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Supporting information

Dually-crosslinked Degradable Polyionic Micelles for Sustained Glucose-responsive Insulin Release

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1. Materials.

Methoxyl poly (ethylene glycol) ($mPEG_{5000}$, Fluka, M_n = 5000 g/mol), Zinc bis[bis(trimethylsilyl)amide] (Aldrich, 97%), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), 3-aminophenylboronic acid (APBA), triethylamine (Et_3N, 99.5%), 3-mercaptopropane-1, 2-diol (MPD) and 3-mercaptopropionic acid (MPA), 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, 98%) were purchased from Energy Chemical (Beijing, China). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Macklin Biochemical (Shanghai, China). Acryloyl carbonate (AC) and 2-dimethylamino-trimethylene carbonate (DMATC) were synthesized according to our previous report [1–3]. Bovine pancreas insulin was purchased from Aladdin Chemistry (Shanghai, China). Streptozocin (STZ, 98%) was purchased from Sangon Biotech (Shanghai, China). Coomassie brilliant blue was purchased from Beyotime Biotechnology (Shanghai, China). All the other reagents and solvents were purchased from Sinopharm Chemical Reagent (Suzhou, China). Dichloromethane was dried by refluxing over CaH_2 under an argon atmosphere before distillation.

2. Synthesis of mPEG-PAC-PDMATC.

The ring-opening polymerization (ROP) of AC was carried out in dried CH_2Cl_2 at 40 ºC using mPEG5000 and isopropanol as the initiators and zinc bis[bis(trimethylsilyl)amide] as a catalyst, followed by DMATC in CH2Cl2 and DBU as a catalyst according to our previous report 36,37. Briefly, in a glove box under a nitrogen atmosphere, a stirred solution of $mPEG_{5000}$ (0.5 g, 0.1 mmol) and AC (0.5 g, 2.5 mmol) in CH_2Cl_2 (5 mL) was rapidly supplemented with zinc bis[bis(trimethylsilyl)amide] (50 mg, 0.13 mmol). The reaction vessel was sealed and placed in an oil bath, where it was stirred for 24 h at 40 ºC. Then the DMATC (0.5 g, 3.4 mmol), CH_2Cl_2 (3.0 mL) and DBU (30 μL) were added under the same condition. Following an additional 24 h of polymerization, the reaction was halted by the addition of two drops of acetic acid. The obtained copolymer was isolated by precipitation into cold diethyl ether, yielding a white flocculent solid dried in vacuo at room temperature (1.05 g, yield: 70%). One sample was taken for the determination of monomer conversion using ^1H nuclear magnetic resonance (^1H NMR).

3. Characterization of polymers.

^1H NMR spectra were recorded on a Bruker ECX 400 (Germany) operating at 400 MHz. $CDCl_3$ and DMSO-d_6 were used as solvents and the chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined using gel permeation chromatography (GPC) instrument (GPC-300, 7.8 × 300 mm, 5 um, 300 Å, Sepax). The measurements were performed using *N*, *N*-

dimethylformamide (GPC grade) as the eluent at a flow rate of 1.0 mL·min^{-1} at 30°C and a series of narrow polystyrene standards for the calibration of the columns. 3 mg·mL^{-1} solution of the copolymer in THF/DMF, filtered through a 0.22 um filter prior to use, was injected for all measurements.

4. Characterization of micelles.

^{1}H NMR spectra were recorded on a Bruker ECX 400 operating at 400 MHz. DMSO-d_6 was used as a solvent and the chemical shifts were calibrated against residual solvent signals. Infrared absorption spectra were recorded on Fourier transform infrared (FTIR) spectra with an IRAffinity-15 spectrophotometer (Japan) within the range of 400-4,000 cm^{-1} at room temperature with KBr pellets. The conformation of insulin was examined using a Mos-500 circular dichroism (CD) spectropolarimeter (Jasco Incorporated, Easton).

5. *In vitro* cyctotoxicity assay.

The MTT assay was used to determine the cytotoxicity of SCM and DCM materials toward L02 cells. The L02 cells were seeded at a density of 10000 cells per well into 96-well plates and allowed to adhere overnight. Subsequently, the cells were treated with varying concentrations of DCM for 24 h. Following this exposure period, the culture medium in each well was replaced with MTT (0.5 mg/mL) and incubated for an additional 2 h prior to the addition of DMSO to dissolve the formazan crystals. The optical density of each well was then measured at 562 nm with a microplate reader. The same method above was used for the SCM cytotoxicity test as a control. Each concentration was tested three times in three independent experiments.

