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Supporting Information

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Bioresponsive Microneedles with a Sheath Structure for H_2O_2 and pH Cascade-Triggered Insulin Delivery

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Materials and Methods

1. Materials. 4-(Bromomethyl) phenylboronic acid was purchased from Boron Molecular. All other chemical reagents were purchased from Sigma-Aldrich. Insulin was purchased from Gibco. $MPEG_{5K}$-Br was synthesized as reported.[1] Poly(vinyl alcohol) (PVA, 89-98 KDa) and polyvinylpyrrolidone (PVP, ~55 KDa) were purchased from Sigma-Aldrich.

2 Synthesis

2.1 Synthesis of $MPEG_{5K}$-$P(DMAEMA)_{6K}$. $MPEG_{5K}$-Br (0.2 g, 0.04 mmol), CuBr (5.7 mg, 0.04 mmol) and 2, 2′-dipyridine (12.5 mg, 0.08 mmol) were added to a round bottom flask and protected with the N_2 atmosphere. To this mixture, THF (2 mL) and DMAEMA (0.2 g, 1.3 mmol) were added sequentially and mixed gently. After three freeze-thaw cycles, the flask was sealed with N_2, immersed in an oil bath and stirred overnight at 60 ^oC. The resultant solution was poured into ethyl acetate (100 mL) and washed with $NaHCO_3$ (0.1 N, 3×50 mL) and dried over anhydrous $NaSO_4$. After filtration and removing the solvent, slightly yellow viscous solid was obtained (0.2 g, yield 50%). 1H-NMR (300 MHz, $CDCl_3$) was shown in Figure S2a.

2.2 Synthesis of $MPEG_{5K}$-$P(DMAEMA\text{-}PBA)_{6K}$. $MPEG_{5K}$-$P(DMAEMA)_{6K}$ (0.11 g, 0.01 mmol) and 4-(bromomethyl)phenylboronic acid (0.5 g, 2.3 mmol) were dissolved in DMF separately and mixed. The mixture was stirred at 60 ^oC overnight and dialysis against H_2O (3×2 L). After filtration and lyophilization, white product was obtained (0.15 g, yield 75 %). 1H-NMR (300 MHz, D_2O) was shown in Figure S3a. Upon comparison of the relative integrations

of two proton signals (7.0-8.0 ppm vs 4.0-4.5 ppm), about 90 % of DMAEMA residuals was substituted by PBA.

2.3 Synthesis of poly(DMAEMA). DMAEMA (0.2 g, 1.3 mmol), CuBr (5.7 mg, 0.04 mmol), ethyl α-bromoisobutyrate (8 mg, 0.04 mmol), and 2, 2′-dipyridine (12.5 mg. 0.08 mmol) were added to a round bottom flask and protected with the N_2 atmosphere. To this mixture, THF (2 mL) and ethyl α-bromoisobutyrate (8 mg, 0.04 mmol) were added sequentially and mixed gently. After three freeze-thaw cycles, the flask was sealed with N_2, immersed in an oil bath and stirred overnight at 60 ^{o}C. The resulted solution was poured into ethyl acetate (100 mL) and washed with $NaHCO_3$ (0.1 N, 3×50 mL) and dried over anhydrous $NaSO_4$. After filtration and removing the solvent, slightly yellow viscous solid was obtained and was used directly (0.14 g, yield 70%). 1H-NMR (300 MHz, $CDCl_3$) was shown in Figure S4a.

2.4 Synthesis of poly(DMAEMA-PBA). Poly(DMAEMA) (0.1 g) and 4-(bromomethyl) phenylboronic acid (0.5 g, 2.3 mmol) were dissolved in DMF separately and mixed. The mixture was stirred at 60 ^{o}C overnight and dialysis against H_2O (3×2 L). After filtrated and lyophilized, white product was obtained. 1H-NMR (300 MHz, D_2O) was shown in Figure S5a. Upon comparison of the relative integrations of two proton signals (7.0-8.0 ppm vs 4.0-4.5 ppm), about 95 % of DMAEMA residuals was substituted by PBA.

2.5 Synthesis of poly(vinyl alcohol) methacrylate. Poly(vinyl alcohol) (1 g) and methyl anhydride (1 g) were dissolved in DMSO (20 mL), and Et_3N (1 mL) was added as a catalyst. The mixture was stirred overnight at room temperature and dialysis against H_2O (3×2 L) and lyophilized to obtain the product. 1H-NMR (300 MHz, d-DMSO) was shown in Figure S11.

2.6 Rhodamine B or FITC labeled insulin or CAT. Rhodamine B isothiocyanate (0.5 mg) dissolved in DMSO (1 mL) was added to insulin (20 mg) dissolved in $NaHCO_3$ aqueous solution (100 mM, 1 mL). The mixture was stirred for one hour and dialyzed against H_2O (3×2 L). The resultant solution was lyophilized to obtain rhodamine B labeled insulin. Other fluorescence-labeled proteins were obtained with the same method. The fluorescence-labeled insulin or CAT were used in the same way as the one not labeled, and the fluorescence images were taken on a fluorescence microscope (Olympus, IX71).

3 H_2O_2 generation rate assay in glucose solution in the presence of GOx-NC or native GOx. The H_2O_2 concentration in solution was evaluated using a fluorometric hydrogen peroxide assay kit according to the manufacturer's protocol (Sigma-Aldrich). Glucose solutions (100 or 400 mg/dL) containing GOx-NC or GOx (0.2 mg/mL) were incubated at 37 °C. Samples (10 μL each tube) were withdrawn and diluted at timed intervals, and the fluorescence intensity was detected.

4 Preparation of insulin-NCs or GOx-NCs. Typically, insulin (2 mg/mL, 5 mL) or GOx (1 mg/mL, 5 mL) and $MPEG_{5K}$-P(DMAEMA-PBA)$_{14K}$ (1 mg/mL, 5 mL) were mixed, and the pH was adjusted to 7.4. During this process, complex micelles were generated, and PVA (1 wt%, 0.5 mL) (for insulin-NC) or PVA methacrylate (1 wt%, 0.5 mL) (for GOx-NC) was added as a stabilizer to obtain insulin-NC without or GOx-NC with further exposure to UV light (365 nm, 6 × 10 s).

5 *In vitro* insulin release from the complex of insulin and poly(DMAEMA-PBA). The complex was suspended in 10 mM PBS at pH 7.4 and allocated to centrifuge tubes. Various amounts of glucose (0, 100 or 400 mg/dL final concentration) and GOx (0.2 mg/mL) were added to the solutions. At predetermined time intervals, solution (20 μL each tube) was

withdrawn and centrifuged, supernatant (10 μL) was stained with Coomassie blue (200 μL), and the absorbance at 595 nm was detected on an Infinite 200 PRO multimode plate reader (Tecan Group Ltd.). The insulin concentration was calibrated by a standard curve.

