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Novel amphiphilic glucose-responsive modified starch micelles for insulin delivery†

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The high pK_a (8.26 to 8.6) of PBA has restricted its glucose-responsiveness in physiological conditions, and the high cytotoxicity of polymers is also a limiting problem in their potential application for insulin delivery. Novel amphiphilic glucose-sensitive dialdehyde starch polymers containing 3-aminophenylboronic acid (APBA) as a glucose-responsive group and mPEGylated dialdehyde starch (mPEG-DAS) with hydrophobic 7-hydroxycoumarin-4-acetic acid (Cou) were synthesized. This dialdehyde starch derivative can self-assemble into mPEG-DAS-APBA-Cou micelles with "shell-core" structures in phosphate-buffered saline solution (PBS). In addition, the drug-loaded micelles can release insulin rapidly in response to hyperglycemia in a physiological environment. The results demonstrated that the mPEG-DAS-APBA-Cou micelles showed notable glucose responsive behavior near the physiological range. The insulin release from the nanocarriers is sensitive to different concentrations of glucose, releasing insulin rapidly under the conditions of 3 mg mL⁻¹ glucose while demonstrating comparatively inert release at 1 mg mL⁻¹ glucose (pH 7.4). MTT assays and hemolysis studies both confirmed that the mPEG-DAS-APBA-Cou micelles have low cytotoxic activity to A549 cells and low blood toxicity. These results suggest that the glucose-sensitive dialdehyde starch micelles (mPEG-DAS-APBA-Cou) have potential applications as a glucose-responsive material for insulin delivery.

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Introduction

Glucose-responsive materials based on phenylboronic acid (PBA) have attracted great interest due to their self-regulated insulin delivery capacity, which can be applied in current clinical diabetes treatment.^{1–4} Various forms of delivery systems based on PBA materials have been constructed, including gels,^{5,6} micelles⁷ and vesicles.⁸ For PBA-containing gel systems, the addition of glucose can induce deswelling instead of swelling. Asher *et al.*⁹ first reported PBA-containing hydrogels in which polyethylene glycol (PEG) or crown ether functional groups were incorporated. In these hydrogels, glucose could simultaneously bind with two boronates of the hydrogel. In addition, with increasing crosslinking density, the swelling degree of the hydrogels gradually decreased. Therefore, much effort has been made to design polymers with glucose-responsiveness behavior. In drug delivery systems, gels with glucose-responsiveness maintain structural integrity as drug

carriers; however, they encounter the dilemma of possible burst release of the drug in the blood, leading to hypoglycemia. In addition, the gels are large in size, up to micron dimensions, and are easily removed by the permeability and retention effect (EPR), which is undesirable for long circulation.¹⁰ Particularly, self-assembled glucose-responsive micelles^{7,11–13} and vesicles^{8,14} with PBA-containing polymers have shown uncommon and notable properties.

Compared to gels, which lack protection from a corona layer,¹⁵ self-assembled micelles are formed with the hydrophilic groups of PEG as an outer shell that is resistant to the approach of proteins to the surfaces of the nanocarriers; this shields the nanocarriers from recognition by the body's immune system^{16,17} and increases blood circulation time *in vivo* by the protection of PEG, providing potential applications as drug carriers for insulin delivery.^{15,18–20} Kim *et al.*^{21,22} synthesized a new class of polymers possessing styreneboroxole and *N*-functionalized maleimide with sugar-responsive behavior. The boroxole-based monomer bound to oligo (ethylene glycol) groups as a chain-transfer agent adjusted to changes in glucose solubility and demonstrated sensitive behavior at reduced glucose concentrations close to physiological conditions. Shi *et al.*⁷ developed drug-loading micelles, which were self-assembled by covalent complexation of phenylboronic acid and glycosyl. The sensitive behavior of the complex micelles required the protection of PEG. Shi *et al.*²³ also reported micelles with favourable

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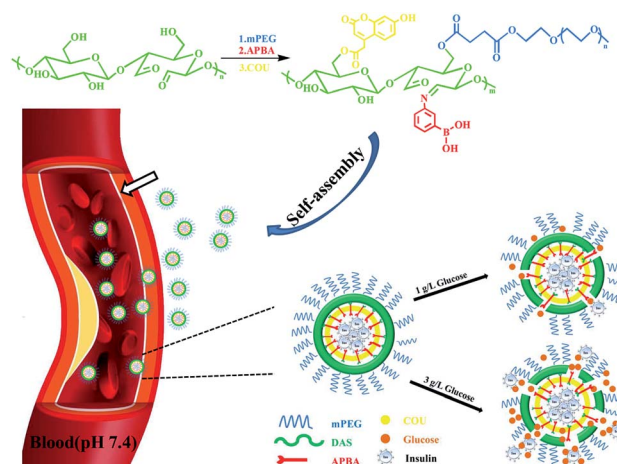


responsiveness based on polyethylene glycol-co-poly(acrylic acid) with modified APBA (PEG-PAA-PBA); the lack of degradation behavior of PAA and PBA limited their potential for degradation behavior *in vivo*. It is necessary to adopt biodegradable and biocompatible materials, such as natural polysaccharides, in place of refractory fragments.

