Supporting Information

Glucose-responsive Dual-nanoparticle-hydrogel-Based Microneedle Patch for Co-Delivery of Insulin and Exendin-4

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1. Materials

All chemicals were obtained from commercial suppliers unless otherwise specified and were used as received. Methoxy polyethylene glycol amine (CH_3O-PEG_{114}-OH, Mw = 5000 Da) was purchased from Ponsure (Shanghai, China). ε-Polylysine and polyvinyl alcohol was purchased from Aladdin (Shanghai, China). Butylamine, β-benzyl L-aspartate-N-carboxyanhydride (BLA-NCA), glucosamine hydrochloride, 3-aminophenylboronic acid, (3-amino-2-fluorophenyl) boronic acid and 4-borono-3-fluorobenzoic acid were purchased from Bide Pharmatech Ltd. (Shanghai, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS) and alizarin red (ARS) were purchased from Aladdin (Shanghai, China). Methylene chloride (CH_2Cl_2), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Macklin Co., Ltd. (Shanghai, China). Insulin from porcine pancreas and exendin-4 were purchased from GL Biochem. Fluorescein isothiocyanate (FITC, 96%) was purchased from Myrell Chemical Technology Co., Ltd. (Shanghai). Cyanine5 (Cy5) isothiocyanate was purchased from Sigma-Aldrich. Cell Counting Kit-8 (CCK-8) were purchased from Biosharp (Hefei, China).

2. Synthesis of PAsp

First, poly(β-benzyl-l-aspartate) (PBLA) was synthesized by ring-opening polymerization (ROP) of BLA-NCA using butylamine as an initiator. Butylamine was dissolved in dry CH_2Cl_2 (50 mL). Then BLA-NCA (3.0 g) was added into above solution quickly. After freeze-degas-thaw cycles, the reaction mixture was stirred at 35℃ for 72 hours. Finally, the mixture was precipitated into excessive cold diethyl ether to obtain the product. The precipitate was dried under vacuum for 24 hours to get PBLA. Then, PBLA was dispersed in NaOH (1 M) and stirred for 10 hours at room temperature for deprotection. After that, the solution was neutralized with 1 M HCl, concentrated by vacuum evaporation and dialyzed against DI water in a dialysis bag. After lyophilization, PAsp was obtained.

3. Synthesis of P(Asp-*co*-AspGA-*co*-AspNTA)

P(Asp-*co*-AspGA-*co*-AspNTA) was synthesized by partial modification of PAsp using glucosamine hydrochloride and nitrilotriacetic acid. First, PAsp was dissolved in water

and then NHS and EDC were added to the solution. The reaction mixture was stirred at 4℃ for 2 hours. Then, glucosamine hydrochloride was added to the solution and the reaction was proceeded for 12 hours at room temperature. Subsequently, the solution was dialyzed against DI water for 48 hours. P(Asp-*co*-AspGA) was obtained after lyophilization. P(Asp-*co*-AspGA) and nitrilotriacetic acid yield P(Asp-*co*-AspGA-*co*-AspNTA) through the same amidation reaction.