6 *In vitro* insulin (or GOx) release from Ins-NCs (or GOx-NCs) from the PVA methacrylate gel (with insulin as an example). Ins-NCs (1 mg/mL) solution and radical initiator were mixed with PVA methacrylate solution (5 wt% in H_2O) and irradiated to form a gel. The gel was added to centrifuge tubes containing glucose (100 or 400 mg/dL). At predetermined time intervals, solution (10 μL each tube) was withdrawn, stained with Coomassie blue (200 μL) and the absorbance at 595 nm was detected on an Infinite 200 PRO multimode plate reader (Tecan Group Ltd.). The insulin concentration was calibrated by a standard curve.

7 Fabrication of microneedle array patch (with MN(G+C+I) as an example). All the MNs in this study were prepared using commercial silicone moldspurchased from Blueacre Technology Ltd. Each MN had a round base of 300 μm in diameter, which tapers over a height of 600 μm to a tip of 5 μm diameter. The MNs were arranged in a 20×20 array with 600 μm tip-tip spacing. First, diluted aqueous solutions of PVA (contain 10 % PVA methacrylate, 3.5 wt% in H_2O, 500 μL), CAT-NG (1 mg in 400 μL H_2O) and a photoinitiator (Irgacure 2959; 5 wt%) were prepared and mixed. After deposition of this solution (100 μL) in a silicone mold, the solution was kept under reduced vacuum for 30 minutes and then transferred to a Hettich Universal 32R centrifuge for 30 min at 2000 rpm. Then, diluted aqueous solutions of PVA: PVP (2:1), PVA methacrylate (5 % in total), GOx-NCs, Ins-NCs and photoinitiator (Irgacure 2959; 5 wt%) were loaded into a mold, and this procedure was repeated for several times until predetermined amount of Ins-NCs was loaded. Finally, the microneedle array patch was dried under vacuum for 2 days. After the desiccation, the MN arrays were carefully peeled off the

silicone mold, and the MNs underwent crosslinking via UV irradiation (365 nm, 9 W/cm^2, BlueWave® 75 UV Curing Spot Lamp) for six cycles of 10-second exposure. The morphology of the MNs was characterized on an FEI Verios 460L field-emission scanning electron microscope.

8 The mechanical strength test. The mechanical strength of microneedles with a stress-strain gauge was determined by pressing a stainless-steel plate against microneedles on an MTS 30G tensile testing machine. The initial gauge was 2.00 mm between the tips of microneedle and the plate, with 10.00 N as the load cell capacity. The speed of the plate approaching microneedles was set as 0.1 mm/s. The failure force of microneedles was recorded as the force at which the needle began to buckle.

9 In vitro cytotoxicity assay. The cytotoxicity of materials to Hela cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Hela cells (5000 cells per well) were plated into 96-well plates and incubated overnight. Then cells were exposed to serial dilutions of carrier materials for 40 h. Subsequently, the medium in each well was replaced with fresh culture medium containing 0.5 mg/mL MTT. The plates were incubated for another 2 h before the addition of DMSO to dissolve the formazan crystals. The absorbance of each individual well was measured at 562 nm with a microplate spectrophotometer. Each drug concentration was tested in triplicate and in three independent experiments.

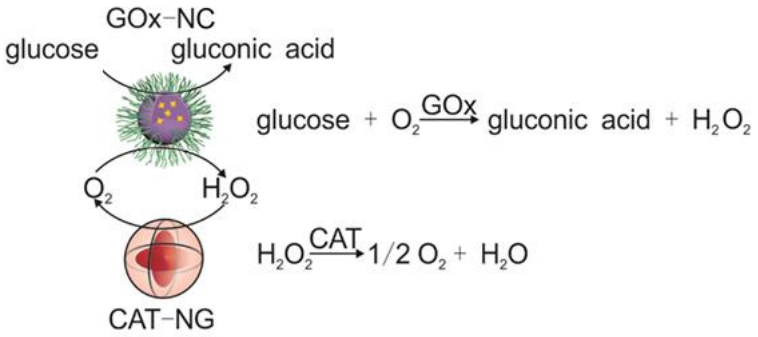
10 *In vivo* studies using streptozotocin-induced diabetic mice. The *in vivo* efficacy of MN-array patches for diabetes treatment was evaluated in adult diabetic mice (male C57B6, age 8 weeks; Jackson Laboratory) induced using streptozotocin. The animal study protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University and the University of North Carolina at Chapel Hill. The blood glucose levels were

measured using tail vein blood samples (~3 μL) of mice using the Clarity GL2Plus glucose meter (Clarity Diagnostics). The mouse glucose levels were consitantly measured before treatment. For each group, five mice were selected to be treated using MN patches or native insulin. The glucose level of each mouse was monitored until stabilization.

11 *In vitro* skin penetration test. To evaluate the *in vitro* skin penetrating ability of MNs, the MNs were inserted into the mouse skin for 10 min. The skin was excised and stained with trypan blue for 10 min before imaging by optical microscopy (Leica EZ4 D stereomicroscope).

12 Statistical analysis. Differences in blood glucose levels between the treated groups and controlled groups were determined by unpaired Student's *t*-test. The results were considered statistically significant if the two-tailed *P*-values were less than 0.05. The statistical approach remained consistent throughout all analyses.

13 Animal experiment. The sample size calculated by power analysis: G*power 3.1. The experiments did not use a method of randomization. The investigators were not blinded to allocation during experiments and outcome assessment.



Scheme S1. Schematic illustration of H_2O_2 generation by GOx-NC and elimination by CAT-NG.

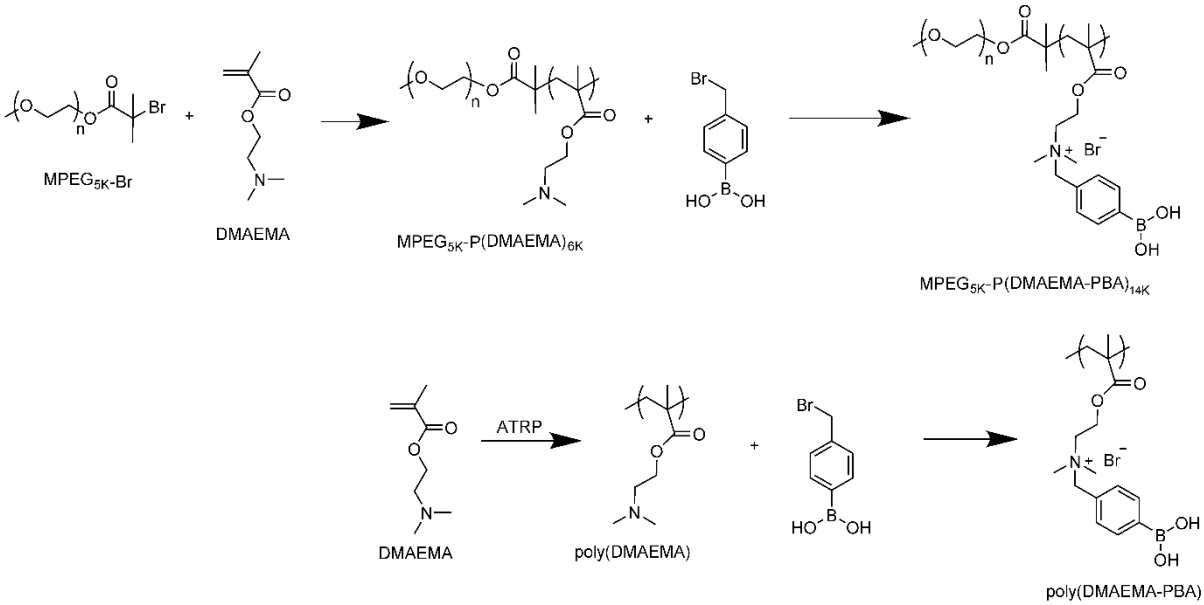


Figure S1. Synthetic routes of polymers utilized in this study.

