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Supplementary Information

Photothermal-Triggered Protein Release from an Injectable Polycaprolactone Microspherical Depot

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Experimental Section

Reagents

PCL (Mn: 10k, 25k, 43k) and PCL diol (2k) was purchased from Sigma-Aldrich and used as received. Gold chloride trihydrate ($HAuCl_4$; 99.9 %) was purchased from Sigma-Aldrich (Australia) and tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was purchased from Ajax Finechem. Poly(ethylene glycol) methyl ether acrylate (OEGA, Aldrich, average *M*n=480 g/mol) and RAFT agent 4-cyano-4-(phenylcarbono-thioylthio) pentatonic acid (CPADB; 97 %) was purchased from Strem Chemicals and used as received. 4,4′-Azobis(4-cyanovaleric acid)（ACVA） was purchased from Sigma Aldrich. 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and bovine serum albumin (BioXtra, A3311) was purchased from Sigma-Aldrich (Australia). Analytical reagent grade of poly (vinyl alcohol) (*M*W=30k-70k, 87-90 % hydrolyzed), dichloromethane (DCM), sodium chloride, acetone, methanol, ethanol were purchased from Merck. Millipore purified water was used in all experiments. For the MTS assay, the phenazine methosulfate (PMS) and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) were purchased from Sigma-Aldrich and Promega Corporation. Horseradish peroxidase (HRP) (Type VI-A, 250-330 units/mg), foetal bovine serum (FBS), and hydrogen peroxide solution (H_2O_2, 30% (W/W)) were purchased from Sigma Aldrich. The LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian cells, (L3224 Molecular probes) was purchased from Life Technologies (Australia). L929 cell culture medium comprised of MEM-GlutaMax, Anti-Anti and NEAA supplement all of which were purchased from Life technologies.

Instrumental methods

NMR analysis was performed with a Bruker Av 400 NMR spectrometer and ^1H NMR spectra were recorded in deuterated chloroform ($CDCl_3$). Gel permeation chromatography (GPC) analysis of the POEGA was performed on Shimadzu system equipped with a CMB-20A controller, a SIL20 HT autosampler, an LC-20AT tandem pump system operating at a combined flow rate of 1 mL/min, a DGI-20A degasser, a CTO-20AC column oven operating at 80 ℃, and a RDI-10A refractive index detector. The samples were run in *N*,*N*-dimethylacetamide (DMAc) containing 0.1 % LiBr over four Waters® Styragel columns (HT5, HT4, HT3 and HT2 each 300 mm × 7.8 mm). Number-average molar mass (M_n) and weight-average molar mass (M_w) were

determined against polystyrene standards ranging from 4430 – 3242000 g/mol using the LabSolutions postrun analysis software (version 5.63). UV-vis spectra were measured from with Cary 50 Bio UV-visible spectrophotometer (Varian Co., USA). GNP size distribution was measured from a Zeta Sizer-Nano instrument (Malvern, UK) and the gold nanoparticle concentration was measured by using a Cary 50 Cary 50 Bio UV-visible spectrophotometer (Varian Co., USA) at room temperature. The transmission electron microscopy (TEM) images were taken from a Tecnai 12 TEM (FEI, Netherlands). Cell imaging was performed using a Nikon TE2000 Eclipse fluorescent microscope with NIS-Elements AR3.2 software. The microspheres were observed on Optical microscope (Nikon, Japan). The scanning electron microscopy (SEM) image was observed using Camscan SEM and energy dispersive spectroscopy (EDS) was measured on x-ray analysis (Aztec, Oxford). The melting temperature (T_m) of PCL microspheres were determined by differential scanning calorimetry (DSC) measurements (Mettler Toledo, Switzerland) under N_2 atmosphere. The ATR-FTIR analysis of POEGA, POEGA-coated gold nanoparticles (PGNP), PCL_{2k} neat and PCL_{2k}@$PGNP_{0.5\%}$ were performed with a Thermo Scientific Nicolet 6700 FT-IR spectrometer and the samples were dried in vacuum overnight before measurement.

1. Polymer synthesis

The POEGA polymer was prepared by Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization method using a molar ratio of [OEGA]/[CPADB]/[ACVA] = 30 / 1 / 0.2 (to 65 % monomer conversion) based on the method of Liu et al.[1] The polymer was purified by precipitation in the cold diethyl ether/cyclohexane (1:1) and dried before being characterized by ^{1}H NMR (degree of polymerisation (DP = 22) and GPC.

