THE FABRICATION AND IDENTIFICATION OF HYALURONIC ACID-FORMULATED IRON OXIDE NANOCLOUDS

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ABSTRACT

We developed a simple emulsion and solvent-evaporation approach for clustering of iron oxide nanoparticles using hyaluronic acid (HA). Notably, this synthesis approach displaying highly production and waste reduction were the greener method. To obtain well-constructed clusters of iron oxide nanoparticles (IONP), the synthetic parameter of IO/HA ratio was investigated and were highly stable in storage at room temperature. In addition, IONP clusters with 500 μM remained the viability of HT29 cells over 84%. Thus, these clusters, which were prepared with the use of hyaluronic acid, can potentially serve as efficient contrast agents for magnetic resonance applications. Furthermore, due to the overexpress CD-44 which has a special interaction with HA on the tumor cell's membrane, these clusters also can potentially enhance the therapeutic effect.

Keyword: Iron oxide nanoparticles, Hyaluronic acid, Magnetic resonance imaging, Clusters

INTRODUCTION

Magnetic resonance (MR) imaging

Magnetic resonance (MR) imaging has been regarded as a non-invasive and powerful imaging tool that yields excellent soft-tissue contrast, has a high spatial resolution, and possesses tomographic capabilities without the hazard of ionising radiation.1 Among the contrast agents used in MR imaging, the practicability of employing superparamagnetic iron oxide (SPIO) nanoparticles as MRI T2-shortening agents for non-invasive cell-labeling or tumor detection in clinical practice has been demonstrated.2-5 In addition, magnetic nanoparticles can be functionalised and concurrently respond to a magnetic field for use as potential theranostic tools.6, 7

Self-assembling clusters

Recently, self-assembling clusters of metal nanoparticles have been extensively investigated in various fields. To prepare these clusters, various ionic surfactants, such as cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate, and polyethyleneimine, have been employed as stabilizers or emulsifiers to form water-dispersed spherical clusters.8-12 It has been demonstrated that SPIO clusters have higher transverse relaxivity values than individual SPIO nanoparticles, and thus they can act as potential contrast agents for T2-weighted MR imaging.8 This phenomenon can also be utilised to develop an ultrasensitive medical MR sensor.13 To develop these magnetic nanoparticles for clinical applications, the surface chemistry of the nanoparticles/clusters needs to be carefully considered because this characteristic greatly influences the particles’ fate within a biological system due to the mechanisms of cell recognition, biodistribution, immune response and nanotoxicity.14, 15 For example, the CTAB ionic surfactant can stabilise the SPIO cluster easily due to the interaction between specific functional groups and iron. However, these ionic surfactants are quite toxic to cells. Qiu and Chen et al. demonstrated that the CTAB coating on Au nanorod surfaces can cause cell apoptosis by altering the mitochondrial membrane potential and increasing the intracellular reactive oxygen species, thereby limiting the biomedical use of these nanorods.16 Thus, a facile method of SPIO cluster development using biocompatible stabilizers is necessary for biomedical applications.

In this study, we demonstrated a facile and greener synthesis to fabricate iron oxide nanoparticles (IONP) cluster formation

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using hyaluronic acid through the adjustment of the ratio of IO/HA. In the conventional procedure for cluster formation, size selection has to be carried out via centrifugation and/or filtration process which usually required time-consuming and low yield production. In comparison with the previous process of cluster preparation using ionic surfactants, non-ionic surfactant-assisted IONP clusters resulted in less cytotoxicity with more biocompatibility for biomedical applications.

**EXPERIMENTAL SECTION**

**Materials**

Iron (III) acetylacetonate (Fe(acac)₃), 1,2-hexadecanediol (90%), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (dimethylsulfoxide) were purchased from Sigma Aldrich (St. Louis, MO, USA). Oleylamine and phenyl ether (99) were obtained from ACROS (New Jersey, USA). Sodium phosphate dibasic (Na₂HPO₄) and sodium chloride (NaCl) were obtained from Tedia (OH, USA). Hyaluronic acid, Potassium chloride (KCl), potassium dihydrogenphosphate (KH₂PO₄), sodium bicarbonate (NaHCO₃) and oleic acid (99%) were obtained from Showa (Tokyo, Japan). The solvents were all dehydrated prior to use. For the cell culture studies, Modified Eagle’s Medium (MEM), fetal bovine serum (FBS) and 0.25% trypsin-EDTA were purchased from Gibco (Gibco-BRL, USA). The penicillin-streptomycin-neomycin solution and 10% formalin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double distilled water was used in all of the aqueous solutions.