4. Synthesis of PEG-*b*-P(Asp-*co*-AspPBA) and PEG-*b*-P(Asp-*co*-AspFPBA)

PEG-*b*-P(Asp-*co*-AspPBA) and PEG-*b*-P(Asp-*co*-AspFPBA) were synthesized by partial modification of PEG-*b*-PAsp. First, Poly(ethylene glycol)-*b*-poly(β-benzyl-l-aspartate) (PEG-*b*-PBLA) was synthesized by ring-opening polymerization of BLA-NCA using mPEG-NH_2 as a macroinitiator. Anhydrous mPEG-NH_2 (0.5 g) was dissolved in dry CH_2Cl_2 (50 mL). Then BLA-NCA (4.4 g) was added into above solution quickly. After freeze-degas-thaw cycles, the reaction mixture was stirred at 35℃ for 72 hours. Finally, the mixture was precipitated into excessive cold diethyl ether. The precipitate was dried under vacuum for 24 hours to get PEG-*b*-PBLA. Then, PEG-*b*-PBLA (2.0 g) was dispersed in NaOH (1 M, 180 mL) and stirred for 10 hours at room temperature for deprotection. After that, the solution was neutralized with 1 M HCl, concentrated by vacuum evaporation and dialyzed against DI water in a dialysis bag (70 kDa). After lyophilization, PEG-*b*-PAsp was obtained. PEG-*b*-P(Asp-*co*-AspPBA) was synthesized by partial modification of PEG-*b*-PAsp using 3-aminophenylboronic acid through amidation reaction. PEG-*b*-P(Asp-*co*-AspFPBA) was synthesized by partial modification of PEG-*b*-PAsp using (3-amino-2-fluorophenyl) boronic acid also through amidation reaction. These experimental procedures are the same as those used in the synthesis of P(Asp-*co*-AspGA).

5. Synthesis of ε-P(Lys-*co*-LysFPBA)

4-Borono-3-fluorobenzoic acid (0.9 g) was dissolved in DMSO and then NHS (1.7 g) and EDC (2.25g) were added to the solution. The reaction mixture was stirred for 2 hours at 4℃. Then, ε-PLys (2.5 g), which was dissolved in water, was added to the solution and the reaction was proceeded for 12 hours at room temperature. Subsequently, the solution was dialyzed against DI water for 48 hours. ε-P(Lys-*co*-

LysFPBA) was obtained after lyophilization.

6. Synthesis of FITC-labeled insulin and Cy5-labeled exendin-4

Insulin was dissolved in sodium carbonate-bicarbonate buffer. Then FITC-NHS was dissolved in DMSO and added to the insulin solution. After an incubation at room temperature overnight, FITC-labeled insulin was purified by dialysis against DI water (1000 Da) and lyophilized. The whole experiment process was protected from light (Cy5-labled exendin-4 was prepared using a similar method).

7. Preparation and Characterization of iNPs and eNPs

P(Asp-*co*-AspAGA-*co*-AspNTA) was dissolved in water and then zinc acetate solution was added to the solution. The reaction mixture was stirred for 1 hours at room temperature. Subsequently, the solution was dialyzed with DI water for 48 hours to remove free zinc ions. P(Asp-*co*-AspAGA-*co*-AspNTA)-Zn was obtained. Subsequently, the solution of insulin and PEG-*b*-P(Asp-*co*-AspPBA) were added to the solution of PEG-*b*-P(Asp-*co*-AspDA) and stirred for 12 hours. Then, the mixed solution was transferred into dialysis bag and dialyzed against PBS (pH 7.4) for 48 hours to obtain iNPs. Through the same method, eNPs were also easily prepared. During the experiment, insulin and PEG-*b*-P(Asp-*co*-AspPBA) were replaced by exendin-4 and PEG-*b*-P(Asp-*co*-AspFPBA).

Dynamic light scattering (DLS) measurements: Approximately 1.5 mL of sample was withdrawn and filtered through a 0.8 μm Millipore filter into scintillation vials. DLS measurements of iNPs and eNPs were performed on a Nanophox.

TEM measurements: TEM sample (10 μL) was first deposited onto a carbon-coated copper grid. After 5 min, excess solution was blotted away with filter paper. Subsequently, 10 μL of 2% (w/v) uranyl acetate (UA) staining solution was applied to the grid; after 1 min, excess stain was blotted dry with filter paper. Finally, the samples were imaged using a Talos F200C transmission electron microscope at an acceleration voltage of 200 kV.

