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Rationally designed magnetic nanoparticles as anticoagulants for blood purification

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ABSTRACT

Heparin-based anticoagulant drugs are widely used for the prevention of blood clotting during extracorporeal circuit (bloodlines or cassette system) and surgical procedures as well as for the treatment of thromboembolic events. However, these anticoagulants are associated with bleeding risks that demand continuous monitoring and neutralization with antidotes. We explore the possibility of utilizing anticoagulants for blood clotting prevention, then removing them before transfusing the blood back to body, thus avoid bleeding risks. Here, we report on the strength of a strategy to solve problems with bleeding risks by rationally designing and using superparamagnetic iron oxide nanoparticles (SPIONs) with layer-by-layer self-assembled heparin. The morphology of these SPIONs was investigated by using dynamic light scattering and transmission electron microscopy. *In vitro* assays demonstrated superior efficacy and safety profiles and significantly mitigated conventional heparin-induced bleeding risks. In addition, the *in vivo* assay in a model animal (dog) proved that it is possible to use magnetic anticoagulant (MAC) in blood purification. The new magnetic anticoagulant drugs may benefit patients undergoing high-risk surgical procedures and may overcome anticoagulant-related bleeding problems to a great extent.

1. Introduction

Haemodialysis treatment requires anticoagulation to prevent thrombosis. Anticoagulation is usually achieved with heparin or its low-molecular-weight derivatives. However, systemic anticoagulation precludes using heparin in patients at high risk of bleeding [1]. Currently, neutralization of anticoagulant activity is an option to prevent the risk of fatal haemorrhage [2]. Although oligonucleotides complementary to the aptamer could act as antidotes capable of efficiently reversing the activity of the anticoagulants [3], protamine is the only antidote approved by the Food and Drug Administration (FDA) and available to reverse the anticoagulant effects of unfractionated heparin [4]. Furthermore, poor reversal of low-molecular-weight heparins [5] limits the use of antidotes in avoiding bleeding risks. Alternatively, heparin-free haemodialysis treatment by either coating heparin [1] or grafting heparin [6] onto dialysis membranes is another procedure used in some dialysis facilities. Here, we report a promising approach utilizing superparamagnetic nano-anticoagulants for blood clotting prevention and then removing them before transfusing the blood back to body by an external magnetic field, thus avoid bleeding risks to certain content.

Due to their excellent optical, mechanical and magnetic properties, magnetic nanoparticles are a source of much interest in the areas of batteries [7], catalysts [8], magnetic resonance imaging [9], gene transfer [10], drug delivery [11], and magnetic drug targeting [12]. Superparamagnetic Fe_3O_4 nanoparticles (SPIONs) are the primary focus in the field of magnetic nanoparticles due to their large magnetic moments, excellent superparamagnetism, and high stability in aqueous media.[13] SPIONs were coated with doxorubicin-conjugated heparin for targeted anticancer drug delivery and magnetic resonance imaging (MRI) contrast agent [14]. Heparin-based SPIONs have been evaluated *in vitro* for the treatment of human CP70 ovarian cancer cells [15]. SPIONs coated with unfractionated heparin had high uptake yields and low toxicity as a new negative contrast agent for the *in vivo* MRI of human mesenchymal stem cells (hMSCs), which offered significant therapeutic potential in the field of regenerative medicine [16]. Non-covalent Fe_3O_4 nanoparticle-heparin hybrid systems were synthesized as the bioactive nanoparticles for uptake [17]. Core-shell poly(vinyl alcohol)/iron oxide magnetic nanoparticles of heparin conjugate were used as recycling anticoagulants, and the anticoagulant activity was evaluated by several parameters including activated partial

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thrombin time, prothrombin time, fibrinogen time, and thrombin time [18]. However, whether and how to use the Fe_3O_4/heparin hybrid nanoparticles as anticoagulants for blood clotting prevention and to avoid bleeding risks remain open questions.

Here, we present a unique strategy to fabricate superparamagnetic Fe_3O_4/heparin hybrid nanoparticles based on a layer-by-layer (LbL) self-assembly technique. Fe_3O_4@PEI endows the magnetic particles with positive charges, and thus, negatively charged heparin can be incorporated onto the hybrid nanoparticles via electronic attractions. The hybrid nanoparticles combine the magnetic property of Fe_3O_4 and the anticoagulant property of heparin and therefore have the potential to be used as a new method for the generation of anticoagulant reagent during haemodialysis.

2. Experimental section

2.1. Synthesis of PEI@Fe3O4 using thermal decomposition

PEI@Fe_3O_4 was synthesized by a modified solvothermal reaction. First, 0.8 g NaOH was added to 20 mL DEG, heated at 120 °C for 1 h in a nitrogen atmosphere, and cooled down to 70 °C to produce a NaOH/DEG stock solution. Then, 1 g PEI and 1 g $FeCl_3{\cdot}H_2O$ were dispersed in 40 mL DEG, which was heated to 220 °C for 30 min under the protection of nitrogen flow and constant stirring. After that, 5 mL NaOH/DEG stock solution was rapidly injected into the hot mixture. The resulting mixture was further heated at 220 °C for 1 h and then allowed to cool to room temperature. With the aid of a magnet, the black products were washed with D.I. water several times to remove the solvent and unreacted chemicals and then stored in normal saline (NS) solution.