The medical treatment for diabetes is a persistent process; hence, it requires baseline drugs that are non-toxic, show sustained release, are biodegradable, and are non-toxic to the body for *in vivo* applications. However, the compatibility between favourable glucose-responsiveness and high biodegradability and biocompatibility is a limitation for drug delivery in current studies. Therefore, designing glucose-sensitive materials is imminently and greatly needed to achieve high biodegradability and biocompatibility in insulin delivery systems.²⁴ Starch, a major natural source of polysaccharides, exhibits nontoxicity, compatibility, and nonimmunogenicity. Thus, it has been widely used in the pharmaceutical field as an excipient for drug delivery systems.^{25,26} Starch has superior advantages of low cost and biodegradability due to hydrolysis and human enzymes in comparison to other polysaccharides.²⁷ In addition, various functional materials can be attained *via* modification of starch due to the large numbers of functional hydroxyl groups in the chains.^{28,29} The use of dialdehydes has been explored to obtain more biocompatible materials;³⁰ aldehyde-functionalized starch has aroused considerable interest due to its minimal toxicity.³¹ The preparation of dialdehyde starches containing aldehyde groups has been achieved by reaction with sodium periodate (NaIO_4).^{32,33} It is noteworthy that micelles can be prepared *via* self-assembly of amphiphilic starch derivatives.^{29,34,35} Lehr *et al.*²⁷ prepared starch-graft-polyethylenimine copolymers for efficient biodegradable gene delivery. Zhang *et al.*³⁶ synthesized grafted copolymers by a reaction between starch and poly(L-glutamic acid) with pH-responsive behavior for controlled release of insulin; this is a fascinating option for synthetic polymers in drug delivery systems.

For most reports focusing on glucose-responsiveness, the high pK_a (8.26 to 8.6) of PBA has restricted its practical applications.^{37,38} However, researchers have attempted to decrease the pK_a of PBA. For example, Yoon *et al.*³⁹ reported a PBA-containing complex with polyol polymer, a stronger acid, and decreased the pK_a of the PBA derivative. Wu *et al.*⁴⁰ introduced amino groups into polymers to enhance the binding affinity of nitrogen and boron and to self-regulate insulin release, even responding positively to glucose in physiological conditions. A subtle relationship may exist in polymers between the structure of coumarins and the pK_a of PBA. Therefore, PBA-based materials are susceptible to glucose-responsiveness at lower pH values than the pK_a of PBA.

Herein, a series of dialdehyde starch derivative polymers (mPEG-DAS-APBA-Cou) were prepared by grafting PEG as the hydrophilic outer shell, Cou as the hydrophobic core and PBA as the glucose-responsive group to a dialdehyde starch backbone. Introducing Cou may decrease the pK_a of a material, and the starch backbone mainly enhances its biodegradable behavior. The polymers were able to self-assemble into spherical micelles in PBS solution. The polymers showed rapid response to



Scheme 1 The formation of insulin-loaded core-shell mPEG-DAS-APBA-Cou micelles and their insulin release behavior under different concentrations of glucose in the blood microenvironment.

glucose due to the hydrophobic micelle core and the apparently low pK_a of PBA in physiological conditions, as illustrated in Scheme 1. The micelles quickly disassembled to release insulin because of the sensitivity of the PBA-based polymer to different concentrations of glucose in the blood microenvironment. The safety and biocompatibility of the micelles as drug carriers were also confirmed by cytotoxicity and hemolysis tests.

Experimental

Materials

Soluble starch (M_w 8.8 kDa) was provided by the Zhejiang Linghu Chemical Reagent Factory. Polyethylene glycol monomethyl ether (mPEG, M_n 1.9 kDa), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), 7-hydroxycoumarin-4-acetic acid, 3-aminophenylboronic, insulin from porcine pancreas and pyrene were purchased from Aladdin Chemistry Co., Ltd. Succinic anhydride, *N*-hydroxysuccinimide, and 4-dimethylaminopyridine (DMAP) were purchased from J&K Reagent Company. All other chemicals were of analytical grade and were used without further purification.

Synthesis and characterization of dialdehyde starch derivatives

Synthesis of mPEGylated modified dialdehyde starch (mPEG-DAS) and APBA-functional dialdehyde starch copolymers (mPEG-DAS-APBA). The mPEGylated dialdehyde starch (mPEG-DAS) was conveniently synthesized by conjugating mPEG-COOH with different oxidation degrees (ODs) of DAS according to previous reports.^{41,42} Briefly, DAS with different ODs (0.2 g), mPEG-COOH (0.38 g), EDC·HCl (0.23 g) and DMAP (0.05 g) were dissolved in DMSO and stirred for 48 h under ambient conditions. Then, to remove DMSO and unreacted substances, the mixture was dialyzed in a dialysis bag (MWCO 10 kDa) for three days. Finally, the solution was lyophilized and the target product mPEGylated dialdehyde starch (mPEG-DAS) was obtained.



mPEG-DAS (0.2 g) and APBA (0.1 g) were dissolved in DMSO and heated to 60 °C for 6 h under nitrogen atmosphere. Then, the mixture was purified in a dialysis bag (MWCO 3.5 kDa) for three days and lyophilized to obtain the product APBA-functional dialdehyde starch (mPEG-DAS-APBA).

