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Glucose and magnetic-responsive approach toward *in situ* nitric oxide bubbles controlled generation for hyperglycemia theranostics

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ABSTRACT

Stimuli-responsive devices that deliver drugs or imaging contrast agents in spatial-, temporal- and dosage-controlled fashions have emerged as the most promising and valuable platform for targeted and controlled drug delivery. However, implementing high performance of these functions in one single delivery carrier remains extremely challenging. Herein, we have developed a sequential strategy for developing glucose and magnetic-responsive microvesicle delivery system, which regulates the glucose levels and spatiotemporally controls the generation of nitric oxide gas free bubbles. It is observed that such injectable microvesicles loaded with enzyme and magnetic nanoparticles can firstly regulate hyperglycemic level based on the enzymatic reactions between glucose oxidase and glucose. In a sequential manner, concomitant magnetic field stimuli enhance the shell permeability while prompts the reaction between H_2O_2 and L-arginine to generate the gasotransmitters nitric oxide, which can be imaged by ultrasound and further delivered for diabetic nephropathy therapy. Therefore, magnetic microvesicles with glucose oxidase may be designed as a novel theranostic approach for restoring glucose homeostasis and spatiotemporally control NO release for maintaining good overall diabetic health.

1. Introduction

Recently, in order to establish more specific and individualized therapies for various pathologies, the promising theranostics paradigm involving a union of diagnostic and therapeutic applications into a single agent offers significant advantages over conventional drug delivery systems [1–3]. These advanced delivery systems combined diagnosis, drug delivery and treatment response are able to deliver a drug and/or contrast agent in spatial-, temporal- and dosage-controlled fashions when they are activated by specific physical (*e.g.*, external triggers like temperature [4], magnetic field [5], ultrasound [6], light [7], electric pulses [8]) or chemical (*e.g.*, endogenous changes in pH [9], enzymes [10], redox gradients [11]) triggers. Various types of carriers including organic polymers [12], inorganic/organic hybrid systems [13], and various nanoparticle [14] delivery systems have been designed to realize above mentioned unpredecendant properties. Magnetic nanoparticles (MNPs), a class of nanomaterial composed of magnetic elements, can be manipulated under the influence of an external alternating magnetic field (AMF) [15]. The magnetic properties of MNPs have been taken advantages in numerous applications related to drug and gene delivery, diagnostics and therapeutics [16–18]. Moreover, when MNPs are elaborately assembled with other multiple carriers, the magnetic composites can be developed as synergistic or sequential drug delivery systems to significantly increase delivery efficacy and reduce side effects [19,20]. However, when developing such magnetic theranostic carriers, it is still challenging to guarantee that the enhanced delivery performance at the target site (spatial control) and at the right time (temporal control) can be precisely obtained by accurate logic trigger codes.

Diabetes is a metabolic disorder that is characterized by the inability of the body to regulate blood glucose levels, among which the type 2 diabetes (non-insulin-dependent) makes up the vast majority in worldwide [21,22]. Nowadays, although a number of novel electronic devices, chemically controlled closed-loop delivery platforms, microgel, nanonetwork, *etc.* [23–25] have been developed to regulate the blood glucose effectively, it is still highly expected to identify hyperglycemia states smartly and intervene with one single dose treatment effectively. Especially, to develop an all-in-one platform combining intensive management of hyperglycemia and decreasing the incidence of complications is much more attracting. Nitric oxide (NO) is one of key biological signaling modulators in diverse physiological processes [26,27]. Deficiencies in NO production or a reduction in its bioavailability has been associated with several pathological conditions [28]. For example, some researchers have provided the evidences that NO availability in diabetes is usually decreased, and it could constitute a factor of the generalized vasculopathy present in diabetic nephropathy [29,30]. However, due to the reactive chemical nature of NO [31,32], the delivery and manipulation of NO to biological system is still a challenge. Based on

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specific enzymatic reactions between glucose and glucose oxidase (GOx), we developed the glucose and magnetic-responsive nitric oxide bubble generation theranostic delivery system to regulate the glucose hemostasis as well as spatiotemporally control NO release and delivery. As shown in Fig. 1, each GOx-MMVs structure consists of L-arginine (NO pro-drug) in the inner core, magnetic nanoparticles in the shell, and GOx assembled on the surface. The GOx-MMVs carrier firstly can be used as a smart glucose stimuli system to decrease the hyperglycemia levels. Then with the help of AMF, spatiotemporally controlling the NO gas generation and delivery can be realized by invoking the reaction of H_2O_2 and L-arginine [33]. The *in situ* generated NO molecules can function as both efficient ultrasound scatters to enhance ultrasound imaging and diabetic nephropathy therapeutic agents. Thus, we expect that by both internal (glucose) and external AMF stimulating, GOx-MMVs could act as effective blood glucose level regulators and spatiotemporal reactor of NO molecules *sin vitro* and in db/db type 2 diabetes mice.

2. Materials and methods

2.1. Materials

The oleic acid-coated supermagnetic iron oxide Fe_3O_4 nanoparticles (MNPs) with a mean diameter of 12 nm were provided by the Jiangsu Key Laboratory for Biomaterials and Devices (China) [34]. Polyvinyl alcohol (PVA) (molecular weight (Mw = 31,000) was obtained from Sigma-Aldrich and poly L-lactic acid (PLLA) (Mw = 30,000) from Shandong Daigang Company (China). Sodium periodate and sodium chlorite were purchased from Shantou Xilong Chemical Company (China). L-arginine and 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Beyotime Institute of Biotechnology (Haimen, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (NHS) sodium salt, and 2-(N-morpholino) ethanesulfonic (MES) acid were purchased from Shanghai Medpep Company (China). Glucose oxidase (GOx, G7141-10,000 U) was purchased from Sigma Aldrich (USA). Chloroform and all the other chemicals were of analytical grade and used without further purification.

