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ORIGINAL CONTRIBUTION

Preparation of multi-responsive micelles for controlled release of insulin

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Abstract The polymeric micelles were prepared by the copolymerization of poly (methoxypolyethylene glycol acrylamide)-macroinitiator (MePEGA) 3-acrylamide phenylboronic acid (APBA) and 2-nitrobenzyl acrylate (NBA) using a surfactant-free miniemulsion reversible addition fragmentation chain transfer (RAFT) polymerization method. The as-prepared micelles were characterized by ^{1}H NMR, transmission electron microscopy (TEM), and dynamic light scattering (DLS). Due to the UV light and glucose-sensitivity of as-prepared micelles, the micelles could be used to encapsulate insulin. The insulin release properties of micelles under UV light irradiation and different glucose concentrations were investigated. The micelles exhibited relative lower cytotoxicity and excellent stability against protein solution. The loaded insulin remained the structure stability after release through CD spectra analysis. These results showed that the micelles are one of effective candidate carriers for insulin release.

Keywords Polymeric micelles · Glucose sensitivity · Insulin · Drug release

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Introduction

At present, diabetes has become a worldwide public health issue. Diabetes is a disease with excessive accumulation of glucose in blood, showing high blood sugars as the main features clinically. Typical types are I diabetes, II diabetes, gestational diabetes, and other special types. Pathogenesis of different diabetes is different, but all are characterized by insufficient insulin secretion [1]. Although gene therapy or insulin-transplant diabetes mellitus has been reported at present, insulin injection still is the most direct and most effective treatment [2]. For the determination of blood glucose levels in the human body, minimally invasive peripheral finger blood collecting is the common method currently [3]. It is an important implication for the diagnosis and treatment for diabetes if the release rate of insulin could be controlled according to the glucose concentration changes in blood. The environmental stimuli-sensitive polymer micelles could be able to response to different environmental stimulus, such as temperature, pH, and light, etc. Moreover, the loaded drugs could be released from these micelles under specific conditions. Consequently, development of glucose-responsive systems enable self-regulated drug delivery while constantly monitoring the blood glucose concentration in diabetic and cancer patients, thus reducing the discomfort of insulin injections and chemotherapy for the patients [4].

Currently, phenylboronic acid (PBA)-based glucose-sensing materials has generated enormous interests for their potential application in the treatment of diabetes and drug delivery. For

instance, 4-(1, 6-dioxo-2, 5-diaza-7-oxamyl) phenylboronic acid (DDOPBA) with a pK_a of 7.8 was fabricated and utilized for the preparation of glucose-sensitive hydrogels operating at physiological pH [5]. PBA and its derivatives are a kind of weak acids with a reported pK_a of 8.2–8.6. In aqueous solution, PBA exists in equilibrium between a triangular form and a tetrahedral form [5]. The tetrahedral form of PBA can complex with *cis*-diol compounds, for example, glucose and form stable but hydrophilic phenylborates [6], which shifts the equilibrium in the direction of increasing hydrophilic form but decreasing the hydrophobic form. Thus, PBA materials are showing glucose responsiveness.

Series of studies on the PBA-based glucose-responsive materials have been reported for the construction of self-regulated insulin delivery systems [7–13]. For example, microgels composed of PBA and poly (*N*-isopropylacrylamide) (PNIPAM) could swell and release insulin in response to glucose [10]. However, these materials displayed effective glucose responsiveness only slightly above the pK_a of PBA, that is, about pH=9.0 [14, 15], or in the presence of glucose with a higher concentration, which hindered their application in glucose-responsive delivery of insulin under the physiological condition [16]. Kataoka et al. [17, 18] synthesized glucose-responsive polymeric gels that could be operated at the physiological pH by using a new PBA derivative DDOPBA possessing an appreciably low pK_a (~7.8). Amino groups were introduced either into the polymer or in the vicinity of the phenylboronic acid moiety to decrease the apparent pK_a value of PBA because of the possible coordination between nitrogen and boron [12, 19, 20]. Yang et al. [21, 22] synthesized an amphiphilic block copolymer PEG-*b*-PPBDE-MA with phenylborate ester as a leaving group in response to glucose in the hydrophobic block. This block copolymer could self-assemble into core-shell micelles, which could be used as a glucose-sensitive drug carrier and successfully realized glucose-responsive release of insulin at neutral pH.