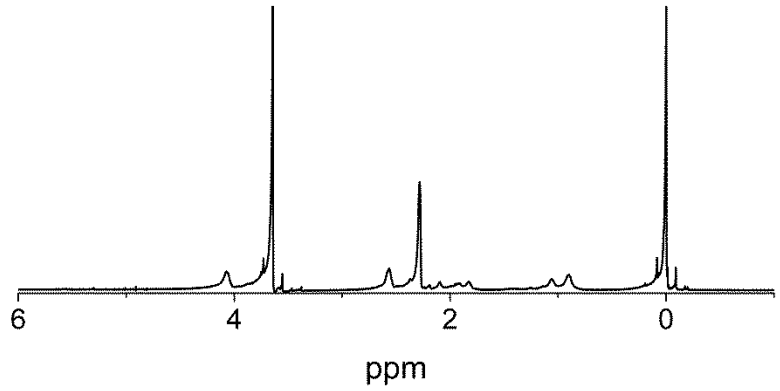


Figure S2a. The 1H-NMR spectrum of $MPEG_{5K}$-$P(DMAEMA)_{6K}$.

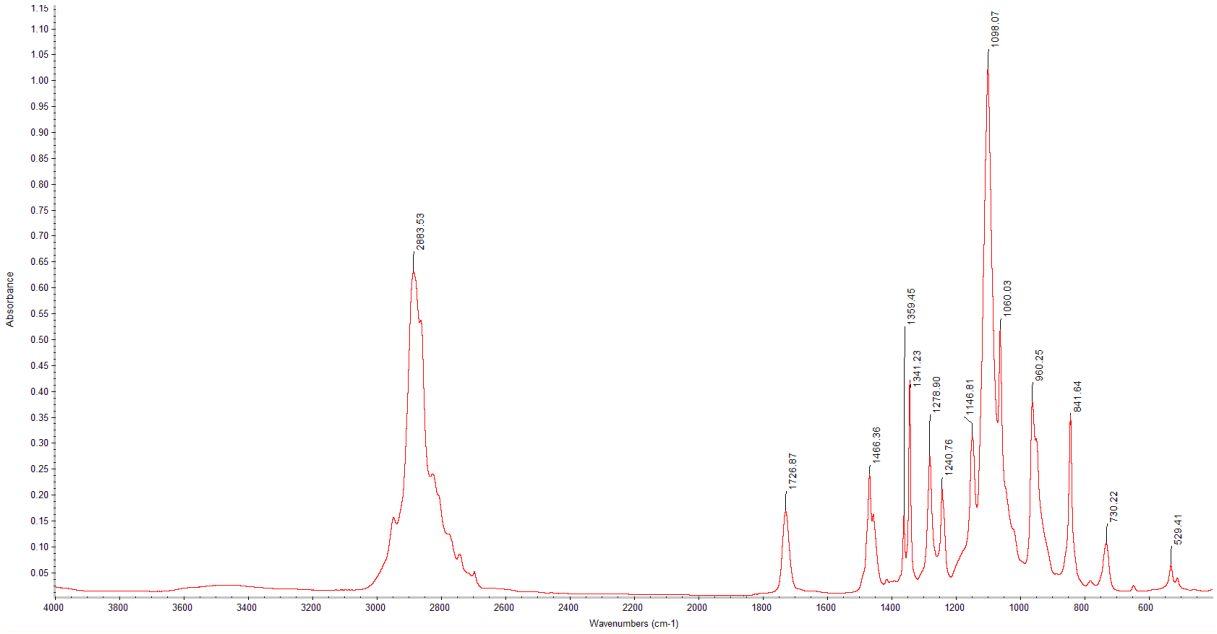


Figure S2b. The FT-IR spectrum of $MPEG_{5K}$-$P(DMAEMA)_{6K}$.

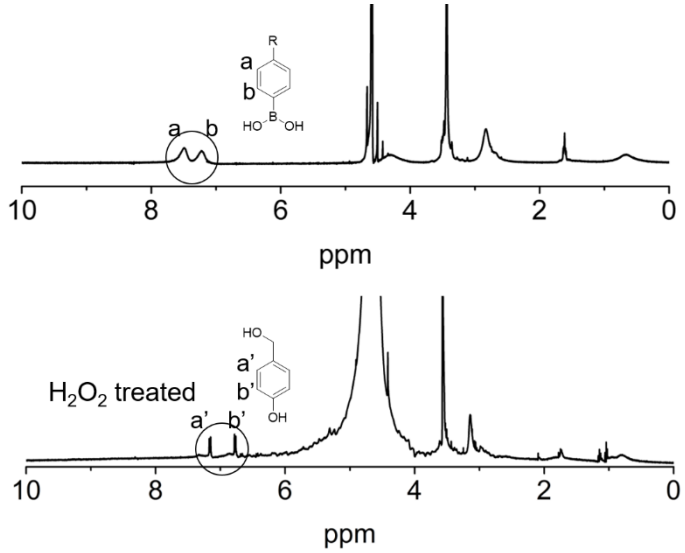


Figure S3a. The 1H-NMR spectra of $MPEG_{5K}$-P(DMAEMA-PBA)$_{14K}$ before and after H_2O_2 (80 mM) treatment.