2.2. Preparation and characterization of enzyme assembled magnetic microvesicles

In order to covalently couple the glucose-specific enzymes (GOx) into the magnetic microvesicles, firstly, polyvinyl alcohol (PVA) polymer (outer shell of the microvesicles) with carboxyl group was synthesized using our previous protocol [35]. Secondly, magnetic microvesicles loaded with L-arginine (MMVs) were fabricated *via* a double-emulsion method. Briefly, chloroform organic solution (10.00 mL) containing PLA (0.50 g) and oleic acid modification Fe_3O_4 superparamagnetic oxide nanoparticles (4 mg/mL, 40 μL) was emulsified with L-arginine (100 mM, 1.00 mL) solution and a little Tween 80 (about 0.05 mL) was added in the organic solution and sonicated continuously for 5 min. The first W/O emulsion is brown and visibly homogeneous. It was then poured into carboxyl group modification PVA solution (3% *w*/*v*) and mixed mechanically for 4 h to form (W/O)/W multiple emulsion MMVs. Thirdly, after separation, MMVs were suspended in MES buffer (50 mM, pH = 5.4). When activated by EDC (0.4 mg/mL) at room temperature, different concentrations of GOx (100, 200, 300, 400, 500, 600, 700, 800 μg/mL) were respectively added to and then incubated with MMVs solution at −4 °C. After 4 h, glucose oxidase modified magnetic vesicles (GOx-MMVs) were collected and washed 3 times with PBS buffer.

The mean size distribution of the GOx-MMVs was measured by particle sizing systems (AccuSizer 780 A, USA) at room temperature. The morphology and structure of the GOx-MMVs were studied by a scanning electron microscope (SEM, FEISirion-200, USA) working under acceleration voltage of 1.00 kV and a transmission electron microscope (TEM, JEOL, JEM-2000EX, Japan). Magnetization properties were studied by using a vibrating sample magnetometer (VSM, LakeShore 7407, USA) in the field H range of ±5000 Oe at room temperature.

The enzyme content adhered to magnetic vesicle was determined by the bicinchoninic acid (BCA) colorimetric protein assay. Briefly, a tertrate buffer (pH = 11.25) containing 25 mM BCA, 3.2 mM $CuSO_4$, and appropriately diluted GOx or GOx-MMVs was incubated at 60 °C for 30 min. After that the solution was cooled to room temperature, absorbance readings at 562 nm were collected by a UV–Vis 3600 spectrophotometer (Shimadzu, Japan). BSA solutions with known concentrations were used as standards. The enzymatic activity of free GOx and GOx-MMVs was tested by the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, USA).

2.3. In vitro autonomous bubble formation

To capture the bubbles formation in real-time, GOx-MMVs samples were incubated with hyperglycemia glucose saline solution (1 mL, 400 mg/dL). The mixture was exposed to an alternating magnetic field (AMF, Shuangpin SPG-06-III) with 390 kHz frequency at room temperature, and then transferred immediately to be observed under the optical microscopy (Olympus cell TIRF, Japan) to record images over time. In order to understand the effect of AMF irradiation on the control of NO production, the various AMF exposure periods (5, 10, 15, 20, 30, 40 min) were investigated. At the same time, a fiber-optic thermometry

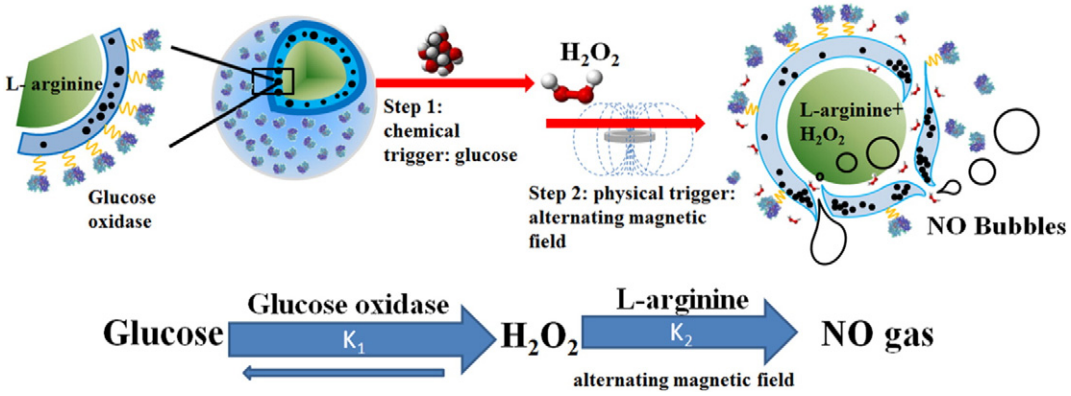

Fig. 1. Schematic diagram of microvesicles encapsulated magnetic nanoparticles and glucose oxidase for dual-stimuli responsive programmable delivery model. Firstly the encapsulated glucose-specific enzyme catalyzes glucose into gluconic acid and H_2O_2. The subsequent alternating magnetic field increases the porosity of the polymer shell, leading to the reaction between L-arginine and H_2O_2 to produce nitric oxides.