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Microfluidic Fabrication of Monodisperse Microcapsules for Glucose-Response at Physiological Temperature

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ABSTRACT

Glucose-responsive systems that can change their volumes or other properties in response to glucose concentration, show great potential as drug delivery carriers for controlled release of insulin for diabetes therapy or targeted delivery of anticancer drugs for cancer treatment. In this study, we report on a novel type of monodisperse phenylboronic acid (PBA)-based microcapsules for glucose-response at physiological temperature (about 37 ^{o}C). Oil-in-water-in-oil (O/W/O) double emulsions generated by microfluidic device are used as templates to synthesize the microcapsules. Poly(*N*-isopropylacrylamide-*co*-3-aminophenylboronic acid-*co*-acrylic acid) (poly(NIPAM-*co*-AAPBA-*co*-AAc)) hydrogel shell of the microcapsule, with PNIPAM component as actuator and AAPBA component as glucose sensor, shows good responsivity to the blood glucose concentration at physiological temperature. To obtain the maximum swelling volume transition of the microcapsule at 37 ^{o}C, the molar ratio of AAc in the hydrogel shell is selected as 2.4 mol%. At physiological temperature, Glucose-responsive microcapsules prepared with 2.4 mol% AAc exhibit reversible and repeated swelling/shrinking responses to glucose concentration changes within the physiological blood glucose concentration range (0.4~3.0 g/L). The glucose-sensitive swelling/deswelling behaviors show good reproducibility with glucose concentration repeatedly varying between 0.4 g/L and 3.0 g/L. Rhodamine B and fluorescein-isothiocyanate-labeled insulin are used as model molecules and model drugs to demonstrate the potential application of the microcapsules for glucose-responsive controlled release. The microcapsules provide a promising and feasible model for developing glucose-responsive sensors and self-regulated delivery systems for diabetes and cancer therapy. Moreover, the microfluidic fabrication approach and research results presented here provide valuable guidance for the design and fabrication of monodisperse glucose-responsive microcapsules.

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1. INTRODUCTION

Over the past few years, sugar-responsive systems have been widely investigated in various fields such as sensors (Alexeev 2003, Takahashi 2005, Egawa 2007, Takaoka 2010), drug delivery systems (Kost 2001, Chu 2004, Tanna 2006), bio-separations (Ito 1997) and micro-reactors (Nayak 2005). As one of the simple sugars, glucose is very important as the target molecule for these systems, due to the key role of glucose in biological functions. For example, diabetes mellitus, one of the most widely spread diseases, is a disorder of glucose regulation which usually results in glucose accumulation in blood. The tumor cells also accumulate glucose faster than normal cells (Ganapathy 2009), where the high glucose concentration is believed to have strong influence on the activity of tumor hexokinase type II promoter (Manna 2010). Glucose-responsive systems that can change their volumes or other properties in response to glucose concentration, show great potential as drug delivery carriers for controlled release of insulin for diabetes therapy (Kataoka 1998) or targeted delivery of anticancer drugs for cancer treatment (Manna 2010). Such glucose-responsive systems enable self-regulated drug delivery while constantly monitoring the blood glucose concentration for diabetes (Kost 2001, Qiu 2001, Miyata 2002, Peppas 2004, Chu 2005, Steil 2005) and cancer, which reduces the pain of patients from insulin injections and chemotherapy.

The glucose-responsive systems are usually based on three glucose-responsive moieties, including glucose oxidase (Chu 2004, Qi 2011), concanavalin A (Kost 2001, Luo 2012, Takahashi 2012), and phenylboronic acid (PBA) derivatives (Kost 2001, Miyata 2002). The glucose oxidase and concanavalin A can specifically interact with glucose, but these natural components are limited by the potential denature problem. The PBA derivatives, although less specific, show greater reliability and longer-term stability than the former two natural components. Based on these functional moieties, glucose-responsive systems in different forms such as bulk hydrogels (Kataoka 1998, Matsumoto 2004, Wang 2010), microgels (Matsumoto 2004, Lapeyre 2006, Zhang 2006, Hoare 2007), and microcapsules (Lapeyre 2009) have been developed as potential self-regulated delivery systems.

Glucose-responsive hydrogels that enable repeated "on-off" release of insulin (Shiino 1995, Kataoka 1998) have been developed and further improved for operating at physiological pH conditions (Matsumoto 2003, Matsumoto 2004). For the demand of drug delivery application and fast response time, it requires scale-down of the bulk hydrogel into micro-scale hydrogel. Glucose-responsive microgels with different compositions have been prepared and further developed for responding at physiological salinity (Kataoka K. 1994, Lapeyre 2006), temperature (Zhang 2008), and pH conditions (Lapeyre 2008), respectively. Compared with the voidless microgels, glucose-responsive microcapsules with hollow structures provide larger inner volumes for drug encapsulation (Chu 2004, De Geest 2006, Levy 2008, Qi 2009, Manna 2010). Those microcapsules, which are mainly fabricated by layer-by-layer techniques, can achieve an increased drug release by decomposing or loosening their multilayer matrix in response to glucose concentration change for diabetes and cancer treatment (De Geest 2006, Qi 2009, Manna 2010). However, compared with cross-linked hydrogel systems with reversible swelling/shrinking changes for continuous glucose response

(Kataoka 1998), those polyelectrolyte microcapsules usually can not repeatedly respond to glucose concentration change due to their decomposition, and few of them are active under physiological temperature and glucose concentration conditions. Moreover, the monodispersity of the microcapsules, which is critical for the precise manipulation of the loading levels and the release kinetics of encapsulated substances (Chu 2007), still need to be improved. Therefore, the development of novel hydrogel-based microcapsules with good monodispersity and repeated glucose-response under physiological temperature and glucose concentration conditions as self-regulated delivery carriers for diabetes and cancer is of both scientific and technological interest.

Microfluidic technology with precise manipulation of emulsion droplets and highly encapsulation efficiency (Utada 2005, Chu 2007, Wang 2011), has already shown great potential in the fabrication of monodisperse microcapsules for encapsulation and delivery (Wang 2009, Liu 2010, Yu 2012). Based on microfluidics, here we report on a simple emulsion-template approach for fabricating monodisperse hydrogel-based microcapsules with repeated glucose-response under physiological temperature and glucose concentration conditions for the first time, aiming for future use as smart delivery systems for the treatment of diabetes and cancer.