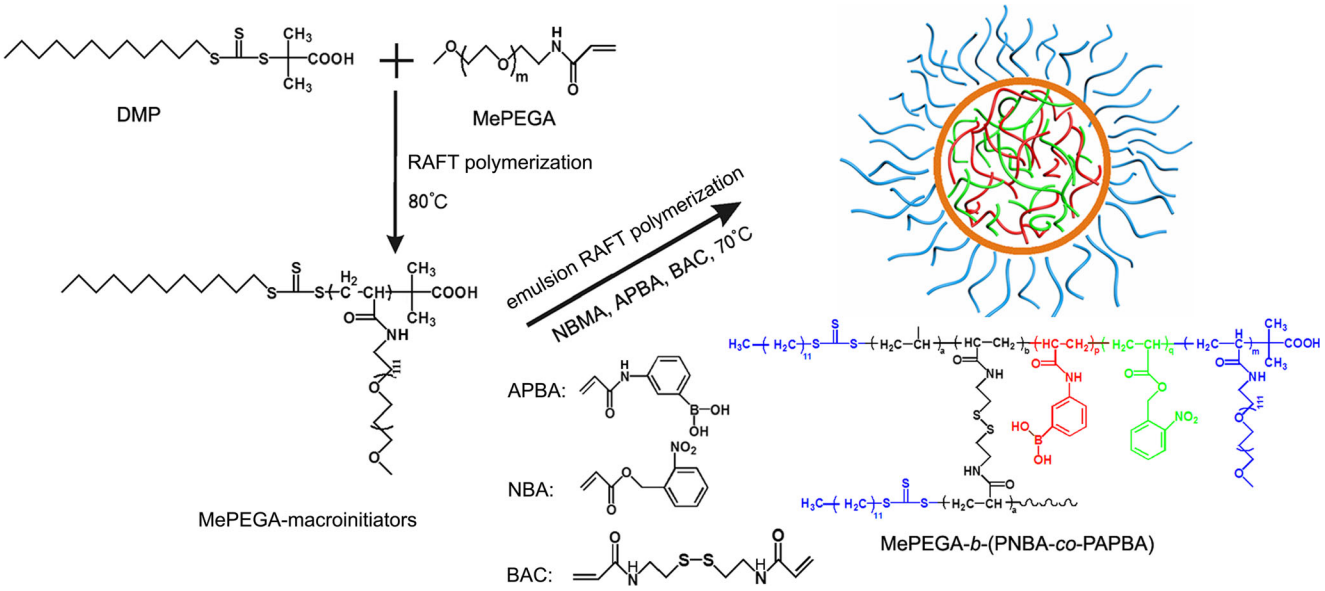
Herein, the glucose-sensitive poly (methoxypolyethylene glycol acrylamide)-*b*-[poly (2-nitrobenzyl acrylate)-*co*-poly (3-acrylamide phenylboronic acid)] (MePEGA-*b*-(PNBA-*co*-PAAPBA)) were synthesized via surfactant-free emulsion RAFT polymerization. The UV and glucose-responsive behaviors of polymer micelles were investigated under various conditions. The as-prepared micelles were designed as self-regulated insulin delivery systems, which could control insulin release in response to the change in the glucose level.

Results and discussion

As shown in Scheme 1, 2-nitrobenzyl acrylate (NBA) monomer is firstly synthesized by reacting acryloyl chloride with 2-nitrobenzyl alcohol. And the MePEGA macroinitiator is prepared via reversible additional-fragmentation chain transfer (RAFT) polymerization, using *S*-1-dodecyl-*S*″-(α, α″-dimethyl-α″-acetic acid) trithiocarbonate (DMP) as a RAFT agent. The structures and composition of MePEGA macroinitiator is confirmed by ^{1}HNMR spectra (Fig. S1). The synthesized MePEGA macroinitiator also can be utilized as stabilizing agent or surfactant for emulsion polymerization due to its amphiphilic property. Then, acrylamide phenylboronic acid (APBA) and NBA monomers are introduced by the polymer chain to form micelles. For further improving the stability of micelles, a small quantity of N, N′-bis(acryloyl)cystamine (BAC) is introduced in the synthesis process. The number average molecular weight (M_n) of as-synthesized MePEGA$_m$-*b*-(PNBA$_q$-*co*-PAAPBA$_p$) (M_n=9.58×10^4 g/mol) is determined by gel permeation chromatographic (GPC) measurement.