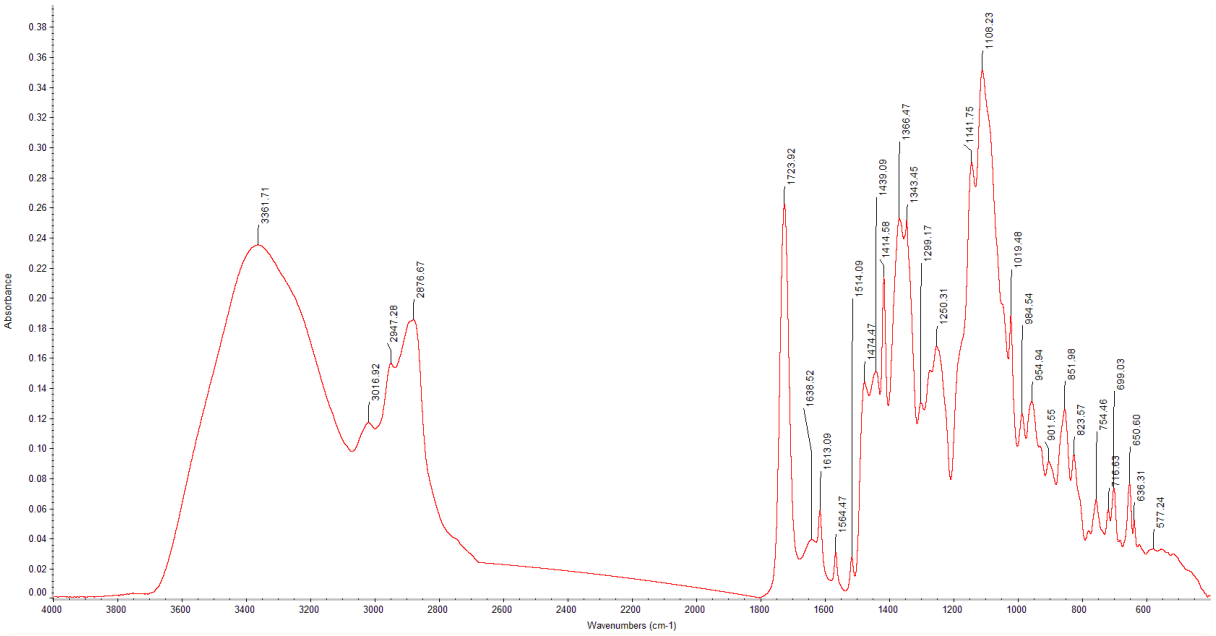


Figure S3b. The FT-IR spectrum of $MPEG_{5K}$-P(DMAEMA-PBA)$_{14K}$.

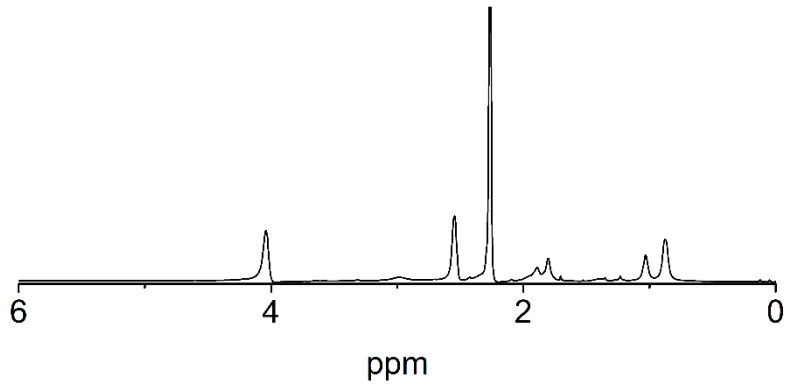


Figure S4a. The 1H-NMR spectrum of poly(DMAEMA) synthesized by ATRP initiated by ethyl α-bromoisobutyrate.

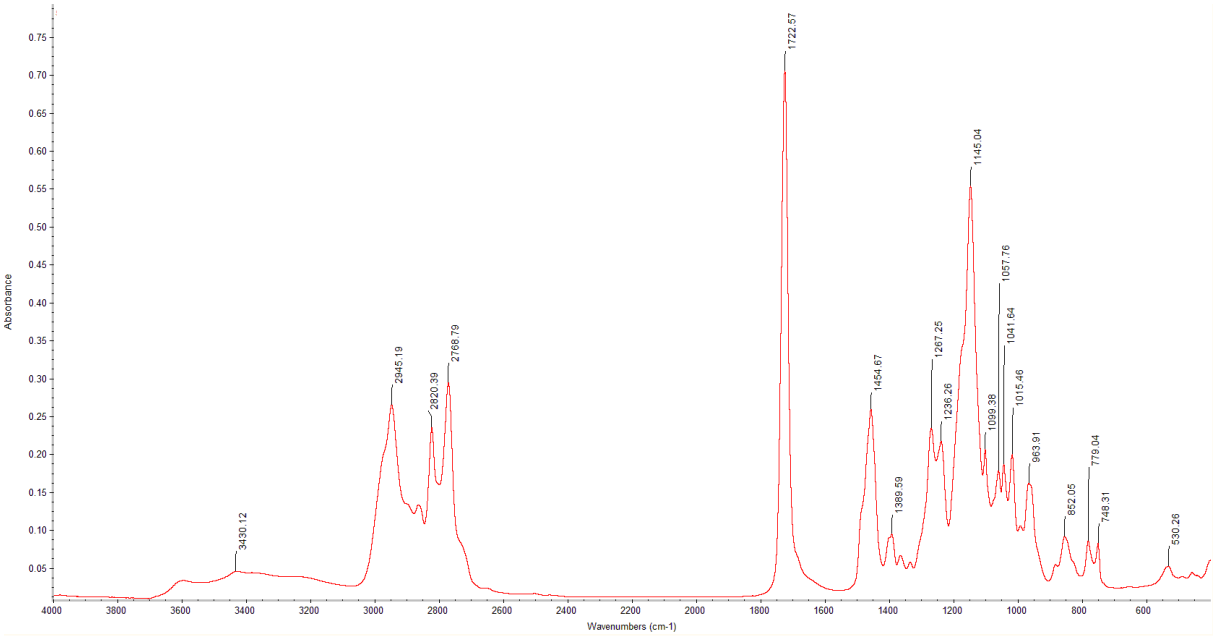


Figure S4b. The FT-IR spectrum of poly(DMAEMA) synthesized by ATRP initiated by ethyl α-bromoisobutyrate.

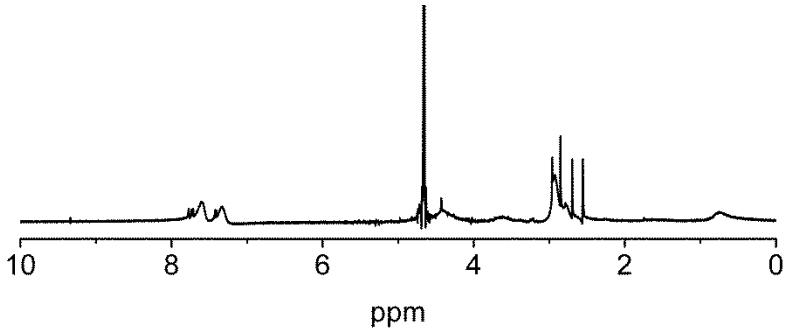


Figure S5a. The 1H-NMR spectrum of poly(DMAEMA-PBA).