Synthesis of Cou-ornamented mPEG-DAS-APBA copolymers (mPEG-DAS-APBA-Cou). Copolymers containing the carboxyl groups of Cou were conveniently coupled with the hydroxyl groups of mPEG-DAS-APBA in the presence of EDC/NHS as described previously.⁴³ Briefly, mPEG-DAS-APBA (0.1 g) and Cou (0.1 g) were dissolved in DMSO containing EDC (0.15 g) under stirring at 45 °C for 12 h. After that, NHS (0.03 g) was added to the above solution. The solution was purified by dialyzing against a deionized water/methanol mixture (1/4 v/v) for 2 days, followed by dialyzing against deionized water for 3 days using dialysis bags (MWCO 3.5 kDa). Finally, Cou-ornamented mPEG-DAS-APBA (mPEG-DAS-APBA-Cou) was obtained by freeze-drying.

Characterization of dialdehyde starch derivatives. Dialdehyde starch derivatives were characterized by ¹H NMR spectra, which were recorded on a Bruker AVANCE III 400 MHz spectrometer (Germany), and by FTIR spectra, which were recorded on an Agilent Technologies Cary 600 Series FTIR spectrometer (USA) at room temperature.

Preparation and characterization of dialdehyde starch derivative micelles

Micellisation of dialdehyde starch derivatives. The micelles of mPEG-DAS-APBA and mPEG-DAS-APBA-Cou were prepared according to literature procedures.⁴⁴ Herein, 20 mL of PBS was slowly dropped into solutions of the mPEG-DAS-APBA and mPEG-DAS-APBA-Cou polymers (20 mg) under gentle shaking at 37 °C over 3 h. Then, the solution was sonicated for 10 min to obtain an optically transparent solution. Finally, the micelles solution was passed through 0.45 nm membrane filters and stored at room temperature.

Characterization of dialdehyde starch derivative micelles. The critical micelle concentration (CMC) was estimated using pyrene as a probe by fluorescence spectrophotometry; the spectra were recorded on a Perkin-Elmer LS55 (USA) instrument at room temperature. Micelle solutions with different concentrations ranging from 10⁻⁴ to 10⁻¹ mg mL⁻¹ were prepared. The fluorescence measurements were acquired at an emission wavelength of 395 nm, and the excitation was monitored from 350 to 400 nm. Dynamic light scattering (DLS) was used to measure the sizes of the micelles. The zeta potentials of the particles in different pH solutions were determined with a Malvern Zetasizer Nano S instrument (Malvern Instruments Ltd.). The morphologies of the micelles were examined using TEM, which was performed with a JEM-2100F instrument (JEOL, FEI, Japan).

In vitro stability study of micelles

The stabilities of the micelles were tested by dispersion in PBS solution (pH 7.4) and gentle shaking at 100 rpm min⁻¹ at physiological temperature. The diameters of the micelles were

measured at intervening times (4 h, 8 h, 24 h and 48 h) using DLS.

Glucose-responsiveness of the complex micelles

The pK_a values of the complex micelles were determined by UV-vis according to a reported method.⁴⁵ The glucose-sensitivity behaviors of the mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles in PBS were measured by DLS. The concentrations of glucose solution were 0, 1 and 3 mg mL⁻¹, referring to the final diluted concentration after adding glucose to the micelle solutions. The micelle solutions were prepared for DLS analysis by filtering as mentioned.

Hemolysis assay

The membrane disruption of red blood cells (RBCs) can be employed to evaluate the blood toxicity of polymers. Fresh human blood samples were obtained from Lanzhou University first affiliated hospital and were centrifuged at 1600 rpm min⁻¹ for 5 min; all experiments were performed in compliance with the approval of the Institutional Authority for Laboratory Animal Care. A549 cells were derived from an assay on rabbit mesenchymal stem cells. Afterwards, the micelle groups were prepared by adding 0.2 mL RBC supernatant to 0.8 mL micelle solution (1 mg mL⁻¹). Then, the micelle systems were incubated at 37 °C for 2 h. Furthermore, the micelle systems were centrifuged at 1600 rpm min⁻¹ for 5 min. Finally, the supernatant (0.5 mL) was added to 5 mL PBS, and the percentages of hemolysis of the RBCs with various samples were calculated *via* the absorbance of released hemoglobin at 570 nm by UV-vis spectrophotometer (Perkin-Elmer Lambda 35, USA) based on the following equation:

$$\% \text{ hemolysis} = \frac{A_{\text{sample}} - A_{\text{negative}}}{A_{\text{positive}} - A_{\text{negative}}} \times 100\%$$

where A_{sample} , A_{negative} , and A_{positive} represent the absorbances measured from the sample, PBS, and water groups, respectively.

