed that the SPIO nanoparticles were spinel-like materials (Figure S1A). It can be seen that SPIO nanoparticles exhibited obvious characteristic peaks located at: \( 2\theta = 30.2^\circ, 35.6^\circ, 43.0^\circ, 53.4^\circ, 57.1^\circ \) and \( 62.5^\circ \), corresponding to the (220), (311), (400), (422), (511) and (440) crystal face of inverse spinel \( \text{Fe}_3\text{O}_4 \), with a face-centered cubic phase. The chemical structure of the DSPE-PEG, DSPE-PEG/VCAM-1, DSPE-PEG/Cy5.5, DSPE-PEG/VCAM-1/Cy5.5 polymers was confirmed by FTIR and \( ^1 \)H NMR, as presented in Figure 1B, 1C.

As shown in Figure 2A, TEM images of SPIO exhibit a good distribution, well crystallography and clear spheres. Meanwhile, the SDP and SDP/Cur nanoparticles are spherical shape or sphere-like morphology with obvious core-shell structure, and uniformly dispersed iron oxide particles visible in the shell (Figure 2B, 2C). For SPIO nanoparticles, the average particle diameter was \( 10.5 \pm 0.78 \) nm (Figure 2D). The size measured by DLS is consistent with the hydrodynamic diameter estimated by TEM. For SDP nanoparticles, the average particle diameter was \( 106.4 \pm 12.7 \) nm (Figure 2E). The diameter of SDP/Cur increased to \( 124.4 \pm 2.48 \) nm (Figure 2F). These results showed that there was no significant difference in the morphology of SDP/Cur after Cur loading. A small size of nanoparticles (10-200 nm) is beneficial to enhance its ability to the tumor/inflamed regions accumulation and retention, and thus to avoid clearance by the body’s defense system-the reticuloendothelial system (RES) [36]. Prior study reported that nanoparticles are easily engulfed by tumor and inflammatory cells via endocytosis, which will maximize efficacy of treatment [37].

**Ultraviolet-visible and fluorescent spectrometry**

Figure 3A shows the UV-vis spectra lines of DSPE-PEG and its derivatives. It can be seen that the UV absorption peak of pure Cur and Cy5.5 appear near 426.5 nm and 673.5 nm, respectively. There is no obvious characteristic absorption peak in DSPE-PEG and SDP.
However, SDP/Cy5.5 and SDP/Cur/Cy5.5 nanoparticles appear a characteristic absorption peak at 673.5 nm, indicating that the NPs maintained their previous optical performance after blending the SDP polymer. In addition, the intensity of UV absorption peak of SDP/Cur/Cy5.5 at the same concentration is slightly lower than pure Cy5.5, indicating that metals have a certain quenching effect on fluorescent molecules. Figure 3B shows the fluorescence spectra lines of DSPE-PEG and its derivatives. Comparing these two curves one can clearly find that the characteristic peaks of SDP/Cy5.5 and SDP/Cur/Cy5.5 nanoparticles at 690 nm are consistent with pure Cy5.5.

Table 1. The encapsulation efficiency (EE%) and drug-loading efficiency (DL%)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total mass (μg)</th>
<th>Added Cur mass (μg)</th>
<th>Measured Cur mass (μg)</th>
<th>Drug-loading efficiency (DL%)</th>
<th>Encapsulation efficiency (EE%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDP/CUR</td>
<td>100</td>
<td>33.3</td>
<td>25.6</td>
<td>25.6 %</td>
<td>76.9%</td>
</tr>
<tr>
<td>SDP-VCAM-1/CUR</td>
<td>100</td>
<td>33.3</td>
<td>25</td>
<td>25.0 %</td>
<td>75.1%</td>
</tr>
</tbody>
</table>

In vitro release study

The UV full wavelength scanning of Cur with different concentration was shown in Figure 3C, and Cur standard curves are shown in Figure S1B. As shown in Table 1, encapsulation efficiency (EE%) and drug-loading efficiency (DL%) of Cur in SDP/Cur nanoparticles were 76.9% and 25.6%, respectively. After modification with VCAM-1 peptide, EE% and DL% of Cur in SDP-VCAM-1/Cur nanoparticles reached 75.1%, and 25.0%, respectively. The cumulative percentages of Cur release in SDP-VCAM-1/Cur nanoparticles were evaluated in PBS (pH 7.4) compared to free CUR, as shown in Figure