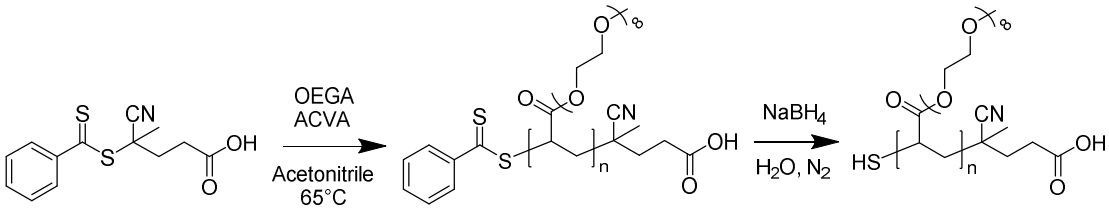

Fig. S1 Synthetics scheme of POEGA by RAFT polymerization and its reduction

2. GNP synthesis

Gold nanoparticles with a mean diameter 46 nm were synthesised by the reduction method.[2] Simply, $HAuCl_4$ solution (567 mL, 0.01 wt %) was heated to boiling using a conical flask under stiring and tri-sodium citrate solution (3.97 mL, 1 wt%) was added quickly. The reaction was stopped after about 30 mins and cooled down at room temperature. The GNPs were then coated with polymer as outlined below.

3. Grafting POEGA onto GNPs surface

The POEGA grafting on GNPs were prepared by a modified method.[3] Simply, $NaBH_4$ aqueous solution (1.0 M) as the reducing agent (molar ratio of $NaBH_4$: dithioester end group was 25:1) were mixed with purified POEGA polymer until the pink colour disappeared under N_2 atmosphere. Then the particles were coated by adding POEGA (0.8 mg) to GNPs solution (0.01 wt%, 100 mL) and mixing for overnight. The solution was centrifuged at 4500 rpm for 1 h. The supernatant was removed and washed by water three times, then replaced by ethanol, DCM three times, respectively. The purified PGNP was stored in DCM before being used.

4. BSA loaded PCL@PGNP microspheres

PCL microspheres were prepared by a modified W/O/W double emulsion solvent extraction/evaporation method.[4] Briefly, BSA 50 mg was dissolved in 0.1 % PVA phosphate buffer saline (PBS, pH=7.4, 0.1 M) solution as inner water phase. The inner phase was emulsified for 20 s with different molecular weight PCL 500 mg (or PCL and PGNP) /methylene chloride solution (oil phase: 15 mL) by sonicator at an output power of 50 W. The resulting first emulsion was then injected at 6 mL/min into a stirred 0.5 % PVA PBS solution (250 mL) as the external water phase to produce the double W/O/W emulsion. The solution was magnetically stirred at 450 rpm in beaker for 30 mins before PBS solution 200 mL with 0.1 % PVA was added and continuously stirred for another 6 h at 25°C. The precipitated microspheres were washed by de-ionized water for three times and then freeze-dried overnight and stored at 4 ^{o}C before use.

5. Characterization of microspheres

The morphology of PCL microspheres was observed by optical microscopy and scanning electron microscopy (SEM). For optical microscopy, the freeze-dried PCL microspheres were observed while dispersed in distilled water on a glass slide. The captured optical images were used to measure the volume mean diameter of the PCL microspheres by the Image Pro Plus software.

6. Drug encapsulation efficiency and loading in microspheres

The BSA encapsulation efficiency and BSA loading per unit weight of microspheres were determined by an extraction method followed by UV spectroscopy. Dried PCL microspheres of 10 mg were dissolved in 1 mL of DCM for 20 mins and 2 mL of PBS was then added. The mixture solution stirred for 10 mins to extract BSA into PBS from the oil phase. The aqueous solution 1 mL was withdrawn after centrifuged and was determined by UV. The absorbance at 278 nm measured was used to determine the protein concentration against standard curve was obtained by determining the known different concentrations of BSA standard solutions. The encapsulation efficiency and loading amount were expressed as the ratio of actual to theoretical BSA content and drug amount per unit microspheres.

$$\%\ \text{Encapsulation efficiency} = \left(\frac{\%\ \text{Drug loading}}{\%\ \text{Theoretical loading}}\right) \times 100 \quad (1)$$

$$\%\ \text{Drug loading} = \left(\frac{\text{Weight of drug in microspheres}}{\text{Weight of microspheres}}\right) \times 100 \quad (2)$$

7. Rheological analysis

The photorheological study was conducted by an ARES photorheometer (TA Instruments, USA) connected to a blue light source (Omnicure Series 2000, USA, 400-500 nm, 0.2 W) via a fibre optic. The molten PCL@PGNP sample was loaded in the centre of two parallel plastic plates of 20 mm diameter with a gap of 0.3 mm. The viscosity (η^*) were measured as a function of time at a constant frequency of 10 rad/s and a strain of 1.0 %. Each sample was loaded and allowed to re-solidify and equilibrate for 30 minutes at 25 ℃ before blue light exposure for 10 minutes.