Insulin loading and glucose-responsive release

Preparation of drug-loaded micelles. The polymers (10 mg) were added to 10 mL insulin solution (0.25 mg mL⁻¹) under constant stirring at 37 °C. Drug-loaded micelles were obtained by dialysis for 24 h using dialysis bags (MWCO 8 kDa). The dosage of insulin was monitored by a UV-vis spectrophotometer at a wavelength of 276 nm. The insulin drug loading efficiency (DLE) and drug loading capacity (DLC) were calculated from the following equations:

$$\text{DLE}\% = \frac{\text{encapsulated insulin}}{\text{total insulin}} \times 100\%$$

$$\text{DLC}\% = \frac{\text{encapsulated insulin}}{\text{micelle weight}} \times 100\%$$

***In vitro* glucose-responsive release of insulin-loaded micelles.** The release of insulin from the micelles *in vitro* was



studied by dialysis. The drug-loaded micelles in PBS with different glucose concentrations of 0, 1 and 3 mg mL⁻¹ were incubated and gently shaken at 37 °C. The released insulin was sampled using an UV-vis absorption spectrometer at 276 nm. Cumulative release is expressed as the total percentage of drug released through the dialysis membrane over time.

Circular dichroism spectroscopy

The structure of the released insulin was determined by circular dichroism (CD) spectra, which were acquired using a DSM1000 CD spectropolarimeter (USA). A standard insulin solution of 1 mg mL⁻¹ was prepared in PBS (pH 7.4) for CD measurements.

Cytotoxicity test

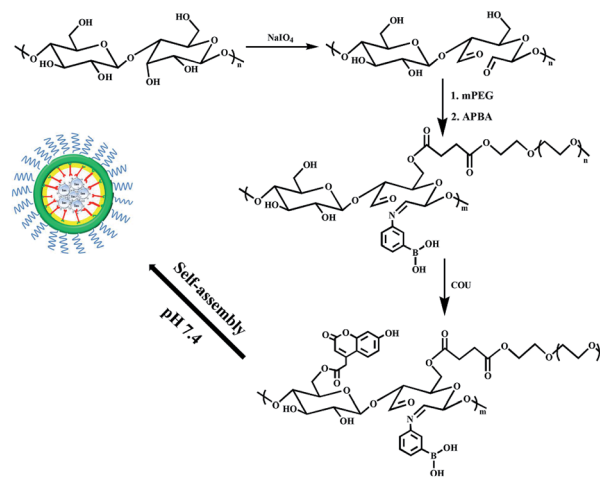
The cell suspension in culture medium was plated at 5×10^4 cells per well in a 96-well plate and incubated in a humidified atmosphere with 5% CO₂ for 24 h at 37 °C. Then, a methyl thiazolyl tetrazolium viability assay (MTT) was carried out with A549 cancer cells from rabbit mesenchymal stem cells. The culture medium was replaced with 200 µL of the prepared culture medium containing blank micelles with concentrations of 1, 5, 10, 50, and 100 µg mL⁻¹ for 24 h and 48 h. MTT solution was added to each well, and the wells were incubated for another 4 h. The optical density was measured using a microplate reader at 490 nm. The cell viability was calculated by means of the percent ratio of the absorbance of the mixtures and micelles to the control.

Results

Synthesis and characterization of dialdehyde starch derivatives

In this work, we successfully synthesized amphiphilic dialdehyde starch derivatives containing hydrophilic mPEG, APBA functional groups and hydrophobic Cou. The dialdehyde starch derivatives of mPEG-DAS-APBA-Cou were synthesized *via* three steps (Scheme 2). Firstly, mPEG-COOH was introduced as a hydrophilic group to dialdehyde starch by an etherification reaction. Then, the conjugated polymer was readily synthesized by reacting the aldehyde groups of mPEG-DAS with PBA *via* Schiff bases (C=N-) due to its high stability. Finally, Cou as the hydrophobic group was attached by esterification reactions of the hydroxyl groups on the dialdehyde starch chains. Dialdehyde starch with different ODs was synthesized and determined by hydroxylamine hydrochloride titration and confirmed by FTIR spectra, as shown in Fig. S1 and Table S1.†

Typical ¹H NMR spectra of the copolymers are presented in Fig. 1. Specifically, the glucose units and aldehyde groups of dialdehyde starch (4.99 to 5.51 and 9.26 ppm, respectively) are shown in Fig. 1B.⁴⁶ The respective assigned peaks of both dialdehyde starch and mPEG could be found in their spectra. According to our published method,⁴⁷ the degree of substitution (DS) of mPEG in mPEG-St polymer was calculated to be 0.25. The protons of the phenyl groups appeared in the range from 6.98 to 7.6 ppm in PBA in Fig. 1A (e, 7.46 ppm), while typical peaks of mPEG from the methylene (-CH₂-CH₂-, b and c) and



Scheme 2 The synthetic scheme of mPEG-DAS-APBA-Cou.

terminal methoxyl (-O-CH₃, a) groups were observed at 3.51 ppm and 3.34 ppm, respectively. Because Cou and PBA contain phenyl groups, the Cou-OH spectrum features absorption peaks at 3.24, as described previously.⁴⁸ The DS of APBA was 0.15 according to the calculated copolymer compositions of the integrated area between the saccharide (5.11 ppm) and APBA (7.46 ppm) signals. Similarly, the DS of the Cou signals at 3.24 ppm was 0.1. In order to prepare mPEG-DAS-APBA polymers with different degrees of cross-linking, a series of experiments was designed with different ODs of mPEG-DAS and APBA. The degrees of cross-linking in the mPEG-DAS-APBA polymers are listed in Table 1. For different ODs of 20%, 40% and 60% of mPEG-DAS, the corresponding degrees of cross-linking were 0.08, 0.15, and 0.17, respectively.