8. Preparation and Rheological characterization of iNPs/eNPs@Gel

First, ε-P(Lys-*co*-LysFPBA) was dissolved in the mixed solution of iNPs and eNPs. The resulting mixture was then added dropwise to the PVA solution, followed by

stirring at room temperature overnight to obtain iNPs/eNPs@Gel. The rheological properties of iNPs/eNPs@Gel were characterized using a rotational rheometer equipped with 40-mm-diameter parallel plates. The plate gap was fixed at 0.5 mm, and all measurements were conducted at a constant temperature of 25°C. Oscillation strain sweep tests were performed by varying the applied strain amplitude from 0.01% to 1000% at a constant angular frequency of 6.28 rad/s. In contrast, oscillation frequency sweep tests were carried out by tuning the angular frequency over a range of 0.01–100 rad/s under a constant applied strain amplitude of 1%.

9. Preparation of MN patches

Microneedle patches were prepared according to the method reported in the literature. Initially, internal air within the iNPs/eNPs@Gel was expelled through centrifugation (3500 rpm for 3 minutes). Subsequently, 100 μL of the air-free iNPs/eNPs@Gel was aspirated and added into a microneedle mold (mold specifications: a 20*20 microneedle array with a microneedle height of 600 μm, a distance of 600 μm between adjacent microneedles, and a base side length of 330 μm for each microneedle). Subsequently, the mold containing iNPs/eNPs@Gel was fixed in a centrifuge tube and centrifuged for 30 min to allow the gel to fill the microneedle cavities. After centrifugation, the filling and centrifugation process was repeated once. The mold was then air-dried at room temperature for 48 hours, and the microneedle patches were obtained by demolding.

10. Mechanical properties of MN patches

The mechanical strength of MN patches was determined using a stress-strain gauge by pressing a stainless steel plate onto the microneedles with a tensile testing machine. The speed at which the plate advanced toward the microneedles was set at 1.2 mm/min. The failure force of the microneedles was recorded as the force at which the needles initiated buckling.

11. Controlled release of insulin and exendin-4

Glucose-responsive drug release experiments in vitro were performed on iNPs, eNPs, iNPs/eNPs@Gel and MN patches. Take iNPs for example, the solution of iNPs was placed into a dialysis bag, which was immersed in buffer solutions with different glucose concentrations (0 g/L, 1 g/L, and 4 g/L). At predetermined time points, 1 mL

of the solution outside the dialysis bag was collected, and its fluorescence intensity was measured using a fluorescence spectrophotometer. The instantaneous release amount and cumulative release amount of insulin at each time point were calculated with reference to the standard curve.

As for the “on – off” controlled release of insulin and exendin-4, the iNPs solution was placed into a dialysis bag (10 KDa), which was immersed in glucose solution (4 g/L) and dialyzed at 37°C for 1 hour. After dialysis in the high-glucose environment, the dialysis bag was transferred to glucose solution (1 g/L) and dialyzed at 37°C for 1 hour. This "high glucose – low glucose" concentration switching cycle was repeated four times. Before each transfer of the dialysis bag, 1 mL of the solution outside the dialysis bag was collected for testing. The fluorescence intensity of the collected external solution was measured using a fluorescence spectrophotometer. The release amount of insulin was calculated with reference to the standard curve. The same experimental method was adopted for the glucose responsive drug release and “on – off” controlled release of eNPs, iNPs/eNPs@Gel and MN patches.

12. Circular dichroism measurement

Circular dichroism (CD) spectra were recorded on Bio-Logic MOS-500 circular dichroism spectrophotometer (France). All the CD spectra were collected in a quartz cuvette of 1 mm path length. The spectra were measured in the wavelength range of 200 - 260 nm to assess the secondary structure of insulin and exendin-4.

13. In vivo study on STZ-induced diabetic mice

Streptozotocin (STZ)-induced adult diabetic mice were used as a model to evaluate the performance of MN patches in vivo. Diabetic mice were randomly divided into 6 groups (n = 5 in each group), and received either subcutaneous injection of different samples or application of microneedle patches. The blood samples were taken from the tail vein of mice, and blood glucose levels were measured via a blood glucose meter. Besides, healthy mice were used as normal controls in the intraperitoneal glucose tolerance test (IPGTT).

