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Alginate-calcium microsphere loaded with thrombin: A new composite biomaterial for hemostatic embolization

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ABSTRACT

To date, transcatheter arterial embolization (TAE) has become a standard treatment to control intracavitary bleeding as an alternative to surgery. Due to excellent biocompatibility and no residual *in vivo*, biodegradable materials are preferred in TAE. However, gelfoam is the only commercially available biodegradable embolic material used to treat blunt trauma of solid abdominal viscera until now, and controversial on its stability and reliability never stopped in the past five decades. In this study, a new biodegradable macromolecule material (thrombin-loaded alginate-calcium microspheres, TACMs) was prepared using electrostatic droplet techniques and a special method was developed for hemostatic embolization. Thrombin was successfully loaded into microspheres with high encapsulation efficiency and drug loading capacity. A burst release of TACMs was observed at early stage and sustained release later on, with the activity of thrombin preserved well. The strength of TACMs mixed thrombus, which was used as embolic agent, increased in a dose-dependent manner after TACMs were added. In addition, the TACMs were verified to be of no cytotoxicity and systemic toxicity, and biodegradable *in vivo*. Finally, the results of preliminary applications revealed that the TACMs could serve as an effective and promising embolic material for blunt trauma and hemorrhage of solid abdominal viscera.

1. Introduction

Q2 Operation was the only rapid method available to control intracavitary bleeding over the past few decades. To date, nonoperative management (NOM) of blunt trauma to solid abdominal viscera in hemodynamically stable or unstable patients with transcatheterarterial embolization (TAE) has become a standard treatment alternative to surgery and been widely accepted by surgeons [1–3]. TAE is the intentional occlusion of vascular structures by introducing embolic agents into the blood vessel through a catheter [4], and then the blood flow in the injured artery of solid abdominal viscera is cut off to achieve devascularization and hemostasis. The embolic materials used in TAE can be categorized into two general classes, according to whether they provide permanent (for progressive diseases, such as tumors) or temporary (for self-limited diseases, such as traumatic lesions) occlusion [5]. Commercially available embolic agents for hemorrhage of solid abdominal viscera include coils, liquids (cyanoacrylate and Onyx liquids), and nonspherical particles, most of which are permanent and non-biodegradable [6,7]. It is reported that the long-term presence of the embolic materials as a foreign body *in vivo* provokes chronic inflammation, thus causing tissue damage [8,9]. To avoid these complications and improve the physiological function of injured organs, biodegradable embolic materials are preferred [10]. Until now, the only commercially available biodegradable embolic material for blunt trauma to solid abdominal viscera is gelatin sponge particles, which have been used as a temporary embolic material in endovascular procedures since 1964 [11]. However, it cannot precisely control the level of embolization [4],

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and ischemic complications are often related to the nontargeted embolization and occlusion of distal small arteries that prevents the formation of collateral circulation [12,13]. In addition, Wu et al. suggest that the gelatin sponge particles, which occlude the injured artery successfully in TAE operation, are rapidly absorbed, leading to an increased risk of rebleeding before final hemostasis [7].

Recently, alginate-calcium microspheres are used as an embolic agent in treating aneurysms, tumors or uterine fibroids in TAE [14,15], and have increasingly attracted researchers' interest due to its drug-loading capacity, biocompatibility, biodegradation potential, low toxicity and mechanical stability [16–21]. Alginate is one of the most commonly used natural macromolecule biomaterials. It is a naturally occurring anionic polymer typically obtained from brown seaweed and contains blocks of (1,4)-linked-β-D-mannuronate (M) and α-L-guluronate (G) residues. In addition, its biodegradability and drug release properties could be regulated by composition, sequence, G-block length, and molecular weight of alginate and by the preparation techniques and methods [17,22,23]. At present, the biodegradable microspheres (including drug-loaded or blank microspheres) achieve the therapeutic purposes mainly by temporary mechanical blocking of the main feeding arterioles of the target organ or tumor, and then the degradation of the microspheres occurs gradually to achieve retreatment of drug delivery or restore the physiological function of the organs as far as possible [5,10]. Until now, there is still no appropriate microsphere products and corresponding effective delivery method for the interventional hemostasis of serious blunt trauma and hemorrhage of solid abdominal viscera.

Thrombin is an effective topical hemostatic agent extracted from animal blood. Once directly contacting with the blood, it catalyzes the fibrinogen into fibrin and promotes the platelet aggregation rapidly to achieve hemostasis. The thrombin has been increasingly widely used in clinical settings in recent years, including treatment of pseudoaneurysm by ultrasound-guided thrombin injection (UGTI) [24]. However, the autologous thrombus or blood clots formed by thrombin are so soft and easy to exfoliate that they can cause inadequate hemostatic effects and rehemorrhage, and eventually lead to failure of TAE [25].