6. *In vitro* hemolysis assay:

0.9 % NaCl was used to wash the erythrocytes three times after the blood sample collection and centrifugation for 10 min at 2000 rpm, and then diluted with the same solution to achieve a volume fraction of 5 %. H-water, known for its ability to damage red blood cells, was used as the positive control (+), while 0.9% NaCl served as the negative control (−). Subsequently, 0.5 mL of the polyionic micelles solution with varying concentrations was combined with 0.5 mL of red blood cells and co-incubated at 37 ºC for 1 h. By centrifuging each sample for 5 min at 3000 rpm, the supernatant was collected and measured the absorbance at 542 nm using a microplate reader. The percentage of hemolysis was determined using the following formula:

$$Hemolysis\ \% = \frac{Abs - Abs(-)}{Abs(+) - Abs(-)} \times 100\%$$

where *Abs, Abs (+)* and *Abs (−)* are the absorbances of the blended sample, H-water, and 0.9% NaCl, respectively.

7. Animal experiment

To establish the type 1 diabetic mice model, healthy mice were intraperitoneally injected with 5.0 mg/mL of streptozocin (STZ) at a dosage of 40 mg/kg for 5 consecutive days. Diabetic mice were identified by tail trimming if their fasting glycemic levels (BGLs) surpassed 16.7 $mmol \cdot L^{-1}$ and remained elevated for one week.

8. *In vivo* glucose tolerance test

An intraperitoneal glucose tolerance test (IPGTT) was conducted to assess the glucose responsiveness of micelles 1 h after the administration of insulin, DCM@insulin, and SCM@insulin. In short, diabetic mice were administered insulin, DCM@insulin and SCM@insulin. After that, a glucose solution in PBS was intraperitoneally injected (dose: 1.5 g/kg), followed by monitoring of the BGLs over time. The glucose tolerance test was conducted on healthy mice for comparison.

9. *In vivo* biodistribution

SCM@insulin and DCM@insulin were prepared to evaluate the biodistribution of each micelle. SCM^{DIR}@insulin and DCM^{DIR}@insulin were subcutaneously injected at 80 U·kg-1 (n=3). At predetermined time intervals (1, 2, 12, or 24 h), mice were anaesthetized and fluorescent images of the nanoparticles were acquired using an IVIS Lumina II system. Mice were sacrificed at 24 h post-administration, and the associated organs were collected for imaging analysis.” in the supporting information.