8. *In vitro* BSA release study under blue light exposure

A 40 mg aliquot of dried microspheres was placed in 4 mL fluorimeter cuvettes and incubated in 2 mL of PBS buffer. The drug release experiments were performed with blue light with 400-500 nm (Omnicure Series 2000, USA) with adjustable power/light intensity at 37℃ and the real time measurement of drug concentration was performed using Cary 50 Bio UV-visible spectrophotometer (Varian Co., USA) fitted with a Cary 50 fiber optic probe. The samples were first placed at 4°C for 4 h and determined the initial burst on Day 0. Then the sample was placed in the incubator at 120 rpm and 37°C, and were exposed blue light on days 1, 3, 7 for 10 mins at each time point. 30 minutes after the 10 minutes blue light exposure, 1 mL of the supernatant was withdrawn and fresh buffer was added back into the tube. The absorbance of supernatant at 278 nm measured was used to determine the cumulative BSA release based on the standard curve. All the samples were conducted duplicate or triple.

9. SDS-PAGE and CD of released BSA

SDS-PAGE and CD was used to determine the primary structure of the BSA released from microspheres *in vitro*. The supernatant was collected from the buffer microspheres mixture used for the *in vitro* release study. For SDS-PAGE, the samples 40 µl (BSA about 0.8 µg) were mixed with 10µl of a SDS (1% w/v), Tris–HCl (pH 6.8) (0.06 mM), glycerol (3 mM), and bromophenol blue (0.01% w/v) solution for staining. The samples were resolved through a NuPAGE 4-12% Bis-Tris gel. Electrophoresis was performed using a BioRad Mini-Protean II electrophoresis setup at a constant voltage (140 V) as described by the manufacturer. The gels were stained with silver using a Silver Stain kit (BioRad). The molecular weight of the detected bands was compared to standards. BSA control samples were also run on the gel to compare that of the released samples. The stained gels were immediately photographed

10. The bioactivity assay of HRP

Based on the enzymatic activity of HRP, the bioactivity is determined by measuring the colour depth at 405 nm with ABTS as hydrogen donor at 25℃. To a solution containing 30% H_2O_2 (5%, v/v) and ABTS (0.5 mg/mL) in 100 µl of a citrate phosphate buffer (0.03 M), was added HRP at different concentrations. After the solution colour became

green, the oxidized $ABTS^{\bullet+}$ product was followed by UV-VIS spectroscopy. The standard curve was obtained by different HRP concentrations and the absorbance at 405 nm measured was used to determine the released HRP bioactivity against a standard curve.

$$ABTS + H_2O_2 \xrightarrow{HRP} 2H_2O + ABTS^{\bullet+}$$

11. H_2O_2 intervention assay

The detection of H_2O_2 elimination by HRP was also studied to prove if the released HRP remain bioactive or not. The effective HRP can eliminate the reactive oxygen species (ROS) formed by hydrogen peroxide (H_2O_2).[5] The cells were incubated in the presence of the test solutions according to the ISO10993 standard. Released HRP (R-HRP) or Fresh HRP (F-HRP) at different concentrations was reacted with 468 μM H_2O_2 added to each well and co-incubated for 24 h. The cell viability of triplicate samples was measured by MTS assay. An additional well was washed with fresh medium and stained with Live-Dead™ kit to stain viable cells (green) or dead cells (red fluorescence) and visualized using a Nikon TE2000 Eclipse fluorescent microscope with NIS-Elements AR 3.2 software.

12. Qualitative HRP release test from skin implanted depot

PCL_{2k}@$PGNP_{0.5\%}$ microspheres (50 mg) loaded with HRP (1.5% w/w) were dispersed in the 1.5% w/w hyaluronic acid solution (950 mg), followed by epidermal injection (150 μL) to the porcine skin (cadaver tissue, fresh belly skin obtained from local butchery). After injection, a small HA encapsulated PCL_{2k}@$PGNP_{0.5\%}$ depot was observed under the skin, which was then immediately exposed to blue light (390 mW/cm^2, 5 minutes). The release of HRP from the implant was qualitatively verified by incubation of the porcine skin for 5 minutes (r.t.) in the 3,3',5,5'-tetramethylbenzidine (TMB) solution, which was indicated by the appearance of blue colour. TMB solution was prepared fresh using 60 mg TMB pre-dissolved in 20 mL ethanol, followed by mixing in 200 mL 0.1 M acetate buffer (pH 5). 10 minutes prior to incubation of the porcine skin, 200 μL of H_2O_2 (30% w/w) was added. For comparison, HRP-loaded PCL_{2k} microspheres (without gold nanoparticles) were also

implanted to the porcine skin and treated equally for irradiation and TMB incubation, while an injected blank 1.5% w/w HA hydrogel served as a negative control.