In this study, we prepared a new embolic material (thrombin-loaded alginate-calcium microspheres, TACMs), which combines the advantages of embolic microspheres with the efficient procoagulant activity of thrombin. In addition, a novel method of using TACMs was developed to treat blunt trauma and hemorrhage of solid abdominal viscera by TAE, including mixing TACMs with whole blood *in vitro* to form a stronger mixed thrombus as embolic agent and delivering the mixed clots containing TACMs by the sandwich method using a catheter. The TACMs were prepared using an electrostatic droplet technique under mild conditions. The size distribution, morphology, pharmacological characteristics, changes in clot strength, toxicity and degradation *in vivo* of the TACMs were investigated, as well as the feasibility of embolization hemostasis for solid abdominal viscera using mixed clots composed of TACMs and whole blood.

2. Materials and methods

2.1. Materials

Sodium alginate (purity ≥ 98%, viscosity 100 cp, average molecular weight 400 kDa, G/M = 0.38) was supplied by Bright Moon Seaweed Group Co. Ltd. (Qingdao, China). Thrombin was purchased from First Biochemical Pharmaceutical Co. Ltd. (Shanghai, China) and calcium chloride was purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Fibrinogen was purchased from Sigma–Aldrich Co. LLC (MO, USA). L929 mouse fibroblast cells were supplied by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell Counting Kit-8 (CCK8) was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

All the protocols for animal experiment in this research were approved by the Institutional Animal Care and Use Committee (IACUC) of the General Hospital of Shenyang Military Region and the animal ethical permission registration no. is 2013-07. All procedures of animal experiment were in full compliance with recommendations on animal studies of the Helsinki Declaration of World Medical Association.

2.2. Preparation of thrombin-loaded alginate-calcium microspheres (TACMs) and alginate-calcium microspheres (ACMs)

Electrostatic droplet technique was adopted to prepare TACMs. Alginate solution (3%, w/v) was prepared by dissolving sodium alginate in 0.9% (w/v) normal saline (NS) and was filtered through 0.8, 0.45 and 0.22 μm membrane filters. Then the thrombin was resolved into the alginate solution, with a concentration of 10 mg/ml. The mixed solution was stored at 4 °C overnight to be deaerated and then extruded through a needle into a gel solution of 2% (w/v) $CaCl_2$, using an electrostatic droplet generator (YD-06, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China), to form calcium-alginate microspheres containing thrombin. The parameters of equipment were as follows: voltage 6.3 kV, frequency 157.3 Hz, syringe needle specification 4.5#, pump speed 7.6 ml/h. The ACMs were prepared following the same steps above but without adding thrombin into the alginate solution.

The surface morphology of the TACMs and ACMs was examined by a JEOL JCM-5000 (JEOL, Tokyo, Japan) scanning electron microscope (SEM). The diameters of 500 individual TACMs were measured under optical microscope. The profiles of size distribution were drawn and the number-average diameter of microspheres was calculated.

2.3. Entrapment efficiency and drug loading of TACMs

Thrombin content was determined by dispersing TACMs (0.1 mg) in 1 ml solution of ethylene diamine tetra-acetic acid (EDTA) ($n = 5$). The EDTA was employed to prompt the break of microspheres and release of thrombin from the microspheres completely. After centrifugation at 5000 rpm for 5 min, concentrations of thrombin in the clear supernatant solution were detected by the ultraviolet (UV) spectrophotometer (Shimadzu 2550, Japan) at 275.2 nm, which was predetermined by standard solutions of thrombin, using fresh EDTA as a blank control. Then, the content of thrombin in microspheres was determined by a standard curve of UV absorption *versus* concentration of thrombin solution. Entrapment efficiency (EE) and drug loading (DL) were calculated as Eqs. (1) and (2):

$$\mathrm{EE}(\%) = \frac{X_t}{X_0} \times 100\% \quad (1)$$

$$\mathrm{DL}(\mathrm{mg/ml}) = \frac{X_t}{V} \quad (2)$$

where X_t is the total amount of thrombin loaded into microspheres and X_0 represents the initial amount of thrombin added in the preparation process, and V stands for the volume of microspheres.

2.4. Drug release and activity retention in vitro

The release behaviors of TACMs were tested as follows. microspheres were put in a group of tubes for 1 ml each, into which 20 ml NS was added as dissolution medium, and then they were put into stirring baskets. The study was carried out at 4 °C with a stirring rate of 100 rpm. At predetermined time points (0, 1, 2, 12,