The composition of MePEGA-*b*-(PNBA-*co*-PAAPBA) micelles is determined by ^{1}HNMR spectra. As shown in Fig. 1a, the peak at about 3.5 ppm is contributed to the protons from methylene (−CH_2-) of PEG blocks. However, the signals from the phenyl protons in the PNBA/PAPBA segment are not founded. Due to the hydrophobic property of PNBA/PAPBA segment, they are assembled into the inner of micelles. Because of the redox sensitive of disulfide bonds, these linkages can be cleaved by dithiothreitol (DTT). The assumption is further confirmed by the ^{1}HNMR analysis. Adding a trace of DTT (10 mM) into the micelles solution, the new peaks at around 5.5 and 8.0 ppm are appeared, which are attributed to methylene in PNBA and phenyl protons in PNBA/PAPBA segment, respectively (Fig. 1b). Therefore, it indicates that micelles with PAPBA and PNBA segment as core, hydrophilic MePEG segment as shell can be formed in the aqueous solution. The diameter of micelles is observed with range from 80 to 120 nm that was determined by transmission electron microscopy (TEM) analysis (Fig. 1c). The mean diameter of micelles is 119 nm that was observed by dynamic light scattering (DLS) measurement. The relatively smaller size of micelles is beneficial to their applications in biology, since nanocarriers with a size under 200 nm are more likely to be taken up by cells [23–25].

The stimuli responsive properties of micelles were further investigated under different conditions. Under UV irradiation at 365 nm for 30 min, the color of micelles dispersion has been turned to yellow (Fig. S2). It is caused by photocleavage of the chromophore of *o*-nitrobenzyl groups. Hydrophobic 2-nitrobenzaldehyde molecules are detached from PNBA, and hydrophobic PNBA chains are converted into hydrophilic poly (acrylic acid) [26]. The nanostructure of micelles based on the hydrophilic-hydrophobic balance is wrecked. However, the formed carboxylic acid group can be reacted with imino groups from MePEGA to form hydrogen bonds. And the photocleaved micelles still can form some aggregates with larger size (Figs. 2a and d) [27].



Scheme 1 Preparation of MePEGA-*b*-(PNBA-*co*-PAAPBA) polymer micelles

It is well known that phenylboronic acid compounds exhibit equilibrium between the uncharged and charged forms in an aqueous milieu of weak alkalescence (pH value near its pK_a). The uncharged form is insoluble while the charged form becomes soluble. In an aqueous solution with a pH value below 8.2, the majority of PAPBA exists in its uncharged