2.2. Synthesis of magnetic anticoagulant by the LbL method

Hep-SPIONs were fabricated by a simple self-assembly method. Typically, a certain amount of heparin sodium was added into 5 mg/mL PEI@Fe_3O_4/NS solution. The mixture was placed in a water bath at a shaking speed of 150 rpm at 25 °C. After 2 h of shaking, the Hep-SPIONs were washed with NS solution several times to remove the unbound sodium heparin and stored in fresh NS solution at 4 °C.

2.3. Characterizations of SPIONs

FT-IR (Fourier transform infrared) spectra were recorded on a Nicolet 560 instrument with a universal ATR sampling accessory in the range of 400–4000 cm^{-1}. Prior to thermal analysis, all of the samples were pre-dried at 30 °C for at least 24 h under vacuum. TGA of the specimens was performed on a TG209F1 (Netzsch Co., Germany), and the samples were heated from 30 to 700 °C at a rate of 10 °C min^{-1}, protected by dry nitrogen. The energy dispersive spectra of the SPIONs were observed using a JSM-7500F (JEOL, Japan). The samples were lyophilized overnight in small vials, attached to the sample supports using carbon tape, and coated with a 7-nm gold layer. The magnetization curve was monitored at room temperature to investigate the dependence of the sample magnetic moment on the applied magnetic field from −20 to 20 kG. The zeta potentials of magnetic nanoparticles were determined by dynamic light scattering (DLS) (ZETA-SIZER, MALVERN Nano-ZS90). The temperature was 25 °C, and the scattering angle was 90°. The nanoparticle solution was diluted to 0.1 mg/mL prior to the test. Transmission electron microscopy (TEM) was performed on a HT-7700 (Hitachi) microscope at an acceleration voltage of 100 kV. The nanoparticles were deposited onto a copper grid from a 0.1% (wt/V) aqueous solution.

2.4. Haemolysis ratio

The haemolysis test was performed as previously reported.[19] In brief, whole blood was added to PBS (pH = 7.4) and then centrifuged at 500*g* for 10 min five times to isolate red blood cells (RBCs). Then, the diluted RBC suspension (~5×10^8 cells/mL) was added to magnetic particles. Deionized water-dispersed RBCs were used as the positive control, and the PBS (pH 7.4)-dispersed RBCs were used as the negative control. All the suspensions were incubated in a rocking shaker at 37 °C for 3 h and then centrifuged at 10,016*g* for 3 min. The absorbances of the released haemoglobin in the suspensions were measured at 540 nm using a UV–vis spectrophotometer. The haemolysis ratio was calculated using the following equation.

$$\text{Haemolysis ratio}\,(\%) = \frac{Suspension_{Abs} - Negative\ control_{Abs}}{Positive\ control_{Abs} - Negative\ control_{Abs}} \times 100 \quad (1)$$

2.5. Clotting times

To evaluate the antithrombogenicity of the magnetic particles, activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) were measured by a CA-530 automated blood coagulation analyser (Sysmex Co., Japan) according to the method described in previous reports [20–22]. In brief, the magnetic particles were incubated in 300 μL of fresh PPP at 37 °C for 30 min. The incubated PPP was drawn out and added to a test cup. The clotting times (APTT, TT and PT) were automatically measured by the blood coagulation analyser by adding the corresponding reagents.

2.6. Complement activation

The complement activation was also evaluated by an ELISA with Human Complement Fragment 3a (C3a) and Human Complement Fragment 5a (C5a) kits (BD Biosciences, USA) [23]. Briefly, the sample was immersed in normal saline overnight and incubated with 250 μL human whole blood for 1 h, and then, the whole blood was withdrawn and centrifuged at 1000 g (4 °C) centrifugal force for 15 min to obtain plasma. For the C3a test, 5 μL of the obtained plasma was diluted 500 times with C3a-sample diluent, and 100 μL of the diluted plasma was then added to an antibody-coated well (provided by the C3a kit); for the C5a test, 10 μL of the obtained plasma was diluted 10 times with C5a-sample diluent, and then, the diluted plasma was added into another antibody-coated well (provided by the C5a kit). Afterward, the detections were performed according to the respective instructions from the manufacturer. Three replicates were averaged, and the results were expressed as the mean ± SD (n = 3).

2.7. Contact activation

The contact activation levels of the magnetic particles, PES and heparin were measured in an enzyme-linked immune sorbent assay (ELISA) with a thrombin-antithrombin III (TAT) kit (Assaypro LLC, USA) and Human Platelet Factor 4 (PF4) kit (Hyphen BioMed, France). The sample (approximately 2 mg dry weight) was immersed in normal saline in a 24-well cell culture plate at 4 °C overnight. Then, the normal saline was removed, and 250 μL of human whole blood was introduced. After being incubated at 37 °C for 1 h, the whole blood was withdrawn and then centrifuged for 10 min at 2500 g (4 °C) to obtain plasma. For the TAT test, 50 μL of the obtained plasma was added into an antibody coated well (provided by the TAT kit); for the PF4 test, 40 μL of the obtained plasma