**Synthesis of iron oxide nanoparticles**

IONP with diameters of approximately 6 nm were synthesised by a high-temperature thermal decomposition method in a nitrogen atmosphere, as previously reported. In brief, the Fe(acac)₃ (0.706 g) and 1,2-hexadecanediol (1.25 g) were mixed in diphenyl ether (20 ml) at 60 °C; the oleic acid (1.4 ml) and oleylamine (1.4 ml) were then added as surfactants into the mixed solution. This reaction was heated under precise control at the rate of 5 °C/ min until it reached 200 °C; the reaction was then maintained at this temperature for 10 minutes. Then, the reaction system was heated at a rate of 2 °C / min to 250 °C and refluxed for 3 hours. Finally, the reaction was precipitated using excess ethanol, centrifuged at 6,000 rpm, vacuum dried and then characterized with an X-Ray Powder Diffractometer (XRD, PANalytical X'Pert Pro MRD, Almelo, The Netherlands).

**Preparation of IONP@HA formulations**

To prepare the HA-assisted IONP (IONP@HA) clusters, the emulsion and solvent-evaporation method was employed; Briefly, controlling the ratio of dried IONP and HA, then were mixed in hexane (0.5 ml) at room temperature, and then 10 ml water was added to this mixture during ultrasonic treatment for 10 minutes to form an O/W emulsion. The hexane was then removed from the emulsified solution, by 240 °C temperature of the hotplate under constant stirring at 600 rpm (CO-PC420D, Corning, USA). After cooling down to the room temperature, the products were dialysed (MWCO: 3500) against water to remove free Surfactant HA.

**Characterization of IONP@HA formulations**

The particle sizes of the IONP@HA formulations were analysed by dynamic light scattering (DLS, ZS 90, Malvern Instruments Ltd.) at 25 °C, and their morphologies were observed by a JEOL 1400 transmission electron microscope (TEM, JEM 1400, JEOL Ltd., Japan) with an accelerated voltage of 120 kV. The iron concentrations of the IONP, IONP@HA clusters were determined quantitatively using an atomic absorbance spectrophotometer (GBC 932, USA).

**Cell culture condition and cell viability assay**

For the in vitro studies, HT29 cells were cultured in T75 flasks with MEM, 10% FBS and penicillin-streptomycin-neomycin and maintained in an incubator under a 5% CO2 atmosphere at 37 °C. To investigate the cytotoxicities of the IONP@HA
clusters, cells were first seeded into 96-well plates at a density of 8,000 cells per well and cultured for 24 h. The nanoparticles were then added into the wells with two-fold serial dilutions from 500 μM to 0 μM in a total volume of 0.1 ml at 37 °C. After a 24-hour incubation, the cells were washed twice with PBS, and then the cell viability was evaluated by MTT assay using a scanning multiwell ELISA reader at 485 nm (SpectraMax® M2e, Molecular Devices, USA).

RESULTS AND DISCUSSION

Characterization of IONP@HA clusters

The IONP was synthesised by a high-temperature thermal decomposition method, which used an oleic acid/oleylamine stabilizer; the XRD measurement demonstrated that the crystalline structure was consistent with the six characteristic peaks of Fe₃O₄.¹⁶ The IONP morphology observed with TEM showed a spherical shape with sizes of 8.10±0.97 nm using the SigmaScan Pro statistic software (Figure 1A). Hyaluronic acid has non-toxicity, low immune response, high biocompatible, biodegradation. More importantly, HA can combine with CD44, a protein express on the cell membrane specially overexpress on the tumor cell. It gives a effective targeting effect.¹⁸ The preparation of the IONP clusters with non-ionic, biocompatible surfactant HA was inspired by the nanocrystal stabilisation and emulsion approaches that lead to the coexistence of two immiscible liquids as a well dispersion under the assistance of the surfactant HA, which is utilised in this oil-in-water (O/W) system.⁸,¹⁹ According to our results, IONP solution can be emulsified and stabilised successfully using HA, which can align at the interface of oil and water to reduce the surface tension of the oil droplets in water.²⁰ Thus, hexane with a low boiling point was utilised to create an oil phase that could easily be removed by evaporation. This phase-transfer process lead to the condensation of IONP and the formation of IONP clusters inside of the HA micelles. IONP@HA clusters were generated after the evaporation of the hexane solvent with a digital-control hotplate at different temperatures. The synthetic conditions and size measurements of the IONP clusters without centrifugation or filtration are shown in Table 1. The IONP@HA clusters that were formed on a 240°C hotplate exhibited spherical clusters with free IONP, and the size of the clusters was decreased as the HA amount was reduced until sample 5. We chose the sample 5,6,7 to take TEM images which had the suitable particle size and lower PDI. As shown in Figure 1B-E, the results were not suitable as we expected. Particle sizes of clusters were also measured by DLS which can directly reveal the real situation of IONP@HA clusters in aqueous solution for further biomedical applications. In addition, the results from DLS can fast reflect the size change from the emulsified bodies to stable nanoclusters after solvent evaporation. The results are summarised in Table 1. The particle sizes of IONP@HA clusters (sample 1 to 5) decreased from 238.5 nm to 83.06 nm and then increased to 128.5 nm as the IO/HA ratio(\\/\\) increased from 1/1 to 30/1. The polydispersity index (PDI) of these eight samples was in the range of 0.180 to 0.446. The IONP@HA clusters prepared with 0.5 ml hexane at 240 °C revealed particle size around 83.06 nm with PDI 0.229 (sample 5). If the volume of IO increased to 15 or 30 mg, the particle size of IONP@HA clusters prepared showed was larger than 83.06 nm and we could significantly see the precipitation of IONP. It might be due to the excess amount of IONP that lead to the spherical clusters cannot be constructed very well.
Interestingly, the volume of oil droplets may have been the main determinant of the size of the IONP clusters, and more hexane may have provided more spaces for IONP agglomeration before solvent removal. If the volume of hexane was increased to more than 1 ml, a large portion of the IONP aggregated as very large spherical clusters (> 190 nm) after the phase-transfer process by HA in aqueous solution. Notably, in the optimal condition, the yield of IONP cluster is 100 % without further centrifugation to remove free IONP. The simple synthetic procedure, highly production and waste reduction were the greener method. Thus, we found that HA can be employed as a nonionic surfactant for the phase-transfer IONP@HA cluster formation, and the amount of HA is critical to form well-architected clusters. The particle size is one of the important concerns for clinical treatment, and the size-dependent enhanced permeability and retention (EPR) effects of the nano-clusters due to the leaky vasculature of the tumor microenvironment can be well observed with particle sizes smaller than 150 nm. In this study, sample 5, which had a particle size of approximately 83.06 nm and a PDI of 0.229, was speculated to be a potential particle with good passive targeting ability and thus employed for the following biological tests.