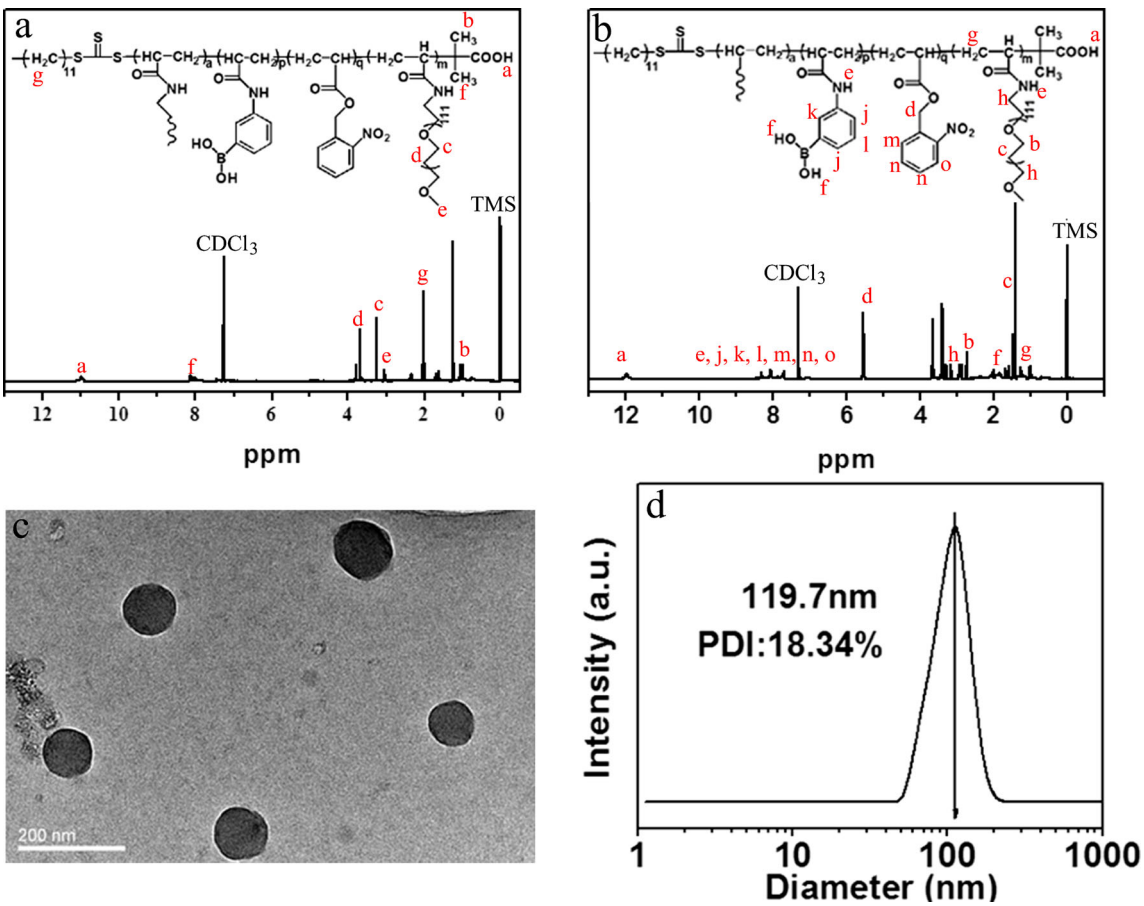


Fig. 1 ^{1}HNMR spectra of MePEGA-*b*-(PNBA-*co*-PAAPBA) micelles *before* (**a**) and *after* (**b**) cleavage of disulfide bonds. TEM image (**c**) and DLS analysis (**d**) of as-prepared micelles