The FTIR spectra of St, DAS and mPEG-DAS-APBA-Cou are shown in Fig. 2. Fig. 2a shows the characteristic peaks of the St sample; a faint adsorption band can be found at 1660 cm⁻¹ that corresponds to starch bone stretching vibrations. Compared with starch, the FTIR spectrum of Fig. 2c shows -C=O- stretching vibrations and a new strong characteristic absorption band at 1106 cm⁻¹ resulting from C-O asymmetric stretching vibrations; moreover, the -C=O- absorption peak was weaker than that in the spectrum of DAS (Fig. 2b).

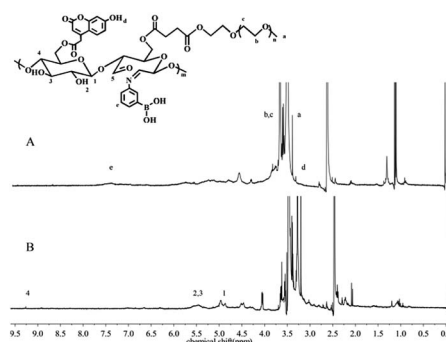


Fig. 1 ¹H NMR spectra of 40% mPEG-DAS-APBA-Cou (A) and 40% mPEG-DAS (B) in DMSO-d₆ solution.



Table 1 Physicochemical properties of synthesized starch derivatives

Sample	Feeding ratio		Graft ratio		Zeta (mV)	CMC (mg mL ⁻¹)	DLE (%)	DLC (%)
	mPEG-DAS/APBA	mPEG-DAS/Cou	APBA	Cou				
20% mPEG-DAS-APBA	1/5	—	0.08	—	-1.39 ± 0.12	1.32 × 10 ⁻³	—	—
40% mPEG-DAS-APBA	1/5	—	0.15	—	-3.39 ± 0.49	8.13 × 10 ⁻³	—	—
60% mPEG-DAS-APBA	1/5	—	0.17	—	-4.09 ± 0.42	3.02 × 10 ⁻²	—	—
mPEG-DAS-APBA-Cou	1/5	1/3	0.15	0.1	-9.96 ± 0.87 (pH 7.4) -6.34 ± 0.32 (pH 5.4)	3.57 × 10 ⁻³	30.4	9.4

Characterization of dialdehyde starch derivative micelles

During the self-assembly process, CMC is a vital characteristic of polymers that not only indicates the self-aggregation behavior of micelles, but also evaluates their stability.⁴⁹ The self-assembly behaviors of different ODs of mPEG-DAS-APBA micelles and mPEG-DAS-APBA-Cou micelles were investigated by fluorimetry. The changes in the intensity ratio of I_{384}/I_{373} , which are dependent upon high sensitivity to hydrophobicity, are plotted in Table 1 and Fig. 3A. It can be observed that the CMC increased with increasing value of OD.

As shown in Fig. 3, TEM and DLS were performed to evaluate the aggregation behaviors of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles as well as their sizes and morphologies. The morphologies of the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou polymeric self-assemblies were spherical, with diameters of about 175 ± 7.5 nm and 113.5 ± 3.5 nm, respectively. On the other hand, the “core-shell” structures of the micelles with different compositions were also very distinct in the TEM observations (Fig. 3C and D). Moreover, the DLS results indicated that the hydrodynamic diameters of the 40% mPEG-DAS-APBA (241.4 nm) and mPEG-DAS-APBA-Cou (177.9 nm) micelles decreased upon addition of hydrophobic Cou, where each of the groups exhibited a monomodal size distribution (Fig. 3B). The zeta potential values of the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou polymers with “core-shell” structures are shown in Table 1. The zeta value of 40% mPEG-DAS-APBA is -25.39 ± 0.49 mV (PBS 7.4), whereas slight changes in the zeta potential of the mPEG-DAS-APBA-

Cou micelles were observed at -9.96 ± 0.87 mV (PBS 7.4) and -6.34 ± 0.32 mV (PBS 5.4).

In vitro stability study of dialdehyde starch derivative micelles

First, the stabilities of the dialdehyde starch derivative micelles were measured by DLS. As shown in Fig. 4A, the stabilities of the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles were investigated at 1 mg mL⁻¹ for 4 h, 8 h, 24 h and 48 h at 37 °C. The 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles changed distinctly from 241.4 nm to around 438.2 nm and from 177.9 nm to around 307 nm after 48 h, respectively. The 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles both showed slight increases in particle size after 8 h and considerable increases after 24 h.

Glucose-responsiveness of dialdehyde starch derivative micelles

The UV absorbance (268 nm) is shown in Fig. 4B; the pK_a values of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou were estimated to be 8.0 and 7.6. It can be clearly seen in Fig. 4C that the particle size of the mPEG-DAS-APBA-Cou micelles remained almost unchanged in 70 min in the presence of 0 g L⁻¹ glucose. Notably, glucose-responsive behavior was observed at 1, 2 and 5 g L⁻¹ in different concentrations of

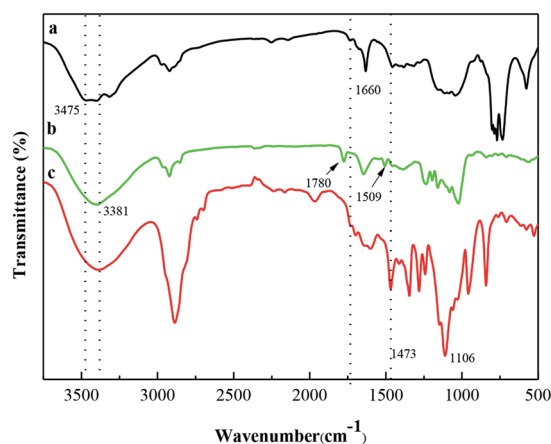


Fig. 2 FTIR spectra of St (a), DAS (b), and mPEG-DAS-APBA-Cou (c).

