

# Research Article **Degraded Hyaluronic Acid-Modified Magnetic Nanoparticles**

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Received 4 October 2019; Revised 16 December 2019; Accepted 21 January 2020; Published 11 February 2020

Academic Editor: Ilaria Armentano

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Superparamagnetic iron oxide nanoparticles (SPIONs) conjugated with hyaluronic acid (HA) functional groups have potential applications as cell targeting materials. However, SPIONs incubated with high-molecular weight HA can result in severe agglomeration. In this work, we found that when modified with degraded HA (hyaluronan oligosaccharides (oHAs)), the nanoparticles were uniformly dispersed with small hydrodynamic sizes, and the oHA-modified SPIONs exerted minimal cytotoxicity. With the same functional groups as HA, the oHA-modified SPIONs may have various biomedical applications.

# 1. Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) have many uses in biomedical research, such as for drug delivery [1], magnetic resonance imaging [2], cancer cell targeting [3], size-related uses [4], magnetism [5], and optical performance [6]. Several SPIONs have been approved by U.S. Food and Drug Administration (FDA) to be the potential treatment of diagnosis and treatment [7]. Surface coating affects the application potential of the SPIONs, and the molecular structure, modification methods, and modification agent proportions lead to significantly different properties [8, 9]. Hydrophilic polymer coating can guarantee the colloidal stability of nanoparticles with electrostatic or steric repulsion, reduce the uptake regulation effect of reticuloendothelial cells, and extend the duration of action of nanoparticles in vitro. As modification of polyethylene glycol (PEG) helps nanoparticles to escape the uptake of the system and then to reach cells and start drug release [10], glutathione (GSH) can lower cytotoxicity and enhance T1 MRI characteristics [11, 12].

Hyaluronic acid (HA) plays an important role in cell proliferation, embryonic development, tumor cell migration, and wound repair [13, 14]. Currently known cell surface HA receptors include CD44 [15], receptor for hyaluronanmediated motility (RHAMM) [16], lymphatic vessel endothelial HA receptor (LYVE-1) [17], layilin [18], and hyaluronan receptor for endocytosis (HARE) [19]. Special RHAMM receptors are distributed on the cell surface, cytoskeleton, and mitochondria. When the cells are stimulated accordingly, the RHAMM receptors stored in the cells are transported to the cell membrane [19]. Therefore, HA-modified SPIONs are expected to have great potential in cell targeting applications; little research work about the modification of HA on SPIONs was reported.

In this work, we found that HA-modified nanoparticles became heavily aggregated; however, degrading highmolecular weight HA via chemical methods [20–22] produced hyaluronan oligosaccharides (oHAs), and the oHA-modified SPIONs were uniformly dispersed with small hydrodynamic sizes. oHA is reported to increase the flexibility of the extracellular matrix and increase cell growth and mobility during



SCHEME 1: The synthesis and probable coating of the SPIONs.

division and differentiation [23, 24]. Therefore, oHA-SPIONs with low toxicity may have applications in various biomedical fields.

#### 2. Experiment

2.1. SPION Preparation and Characterization. The raw materials used and their synthesis were reported in our previously published work [25]. In brief, SPIONs were synthesized by decomposing 0.7 g of Fe(acac)<sub>3</sub> (Tokyo Chemical Industry, Japan) in 15.0 g of polyethylene glycol (PEG; Aladdin, China) mixed with 0.3 g of polyetherimide (PEI; Aladdin) at  $260^{\circ}$ C for 1 h in an argon atmosphere. The reactants were cooled to  $60^{\circ}$ C, then washed three times successively with toluene and acetone. The SPIONs were then collected using a magnet placed under the container.

2.2. HA Degradation. HA (80 mg) was dissolved in 10 mL of water, then fully dissolved at  $4^{\circ}$ C overnight. Next, 1 mL of 16% sodium hypochlorite was added every 6 hours for the next 24 hours; then, the pH was adjusted to approximately 7.0 using 0.1 M HCl.

2.3. Modification of HA and oHA on the SPIONs. HA (20 mg, undegraded) and 10, 20, and 40 mg oHA (hereafter referred to as "m(SPIONs):m(oHA)") at ratios of 2:1, 1:1, and 1:2 were mixed with 20 mL of 1 mg/mL of the SPION aqueous dispersions (Scheme 1). The reaction was carried out for 5 h in a shaker at 60 rpm and 4°C. After incubating overnight at 4°C in a refrigerator, the mixture was dialyzed against deionized water for 120 h (MWCO 100,000 dialysis bag,

SpectrumLabs, USA). Samples were kept in a refrigerator at 4°C for later use.