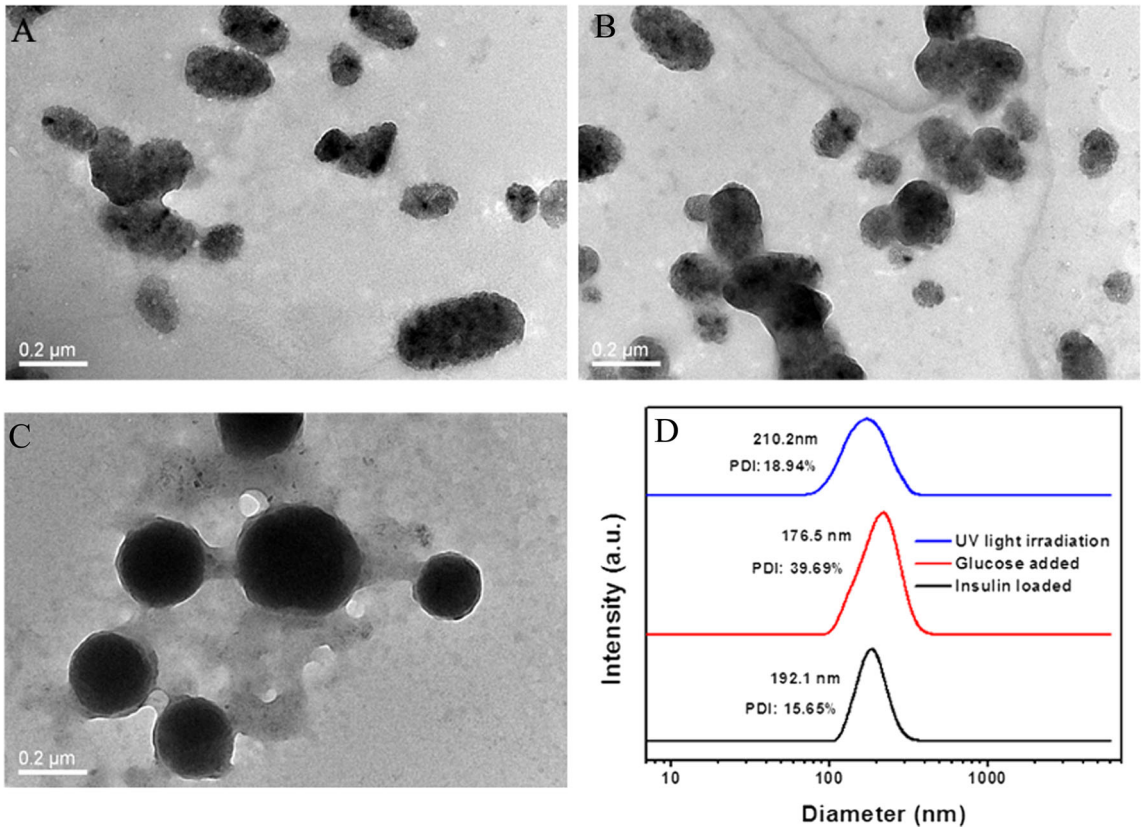


Fig. 2 TEM images of the as-prepared micelles after UV irradiation for 10 min (λ_{max}=365 nm, 75 mW/cm^2, pH=7.0, $C_{glucose}$=0.0 mmoL/L) (**a**), with the addition of glucose ($C_{glucose}$=15.0 mmol/L, pH=7.0) (**b**) and insulin-loaded micelles (pH=7.0, $C_{glucose}$=0.0 mmoL/L) (**c**). DLS analysis of the as-prepared micelles from **a**, **b**, and **c** samples

form while only a small quantity of PAPBA is charged. PAPBA can be acted as a kind of Lewis acid. When glucose molecules are introduced, the negatively charged PAPBA can combine with 1, 2-diols of glucose and form stable and soluble phenylborate [28], which drives the equilibrium to the side for forming more combination. Meanwhile, this process also improves the solubility of the PAPBA units in the aqueous media. The size of resultant micelles increases as well due to the complex of glucose and PAPBA segments, as shown in Fig. 2b and d [29, 30].

Insulin is hydrophilic drug in acid solution (pH=2.0–3.0) and basic solution (pH=8.0–8.5), but it is hydrophobic in the range of pH=5.3–5.4 (the isoelectric point of insulin). During the preparation of micelles, the majority of insulin in the aqueous media is insoluble. Due to the hydrophobic interaction between the inner of micelles and insulin, the hydrophobic

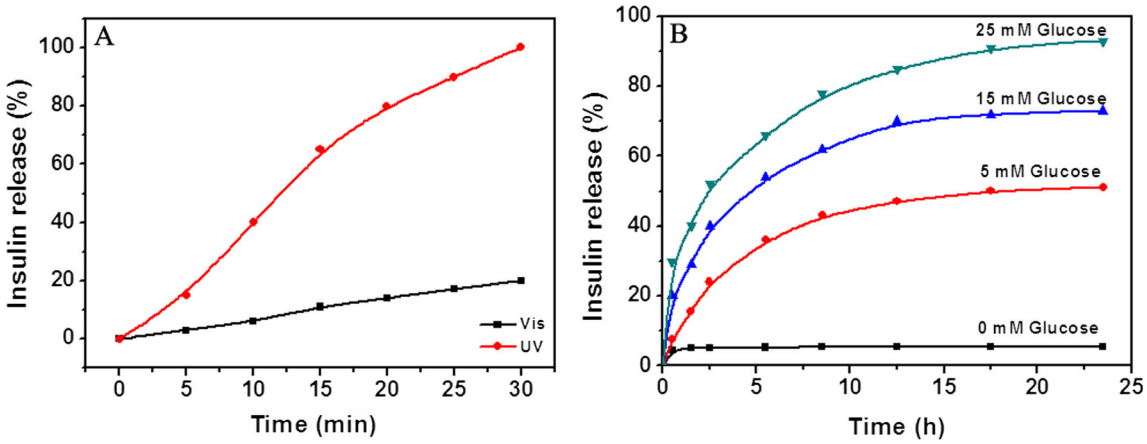


Fig. 3 The insulin release profiles against under UV and visible-light irradiation (**a**) and different concentration of glucose (**b**)