14. In vitro and in vivo toxicity evaluation

For in vitro toxicity evaluation, the cytotoxicity of different samples (iNPs, eNPs and

Gel) was evaluated using Cell Counting Kit-8 (CCK-8) assay. Briefly, 3T3 cells were seeded into 96-well plates. After 24 h, the medium was replaced with fresh medium. Subsequently, iNPs, eNPs and Gel were added to each well respectively and incubated at 37℃ for 24 hours. Afterwards, the CCK-8 was added to each well and incubated at 37℃ for 2 hours. Absorbance at 450 nm was detected to calculate the cell viability.

The hemocompatibility of samples was evaluated by a hemolysis assay. 1 mL of anticoagulant rat's blood was washed with PBS for 3~5 times, and RBCs were collected by centrifugation, then diluted with PBS to a 5% (v/v) suspension. 0.2 mL RBC suspension was co-incubated with 0.8 mL sample extracts in a 96-well plate at 37℃ for 2 hours. Absorbance at 545 nm was detected by a microplate reader.

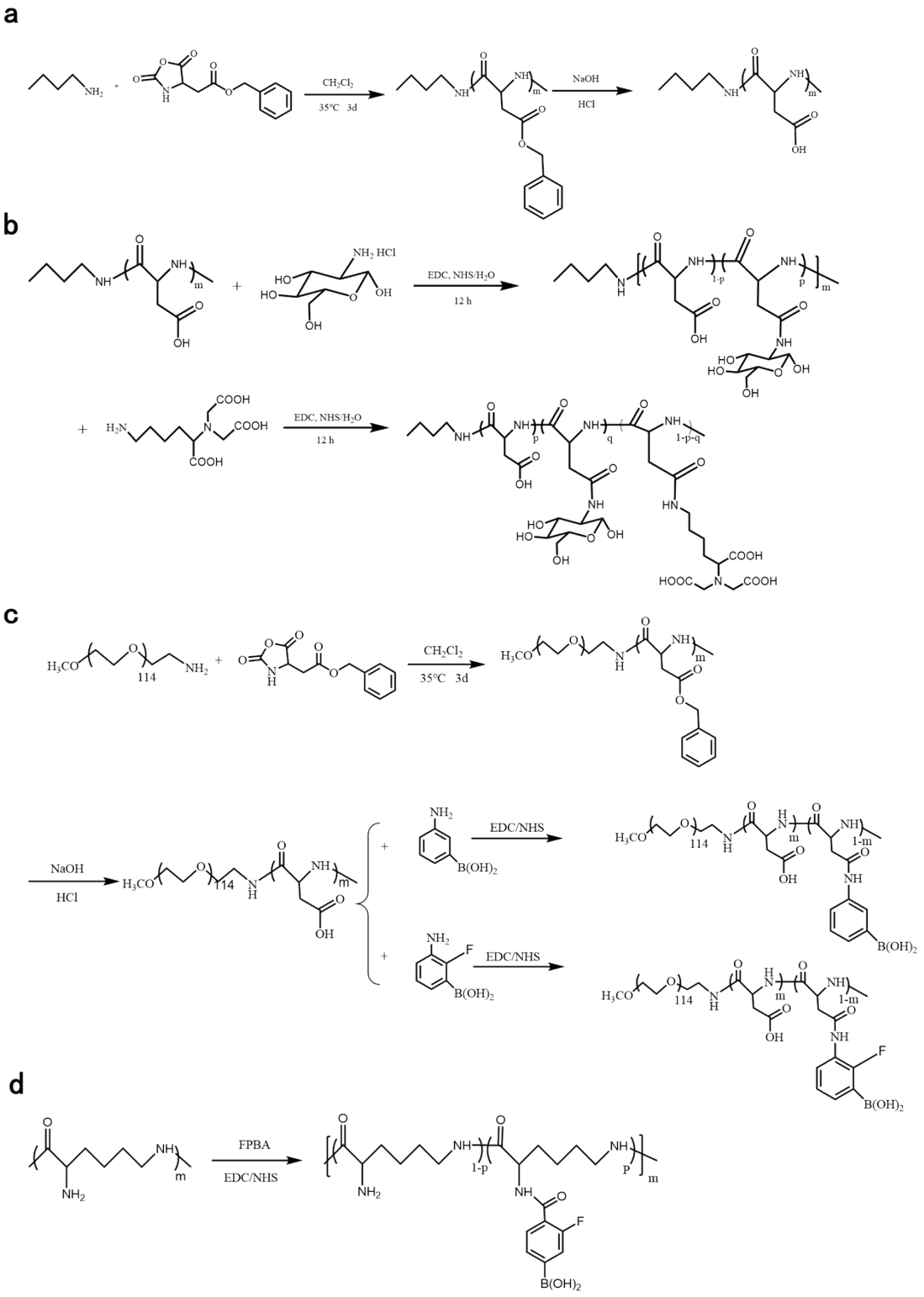


Fig. S1. Synthesis routes of (a) PAsp, (b) P(Asp-*co*-AspGA-*co*-AspNTA), (c) PEG-*b*-P(Asp-*co*-AspPBA) and PEG-*b*-P(Asp-*co*-AspFPBA), (d) ε-P(Lys-*co*-LysFPBA).

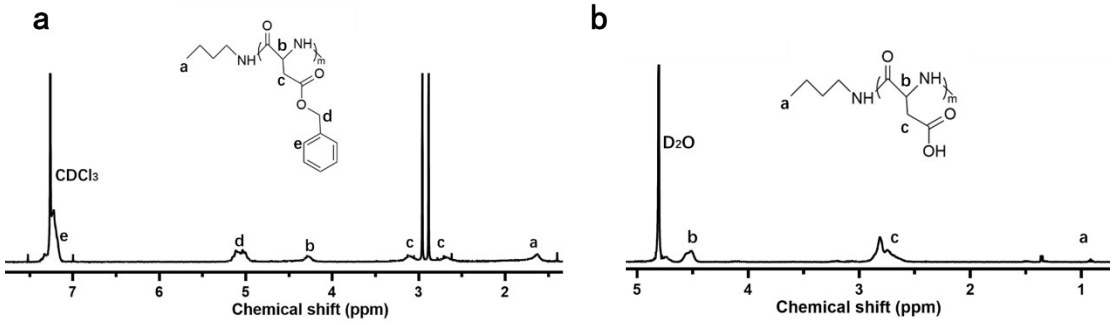


Fig. S2. ^{1}H NMR results of (a) PBLA and (b) PAsp.

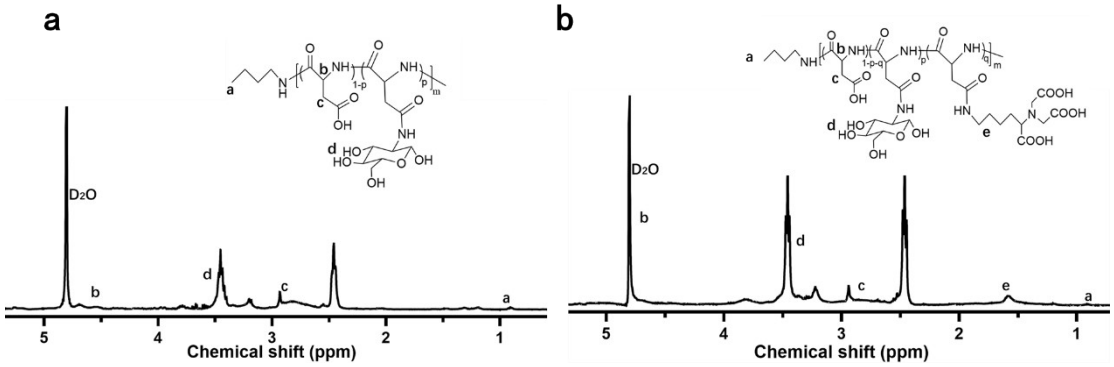


Fig. S3. ^{1}H NMR results of (a) P(Asp-*co*-AspGA) and (b) P(Asp-*co*-AspGA-*co*-AspNTA).

