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A mussel-inspired approach towards heparin-immobilized cellulose gel beads for selective removal of low density lipoprotein from whole blood

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ABSTRACT

In this study, we report a mussel-inspired approach to fabricate heparin-immobilized cellulose (HeTaCe) gel beads with self-anticoagulative and biocompatible properties which can selectively remove low density lipoprotein (LDL) from whole blood directly. First, a phase inversion technique was applied to prepare cellulose gel beads. Then the as-prepared gel beads were dipped into a mixed solution of heparin and tannic acid in phosphate buffered saline (PBS, pH 8.5) to obtain HeTaCe gel beads. Blood compatibility experiments indicated that the HeTaCe gel beads could suppress complement activation as well as contact activation and prolong the clotting times to the upper detect limits (activated partial thromboplastin time > 600 s and thrombin time > 180 s) of the automated blood coagulation analyzer. An ideal adsorption capacity of LDL *in vitro* was achieved by the HeTaCe gel beads with an amount of 79.1 mg/g. Besides, dynamic column adsorption test further demonstrated a selective adsorption of LDL without a significant reduction of high density lipoprotein (HDL) in a simulative hemoperfusion system. It is believed that the HeTaCe gel beads will be quite appealing to future clinical practice aiming at lowering LDL and improving the outcomes of patients with high cardiovascular risk.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in most countries and is greatly associated with dyslipidemia including hypertriglyceridemia and hypercholesterolemia (Benjamin et al., 2018; Commission, 2014). By 2035, an estimation of greater than 130 million adults in the U.S. population (45.1%) will have had some forms of CVD, and the total cost of it will have reached $1.1 trillion (Benjamin et al., 2018). During the past decades, it has been well established that the elevated low density lipoprotein cholesterol (LDL-c) is a major risk factor of CVD for its association with the development and progression of coronary artery disease, stroke, heart failure and peripheral arterial disease (Stamler, Wentworth, & Neaton, 1986; Wilson et al., 1998). Decrease of LDL-c has been shown to reduce the risk of CVD and death. Statins are the primary agents to lower LDL-c now, and the efficacy of statins has been proven in both the treatment and secondary prevention of patients with cardiovascular disease and cerebrovascular disease (Grundy et al., 2002; Smith et al., 2011). Moreover, several previous "more versus less statins" trials demonstrated that high-intensity statin therapy significantly reduced cardiovascular events compared with moderate-intensity statin therapy among both western and eastern patients with CVD (Baigent et al., 2010; Isao Taguchi, Iwata, & Takashima, 2018; LaRosa et al., 2005). However, statins do not always work. On the one hand, many individuals with familial hypercholesterolemia (FH) do not achieve recommended LDL-c targets despite of the sufficient use of LDL-lowering drugs with proper lifestyle modification (Waters et al., 2009). FH is an autosomal codominant disorder of lipoprotein metabolism that is characterized by abnormally high levels of serum LDL-c and early onset of atherosclerosis and cardiovascular death (Goldberg et al., 2011). On the other hand, high-intensity statin therapy can lead to a few severe adverse effects such as an elevation of hepatic enzymes and rhabdomyolysis (Lim, Oh, Sakuma, & Koh, 2014; Nissen et al., 2006). Therefore, an alternative solution to the failure of statins in these occasions is critically required.

To date, low density lipoprotein apheresis (LDL-a) has been well known as an effective strategy to lower LDL levels and improve the survival rates of FH patients. The safety and efficacy of various kinds of LDL-a therapies, including dextran sulfate adsorption (DSA), heparin-induced extracorporeal LDL precipitation (HELP), immunoadsorption and polyacrylamide adsorption (direct adsorption of lipoproteins,

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DALI) have also been confirmed (Thompson & Grp, 2008). DSA and HELP are commercially available at present in the US. Nevertheless, it must be noted that both of the two techniques involve an initial separation of plasma from whole blood and a reinfusion of the processed blood, which unfortunately limit their clinical applications because of complicated procedures. Situations in China can be worse. Although plasma exchange and double-filtration plasmapheresis are available in most medical centers, both of them have obvious shortages like high expenses, unnecessary waste of blood product and cumbersome operation procedures. So, it is of great importance to develop a novel LDL adsorbent, which can selectively remove LDL from whole blood directly in order to overcome the drawbacks of the existing techniques and to prevent the adverse effects of high-dose statin therapy.

Most relevant studies have focused on anionic polymer materials during the past two decades because negatively charged ligands can selectively bind the positively charged apolipoprotein B100 containing lipoproteins (Cao et al., 2011; Fang et al., 2017; Li et al., 2004; Murabayashi, Nishide, & Mitamura, 2002). Meanwhile, several heparin-based biomaterials have also been developed to remove LDL from plasma (Hou, Zhang, & Cao, 2013; Li et al., 2014; Liu et al., 2015). Heparin is widely recognized as one of the most popular agents against blood clotting and thrombosis formation in various clinical scenarios including hemoperfusion and hemodialysis (Rabenstein, 2002). Currently, heparin-based polymer materials have drawn more and more attention worldwide since many works suggested that the bioactivities of heparin remained when it was attached to diverse substrates (She et al., 2013; Zhao, Liu, Zhang, Su, & Zhao, 2018). Nevertheless, several drawbacks of the previously developed heparin-based LDL adsorbents need to be overcome to achieve better practical applications. Firstly, the synthetic procedures of the heparin-based adsorbents were quite complicated and the adsorption capacities were relatively low in some works. Secondly, the majority of related studies paid little attention on biocompatibility evaluation of the developed adsorbents and overlooked the problem that hemoperfusion, where the blood flow was reduced to about 220 mL/min to achieve better clearance, was at higher risk of blood coagulation and thrombogenesis than common hemodialysis. Thirdly and most importantly, all the adsorbents were designed to eliminate the elevated LDL from plasma, making it impossible to utilize these techniques in the future whole blood hemoperfusion.