2. Strategy for the design and fabrication of the glucose-responsive microcapsules

To construct a totally synthetic microcapsule with long-term stability and repeated glucose response, glucose-responsive 3-acrylamidophenylboronic acid (AAPBA) and thermo-responsive poly(*N*-isopropylacrylamide) (PNIPAM) are respectively employed as the glucose sensor and actuator for constructing the microcapsule shell. The sensor AAPBA can reversibly form complex with cis-diol such as glucose (Hoare 2007), as shown in Fig. 1. In aqueous solution, PBA derivatives exist in equilibrium between an uncharged form and a charged form, both of which can react reversibly with glucose. Especially, only the charged form can stably complex with glucose through reversible covalent bonding, whereas the uncharged form is highly susceptible to hydrolysis (Lapeyre 2006). The actuator PNIPAM is a famous thermo-responsive material that can reversibly switch between a swollen and a shrunken state via temperature changes, exhibiting a volume phase transition temperature (VPTT) (~32 ^{o}C) close to the physiological temperature (37 ^{o}C). However, incorporation of hydrophobic PBA moiety and PNIPAM into microcapsule shell makes its VPTT lower than 32 ^{o}C. So certain amount of hydrophilic acrylic acid (AAc) is used for VPTT adjustment to make the microcapsule achieve a maximum swelling/shrinking volume change in response to glucose concentration change at 37 ^{o}C.

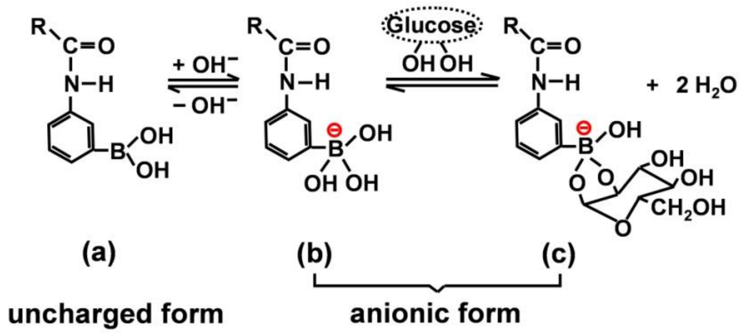

Fig. 1 Representation of the complex between the phenylboronic acid and glucose in aqueous solution

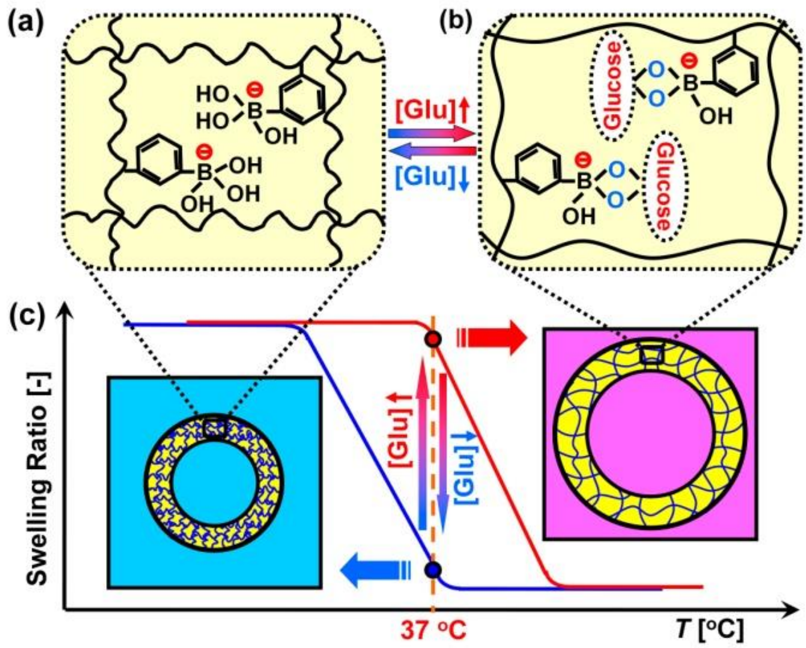

Fig. 2 Schematic illustration of the proposed glucose-responsive microcapsule with reversible glucose-induced swelling/shrinking behavior

The concept of the proposed glucose-responsive hollow microcapsule is schematically illustrated in Fig. 2. In an environment with pH value close to the pK_a of AAPBA moiety (pK_a = 8.6) (Kataoka K. 1994), where the PBA is supposed to present in both the uncharged and the charged forms, the glucose-responsive microcapsule is initially shrunken at 37 ^{o}C. When the glucose concentration increases, the charged form of PBA in the hydrogel shell forms stable complex with glucose through reversible covalent bonding. The complex consumes charged PBA forms and shifts the dissociation equilibrium of PBA, which converts more hydrophobic and uncharged PBA groups into hydrophilic and charged phenylborate ions (Kataoka 1998). This makes the VPTT of the microcapsule shift to a higher temperature and builds up a Donnan potential, results in a glucose-induced swelling of the microcapsule at 37 ^{o}C. During the glucose-response process, the PNIPAM-based VPTT shift can exhibit a thermal phase transition amplification of the glucose-induced swelling behavior (Hoare 2007). Similarly, decrease in glucose concentration causes a glucose-induced shrinking of the microcapsule, due to the decomposition of the PBA-glucose complex. The microcapsules with reversible glucose-responsive swelling/shrinking behaviors under physiological conditions will show great potential as glucose-sensors and self-regulated delivery systems for diabetes and cancer.

2.1 Preparation of monodisperse double emulsions and glucose-responsive microcapsules

Monodisperse double emulsions from microfluidics were used as templates for the synthesis of glucose-responsive microcapsules. The glass-capillary microfluidic device (Fig. 3b) for generating double emulsions was fabricated according to literatures (Chu 2007, Wang 2011). The outer diameter of cylindrical capillaries (AIT glass), and the inner dimension of square capillary tubes (Vitrocom) were both 1.0 mm. Three cylindrical capillaries, which were respectively used as the injection tube, transition tube, and collection tube, were aligned coaxially inside the square capillaries. The inner diameters of the injection tube, transition tube, and collection tube were 550 μm, 150 μm, and 300 μm, respectively. The end of injection tube and transition tube were tapered by a micropuller (Narishige, Japan) and then adjusted by a microforge (Narishige, Japan).

Typically, deionized water (4 mL) containing monomer NIPAM (0.4074 g) (*N*-Isopropylacrylamide, Sigma-Aldrich), AAPBA (0.0716 g) and cross-linker MBA (0.0308 g) (*N*,*N'*-methylene-*bis*-acrylamide), initiator V50 (0.0217 g) (2,2'-azobis(2-amidinopropane dihydro-chloride), glycerin (0.2 g, 5 % (w/v)), and Pluronic F127 (0.04 g, 1 % (w/v)) (Sigma-Aldrich) were used as the middle fluid to construct the glucose-responsive poly(NIPAM-*co*-AAPBA) (PNA) shell of the microcapsules. The AAPBA was synthesized according to the procedure reported by Kitano et al (Kitano 1992, De Geest 2006, Lapeyre 2006). The molar ratio of NIPAM to AAPBA was kept at 9:1. To adjust the volume phase transition behavior of the microcapsule, different amount of AAc (0, 2.5, 3, 3.5, 4.5 mol%, with respect to the total moles of NIPAM and AAPBA) were added into the middle fluid to fabricate the poly(NIPAM-*co*-AAPBA-*co*-AAc) (PNAA) microcapsule with tunable VPTT (Fig. 3a).