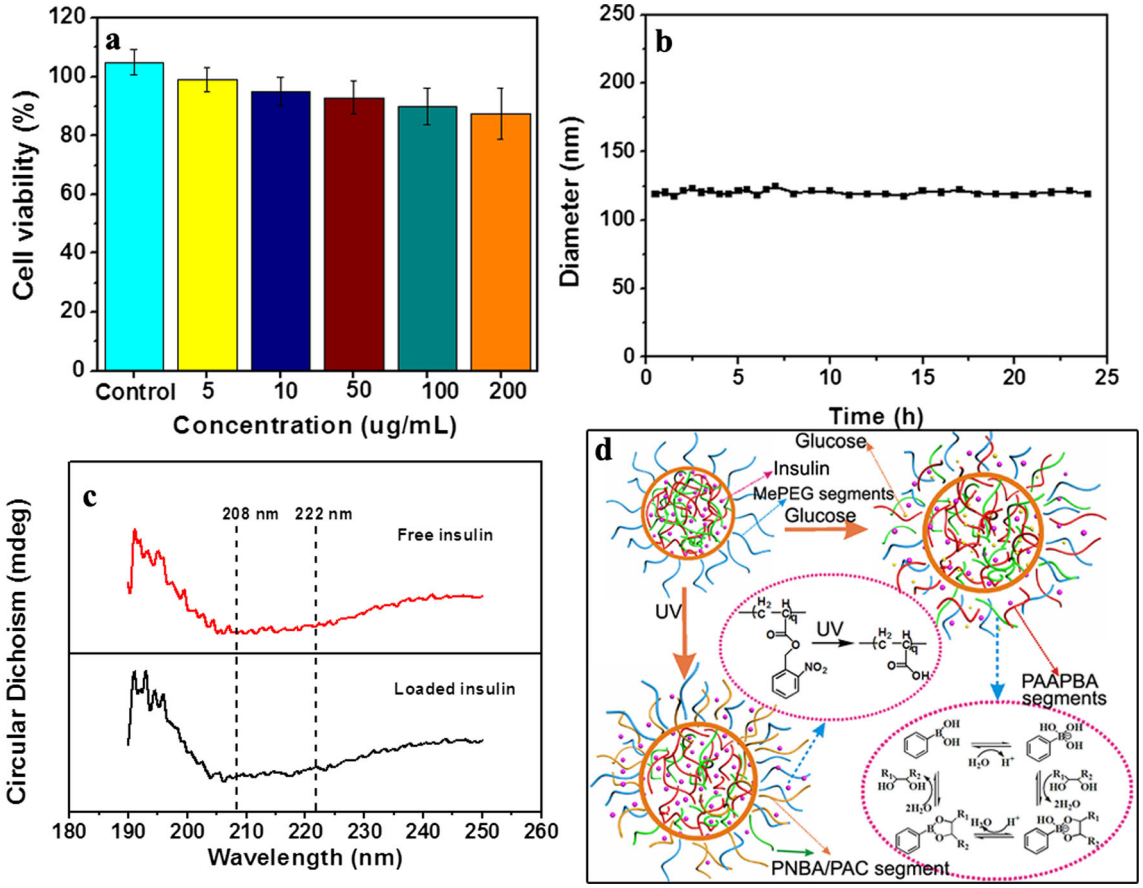


Fig. 4 Viability of HeLa cells against micelles for 24 h by MTT assay, data are presented as average standard deviation (n=5) (**a**). Stability of MePEGA-*b*-(PNBA-*co*-PAAPBA) micelles in 100 % FBS at 37 °C against incubating time (**b**). Circular dichroism (CD) spectra of insulin for free and released insulin from micelles (**c**). Schematic illustration of the evolution of micelles under different conditions (**d**)

insulin molecules can be loaded into the inner of micelles. The size of insulin-loaded micelles increases slightly (~192.1 nm) compared with that of the original micelles, as shown in Figs. 2c and d. In this report, the insulin loading capacity is around 25.6 % that was determined by UV-vis spectroscopy.