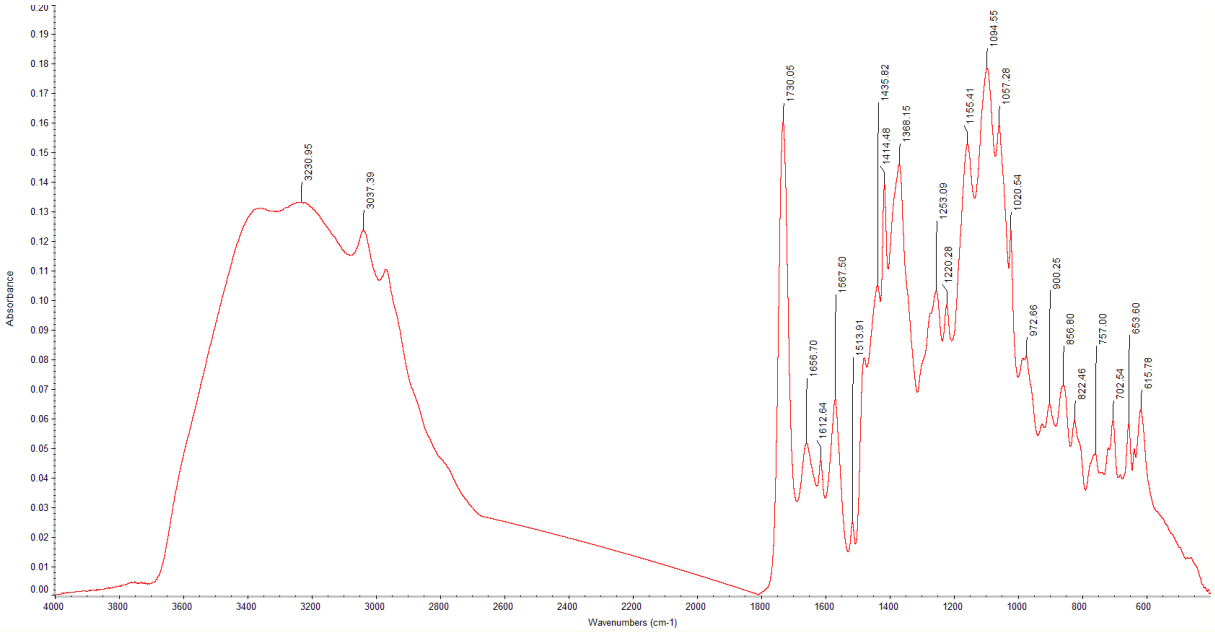


Figure S5b. The FT-IR spectrum of poly(DMAEMA-PBA).

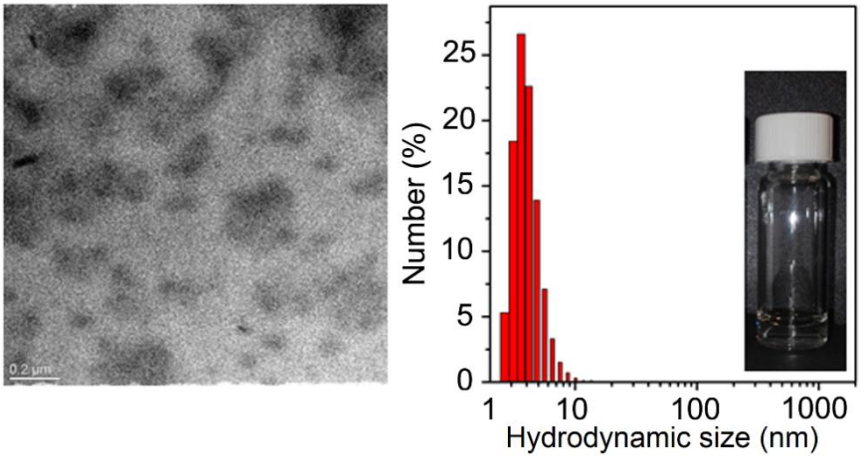


Figure S6. Representative TEM image and size distribution of Ins-NCs after treatment with PBS containing glucose (400 mg/dL) in the presence of GOx.

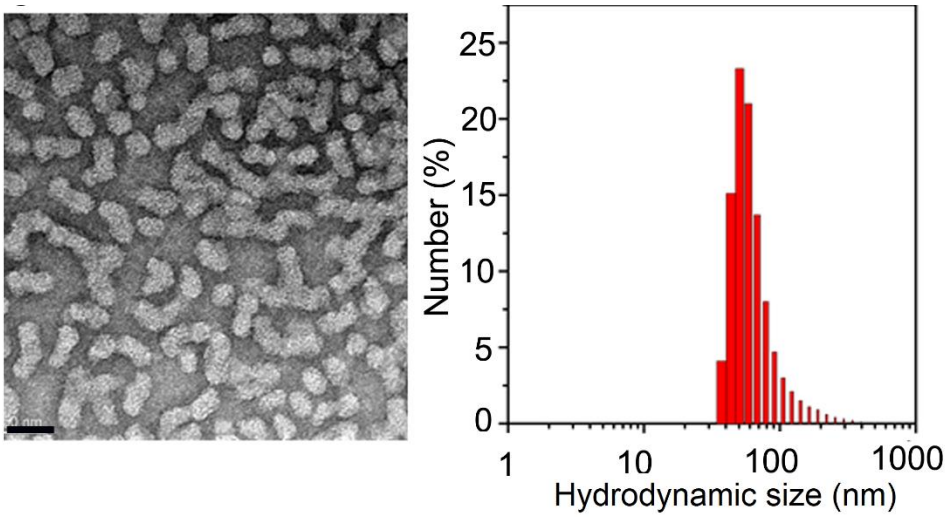


Figure S7. Representative TEM images and size distribution of GOx-NCs. Scale bar, 50 nm.

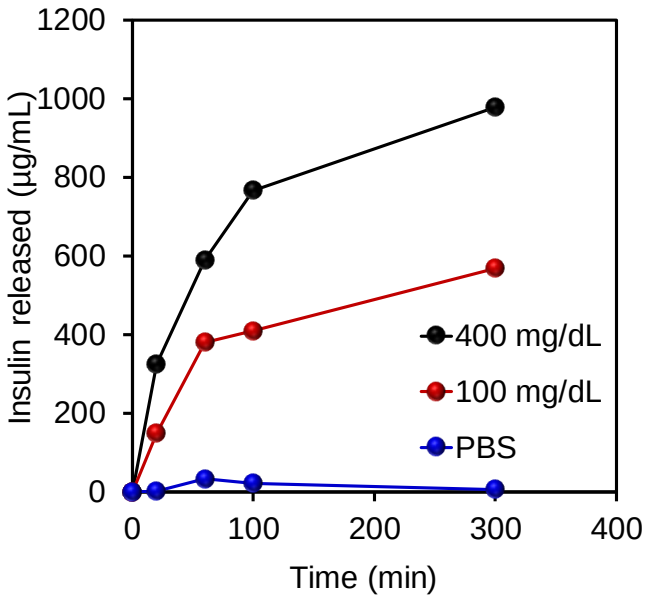


Figure S8. Insulin release profile from complex formed between insulin and poly(DMAEMA-PBA) in PBS at pH 7.4 in the presence of GOx (0.2 mg/mL) with different glucose concentration (400, 100 and 0 mg/dL).