Figure 3. A. UV-vis spectra of different nanoparticles. B. PL spectra of different nanoparticles. C. UV-vis spectra of Cur at various concentrations. From this fit a wavelength of maximal absorption of ≈426.5 nm is obtained. D. Drug release profile of complexes at pH = 7.4. Data were given as the mean ± SD (n = 3).
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**3D.** The free Cur exhibited a “release burst” within the initial 4 hours and had a cumulative release percentage of 68%. The Cur in SDP-VCAM-1/Cur nanoparticles displayed a relatively more rapid release and is followed by a relative slow release. About 32% of the Cur from these NPs was released during first 4 hours, and about 80% over the 24-hour period. The faster cumulative Cur release at the early stage was caused by the hydrophobic Cur molecules adsorbed on the outer surface of the SDP nanoparticles. Afterward, the rate of drug release declined, nearly 20% of Cur were unreleased in SDP nanoparticles even after 24 h. The sustained release of Cur was due to larger amounts of assembled Cur (hydrophobic molecules) being encapsulated in DSPE-PEG.

**VSM**

Magnetic response is one of the important characteristic parameters to test magnetic materials [38]. Meanwhile, the hysteresis loops reflect the ability of magnetic materials to respond to an external magnetic field. As shown in Figure 4A, the magnetic hysteresis curves of all samples basically pass through the origin, indicating that the hysteresis loop were close to zero and have the superparamagnetic behavior. The saturation magnetization was found to be 56 emu/g for SPIO, which proves that the monodisperse SPIO have high saturation magnetization. The saturation magnetic strength of SDP decreases to 10 emu/g, which is due to the polymer coating. However, it still reserved superparamagnetic response to the external magnetic field and can be used for following magnetic resonance imaging.

**Thermogravimetric analysis**

The content of SPIO in SDP nanoparticles was analyzed by thermogravimetric analysis. As shown in Figure 4B, the weight loss of 70% in the temperature range of 150°C to 500°C corresponded to high temperature decomposition of DSPE-PEG. The remaining mass of SDP nanoparticles after 600°C belongs to SPIO, thus it can be calculated that the entrapment efficacy of SPIO in SDP-VCAM-1/Cy5.5 nanoparticles is 28%. Therefore, the SPIO concentrations of SDP nanoparticles were
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Figure 5. RAW264.7 cells viability after incubation with SDP/Cy5.5, SDP/Cur/Cy5.5 and SDP-VCAM-1/Cur/Cy5.5 at various concentrations (0.01-1 mg/mL) for 24 h by CCK-8 assay. Data were given as mean ± SD (n = 3).

more than adequate, which could be used as a perfect contrast agent for imaging and diagnosis. These results were consistent with the previous VSM results.

Magnetic resonance imaging of NPs

To assess the contrast enhancement of the SPIO nanoparticles, five different concentrations of Fe were used: (0.11 mM, 0.22 mM, 0.44 mM, 0.89 mM and 1.78 mM). As shown in Figure 4C, the imaging effect increased gradually as a function of added SPIO concentration. When the concentration is above 0.44 mM, it still has a good imaging effect. When the concentration is lower than 0.44 mM, the imaging effect is not suitable for complex clinical imaging. Meanwhile, MR signal (1/T2*) exhibited a linear upward tendency (Figure S1C). In addition, it can be found that the R2 relaxation rate was calculated to be 64.7 mM-1S-1, indicating that the modified SPIO still retained the original crystal structures [39]. These results indicated that SDP has potential clinical application value as a CT imaging contrast agent.

Cytotoxicity of nanoparticles

The cytotoxicity of nanoparticles is an important index influencing the application of biological research, so the cytotoxicity of the nanoparticles was evaluated in Mouse macrophage cell line (RAW264.7) using the CCK8 assay. The viability of macrophage cell after incubation with SDP/Cy5.5, SDP/Cur/Cy5.5 and SDP-VCAM-1/Cur/Cy5.5 nanoparticles at varying concentrations was shown in Figure 5. No significant difference in cell viability was observed at various concentrations. When the concentration of nanoparticles was lower than 0.5 mg/mL, over 90% of cell viability was maintained, which satisfying the toxicity requirements as a contrast agent, providing a good guarantee for further biological experimental designs. Thus, the prepared nanoparticles did not induce obvious cytotoxicity, and showed excellent biocompatibility in vitro.