13. *In vitro* cytotoxicity evaluation of PCL_{2k} and $PCL_{2k}@PGNP_{0.5\%}$ Microspheres (MS)

L929 fibroblasts were cultured and maintained in MEM supplemented with 10% (v/v) FBS, NEAA and Anti-Anti, and incubated at 37℃ with 5% CO_2. The extraction solution (L929 medium with double-strength Anti-Anti) was based on ISO10993-5 standard biological evaluation of medical devices. The microspheres were extracted (200 mg/mL) in a closed container at 37℃ with agitation for 66 hrs using aseptic technique and the extraction solution applied to L929 cells neat and in a series of halving dilutions. *In vitro* cytotoxicity was evaluated using the MTS assay. 2 x 10^4 cells/well were incubated in 96-well plates in the presence of various concentrations (200, 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8 mg/mL) of PCL_{2k} and $PCL_{2k}@PGNP_{0.5\%}$ microspheres for 24 h. The medium was removed and washed with fresh medium. Then the MTS working solution was added left for 4 hrs at 37℃ and absorbance was read on a plate reader at 490 nm and 655 nm. Each measurement was performed in quintuplicate.

Experimental results

Table S1. characteristic of polymer used in this study

Sample	DP		Molecular weight (g/mol)		Đ
	Target	NMR	NMR M_n	GPC M_n	
POEGA	20	22	11320	10840	1.13

Table S2. Properties of unmodified GNPs and polymer modified PGNP (POEGA-GNPs)

	UV λmax [nm]	**Size UV** [nm]	**Size DLS** [nm]
Unmodified GNPs	530	46	45.46 ± 1.06
PGNP	535	58	51.42 ± 0.64