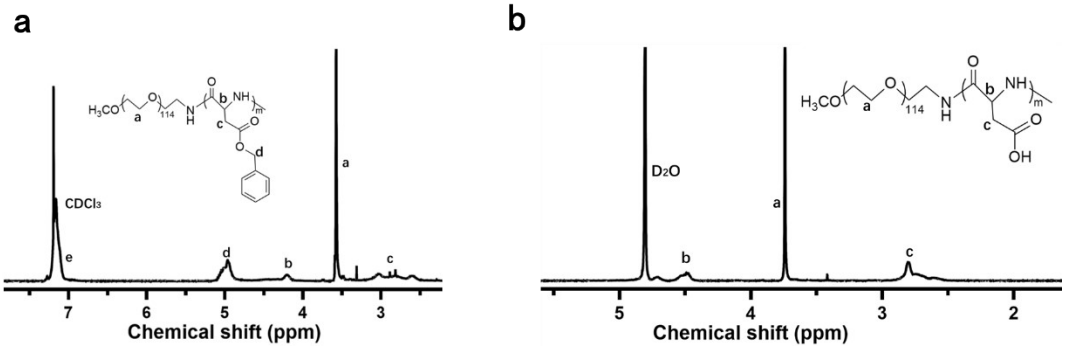
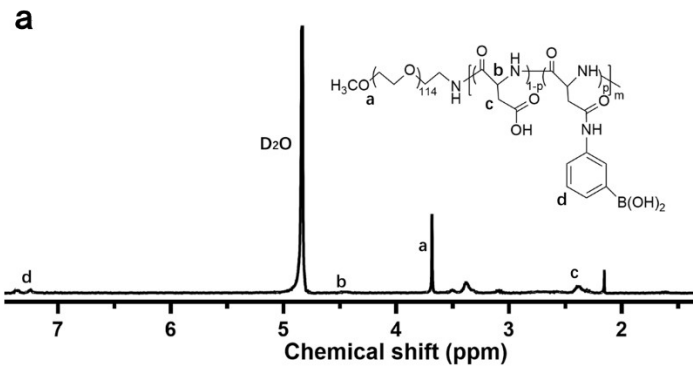


Fig. S4. ^{1}H NMR results of (a) PEG-*b*-PBLA and (b) PEG-*b*-PAsp.



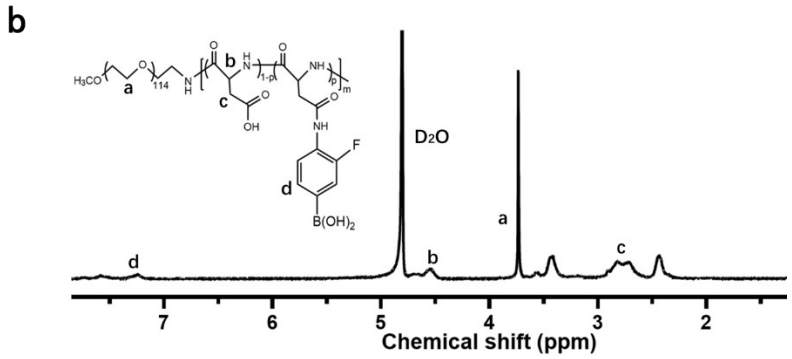


Fig. S5. [1]H NMR results of (a) PEG-*b*-P(Asp-*co*-AspPBA) and (b) PEG-*b*-P(Asp-*co*-AspFPBA).