2.4. Material Characterization. The samples were characterized using a Zetasizer Nano ZS90 (Malvern Instruments), a Quantum Design MPMS XL-7 superconducting quantum interference device, a PL-GPC 50 gel permeation chromatograph, a JEM-2100F transmission electron microscope (TEM), X-ray photoelectron spectroscopy (XPS), and a thermal gravimetric analyzer. The crystal structure of the nanoparticles was confirmed using a PANalytical X'Pert PRO powder X-ray diffractometer (XRD) with CuK $\alpha$  radiation ( $\lambda = 0.15406$  nm) with a scanning step of 0.017° in the 2 $\theta$ range of 20-60° at room temperature.

The X-ray photoelectron spectroscopy (XPS) of the dried sample was performed in vacuum on Thermo ESCALAB 250 to further characterize the coating materials. XPS is a technique which detects the organic surface elements of particles up to a 10 nm thickness or inorganic surface elements of particles up to 3 nm.

2.5. Cytotoxicity Assay. The PC-12 cell line, derived from a transplantable male rat adrenal pheochromocytoma, was purchased from the Chinese Academy of Sciences Stem Cell Bank (Shanghai, China). The cytotoxicities of the SPIONs and oHA-SPIONs on the PC-12 cell lines were evaluated via CCK-8 assay after incubation with different concentrations (0, 5, 10, 25, 50, 80, 100, and 200 [Fe]  $\mu$ g/mL). PC-12 cells were seeded into 96-well culture plates at a density of 8000 cells per well and incubated



FIGURE 1: Molecular structure of hyaluronic acid.



FIGURE 2: Molecular weight distributions before and after HA degradation.

at 37°C in 5% CO<sub>2</sub> for 24 h. Next, 0–200 [Fe]  $\mu$ g/mL of SPIONs and oHA-SPIONs in complete RPMI (Roswell Park Memorial Institute) medium 1640 were added and incubated for another 24 h. The cells were then washed three times with 0.01 M phosphate-buffered saline; then, complete culture medium containing 10% CCK-8 was added. After incubating for 3 hours, the absorbance was measured at 450 nm using a microplate reader, and cell viability was calculated by absorbance to evaluate the cytotoxicity of the SPIONs.

#### 3. Results and Discussion

3.1. Molecular Weight Measurement. Figure 1 shows the molecular structure of hyaluronic acid. After degrading high-molecular weight HA in sodium hypochlorite, the molecular weight change of the HA was tested under the aqueous environment of PL-GPC 50 (Figure 2). The molecular weight of HA before degradation was between 200 kDa and 2.5 MDa, the distribution was uneven, and the viscosity

was thick. The molecular weight of oHA after HA degradation was concentrated around 26 kDa, and the viscosity was low with good fluidity.

3.2. Morphological Characterization, Colloidal Stability, and Crystalline Structure of the Nanoparticles. Figure 3 shows TEM photographs and particle size distributions of the SPIONs, HA-SPIONs, and oHA-SPIONs. The HAmodified nanoparticles showed significant agglomeration (Figure 3(b)), while the unmodified and oHA-modified nanoparticles (Figures 3(a) and 3(c)-3(e)) had good dispersibility and a uniform particle size distribution. The iron oxide core sizes of the particles were calculated using ImageJ software to measure 200 nanoparticles. The average particle sizes of the SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2 were  $7.93 \pm 2.52$ ,  $9.47 \pm 2.18$ ,  $10.19 \pm$ 2.34, and  $11.09 \pm 2.48$  nm, respectively (Figures 3(a) and 3(c)-3(e)). A little aggregation might take place after the modification of oHA.

3.3. Hydrodynamic Size Distribution Profiles and Zeta Potentials. Figure 4 shows the hydrodynamic sizes of the SPIONs, HA-SPIONs, and oHA-SPIONs. The highmolecular weight HA-modified nanoparticles showed severe agglomeration (Figure 4(a)), and the hydrodynamic size profile showed double peaks of approximately 128.2 nm and 5569.4 nm. These data were measured after filtering the HA-SPION aqueous dispersion through a  $0.22 \,\mu m$  filter, suggesting that the nanoparticles or HA molecular agglomerated even after being uniformly dispersed under force. The average hydrodynamic sizes of the SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2 were 24.2, 26.7, 32.1, and 69.1 nm, and their zeta potential values were +24.3, -12.1, -17.6, and -23.4 mV, respectively. The increment of hydrodynamic sizes and the decrease of zeta potentials show the modification of oHA on the SPION surface [26, 27]; the negative zeta potentials are caused by ionized -COOH groups stemmed from oHA [28].

3.4. Surface Characterization of the Nanoparticles. To confirm that the oHA was grafted onto the SPIONs, samples of freeze-dried oHA-SPIONs were collected and tested via



FIGURE 3: TEM images, size distributions, and XRD spectra of the SPIONs (a), HA-SPIONs (b), oHA-SPIONs-2:1 (c), oHA-SPIONs-1:1 (d), and oHA-SPIONs-1:2 (e).