Table 1 Size distribution of cluster samples evaluated by DLS

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>IO/HA (w/w)</th>
<th>Size (d_{nm})</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/1</td>
<td>238.5</td>
<td>0.334</td>
</tr>
<tr>
<td>2</td>
<td>5/2</td>
<td>207.1</td>
<td>0.446</td>
</tr>
<tr>
<td>3</td>
<td>5/1</td>
<td>187.0</td>
<td>0.312</td>
</tr>
<tr>
<td>4</td>
<td>10/1</td>
<td>143.7</td>
<td>0.387</td>
</tr>
<tr>
<td>5</td>
<td>15/1</td>
<td>83.06</td>
<td>0.229</td>
</tr>
<tr>
<td>6</td>
<td>20/1</td>
<td>104.3</td>
<td>0.180</td>
</tr>
<tr>
<td>7</td>
<td>25/1</td>
<td>121.4</td>
<td>0.213</td>
</tr>
<tr>
<td>8</td>
<td>30/1</td>
<td>128.5</td>
<td>0.214</td>
</tr>
<tr>
<td>9</td>
<td>50/1</td>
<td>136.3</td>
<td>0.149</td>
</tr>
</tbody>
</table>

Size stability

Good size stability of IONP@HA clusters in aqueous solution is required for biomedical applications. It was observed that the particle size of IONP@HA clusters remained below 100 nm with a polydispersity of less than 0.3 for over 17 days in storage at room temperature, which indicates that the HA-stabilised IONP clusters present good stability without significant aggregation in aqueous solution at room temperature. The importance of surfactants on a nanoparticle’s surface for biomedical applications has been previously reported. For the optimized IONP@HA clusters, their stability may be due to the full passivation of the
surface of the clusters by the HA surfactant. Furthermore, according to these results, it can be used to clinical treatment and in vitro applications in the future.

**Cytotoxicity and cellular uptake of IONP@HA clusters**

To evaluate the cytotoxicity of the IONP@HA clusters, cells were incubated with various concentrations of IONP@HA clusters from sample 5 for 24 hours and then quantitatively analysed by MTT assay. As shown in Figure 2, HT29 is a human colorectal adenocarcinoma cell line with epithelial morphology. It is also used as an in vitro model to study absorption, transport, and secretion by intestinal cells. IONP@HA clusters obviously revealed the viability over 100% as the iron concentration over 15.625 μg/ml. Similar results were also observed as our previous work using Pluronic F127-encapsulated IONP.22 The increased metabolic activity may be due to the availability of additional free iron released from the IONP in the cells, which can be used to increase cellular metabolic activity in lower concentration.24 Thus, IONP@HA clusters were revealed to be almost non-toxic to the HT29 cells.

Figure 2. Cell viability of the cells treated with IONP@HA clusters as evaluated by the MTT assay in HT29 cells. Data are presented as means ± standard deviation.

**CONCLUSIONS**

In conclusion, we developed a simple emulsion and solvent-evaporation approach for clustering of iron oxide nanoparticles using nonionic surfactant. This synthetic approach displaying a high degree of production and waste reduction were the environmentally friendly method and hyaluronic acid employed to stabilise IONP clusters can minimise the cytotoxicity from the conventional ionic surfactant. By adjusting the ratio of IO/HA, we can efficiently optimize the size and uniformity of IONP@HA clusters. However, magnetic iron oxide nanoparticles have been used clinically, and numerous have already received US Food Drug Administration approval for clinical use. Furthermore, the tumor growth was effectively inhibited by the NIR-induced hyperthermia of the spherical Fe3O4 nanoparticles. The photothermal effect of magnetic iron oxide may be well utilized as an efficient strategy in clinical cancer therapies.

**REFERENCES**