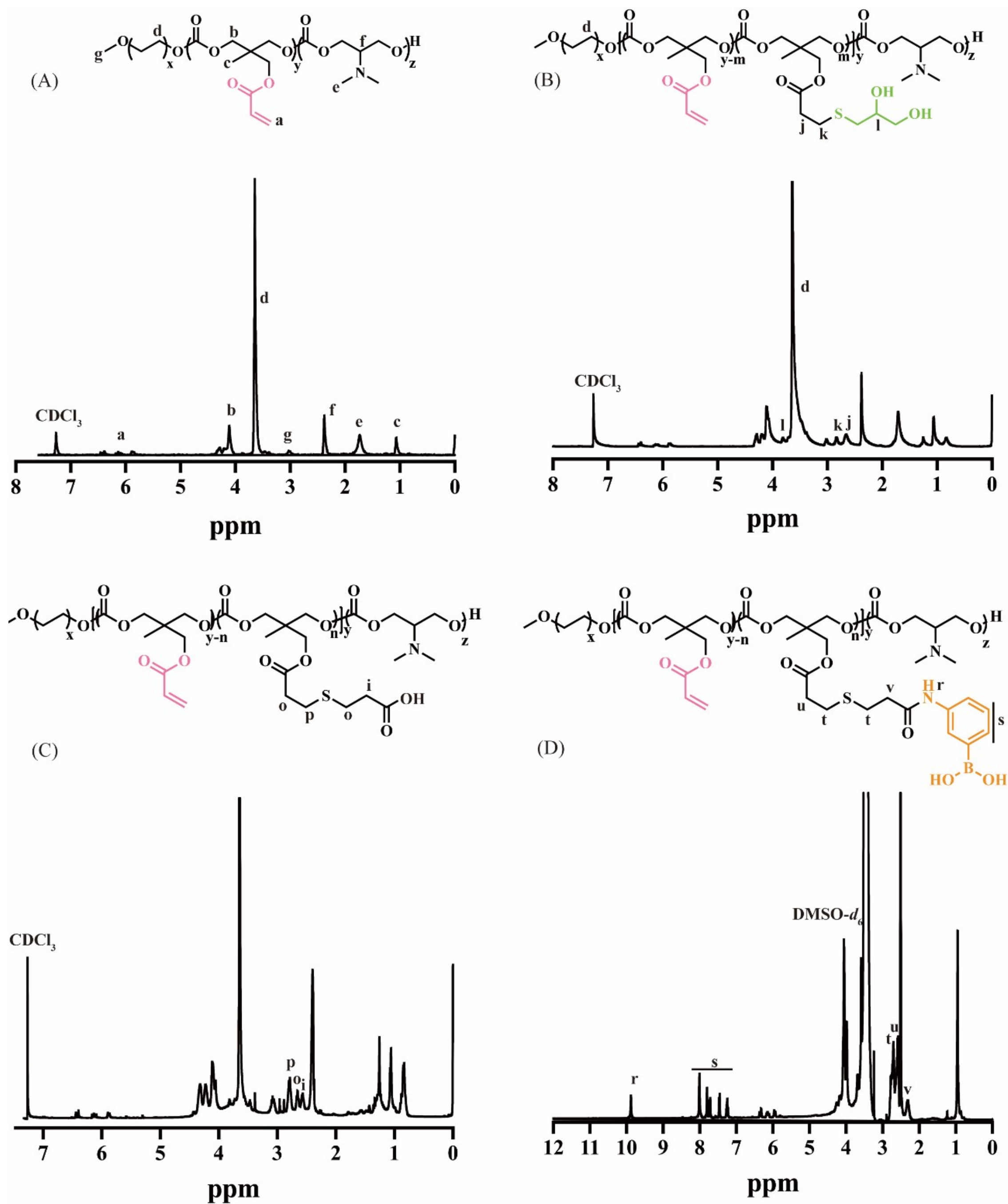

Figure S1. ^{1}H NMR spectra (400 MHz) of (A) mPEG-PAC-PDMATC ($CDCl_3$), (B) mPEG-P(AC-co-MPD)-PDMATC ($CDCl_3$), (C) mPEG-P(AC-co-MPA)-PDMATC ($CDCl_3$) and (D) mPEG-P(AC-co-MAPBA)-PDMATC (DMSO-d_6).

Table S1. Characteristics of triblock polymers

Polymers	M_n (kg/mol)			M_w/M_n	Yield
	Theoretical	^{1}H NMR	GPC	GPC	(%)
mPEG-PAC$_{3.6k}$-PDMATC$_{1.4k}$	5.0-4.0-5.0	5.0-3.6-1.4	10000	1.18	70
mPEG-P(AC$_{3.6k}$-co-MPD$_{0.9k}$)-PDMATC$_{1.4k}$	5.0-(3.6-co-0.9)-1.4	5.0-(3.6-co-0.9)-1.4	11100	1.23	89
mPEG-P(AC$_{3.6k}$-co-MAPBA$_{1.9k}$)-PDMATC$_{1.4k}$	5.0-(3.6-co-1.9)-1.4	5.0-(3.6-co-1.9)-1.4	13000	1.06	72

Table S2. Characteristics of insulin loaded polyionic micelles

Entry	LC%		EE%	Size (nm)	PDI
	Theoretical	Determined			
1	5	4.7	97.9	110 ± 4.4	0.17
2	10	7.8	84.2	110 ± 4.5	0.15
3	15	10.0	74.4	120 ± 1.7	0.20
4	20	14.8	86.7	110 ± 2.5	0.18

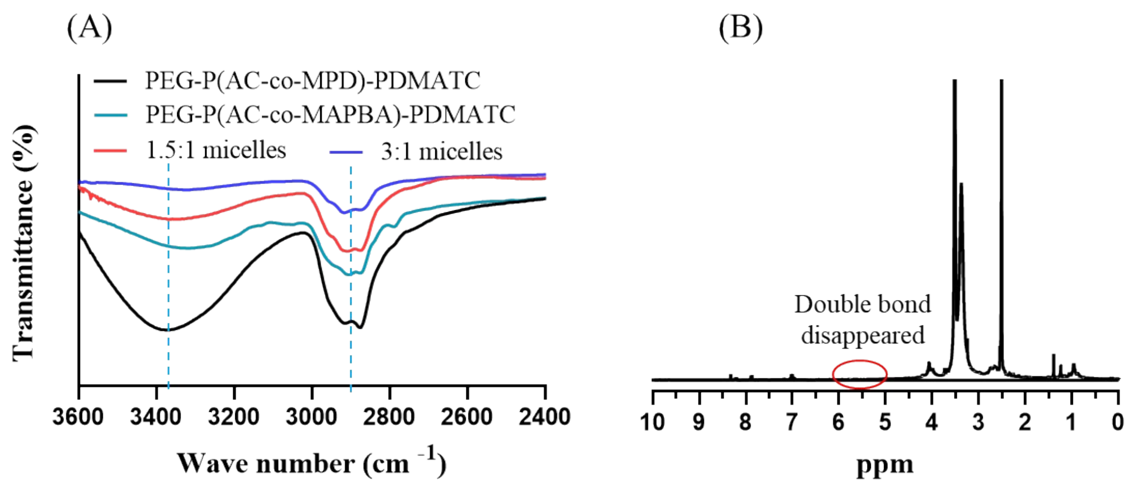

Figure S2. (A) IR of micelles after UV crosslinking with molar ratio of MPD: APBA in 1.5:1 and 3:1. (B) ^{1}H NMR spectra of micelles after UV crosslinking (400 M, DMSO-d_6).