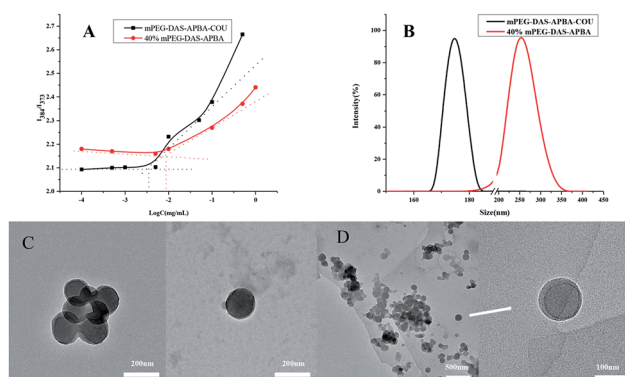


Fig. 3 Characterization of 40% mPEG-DAS-APBA micelles and mPEG-DAS-APBA-Cou micelles. Variations of intensity ratio (I_{384}/I_{373}) versus logarithm of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles (A), size distributions of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles (B), TEM image of the 40% mPEG-DAS-APBA micelles (C), TEM image of the mPEG-DAS-APBA-Cou micelles (D).



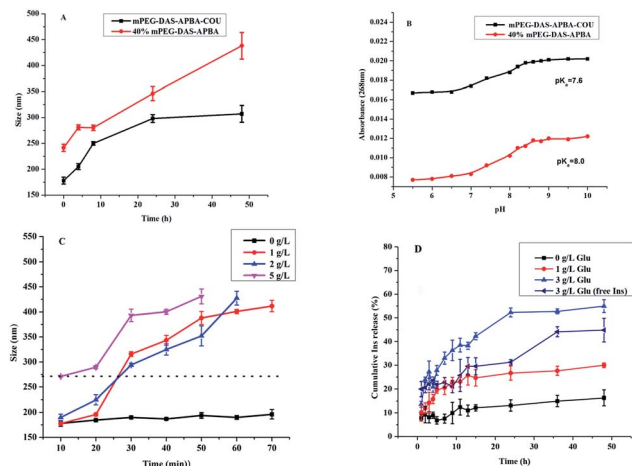


Fig. 4 *In vitro* stability studies of 40% mPEG-DAS-APBA micelles and mPEG-DAS-APBA-Cou micelles (A). The pK_a values of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou were determined to be 7.6 and 8.0 by the absorbance change at 268 nm that occurs upon conversion from the trigonal form (low pH) to the tetrahedral form (high pH) (B). Glucose-responsiveness of mPEG-DAS-APBA-Cou micelles in the presence of glucose with different concentrations of 0, 1, 2 and 5 $g\ L^{-1}$ Glu (C). *In vitro* cumulative release of insulin (pH 7.4). mPEG-DAS-APBA-Cou micelles and free insulin in different glucose media (0, 1 and 3 $mg\ mL^{-1}$) (D).

glucose solution; the micelles remained stable in size in the presence of 1 $g\ L^{-1}$ glucose and changed slightly in the presence of 2 $g\ L^{-1}$ glucose in 10 min. Then, the micelles completely disintegrated in 20 to 30 min in the presence of 1 and 2 $g\ L^{-1}$ glucose. Finally, at a concentration of 5 $g\ L^{-1}$ glucose, the complex micelles completely disintegrated at the outset.

Hemolysis assay

As shown in Fig. 5, compared with the positive control, the dialdehyde starch derivative micelles had lower hemolytic activity. We found that 60% mPEG-DAS-APBA showed a higher hemolytic activity value of 7.93%, whereas 40% mPEG-DAS-APBA (2.00%) exhibited the lowest hemolysis value. Compared to mPEG-DAS-APBA-Cou (4.15%), the hemolytic activity was slightly greater than that of 40% mPEG-DAS-APBA. In addition, a photograph showed that RBCs were released into the supernatant after treatment with dialdehyde starch derivative micelles. However, a slight red color was observed for the dialdehyde starch derivative micelles-treated cells.

Insulin loading and glucose-responsive release

The results are shown in Table 1. The drug loading efficiency (DLE) and drug loading content (DLC) of the insulin-loaded micelles were found to be 30.4% and 9.4% with respect to the standard curve of insulin, respectively.