The loaded insulin can be released under the certain external conditions. As shown in Fig. 3a, it reveals the insulin release rate under the light irradiation. Under the visible-light irradiation, only ~20 % of loaded insulin can be released after 30 min. However, near 98 % of loaded insulin can be released under UV light irradiation (λ_{max}=365 nm, 75 mW/cm^2). The possible for this is PNBA converted into hydrophilic poly (acryl acid) since 2-nitrobenzaldehyde molecules are detached from PNBA under UV light irradiation; the structure of micelles is wrecked, and the hydrophilic-hydrophobic balance of micelles is disturbed as well. The insulin release profiles also exhibit the glucose-responsive characteristics. As shown in Fig. 3b, the loaded insulin release can be ignored under dark and glucose concentration at 0 mM. Only approximately 5 % of insulin is released in the solution after 24 h. With increasing glucose concentration from 5 to 25 mM, the cumulative amount of released insulin increase obviously. The cumulative amount of released insulin is around 93 % in the buffer solution with 25 mM glucose.

The relative cytotoxicity of micelles was estimated by MTT viability assay against HeLa cells [31]. Figure 4a shows the cell viability after 24 h of incubation with micelles at the different concentrations. When the concentration of micelles increased to 200 μg mL^{-1}, the viability of cells is still kept above 80 %, indicating low toxicity of the as-prepared micelles. It partly owes to limited organic solvents that were involved during the surfactant-free miniemulsion RAFT polymerization and excellent biocompatibility of PEG chains at the surface of micelles [32–34]. Inspired by the delicate composition and structure of most outer-cell membranes [24, 35–37], the protein-adsorption-resistance property of as-prepared micelles has been investigated and utilized in creating biomimetic surface/interface. Stability of as-prepared micelles against 100 % fetal bovine serum FBS solution was investigated by measuring the diameter change as shown in Fig. 4b. The as-prepared micelles almost retained their original size even after incubating for 24 h in 100 % FBS, due to its great stability against protein solution. Thus supports its potential stability when applied in vivo.

The activity of the released insulin was assayed for the structure stability analysis of released insulin using CD spectra. Twenty percent micelles containing insulin was prepared and allowed to release insulin for 2 days at 37 °C. CD spectrum of the released insulin was compared with native insulin dissolved in 0.05*N* HCl. Native insulin showed two negative bands at 208 and 222 nm, respectively, in the far-UV region (Fig. 4c), which matched with the previous observations [38, 39]. The band at 208 nm corresponds to the α-helix structure, and the band at 222 nm corresponds to β-pleated sheet structure. Released insulin also displayed similar spectrum as that of the native insulin, indicating secondary structure of the insulin remains unaltered after the release.

The possible process for the encapsulation and release of insulin from as-prepared micelles in aqueous solution is demonstrated in Fig. 4d. The hydrophobic insulin drug can be entrapped in the hydrophobic core of micelles. Under the UV light irradiation, the photocleavage of PNBA segment in the inner of micelles lead to release insulin faster. Meanwhile, adding certain amount of glucose into media, the release rate also can be accelerated due to the affinity reaction between the glucose molecules and phenylboronic acid segment of PAPBA. Therefore, the as-prepared micelles are expected to load and release of insulin under external stimuli.

Conclusion

The polymeric micelles were prepared by the copolymerization of poly (methoxypolyethylene glycol acrylamide)-macroinitiator (MePEGA) 3-acrylamide phenylboronic acid (APBA) and 2-nitrobenzyl acrylate (NBA). Due to the UV light and glucose-sensitivity of as-prepared micelles, the micelles could be used to encapsulate insulin. The insulin release properties of micelles under UV light irradiation and different glucose concentration were investigated. The results showed that this kind of micelles could be used to fabricate micelles platforms for repeated and effective on-off drug release.

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