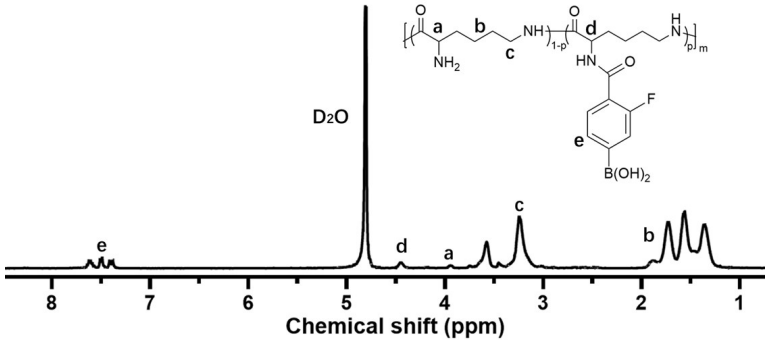


Fig. S6. [1]H NMR result of ε-P(Lys-*co*-LysFPBA).

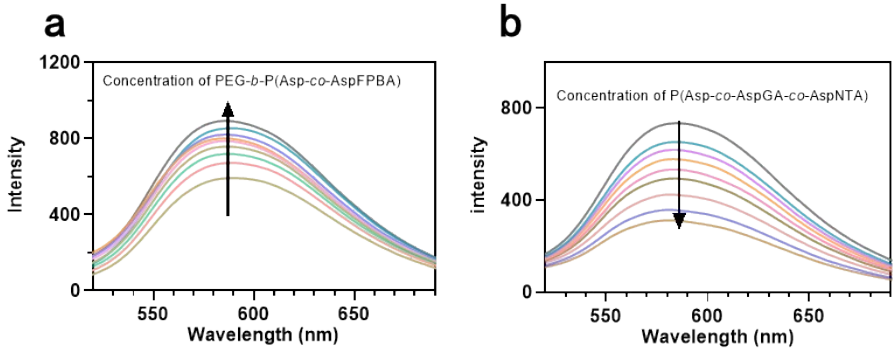


Fig. S7. (a) Variations in ARS fluorescence spectra with the concentration of PEG-*b*-P(Asp-*co*-AspFPBA). (b) Variations in ARS fluorescence spectra with the concentration of P(Asp-*co*-AspGA-*co*-AspNTA).

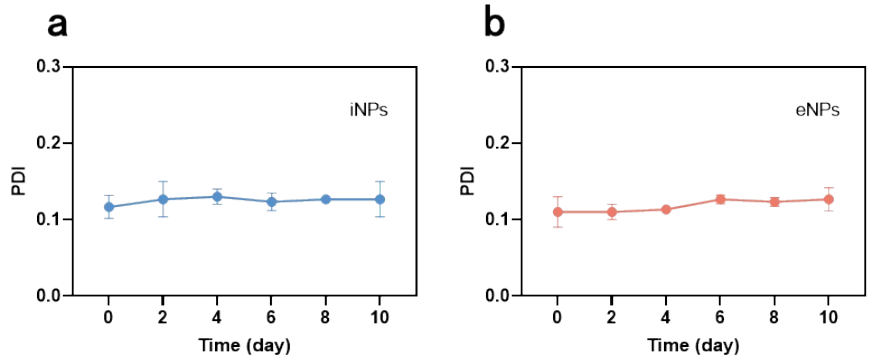


Fig. S8. Variation of PDI values of **(a)** iNPs and (b) eNPs in PBS (pH 7.4) at 4°C as a function of time.