XPS. The spectrum decomposition was performed using the XPSPEAK41 program with Gaussian functions after subtraction of a Shirley background; the ratio between the Lorentzian and Gaussian functions is 60%. Figure 5(a) shows the XPS spectra of the oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, showing peaks at 711.0 eV, 530.0 eV, 400.0 eV, and 286.0 eV, corresponding to Fe 2p, O 1s, N 1s, and C 1s [29], respectively. Figure 5(b) shows the fitted peaks of N 1s for the oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2. The peaks at 399.2 eV, 400.69 eV, and 401.1 eV correspond to the C-N, C=O-N, and C-NH<sub>3</sub> groups, respectively. The XPS results showed that the binding energies of oHA were the same as those of HA, indicating that their structures were not obviously changed after degradation. The C=O-N group indicated that the SPION surface was modified with oHA



FIGURE 4: Hydrodynamic size distribution profiles of the HA-SPIONs (a) and SPIONs and oHA-SPIONs (c); zeta potentials of the HA-SPIONs (b) and SPIONs and oHA-SPIONs (d).

[30, 31]. As the weight proportion of the oHA increased during modification, the C-N bond proportion decreased (Figure 5(b)), and the C=O-N and C-NH<sub>3</sub> group proportions increased accordingly.

3.5. Magnetic Properties and Thermogravimetric Analysis of the Nanoparticles. Figure 6 shows the magnetic properties and thermogravimetric analysis of the SPIONs and oHA-SPIONs [32]. Assuming that the weight of oHA on the oHA-SPIONs equals A, the SPIONs weight is B, and the oHA-SPIONs weight is C, the formulas,  $(A + B)/C = W_1$ and  $B/(C - A) = W_2$ , become  $A/C = (W_1 - W_2)/(1 - W_2)$ . This formula can be used to determine the amounts of oHA modified on the SPIONs as 20.2%, 32.3%, and 38.6% for the oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively.

The saturation magnetizations of the SPIONs and the oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2 were 55.0, 52.34, 42.5, and 33.68 emu/g, respectively. From the thermogravimetric analyses (Figure 6(a)), it can be calculated that there are approximately 85.10%, 75.15%, 63.18%, and 56.76% of pure iron oxide in

SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively, so the saturation magnetizations of pure iron oxide nanoparticles can be recalculated as 65, 69, 67, and 60 emu/g, respectively. The four nanoparticles had a small coercivity (<25 Oe) at 300 K (Figure 6, b-1); thus, all nanoparticles showed excellent superparamagnetic properties [33]. As the organic layer on the nanoparticle surface increased, the saturation magnetization decreased.

3.6. Cytotoxicity of SPIONs and oHA-SPIONs In Vitro. In vitro cytotoxicities of the SPIONs and oHA-SPIONs-1:2 were measured via CCK-8. The cell viabilities of the SPIONs and oHA-SPIONs-1:2 were greater than 85%, so SPIONs and oHA-SPIONs-1:2 had low cytotoxicity (Figure 7).

#### 4. Conclusion

We synthesized superparamagnetic iron oxide nanoparticles conjugated with HA and oHA. Modifying high-molecular weight HA on the nanoparticle surface caused severe agglomeration, but the degraded HA did not affect the



FIGURE 5: (a) XPS spectra: survey of the oHA-SPIONs, where a-1, b-1, and c-1 correspond to oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively. (b) XPS spectra: N 1s of the oHA-SPIONs wherein a-2, b-2, and c-2 correspond to oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively. (c) XPS spectra: Fe 2p of the oHA-SPIONs wherein a-3, b-3, and c-3 correspond to oHA-SPIONs-2:1, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively.



FIGURE 6: (a) Thermogravimetric curves of the SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2 from 0 to 600°C. (b) Hysteresis loops of the SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2 at 300 K. b1–b4 are the partial enlarged views of (b), where b-1, b-2, b-3, and b-4 correspond to the SPIONs, oHA-SPIONs-2:1, oHA-SPIONs-1:1, and oHA-SPIONs-1:2, respectively.



FIGURE 7: Cell viabilities of PC-12 cells after incubation with 0–200  $\mu$ g/mL SPIONs or oHA-SPIONs-1:2 for 24 h. Statistical significance was evaluated using one-way analysis of variance followed by post hoc comparison of the means using Fisher's least significant difference test, n = 6. \*P < 0.05, compared with the control group.

dispersibility of the nanoparticles. The nanoparticles were uniformly dispersed with high modification and low cytotoxicity, which satisfies the requirements for surface modification of the nanomaterials. This work provides a reference for modifying nanoparticles with functional groups of highly viscous materials, such as HA, while maintaining a small hydrodynamic size.

### **Data Availability**

All data are provided in full in the results section of the manuscript. Authors can find all the data in the results section for further analyses.

### **Conflicts of Interest**

The authors declare no competing financial interest.

## Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 51562007).

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