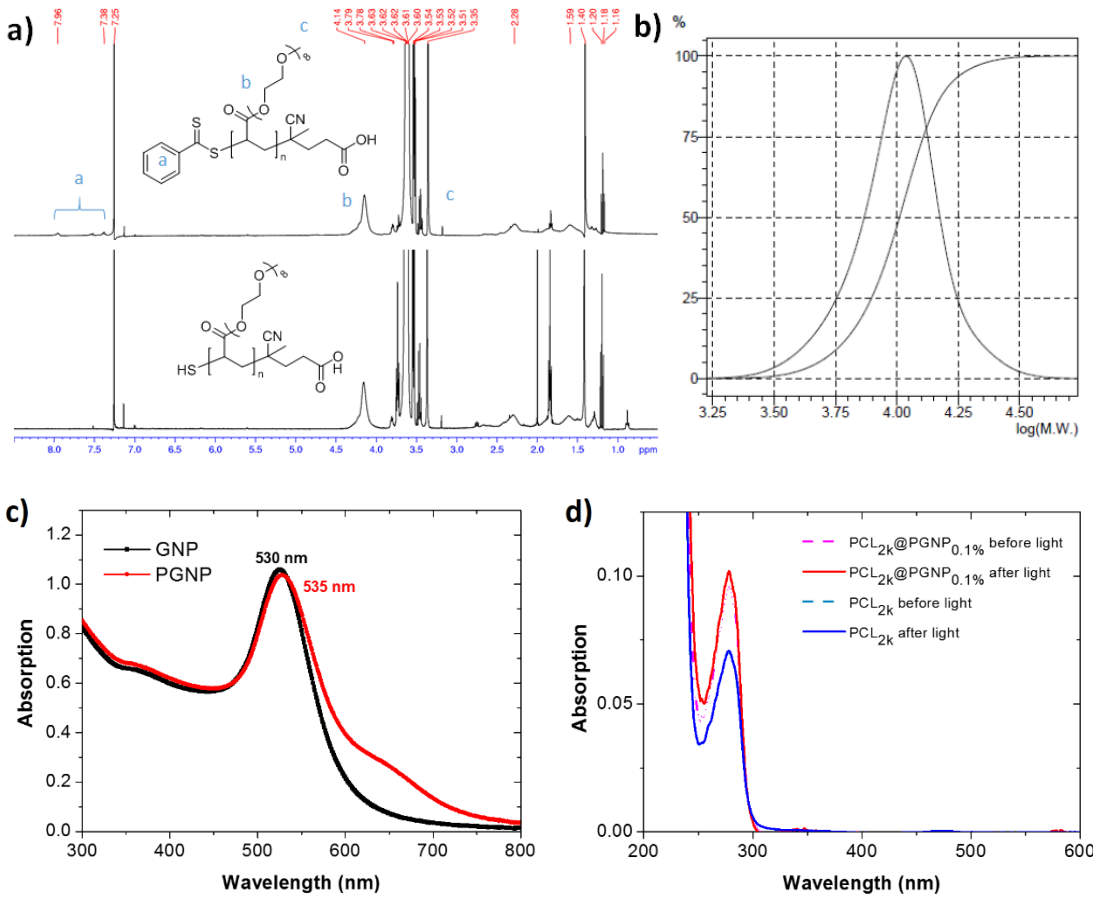

Figure S2 (a) ^{1}H-NMR of POEGA before (top) and after aminolysis (bottom); (b) GPC of POEGA ; (c) UV-Vis absorption of GNPs and PGNPs; (d) The concentration of BSA released from PCL microspherical depot (with and without PGNP) was measured by UV-Vis absorption at 279 nm before and after blue light exposure (day 0).

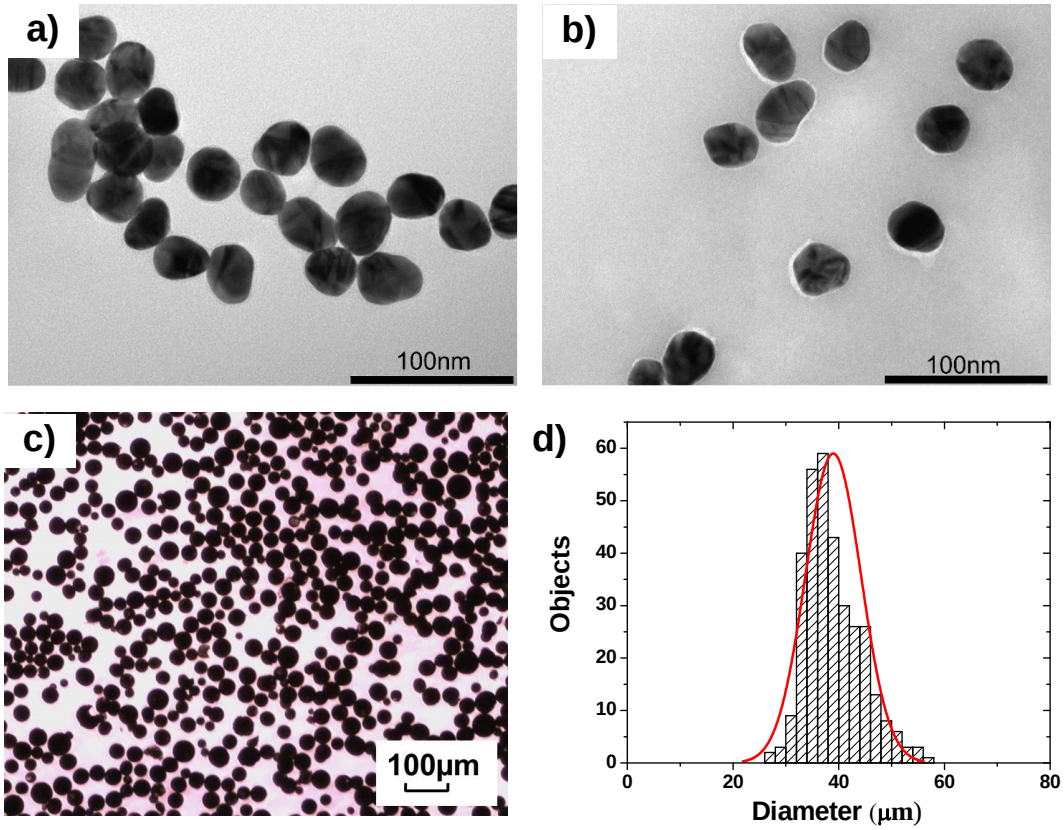

Fig S3. (a) TEM images of unmodified GNPs and (b) GNPs coated with POEGA; (c) the optical micrograph of PCL_{2k} microspheres; (d) the size distribution of PCL_{2k} microspheres.