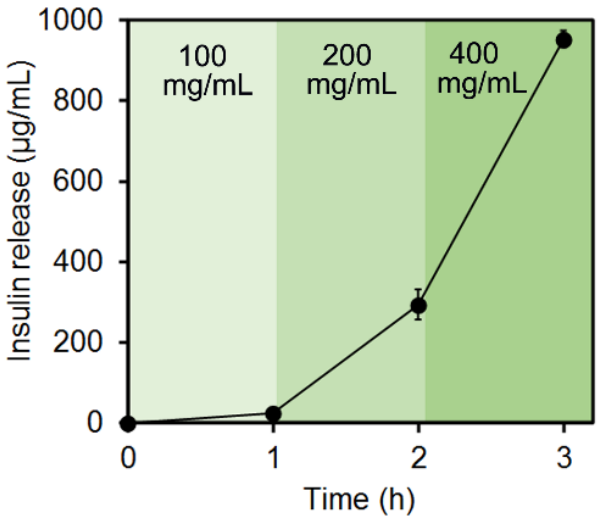


Figure S9. Self-regulated insulin release profile as a function of glucose concentration. Data points represent mean ± SD (n = 3). Error bars indicate SD.

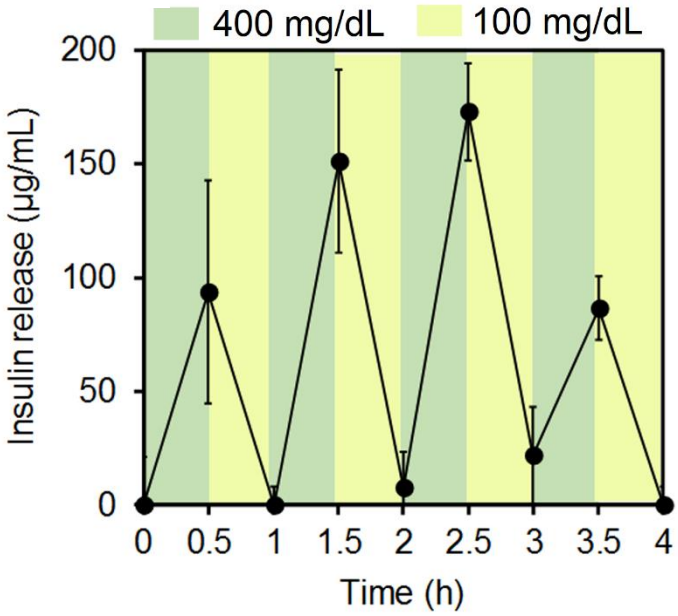


Figure S10. Pulsatile insulin release profile as a function of glucose concentrations over time. Data points represent mean ± SD (n = 3). Error bars indicate SD.

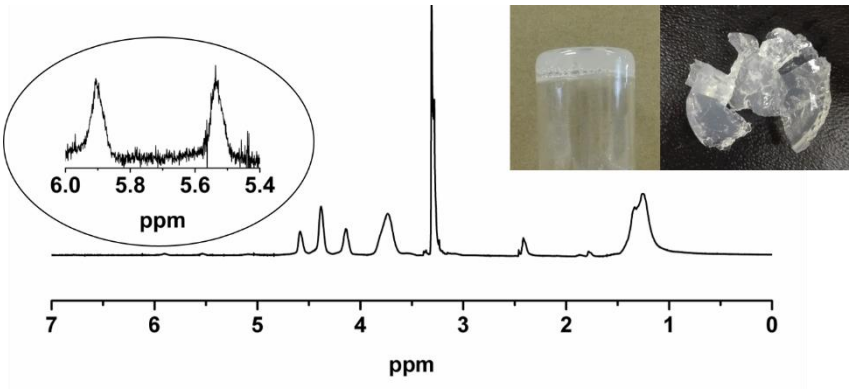


Figure S11. 1H-NMR of PVA methacrylate and its gel in aqueous solution.

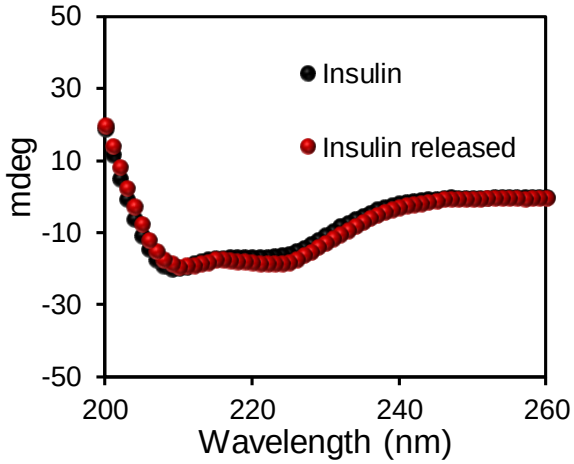


Figure S12. CD spectra of native insulin solution and insulin released from the gels incubated with 400 mg/dL glucose.

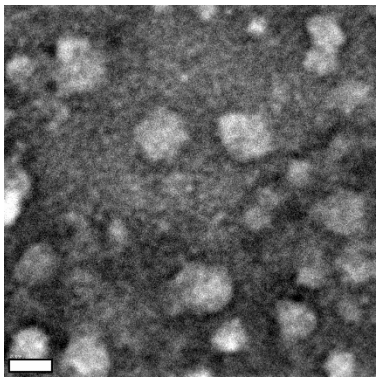


Figure S13. Representative TEM image of CAT-NG. Scale bar, 20 nm.

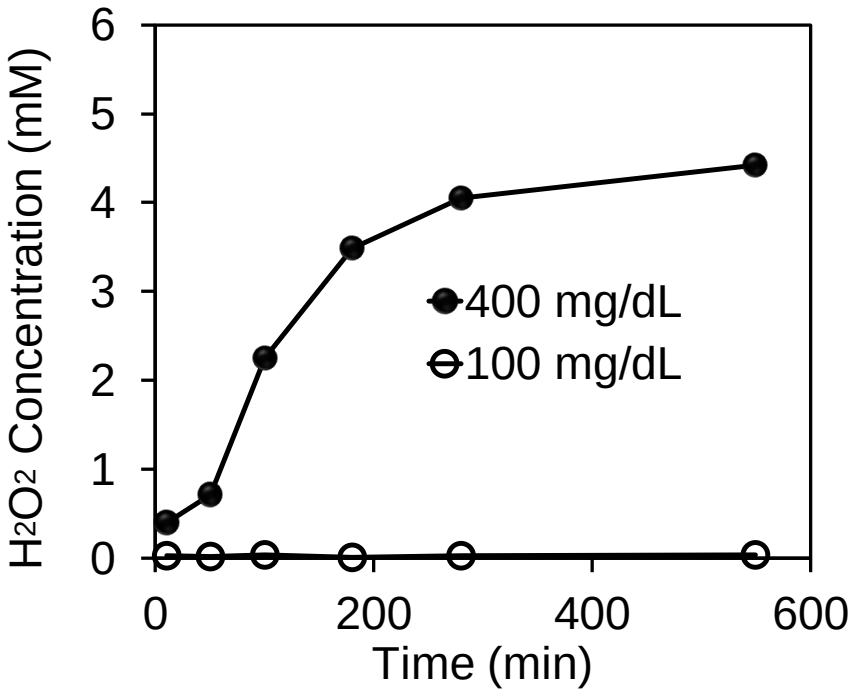


Figure S14. The H_2O_2 generation rate *via* glucose oxidation as catalyzed by GOx-NC in the presence of CAT-NG in glucose solution (100 or 400 mg/dL) in PBS with an initial pH at 7.4. The concentration of GOx and CAT was set to 0.2 mg/mL GOx-eq. concentration and 0.08 mg/mL CAT-eq. concentration, respectively.