Blood compatibility assay

To evaluate whether the prepared nanoparticles were suitable for intravenous administration, the hemolytic test was conducted using mouse red cell hemolysis test in vitro. As shown in Figure 6A, the hemolysis ratio increased with the increase of the concentration of NPs. Furthermore, the hemolysis rate of SDP-VCAM-1/Cur/Cy5.5 was slightly higher compared with SDP/Cy5.5. In addition, hemolysis rate of SDP-VCAM-1/Cur/Cy5.5 nanoparticles was 7.5% at the concentration of 0.5 µg/mL. According to previous reports in the literature, the hemolysis rate of materials was higher than 5%, which will cause hemolysis to red blood cells (RBCs) [40]. This result indicates that NPs ranging from 0.1 to 0.5 mg/µL had a minor effect on the RBCs, showing the good hemocompatibility.

APTT was used to assess endogenous coagulation pathway and PT was used to evaluate exogenous coagulation pathway, which are important index of material hemocompatibility evaluation. The effect of NPs on the coagulation times and blood PLT level (APPT and PT) are presented in Figure 6B. There was not a significant difference in APTT and PT in SDP-VCAM-1/Cur/Cy5.5 compared with PBS at concentrations below 0.5 mg/µL. As reported in the literature, the normal ranges for APPT and PT were 11-14 s and 27-40 s [41]. Our results showed the SDP-VCAM-1/Cur/Cy5.5 at concentrations < 0.5 mg/µL did not induce intrinsic coagulation pathway and extrinsic coagulation pathway, exhibiting satisfactory hemocompatibility.

Cellular uptake tests

To verify the targeting of nanoparticles in biological applications, the cellular uptake behavior of nanoparticles by RAW264.7 cell over 12 h
time period was monitored by the Confocal laser scanning microscopy (CLSM). As shown in Figure 7, the nuclei were stained with DAPI (blue), and the red fluorescent nanoparticles gather in the nuclear area. It is clear from CLSM image that the SDP/Cy5.5 nanoparticles have a small amount of fluorescence at excitation wavelength 678 nm, while the SDP-VCAM-1/Cur/Cy5.5 nanoparticles have a large amount of fluorescence, which confirm the effectiveness of the VCAM-1 target molecules. All results showed that the prepared SDP-VCAM-1/Cur/Cy5.5 nanoparticles have a
Cy5.5 nanoparticles have certain targeting ability in vitro and can be selectively taken up by macrophages.

Conclusions

Multifunctional SDP-VCAM-1/Cur/Cy5.5 nanoparticles were successfully constructed by conjugation of Cy5.5 and VCAM-1 into the DSPE-PEG coated SPIO and Cur for MR/fluorescence dual-modal imaging and chemotherapy of glioma. All prepared nanoparticles were characterized by their FT-IR, 1H-NMR spectra and mass spectral data, which confirmed the successfully loading of Cy5.5 and VCAM into the SDP nanoparticles. The TEM images and DLS dates confirmed the nanoparticles were uniform size and good dispersion with an obvious core-shell structure. The fluorescence spectra confirmed nanoparticles exhibited better stability and higher fluorescent intensity. The obtained nanoparticles showed super para-magnetism and presented good MRI imaging efficiency. The nanoparticles exhibited a sustained release of drugs compared with free curcumin. The CCK-8 assay confirmed that the nanoparticles have no effect on cell cytotoxicity and indicate the biocompatibility, which would be suitable for biomedical applications. Furthermore, the confoocal imaging data confirmed efficient targeting of the nanoparticles to the macrophages. Meanwhile, the prepared nanoparticles at concentrations < ~0.5 mg/µL exhibited good hemocompatibility, with a hemolysis rate less than 5%. Our results demonstrate that the prepared multifunctional nanoparticles are appropriate for use in pathological event in atherosclerosis, which provide a novel therapeutic way for atherosclerosis.

Disclosure of conflict of interest

None.

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References


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[34] Li L, Wu C, Pan L, Li X, Kuang A, Cai H and Tian R. Bombesin-functionalized superparamagnet-


cha I, Dörje F, Odenbach S, Lyer S, Lee G and Alexiou C. Development of a lauric acid/albu-


[38] Lin WC, Chuang CC, Chang CJ, Chiu YH and Tang CM. The effect of electrode topography on the magnetic properties and MRI applica-


[40] Song B, Yang L, Han L and Jia L. Metal ion-che-
lated tannic acid coating for hemostatic dress-

Figure S1. A. XRD of SPIO. B. The standard curve of Cur. C. $T_2$ relaxation rate ($1/T_2$) against Fe concentrations of SDP.