Soybean oil containing 5 % (w/v) PGPR 90 (Polyglycerol polyricinoleate, Danisco, Denmark) and 0.2 % (w/v) $CaCO_3$ nanoparticles for emulsion stabilization, and soybean oil containing 5 % (w/v) PGPR 90 and 1 % (w/v) photo-initiator BDK (Benzil Dimethyl Ketal) were used as the outer and inner fluids, respectively. O/W/O double emulsions were generated by separately pumping the inner, middle and outer fluids into the injection tube, transition tube and collection tube of the microfluidic device (Fig. 3b) and then collected in a container. The flow rates of the inner, middle and outer fluids are respectively 500 μL/h, 600 μL/h, and 3000 μL/h. The inner oil core of the double emulsions was labeled with Sudan Red to emphasize the core-shell structure. The collected O/W/O double emulsions were converted into microcapsules by polymerization under UV irradiation for 20 min in an ice-water bath. Under UV light, monomers contained in the middle aqueous phase of the double emulsions were polymerized to build the glucose-responsive hydrogel shell of the microcapsule (Fig. 3c). A 250 W UV lamp with an illuminance spectrum of 250~450 nm was employed to produce UV light. After washing with isopropanol and deionized water for several times, these microcapsules were redispersed in deionized water for further characterization.

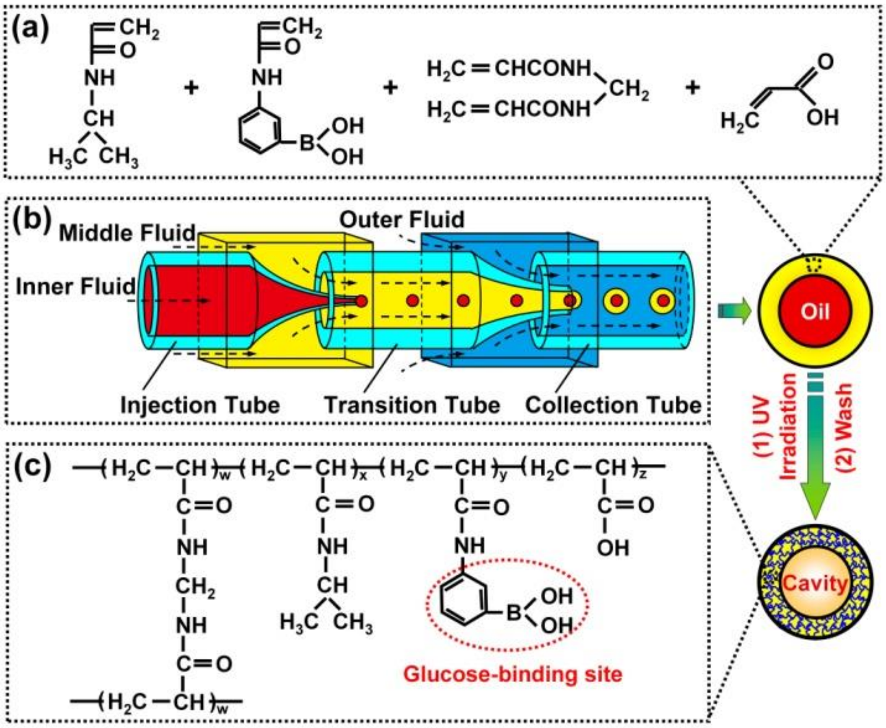

Fig. 3 Schematic illustration of the fabrication process of glucose-responsive microcapsule

2.2 Morphological characterization of double emulsions and glucose-responsive microcapsules

The generation process of O/W/O double emulsions in the microfluidic device was observed by high-speed digital camera (Phantom Micro 3, Vision Research, USA). Optical images of O/W/O double emulsions and glucose-responsive microcapsules were obtained by optical microscope (BX 61, Olympus, Japan).

To determine the size distribution and monodispersity of the double emulsions and microcapsules, an index called coefficient of variation (*CV*), defined as the ratio of the standard deviation of size distribution to its arithmetic mean, was introduced as follows:

$$CV = 100\% \times \left(\sum_{i=1}^{N} \frac{(D_i - \overline{D_n})^2}{N-1} \right)^{\frac{1}{2}} \Big/ \overline{D_n} \quad (1)$$

Where D_i is the diameter of the *i*th sample (μm), $\overline{D_n}$ is the arithmetic average diameter of the samples (μm), and *N* is the total number of measured samples. The inner and outer diameter of the double emulsions and microcapsules were measured by using automatic analytic software. Samples with *CV* value less than 5 % are defined as monodisperse.

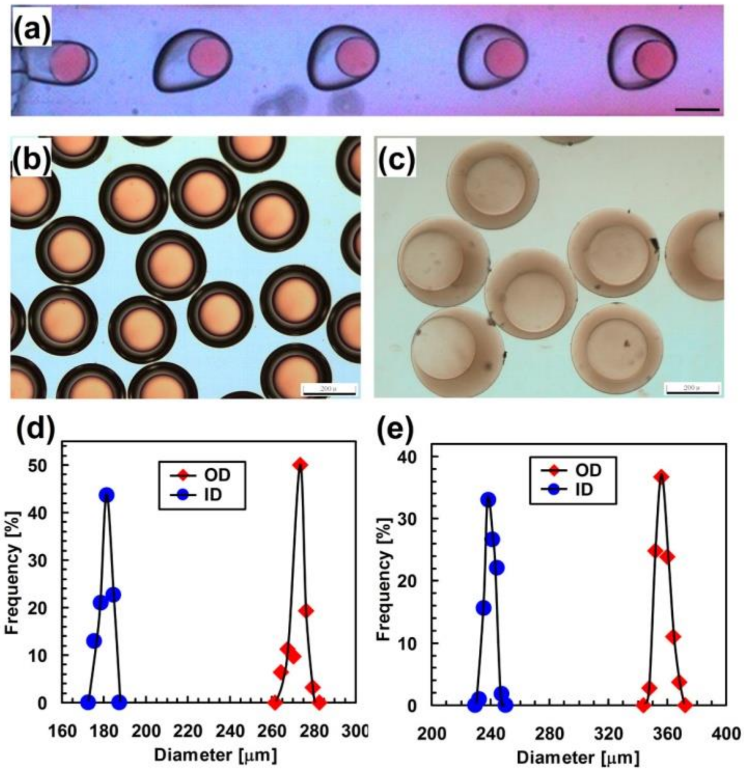

Fig. 4 Characterization of the double emulsion templates and the resulted glucose-responsive microcapsules. The scale bars are 200 μm