Fig. 4D shows that the cumulative release of insulin-loaded mPEG-DAS-APBA-Cou polymers and free insulin upon exposure to different glucose media (0, 1 and 3 $mg\ mL^{-1}$) at 37 °C (pH 7.4) increased with increasing glucose concentration. As indicated in Fig. 6A, little conformational change was detected

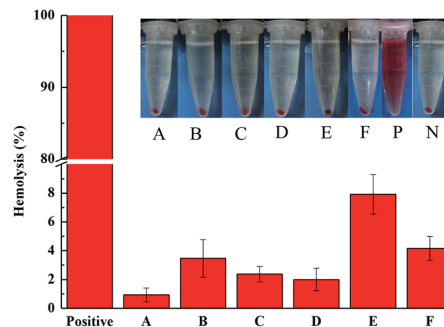


Fig. 5 Percent hemolysis of RBCs incubated with dialdehyde starch derivative micelle systems. Inset photograph: RBCs treated with 40% DAS (A), 40% DAS-APBA (B), 20% mPEG-DAS-APBA (C), 40% mPEG-DAS-APBA (D), 60% mPEG-DAS-APBA (E), and mPEG-DAS-APBA-Cou (F) compared to water and PBS (7.4) groups. P, positive; N, negative.

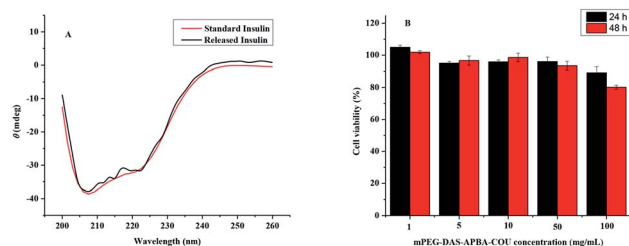


Fig. 6 UV-CD spectra of standard insulin and released insulin (A), viability of A549 lung cells after incubation with blank mPEG-DAS-APBA-Cou micelles at different concentrations for 24 h and 48 h (B).

for the insulin released from mPEG-DAS-APBA-Cou compared with standard insulin (pH 7.4); the $[\phi]_{208}/[\phi]_{223}$ ratios for standard insulin and released insulin were 1.24 and 1.17, respectively.

Cytotoxicity test

To evaluate the potential toxicity of the copolymers, an MTT assay was performed to measure the cell cytotoxicity of the micelles in A549 lung cancer cells for 24 h and 48 h (Fig. 6B). Fig. 6B shows that the cells were exposed to various concentrations of the blank mPEG-DAS-APBA-Cou and incubated for 24 and 48 h. More than 80% A549 lung cancer cell viability was retained at all concentrations from 1 to 100 $mg\ mL^{-1}$ after 48 h cultivation as the nanoparticle concentration increased.

Discussion

In the present study, we developed a novel amphiphilic glucose-sensitive dialdehyde starch polymer composed of APBA and Cou with an mPEG-DAS molecular skeleton for insulin release in diabetes treatment. On the basis of the 1H NMR and FTIR spectral analyses, we confirmed that the targeted dialdehyde starch derivatives were successfully synthesized. During the self-assembly process, the CMC value of 20% mPEG-DAS-APBA provided a lower value than 60% mPEG-DAS-APBA owing to the



different ODs based on different grafting ratios of hydrophobic PBA. The CMC value of 40% mPEG-DAS-APBA-Cou was lower than that of 40% mPEG-DAS-APBA due to the presence of hydrophobic Cou groups; a lower CMC results in higher micelle stability.⁵⁰

The morphologies of the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou polymeric self-assemblies were spherical. The “core-shell” structures of the micelles with different compositions were also very distinct in the TEM observations. However, the micelles showed lower dispersibility in the dry state; this can be attributed to the lower ratio of hydrophilic groups grafted onto dialdehyde starch, whose chain segments tangle easily. Moreover, the DLS results indicated that the hydrodynamic diameters of the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles were reduced upon addition of hydrophobic Cou, where each of the groups exhibited a monomodal size distribution. The interactions of electrostatic, hydrophobic and hydrogen bonds, as well as existing intermolecular forces, may lead to micellization in aqueous solution.

The stability of the dialdehyde starch derivative micelles was measured by DLS because the physiological stability of polymeric micelles is of importance for long-term storage, transportation, and scalable processing in drug carrier applications.⁵¹ Because the micellar structure is susceptible to exposure of the hydrophobic core, the 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou micelles showed a slight increase in particle size after 8 h and 24 h due to the appearance of abundant aggregation of the cores. Compared with the 40% mPEG-DAS-APBA micelles, the mPEG-DAS-APBA-Cou micelles indicated preferable stability. These results confirmed that the mPEG-DAS-APBA-Cou micelles have extraordinarily enhanced stability, which is propitious for a drug carrier.