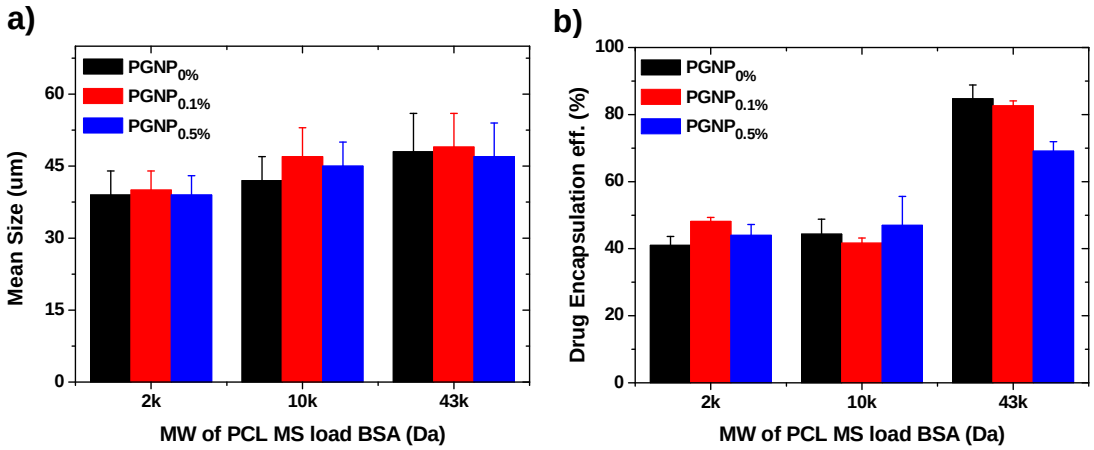

Figure S4. (a) The size distribution and (b) the drug encapsulation efficiency of BSA loaded PCL microspheres.

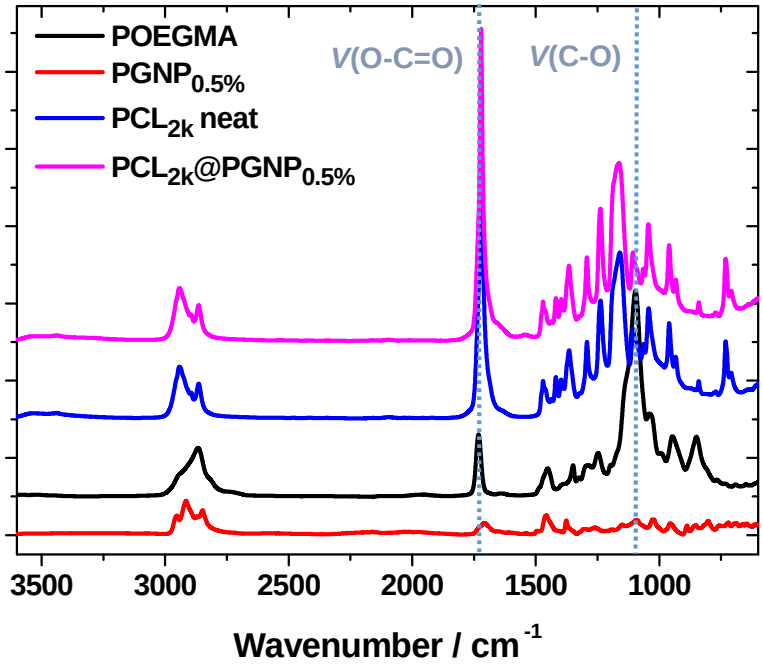

Figure S5. ATR-FTIR spectra of POEGA, the POEGA-coated gold nanoparticles (GNPs), the PCL_{2k} neat and PCL_{2k}@$PGNP_{0.5\%}$. The ester (O-C=O) group from the polymer and coated AuNPs was characterized by its absorption at 1731 cm^{-1}, while the ether group (C-O) was characterized by its absorption at1096 cm^{-1}.