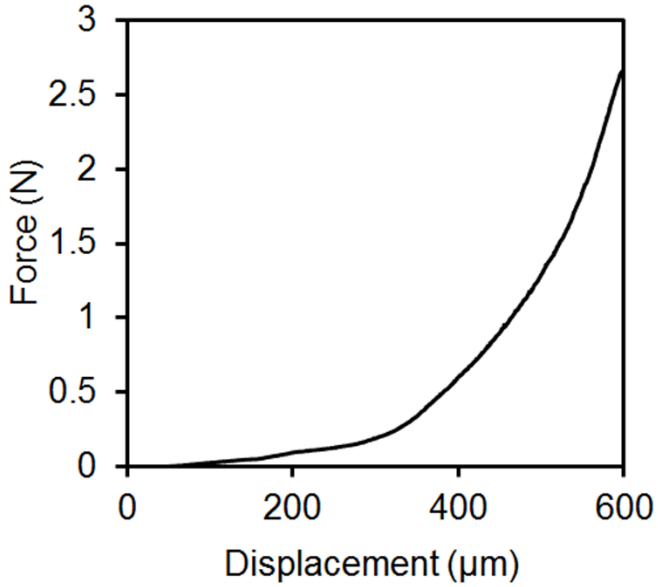


Figure S15. The mechanical strength test of microneedle.

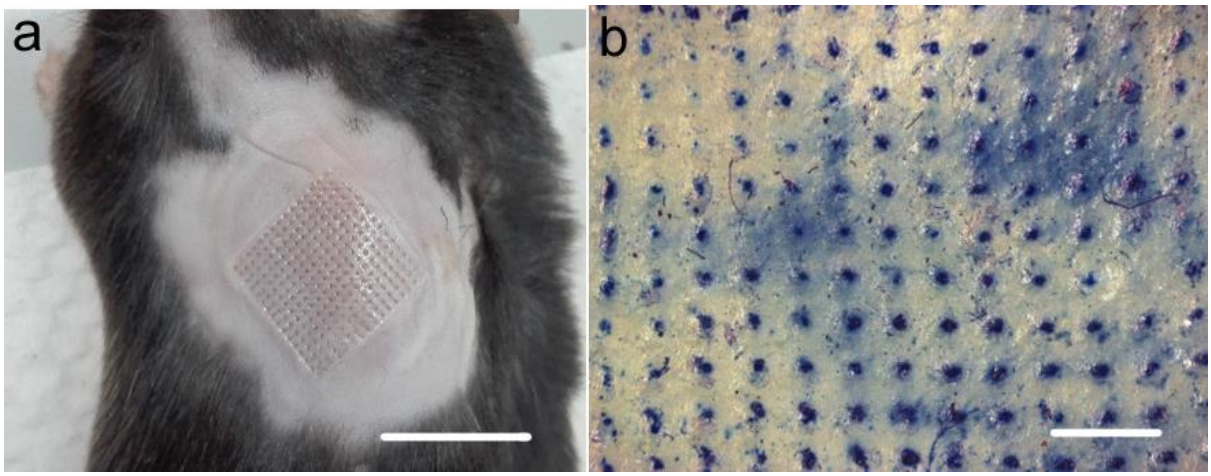


Figure S16. Images of a mouse treated by MN (a) and the trypan blue staining (b). Scale bars, 1 cm for (a) and 600 μm for (b).

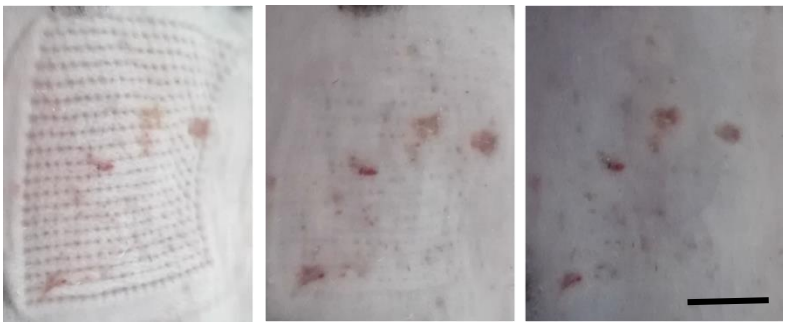


Figure S17. Skin puncture marks at 0, 5 and 120 min post-treatment of MNs. Scale bar, 0.5 cm.

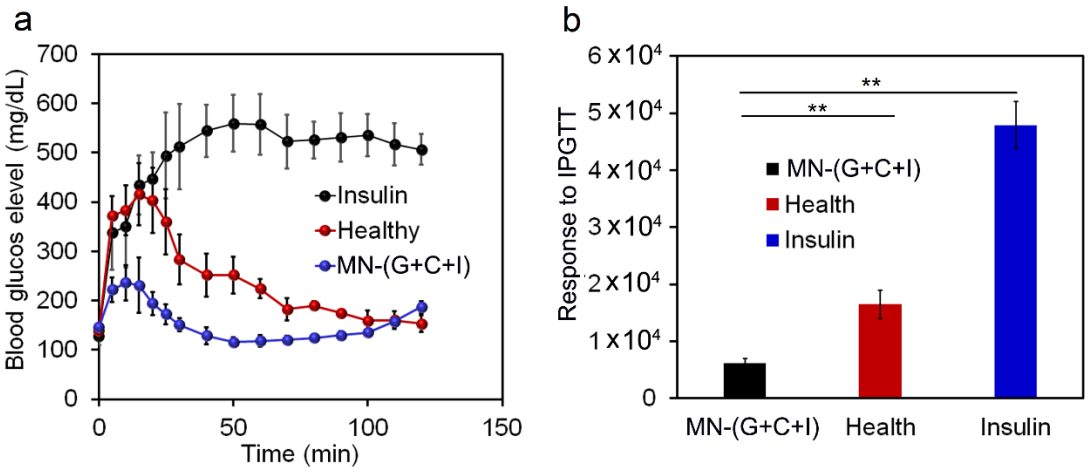


Figure S18. IPGTT and responsiveness. (a) *In vivo* glucose tolerance test toward diabetic mice at one-hour post-treatment of MN-(G+C+I) or subcutaneously injected with insulin. Healthy mice were used as the control. (b) Responsiveness was calculated based on the area under the curve (AUC) in 120 min, with the baseline set at the 0-min blood glucose reading. Data points represent mean ± SD (n = 5). $^{**}P < 0.01$ (analyzed by one-way ANOVA) for MN-(G+C+I) compared with control groups.