The generation process of O/W/O double emulsions in microfluidic device, which are used as templates for the synthesis of glucose-responsive microcapsules, is shown in Fig. 4a. Figs. 4b and 4c present the representative optical micrographs of O/W/O double emulsions and the resulted glucose-responsive microcapsules in isopropanol at 25 ^{o}C, respectively. These double emulsions show clear core/shell structures with oil core containing Sudan Red (Fig. 4b), and uniform size with narrow size distribution (Fig. 4d). The calculated *CV* values for the inner diameters (ID) and outer diameters (OD) of O/W/O double emulsions are 1.25 % and 1.38 % respectively, indicating good monodispersity of the emulsion droplets. Using these uniform double emulsions as templates, glucose-responsive microcapsules with hollow structures can be prepared by UV-initiated polymerizing the monomer-containing middle aqueous layer of the double emulsions and washing off the oil core (Fig. 4c). These microcapsules also show narrow size distributions, and slightly increased ID and OD values that resulted from the swollen hydrogel shell in isopropanol at 25 ^{o}C (Fig. 4e). The *CV* values for the ID and OD of the microcapsules are 1.23 % and 1.38 %, respectively, also showing highly monodisperse structures. The eccentric hollow cavity is resulted from the density difference between the inner oil core and the middle aqueous layer of emulsion templates. More uniform shell could be obtained by matching the densities of inner fluid and middle fluid. The results show that the approach presented here provides an effective and promising microfluidic strategy to fabricate monodisperse glucose-responsive hydrogel microcapsules with hollow structures.

2.3 Study on the effect of glucose concentration on the equilibrium swelling/shrinking behaviors of PNA microcapsules

To mimic the relevant physiological blood glucose concentrations, glucose concentration range of 0.4~3.0 g/L are selected, where 0.4 g/L of glucose is the hypoglycemic limit, 0.7~1.3 g/L is the normal range, 3.0 g/L is the hyperglycemic limit for research (Peppas 2004, Zhang 2008), and 4.5 g/L is the concentration where tumor hexokinase type II promoter shows the maximal activity (Rempel 1996). To study the effect of glucose concentration on the equilibrium swelling/shrinking behaviors of PNA microcapsule, Na_2CO_3-$NaHCO_3$ buffer solution (pH = 8.77, T = 37 ^oC) containing glucose with different concentrations (0, 0.4, 1.0 and 3.0 g/L), which we referred as the glucose buffer solution (GBS), were used as the medium.

The temperature-dependent equilibrium volume changes of the microcapsules in 0, 0.4, 1.0 and 3.0 g/L GBS were respectively studied by optical microscope (BX 61, Olympus, Japan) equipped with a thermostatic stage system (TS 62, Instec, USA) and a CCD camera within the temperature range from 12 ^oC to 40 ^oC. Briefly, the samples were first kept in GBS at room temperature for 12 h to reach their swelling/shrinking equilibrium state. Then, the temperature-dependent equilibrium volume changes of the microcapsules in different GBS were respectively studied by stepwise increasing temperature from 12 ^oC to 40 ^oC. At each temperature, the microcapsules were equilibrated for 15 min before the measurements were made. The swelling ratio of microcapsules, defined as the ratio of microcapsule volume at temperature T to that at initial temperature T_0, was measured to evaluate the equilibrium swelling/shrinking behaviors.

The AAPBA moiety in the hydrogel shell of the PNA microcapsules can work as glucose-sensor to make the PNA microcapsules glucose-responsive. The effect of glucose concentration on the equilibrium swelling/shrinking behaviors of PNA microcapsules is shown in Fig. 5. In all cases, the samples display decreasing volumes with increasing temperature, due to the thermo-responsive volume phase transition of the PNIPAM networks in the microcapsule shell. Incorporation of hydrophobic AAPBA into PNIPAM networks makes the VPTT of PNA microcapsules in pure water shift to a lower temperature (~22.5 ^oC). When placed in 0 g/L GBS with pH close to the pK_a of AAPBA, more AAPBA moieties become hydrophilic and charged form due to ionization, which causes a higher VPTT (~27.2 ^oC) and more swollen state of the microcapsules than those in pure water. Upon stepwise increasing the glucose concentration of GBS from 0 to 3.0 g/L, the PNA microcapsules exhibit a glucose-induced swelling behavior that is in direct proportion to the glucose concentration, due to the complex of AAPBA moieties with glucose. These results indicate both the good thermo-responsive property and glucose sensitivity of the PNA microcapsules. However, although the complex of AAPBA with 0.4 g/L~3.0 g/L glucose results in higher VPTT and more swelling state of the PNA microcapsule, it is still difficult to make their T_{opt} locate at 37 ^oC; because all PNA microcapsules exhibit shrinking at 37 ^oC, as shown in Fig. 5. To solve this problem, hydrophilic AAc is introduced into the PNA microcapsule for further VPTT adjustment.

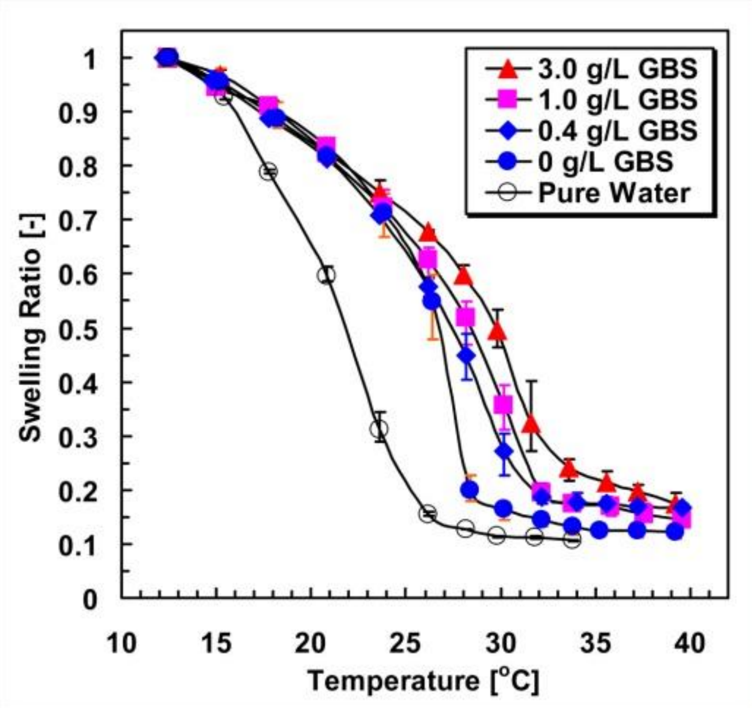

Fig. 5 Temperature-dependent volume changes of PNA microcapsules in GBS containing different glucose concentrations