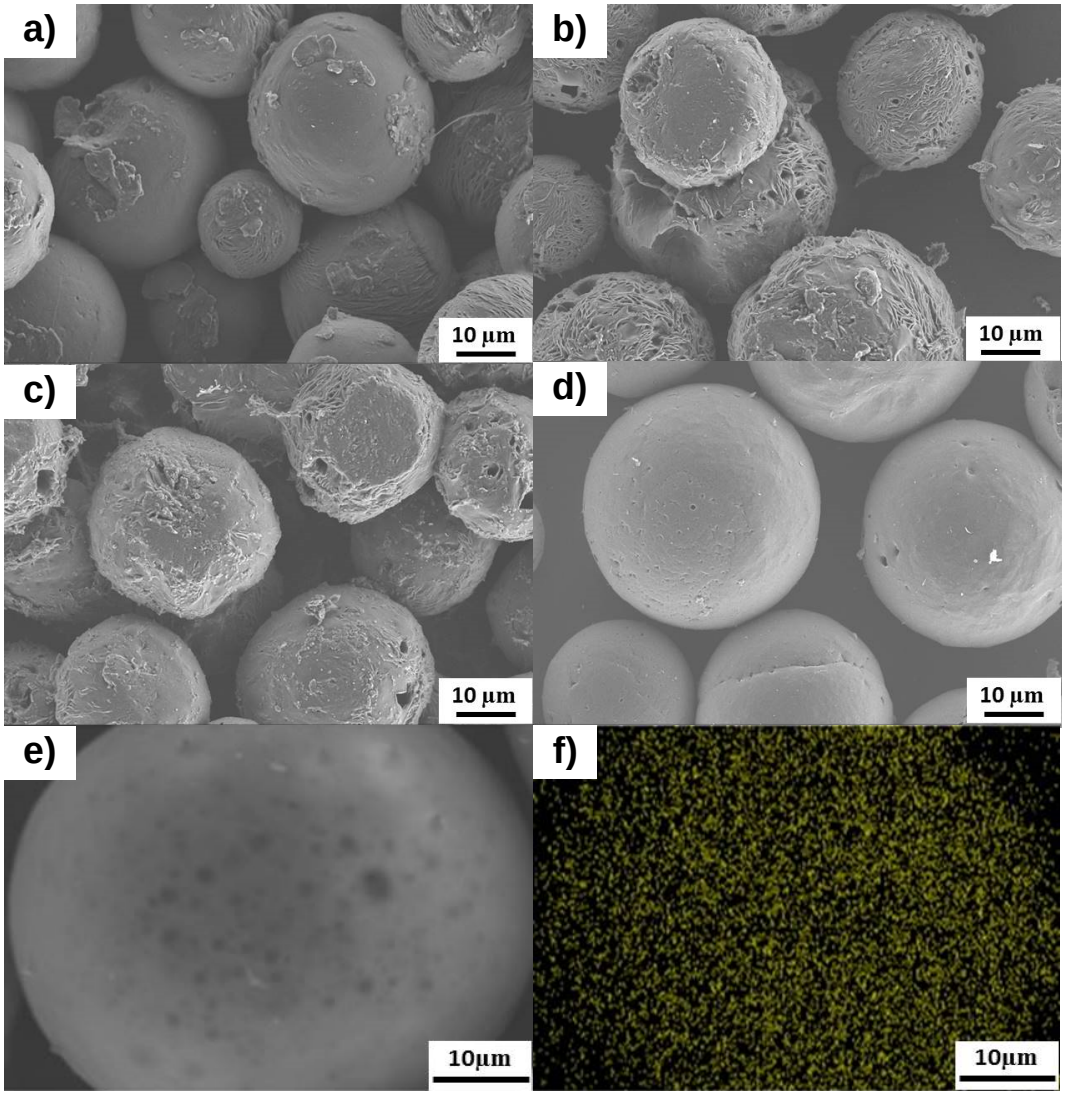

Figure S6. SEM images of (a) PCL_{2k} microspheres without BSA; (b) PCL_{2k} microspheres loaded BSA; (c) PCL_{2k}@$PGNP_{0.5\%}$ microspheres loaded BSA; (d) PCL_{10k}@$PGNP_{0.5\%}$ microspheres loaded BSA; (e) EDS image and (f) EDS analysis of element mapping of Au of PCL_{10k}@$PGNP_{0.5\%}$ microsphere surface.

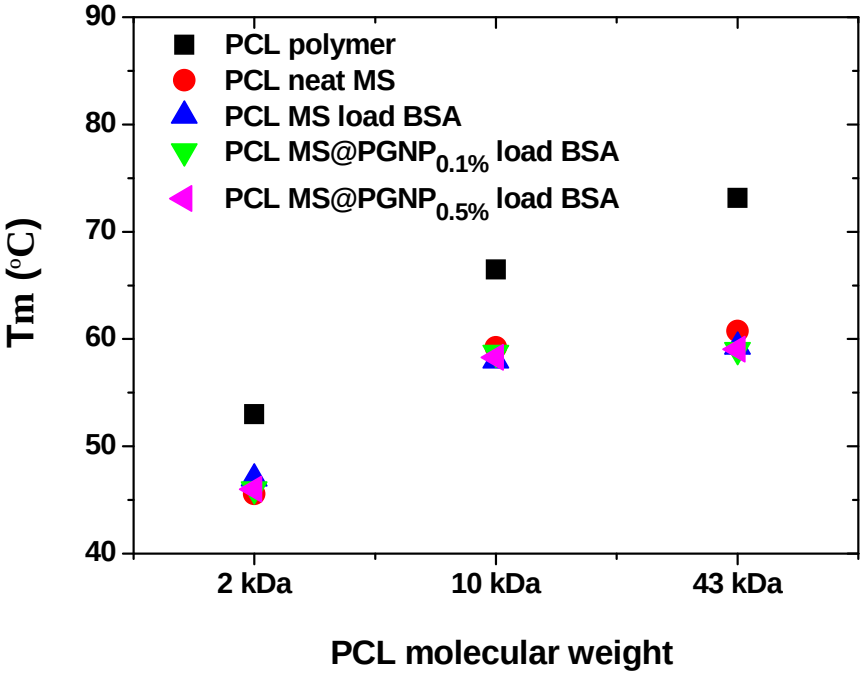

Figure S7. DSC analysis of PCL polymer and PCL microspheres. The reduction in melting temperature (T_m) is predominantly caused by the microspheres formation (higher surface area).