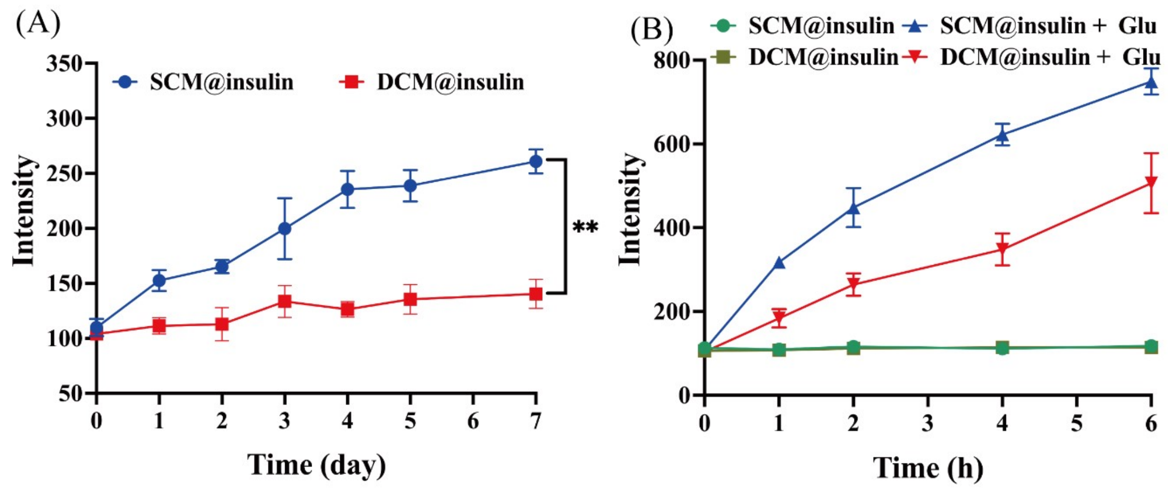

Figure S3. (A) Long-term size stabilities of SCM@insulin and DCM@insulin. (B) Stability of different formulations with or without glucose solution (glucose: 4 mg/mL).

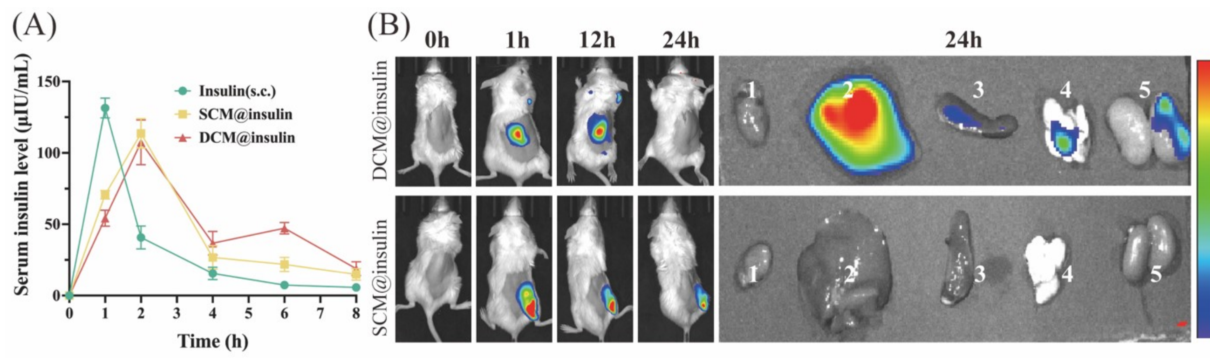

Figure S4. (A) Serum insulin levels versus time profiles of diabetic mice after subcutaneous of SCM@insulin and DCM@insulin. (B) Live animal imaging observation as well as isolated tissue distribution of SCM@insulin and DCM@insulin in diabetic mice. 1: Heart, 2: Liver, 3: Spleen, 4: Lung, 5: Kidney.

1 X. Zhang, M. Cai, Z. Zhong and R. Zhuo, Macromol. *Rapid Commun.*, 2012, **33**, 693–697.
2 Y. Ma, Q. Li, J. Yang, Y. Cheng, C. Li, C. Zhao, W. Chen, D. Huang and H. Qian, *Biomater. Sci.*, 2023, **11**, 975–984.
3 W. Chen, H. Yang, R. Wang, R. Cheng, F. Meng, W. Wei and Z. Zhong, *Macromolecules*, 2010, **43**, 201–207.