2.4 Effect of AAc molar ratio on the equilibrium swelling/shrinking behaviors of PNAA microcapsules in 0.4 g/L GBS and 3.0 g/L GBS

To obtain a good glucose response at physiological temperature, the T_{opt} of the PNAA microcapsules, defined as the temperature at which the microcapsules achieve the maximum swelling/shrinking volume change in response to glucose concentration change from 0.4 g/L to 3.0 g/L, should be located at 37 ^{o}C. However, incorporation of hydrophobic AAPBA into the PNIPAM networks usually leads to VPTT lower than 32 ^{o}C, so hydrophilic acrylic acid (AAc) is introduced into the microcapsules to adjust the VPTT as well as the T_{opt}. The temperature-dependent equilibrium volume changes of PNAA microcapsules with 2.5, 3.0, 3.5, and 4.0 mol% AAc in 0.4 g/L GBS and 3.0 g/L GBS were respectively studied by the same optical microscope within the temperature range from 12 ^{o}C to 60 ^{o}C, similarly as mentioned above.

The effect of AAc molar ratio (2.5, 3.0, 3.5 and 4.5 mol%) on the equilibrium swelling/shrinking behaviors of PNAA microcapsules is shown in Fig. 6. With increasing the molar ratio of AAc from 2.5 to 4.5 mol% stepwise, the VPTT of the PNAA microcapsule increases in proportion to the AAc content. Moreover, at the same temperature, all PNAA microcapsules show a more swelling state in 3.0 g/L GBS than those in 0.4 g/L GBS, also indicating a good glucose-sensitivity.

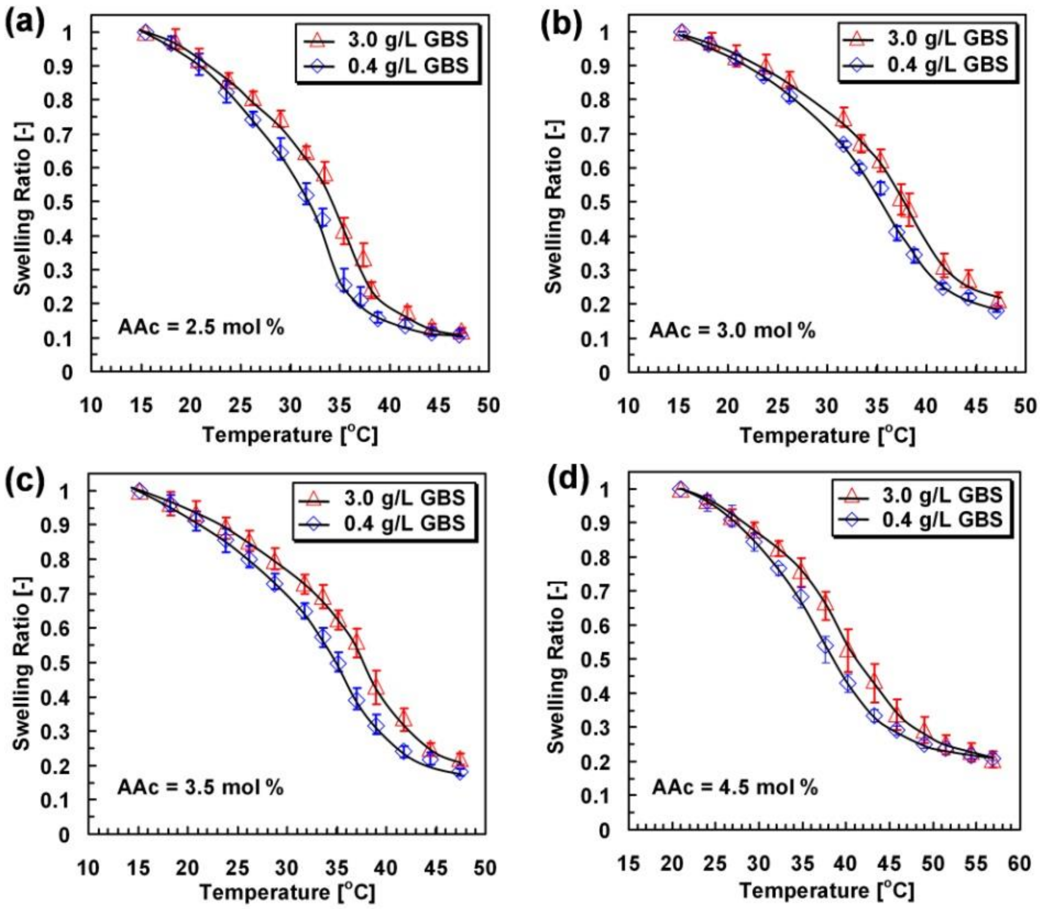

Fig. 6 Temperature-dependent volume changes of PNAA microcapsules with different molar ratios of AAc in 0.4 g/L and 3.0 g/L GBS, respectively

To evaluate the glucose-responsive swelling/shrinking behaviors of PNAA microcapsules, a parameter defined as $R_{T,3.0}/R_{T,0.4}$ is introduced, where $R_{T,0.4}$ and $R_{T,3.0}$ are respectively the swelling ratios of PNAA microcapsules in 0.4 g/L and 3.0 g/L GBS at temperature T. So, T_{opt} is the temperature where $R_{T,3.0}/R_{T,0.4}$ gets the maximum value. The $R_{T,3.0}/R_{T,0.4}$ values of PNAA microcapsules with different AAc contents in the temperature range from 10 ^{o}C to 55 ^{o}C are plotted in Fig. 7a. For the PNAA microcapsules containing 0, 2.5, 3.0, 3.5 and 4.5 mol% AAc, the location of their maximum $R_{T,3.0}/R_{T,0.4}$ values indicates that the T_{opt} value increases with increasing molar ratio of AAc. Fig. 7b shows the direct linear proportion of T_{opt} to AAc molar ratio, from which we can determine the AAc content required for construct microcapsule with T_{opt} at 37 ^{o}C is about 2.4 mol%.