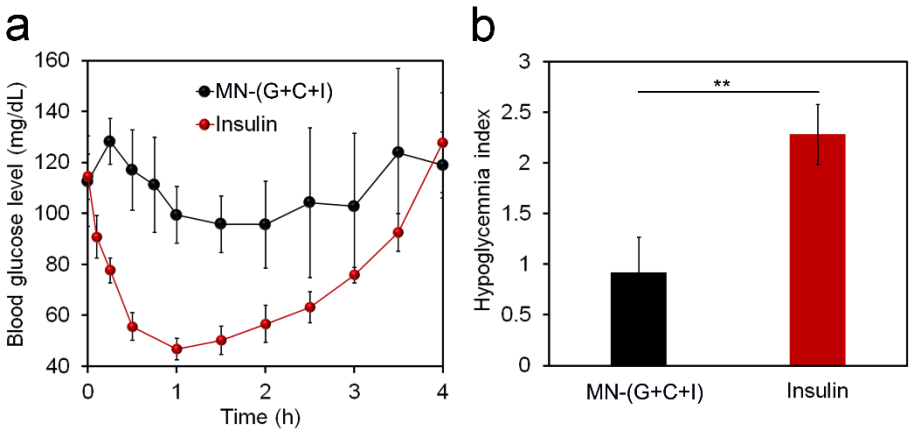


Figure S19. Hypoglycemic test of MN on healthy mice. (a) Blood glucose levels change of healthy mice treated with MN array patch or subcutaneously injected insulin. The treatment was given at 0 min. (b) Quantification of the hypoglycemia index, identified as the difference between the initial and nadir blood glucose readings divided by the time at which nadir was reached. Data points represent mean ± SD (n = 5). $^{**}P < 0.01$ (analyzed by two-tailed Student's *t*-test) for MN-(G+C+I) compared with the insulin-treated group.

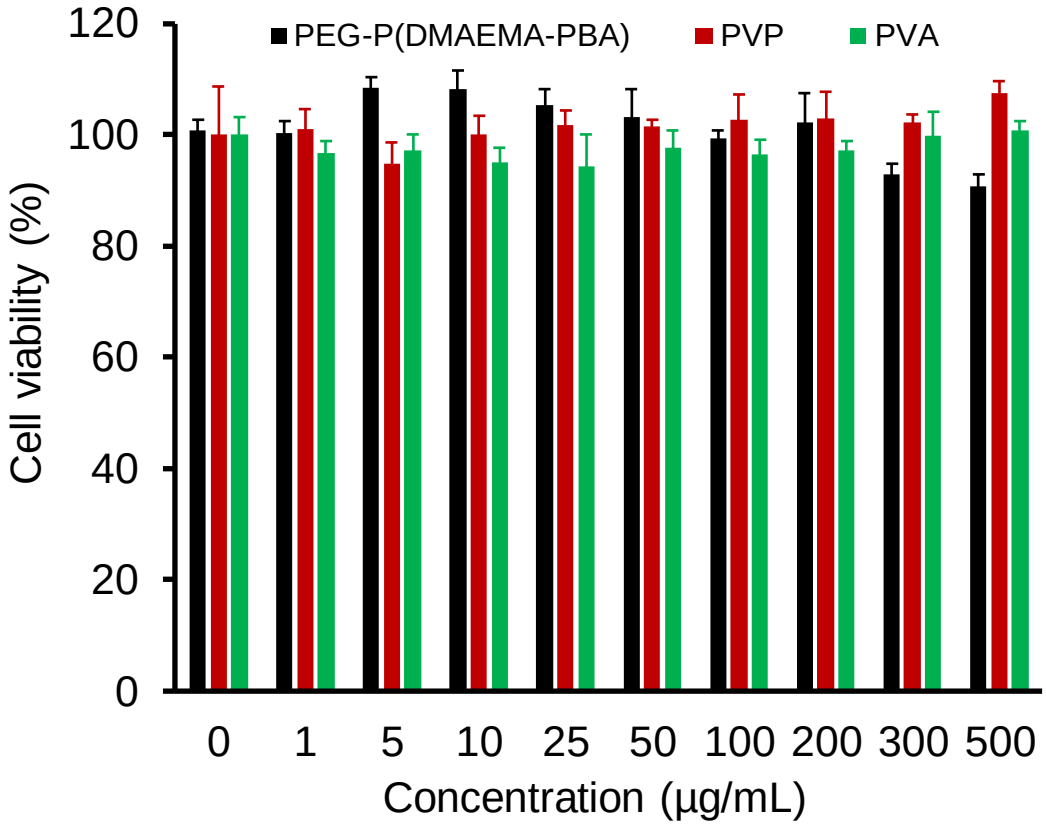


Figure S20. Cytotoxicity evaluation of the matrix materials utilized in this study toward Hela cell.

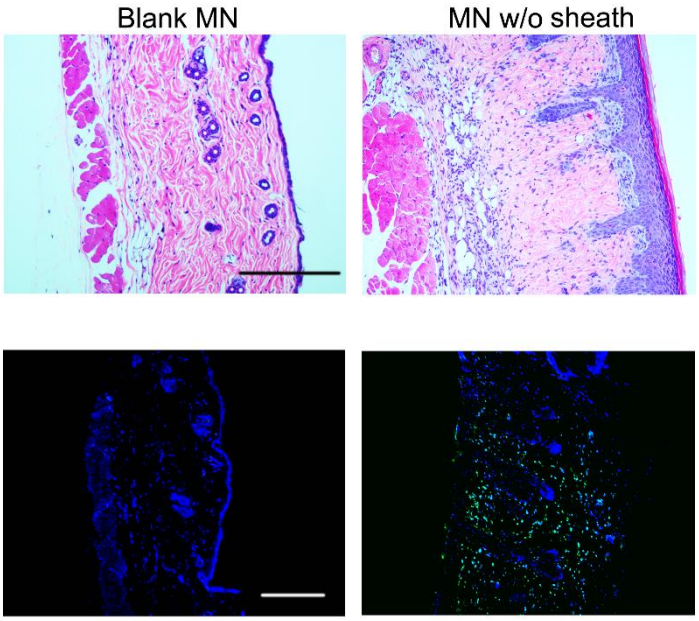


Figure S21. H&E staining and immunohistologic staining results of skins treated by blank MN and MN-(G+I). Scale bars, 300 μm.

Reference

[1] J. Q. Wang, W. W. Mao, L. L. Lock, J. B. Tang, M. H. Sui, W. L. Sun, H. G. Cui, D. Xu, Y. Q. Shen, *ACS Nano* **2015**, *9*, 7195.