Cellulose is the most abundant naturally existing polymer of glucose on earth and commonly recognized as an environmentally friendly and biocompatible product. So far, a number of cellulose-based materials have been developed over a wide range of applications in tissue engineering, controllable delivery system, blood purification, sensor, as well as water treatment (Chang & Zhang, 2011). Mussel-inspired technique refers to an approach where polyphenol self-polymerization, a result of a combination of non-covalent and covalent chemical interactions with the substrates, is used to form thin and surface-adherent films onto biomaterial surface (Lee, Dellatore, Miller, & Messersmith, 2007). Tannic acid (TA) is one of polyphenols that has been shown to make coatings by covalent cross-linking or cooperation with metal ions (Kim et al., 2015; Krogsgaard, Andersen, & Birkedal, 2014). The large number of pyrogallol groups on TA emulates polyphenolic nature of the mussel foot protein. It is widely reported that oxidative covalent cross-links can be formed with the increase of pH values, making it possible to prepare tannic acid and heparin coating onto the surface of cellulose gel beads (Krogsgaard, Behrens, Pedersen, & Birkedal, 2013, 2014). Herein, we conducted a one-pot mussel-inspired approach to synthesize HeTaCe gel beads which aimed to selectively remove LDL from whole blood. Immobilized heparin onto cellulose gel beads through a mussel-inspired approach provided plentiful functional groups ($-COO^-$ and $-SO_3^-$) to adsorb LDL and served as an anticoagulation agent concurrently. Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and thermo-gravimetric analysis (TGA) were performed to characterize the chemical compositions of the gel beads. Meanwhile, blood routine test, hemolysis test and clotting time test were utilized to evaluate the safety of HeTaCe gel beads. Hypercholesterolemic plasma simulated by diluted LDL/PBS mixed solution and whole blood were respectively used to determine the adsorption capacity and selectivity of low density lipoprotein *in vitro*.

2. Experimental section

2.1. Materials

α-Cellulose (90 μm, AR), lithium chloride (99.9% metals basis) and heparin sodium salt (185 USP units/mg) were purchased from Aladdin chemistry Co. Ltd. Ethyl alcohol (AR), methanol (AR) and *N,N*-dimethylacetamide (DMAc, AR) were supplied by Chengdu Kelong Inc. (Chengdu, China) and used as received. Best-reagent Inc. supplied tannic acid, which was extracted from Chinese gallnut and had 10 galloyl units per molecule (see Fig. S1). Native human low density lipoprotein (LDL, 99%) was purchased from Cell Sciences Inc. Deionized water was used throughout the study.

2.2. Preparation of cellulose gel beads

A phase inversion technique inspired by our several previous reports (Huang et al., 2016; Song et al., 2018) was applied to prepare cellulose gel beads. In brief, cellulose (3 wt.%) was suspended in 8 wt.% lithium chloride/*N,N*-dimethylacetamide (LiCl/DMAc) solution and placed at room temperature over 24 h to obtain stable non-degrading cellulose solution (McCormick, Callais, & Hutchinson, 1985). Eventually, the cellulose solution was injected into ethyl alcohol with a 0.6 mm-diameter syringe needle to prepare cellulose gel beads, followed by repeated procedures to elute the residual solvent thoroughly with DI water.

2.3. Preparation of HeTaCe gel beads

HeTaCe gel beads were prepared by a mussel-inspired approach and the synthesis procedure is shown in Fig. 1. The mixed solutions of heparin and tannic acid in phosphate buffered saline (PBS, pH 8.5) with various concentrations were prepared first. Then, the as-prepared cellulose gel beads were added into the solutions mentioned above. The reaction was carried out for 24 h at room temperature with stirring. Finally, the gel beads were washed by fresh DI water to remove residual solvent and unreacted chemicals. The details of sample names and chemical dosages are shown in Table 1 (for example, $He_{10}Ta_1Ce$ represents the concentration of heparin in PBS is 10 mg/mL and that of tannic is 1 mg/mL).

2.4. Characterization of the gel beads

The FTIR spectra of the gel beads were obtained on a FTIR spectrometer (Nicolet 560 FT-IR, USA). TGA/DSC 3 + STAR^e System was used to evaluate the thermostability of the gel beads. Furthermore, the chemical compositions of the gel beads were characterized by X-ray photoelectron spectroscopy and the water uptake behaviors were determined using DI water as a water uptake medium. The detailed procedures are shown in Supporting Information (SI).

2.5. Blood compatibility experiments

Fresh blood from a healthy volunteer (male, 25 years old) was collected using vacuum tubes (5 mL, Terumo Co.) containing citrate /phosphate/dextrose/adenine-1 mixed solution as anticoagulant (anticoagulant to blood ratio, v/v, 1:9). All the blood compatibility experiments were conducted in compliance with the relevant laws and institutional guidelines.

In this work, we performed blood routine and hemolysis ratio tests to investigate the impact of the gel beads on blood cells. Complement