As prospective self-regulated materials for insulin delivery, PBA-based polymer materials have been widely studied. However, favourable response to different glucose concentrations is difficult under physiological conditions for PBA-based nanopolymers because of the high pK_a of PBA.^{37,38} Dialdehyde starch derivative polymers were prepared to build a platform to attach Cou, which is more acidic than the phenylboronic acid derivative in the polymer. From the UV absorbance spectra (268 nm) shown in Fig. 4B, the pK_a values of 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou imply that the boronate ester would remain stable at pH values between 8.0 and 7.6. Particularly, the pK_a value of mPEG-DAS-APBA-Cou was close to physiological conditions. As the glucose sensitivity in complexation with polyol compounds increased under physiological conditions, the pK_a values of the PBA-based materials apparently decreased.⁵² The mPEG-DAS-APBA micelles with different ODs displayed diverse glucose-responsiveness behaviors, showing that the complex micelles with high contents of dialdehyde groups were more sensitive to glucose; this would lead to uncontrolled release, as briefly shown in Fig. S2.† In this study, the micelles with glucose attached indeed manifested water-solubility. These phenomena completely clarified the disintegrative process in the micelles “core-shell” structure and also indicated the high solubility of the amphipathic nanoparticles. The polymer was expected to form micelles with

enhanced glucose-responsiveness for applications in self-regulated insulin delivery close to physiological range.

Determining the blood compatibility and cytotoxicity of dialdehyde starch derivative micelle systems is the primary screening for their *in vivo* applications.⁵³ Because the hydrophobic polymers are combined with human blood, the possibility of absorptivity of plasma proteins will be enhanced. As a result, an embolism or thrombosis at the blood-contacting side of the polymer interface in the bloodstream introduces blood platelet activation.^{54–56} As shown in Fig. 5, compared with the positive control, the dialdehyde starch derivative micelles had lower hemolytic activity. Thus, these results also indicate that 40% mPEG-DAS-APBA and mPEG-DAS-APBA-Cou can prevent hemolytic activity by blocking the hydration layer around the polymer surfaces and electrostatic interactions with the RBC membrane, improving their blood compatibility. To evaluate the potential cytotoxicities of the copolymers, the cytotoxicities of the micelles toward A549 lung cancer cells were determined by MTT assays for 24 h and 48 h, respectively (Fig. 6B). The blank mPEG-DAS-APBA-Cou micelles demonstrated low toxicity and good cytocompatibility with the backbone of DAS. Compared with the PBA-based material reported by Yang *et al.*,¹¹ blank mPEG-DAS-APBA-Cou had lower toxicity. The formation of hemocompatible and cytocompatible polymers has been recognized as an essential characteristic to maintain their protein resistance properties⁵⁷ and low cytotoxicity. Therefore, mPEG-DAS-APBA-Cou has better cytocompatibility for drug delivery.

The practicability of the mPEG-DAS-APBA-Cou micelles as a novel nanocarrier showed sensitivity to glucose and achieved controlled release of insulin. Insulin was loaded into mPEG-DAS-APBA-Cou nanoparticles on the basis of hydrogen bonding, electrostatic and hydrophilic-hydrophobic interactions. The drug loading efficiency (DLE) and drug loading content (DLC) of the micelles are shown in Table 1 with respect to the standard curve of insulin. Fig. 4D exhibits the cumulative release of insulin-loaded polymers upon exposure to different glucose media (0, 1 and 3 mg mL^{−1}) at 37 °C (pH 7.4). The insulin was released from drug-loaded mPEG-DAS-APBA-Cou nanoparticles in the glucose-free medium because the nanoparticle surface was covered with insulin. With increasing glucose concentration (0 to 3 g L^{−1}), the amount of released insulin significantly increased because of the higher glucose concentration. The more APBA moieties dissociated from the mPEG-DAS-APBA-Cou, the more the moieties were associated with higher concentrations of glucose. Compared with glucose in 1 mg L^{−1} and 3 mg L^{−1} treatment, there was a close burst release phenomenon for mPEG-DAS-APBA-Cou within the first 5.0 h, which was ascribed to the adsorption of insulin on the surface of the nanoparticles. Then, the insulin release rate became slow; it finally reached a plateau phase in the same time within 13.0 h and approximately increased by 25.0%. At only 3 g L^{−1} glucose concentration, the structure of the nanoparticles was able to break and quickly release insulin. Compared with a report by Zhang *et al.*⁵⁸ on the cumulative release of insulin-loaded polymers, mPEG-DAS-APBA-Cou showed a high amount of release in a short treatment time.



A convenient technique, CD spectroscopy is the best way to evaluate the conformational changes and self-association of insulin.⁵⁹ The ratio of the band at 208 nm arising from the α -helix structure and that at 223 nm arising from the β -structure ($[\phi]_{208}/[\phi]_{223}$) can qualitatively measure the overall conformational structure of insulin (Fig. 6A). According to the spectral characteristics, the structure of the released insulin has not been distorted.

Conclusions

Novel amphiphilic glucose-sensitive dialdehyde starch polymer micelles with “shell–core” structures were synthesized by Schiff base bonds. Their DLC and DLE values were as high as 9.4% and 30.4%, respectively. Due to the carbohydrate moieties introduced into the polymers, the mPEG-DAS-APBA-Cou nanoparticles had low cytotoxic activity and hemolytic activity. The nanocarriers, whose pK_a was reduced by the introduction of Cou, showed notable glucose responsive behaviour; they released insulin rapidly in 3 mg mL⁻¹ glucose (pH 7.4), with comparatively inert release in 1 mg mL⁻¹ glucose (pH 7.4). The glucose-sensitive insulin release could be adjusted by changing the glucose medium. Therefore, the amphiphilic mPEG-DAS-APBA-Cou nanoparticles have potential applicability as a glucose-responsive material for insulin delivery.

Conflicts of interest

There are no conflicts to declare.

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