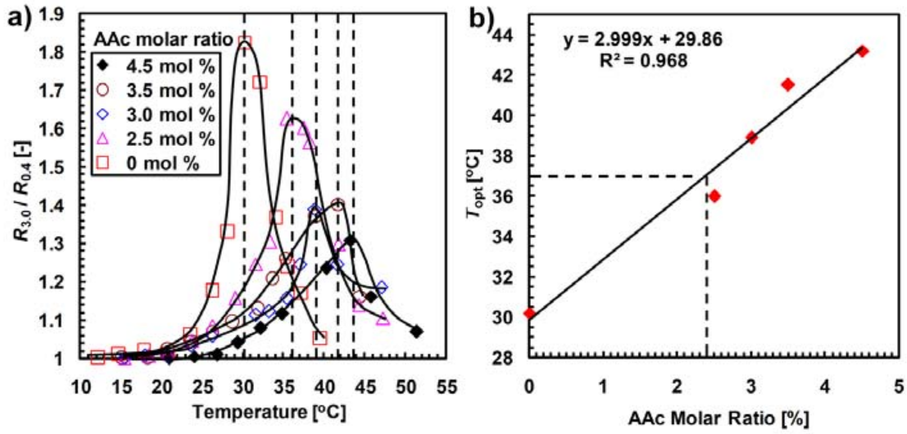

Fig. 7 The $R_{T,3.0}/R_{T,0.4}$ of PNAA microcapsules with different molar ratio of AAc as a function of temperature (a) and the T_{opt} of these microcapsules as a function of the molar ratio of AAc (b).

2.5 Study on the reversible and repeated glucose-responsive swelling/shrinking behaviors of PNAA microcapsules with 2.4 mol% AAc at 37 °C

PNAA microcapsules with 2.4 mol% AAc were fabricated to achieve a good glucose-responsive swelling/shrinking volume change at physiological temperature. Their reversible glucose-responsive dynamic volume changes at 37 °C were investigated by repeatedly transferring the microcapsules between 0.4 g/L GBS and 3.0 g/L GBS. Their dynamic swelling volume change with increasing glucose concentration (0.4 to 3.0 g/L) and dynamic shrinking volume change with decreasing glucose concentration (3.0 to 0.4 g/L) at 37 °C were respectively studied by the same optical microscope as mentioned above. The reversible glucose-responsive static volume changes of the PNAA microcapsules that respectively equilibrated in 3.0 and 4.5 g/L GBS at 37 °C were also studied by using the same optical microscope.

Fig. 8 shows the glucose-induced swelling (Fig. 8a) and shrinking (Fig. 8b) behaviors of PNAA microcapsules in response to glucose concentration changes between 0.4 g/L and 3.0 g/L at 37 °C. At 37 °C, the PNAA microcapsule is initially in a shrunken state (Fig. 8a1). When suddenly increasing glucose concentration from 0.4 g/L to 3.0 g/L, the PNAA microcapsule changes from the shrunken state to a swollen state dramatically (Figs. 8a2-a4), because more AAPBA moieties complex with glucose. Contrarily, when suddenly decreasing glucose concentration from 3.0 g/L back to 0.4 g/L, the PNAA microcapsule returns to the shrunken state again (Figs. 8b1-b4). In both of the swelling (Figs. 8a1-a2) and shrinking (Figs. 8b1-b2) process, the microcapsule exhibits an obvious volume change within t = 0.75 min after glucose concentration change, indicating fast glucose-responsive swelling/shrinking behaviors. Moreover, the reversible volume changes of the PNAA microcapsules that respectively equilibrated in 3.0 and 4.5 g/L at 37 °C (Fig. 8c) exhibit a more swollen microcapsule in 4.5 g/L than that in 3.0 g/L, showing a ~1.3 times volume change. The reversible volume increase of the PNAA microcapsules from 0 to 3.0, and to 4.5 g/L GBS indicates a good glucose-responsive volume change behavior in proportion to the glucose concentration. These results show the good and reversible swelling/shrinking response of the PNAA microcapsules to changes in the glucose concentration at physiological temperature.

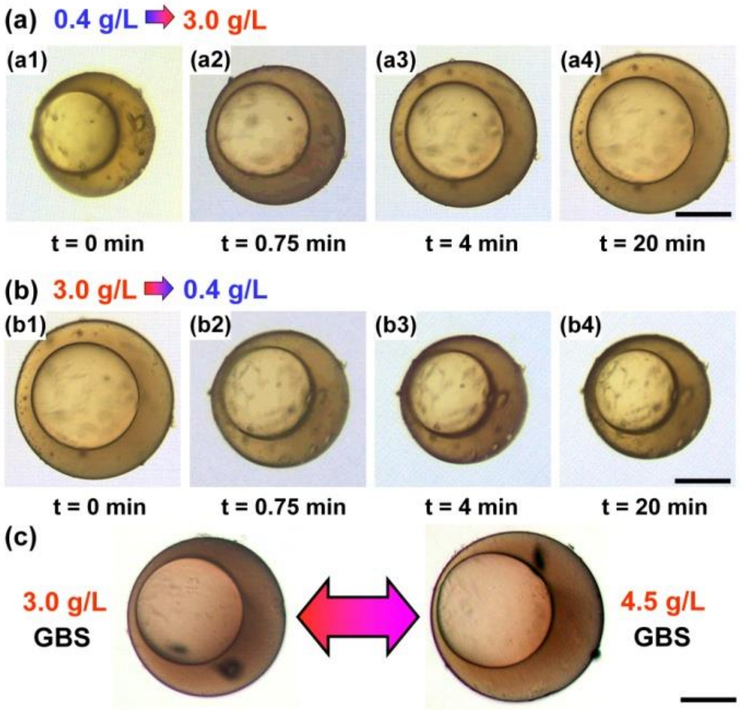

Fig. 8 Optical micrographs of the reversible glucose-induced swelling/shrinking behaviors of PNAA microcapsules with 2.4 mol% AAc in response to glucose concentration change at 37 °C. The scale bars are 100 μm

To investigate the ability of the PNAA microcapsules for repeated glucose response, their glucose-responsive swelling/shrinking behaviors were studied by repeatedly transferring the microcapsules between 0.4 g/L GBS and 3.0 g/L GBS at 37 °C (Fig. 9). Each volume change process was recorded for 20 min to reach their equilibrium swelling/shrinking state. As shown in Fig. 9, the sizes of the microcapsules and their hollow cavities increase with suddenly increasing glucose concentration from 0.4 g/L to 3.0 g/L and decrease with suddenly decreasing glucose concentration from 3.0 g/L to 0.4 g/L, exhibiting reversible and repeated glucose-induced swelling/shrinking behaviors with ~2.6 times volume change. The glucose-induced swelling/shrinking behaviors enables these microcapsules as potential self-regulated delivery systems for controlled drug release.