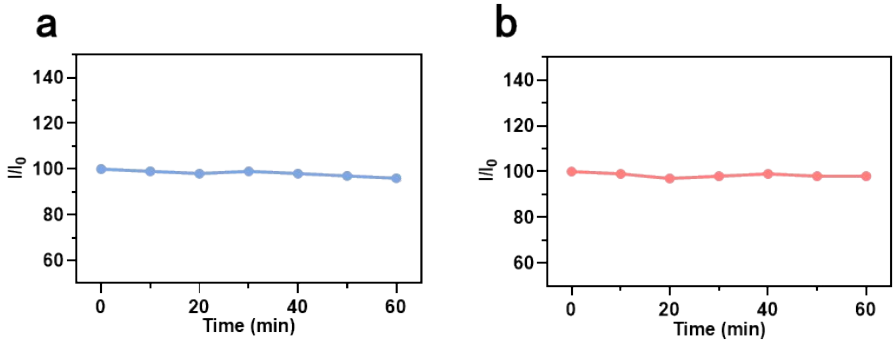


Fig. S9. Variation of light scattering intensities of (a) iNPs and (b) eNPs in 0 g/L glucose solutions over time.

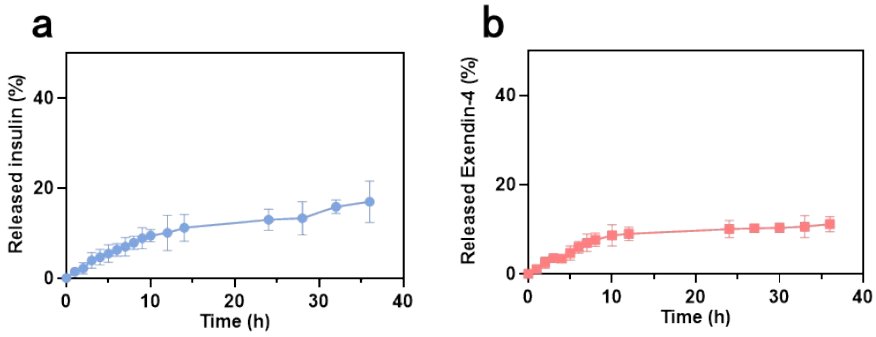


Fig. S10. In vitro release of insulin (a) and exendin-4 (b) from MN patch at 37°C in 0 g/L glucose solutions.

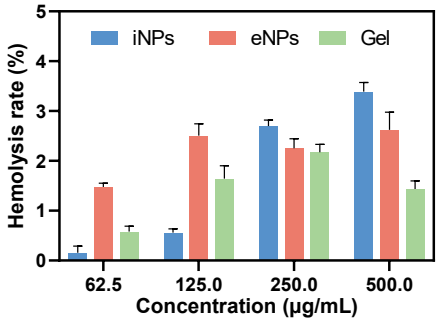


Fig. S11. Hemolysis rate of iNPs, eNPs, and Gel at different concentrations.

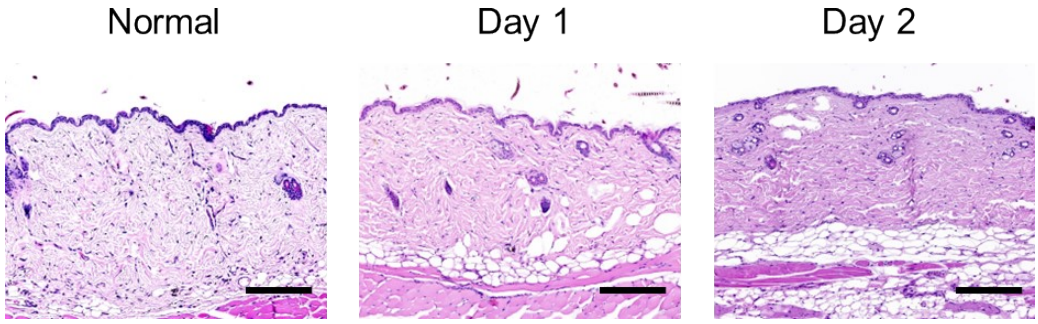


Fig. S12. H&E-stained sections of mice skin treated with MN patches after recovery over time. Scale bar, 200 μm..