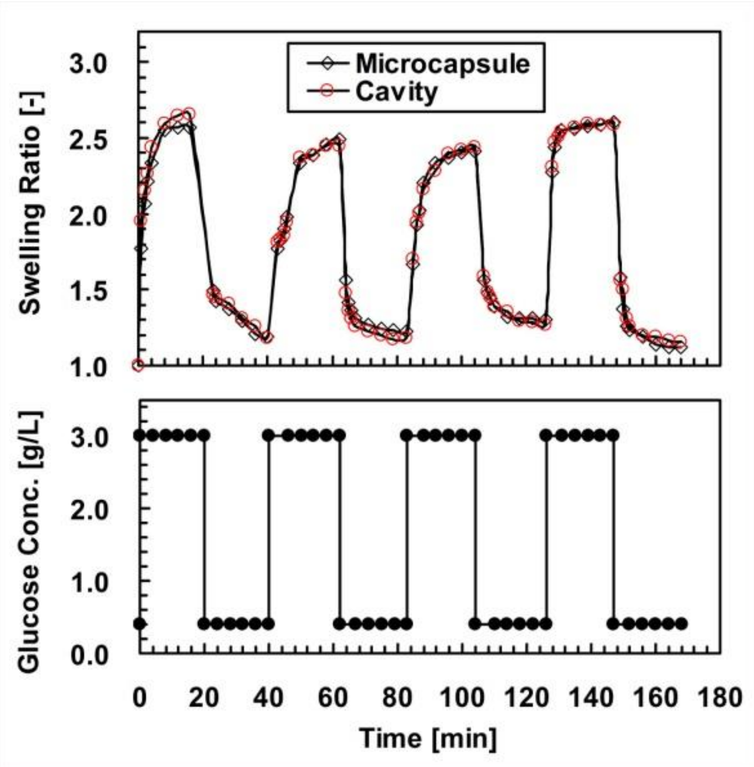

Fig. 9 The repeated and reversible swelling/shrinking behaviors of PNAA microcapsules with 2.4 mol% AAc in response to repeated changes of glucose concentration between 0.4 g/L and 3.0 g/L at 37 ^{o}C.

2.6 *Study on the glucose-responsive drug release behaviors of PNAA microcapsules with 2.4 mol% AAc at 37 ^{o}C*

To demonstrate the potential application of the PNAA microcapsules for drug delivery, their glucose-responsive drug release behaviors at 37 ^{o}C were investigated by respectively using fluorescent rhodamine B and FITC-insulin as model molecules and model drugs.

The glucose-responsive dynamic release behavior of rhodamine B molecules from the PNAA microcapsules was minitored by Confocal Laser Scanning Microscope (CLSM, SP5-II, Leica). For drug encapsulation, the PNAA microcapsules were immersed into 0.4 g/L GBS with 10 g/L rhodamine B at 4 ^{o}C overnight. The microcapsule samples were then placed into a glass-holder and washed with 0.4 g/L GBS several times to remove unloaded model molecules outside the microcapsules. After that, the PNAA microcapsules were quickly transferred into 0.4 g/L GBS and then moved into the observation field of CLSM. The release behavior of rhodamine B molecules from PNAA microcapsules in 0.4 g/L GBS was first monitored. After that, 0.4 g/L GBS was removed from the glass-holder and 3.0 g/L GBS was rapidly added. Then, the release behavior of rhodamine B molecules from the microcapsules in 3.0 g/L GBS was minitored.

The glucose-responsive static release behaviors of FITC-insulin from the PNAA microcapsules in 0.4 g/L GBS and 3.0 g/L GBS respectively were minitored by the same CLSM. The microcapsules were first immersed into 0.4 g/L GBS with 2 g/L FITC-insulin at 4 ^{o}C overnight for drug encapsulation for investigating the release behavior in 0.4 g/L GBS. After washing the microcapsules with 0.4 g/L GBS several times, the release behavior of FITC-insulin from PNAA microcapsules in 0.4 g/L GBS was minitored. Similarly, to study the release behavior of FITC-insulin from PNAA microcapsules in 3.0 g/L GBS, 3.0 g/L GBS was used instead of 0.4 g/L GBS for drug encapsulation and washing.

To quantitatively analyze the release behaviors, average fluorescence intensity of a certain region that reflects the release of the fluorescent molecules and drugs was estimated by Leica Analysis Software mounted on the CLSM. For rhodamine B with low molecular weight that could easily pass through the cross-linked hydrogel shell of PNAA microcapsule, the increase of average fluorescence intensity of a region that covers the direct neighborhood of a typical PNAA microcapsule was estimated for monitoring the release behavior. For FITC-insulin with relative higher molecular weight, the decrease of average fluorescence intensity of a region that covers a typical PNAA microcapsule, was estimated for monitoring the release behavior. The decrease of average fluorescence intensity was characterized by the ratio of fluorescence intensity at time *t* to that at the beginning. A thermostatic stage that mounted on the CLSM was used for temperature control throughout the experiments.

Fig. 10 shows the glucose-responsive dynamic release behavior of rhodamine B from the PNAA microcapsules at 37 ^{o}C. In the drug release experiments, the microcapsules are first equilibrated in 0.4 g/L GBS containing high concentration of rhodamine B for drug loading. After washing with 0.4 g/L GBS solution, the microcapsules are quickly transferred into 0.4 g/L GBS without rhodamine B and then moved into the observation field of CLSM for monitoring the drug release process. This operation process takes ~20 s, so the initial drug release behavior that causes the increase of fluorescence intensity at the very beginning is missed. During the first 10 min, the fluorescence intensity that reflects the amount of rhodamine B released from the microcapsule keeps at a low level, indicating a sustained release with slow rate in 0.4 g/L GBS. The slight decrease of the fluorescence intensity with time is mainly due to the diffusion of the released rhodamine B from the monitoring region into surrounding enviroment; because this diffusion rate is faster than the release rate of rhodamine B across the crosslinked hydrogel shell. Meanwhile, the slightly decreased concentration of rhodamine B inside the microcapsule during the release process also causes a slightly decreased release rate. After suddenly increasing the glucose concentration from 0.4 g/L to 3.0 g/L, the PNAA microcapsule undergoes a glucose-induced swelling volume change, which leads to a rapid increase of fluorescence intensity, indicating a suddenly enhanced release of rhodamine B with a faster rate. The increased release is attribute to the enlarged space size delineated by the polymer networks in the crosslinked shell caused by the glucose-induced swelling volume change. The enlarged spaces delineated by the polymer networks provide larger channels with less resistance for faster release of rhodamine B molecules. After the PNAA microcapsules reach equilibrium swollen state in 3.0 g/L GBS, sustained release of rhodamine B with a relatively faster rate than that in 0.4 g/L GBS is observed. The results show that, the glucose-responsive swelling/shrinking behaviors of PNAA microcapsules enable control of shell permeability for glucose-responsive controlled drug release.

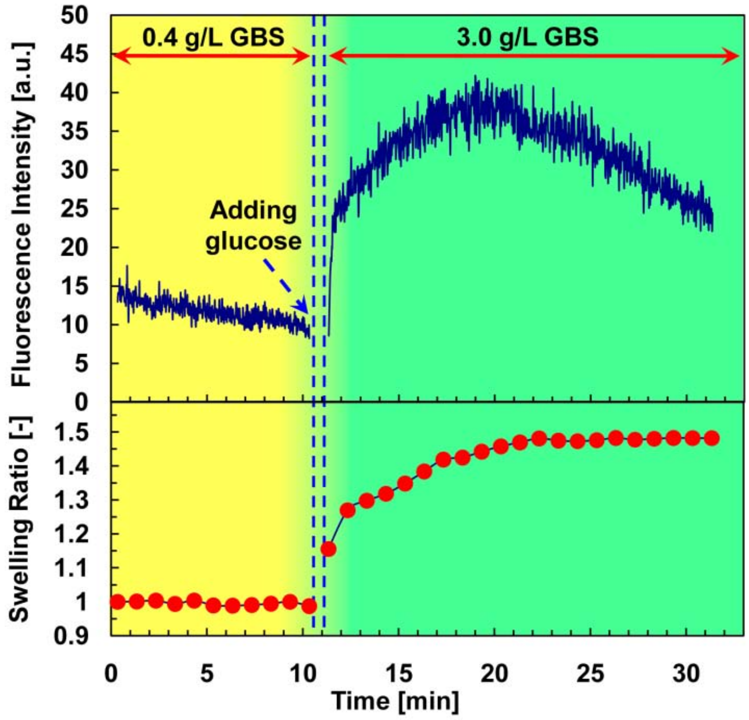

Fig. 10 The glucose-responsive release behaviors of rhodamine B from PNAA microcapsules with 2.4 mol% AAc at 37 °C. Lower: glucose-responsive swelling; Upper: glucose-responsive release, in which the fluorescence intensity is measured in the region that covers the direct neighborhood of PNAA microcapsules

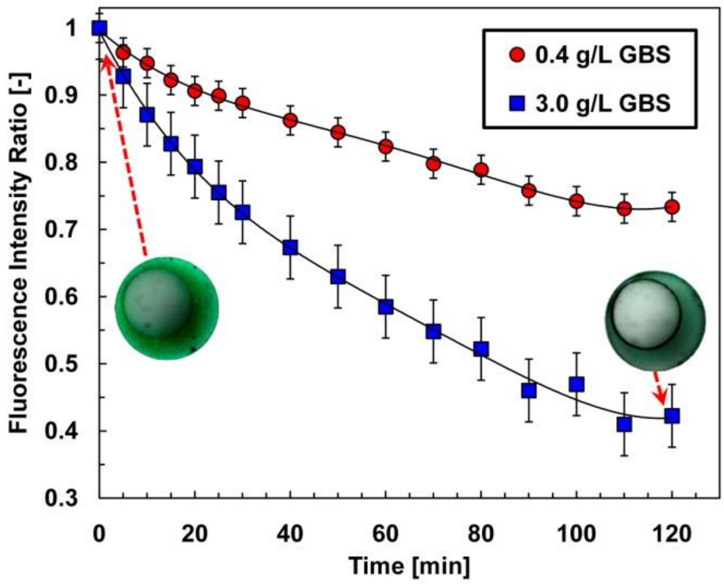

Fig. 11 The glucose-responsive release behaviors of FITC-insulin from PNAA microcapsules with 2.4 mol% AAc at 37 °C

Fig. 11 shows the release behaviors of FITC-insulin from the PNAA microcapsules that respectively immersed in 0.4 g/L GBS and 3.0 g/L GBS at 37 °C. For the PNAA microcapsule with more swollen shell placed in 3.0 g/L, the fluorescence intensity that reflects the amount of FITC-insulin remained in the microcapsule shows a faster decrease than that in 0.4 g/L GBS, indicating a faster release of FITC-insulin in 3.0 g/L. Since the PNAA microcapsules exhibit more swollen state in 4.5 g/L GBS than those in 3.0 g/L GBS, the PNAA microcapsules that enable drug release in response to glucose concentration change from 0.4~3.0 g/L, can also be used for glucose-responsive drug release in response to glucose concentration change from 0.4~4.5 g/L. These PNAA microcapsules with glucose-responsive drug release behaviors show great potential as self-regulated delivery systems for diabetes therapy and cancer treatment.

3. CONCLUSIONS

In this work, hydrogel-based hollow microcapsules with good monodispersity and repeated glucose-response under physiological conditions has been successfully developed by a simple microfluidic emulsion-template approach. In the hydrogel shell of the microcapsule, glucose-responsive AAPBA and thermo-responsive PNIPAM network are respectively used as the glucose sensor and actuator, and 2.4 mol% AAc is used for VPTT adjustment. The prepared hollow PNAA microcapsules with thermo-responsive and glucose-responsive poly(NIPAM-*co*-AAPBA-*co*-AAc) shell exhibit reversible and repeated swelling/shrinking responses to glucose concentration changes within the physiological blood glucose concentration range at 37 °C. Potential applications of the PNAA microcapsules for drug delivery have been demonstrated by respectively using rhodamine B and FITC-insulin as model molecule and model drug for glucose-responsive controlled release. The proposed hollow microcapsule provide a promising and feasible model for developing glucose-responsive sensors and self-regulated delivery systems for diabetes and cancer therapy. Since the responsiveness of the microcapsules fabricated from microfluidics can be tuned by adding functional monomers in the emulsion templates for copolymerization, future work must focus on refining the glucose-responsive microcapsules with modified AAPBA moieties (Matsumoto 2003) for more proper operations under physiological pH concentration. Moreover, the quality of controlled release can be further improved by preparing a more uniform capsule shell via matching the densities of inner fluid and middle fluid (Liu 2011), or using an external electric field (Bei 2009). In addition, a smaller capsule size would be required for applying these systems in biomedical fields, which can be achieved with advanced microfluidic and nanofluidic techniques (Whitesides 2006). We believe that the simple microfluidic approach and research results presented here provide valuable guidance for the design and fabrication of monodisperse glucose-responsive microcapsules.

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