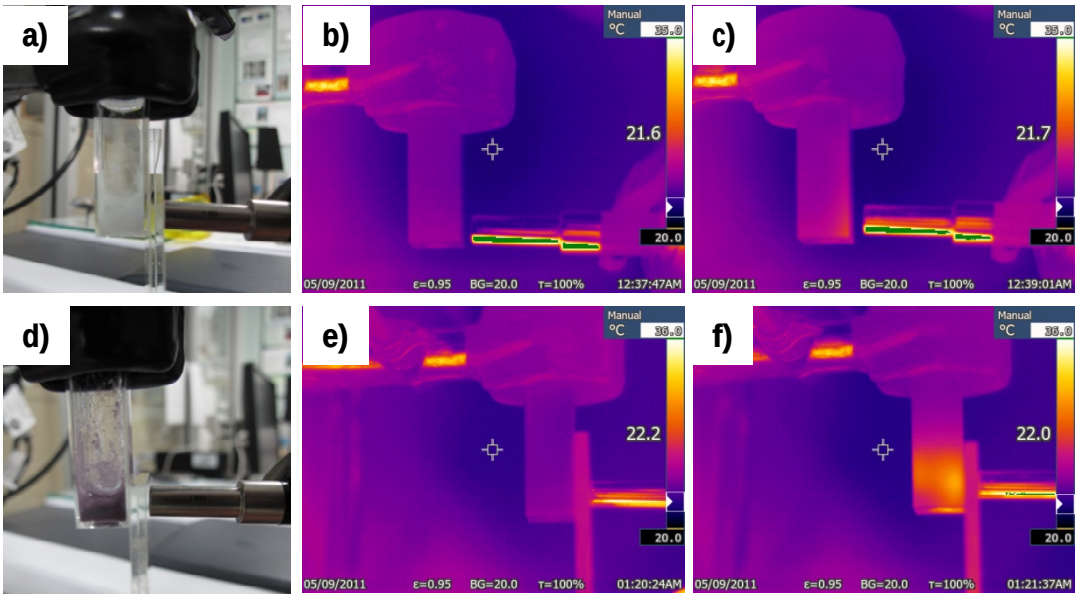

Figure S8. The thermal imaging of the microparticles upon exposure to visible light (a) the PCL_{2k} microsphere alone and their thermal imaging before (b) and during (c) light exposure; (d) the PCL_{2k} @ $PGNP_{0.1\%}$ and their thermal imaging before (e) and during (f) the light exposure. Temperature difference measured for PCL_{2k} and PCL_{2k} @ $PGNP_{0.1\%}$ before and during light exposure was 0.4^{o}C and 6.2 ^{o}C, respectively.

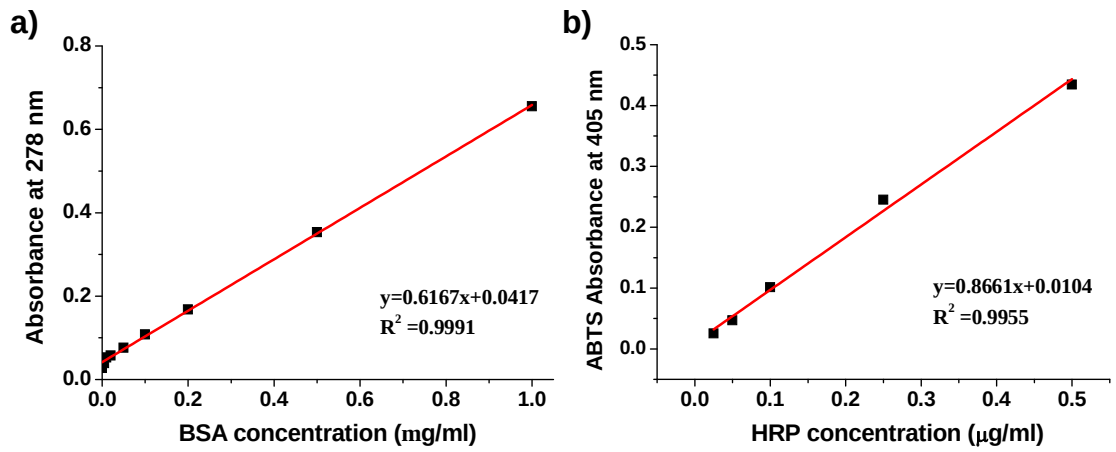

Figure S9. (a) The UV-Vis calibration of standard BSA in PBS (pH=7.4, 0.01M) at 25ºC (b) Calibration of standard HRP enzyme by using a fluorescence plate reader at 405 nm, H_2O_2 and ABTS were used as substrates.

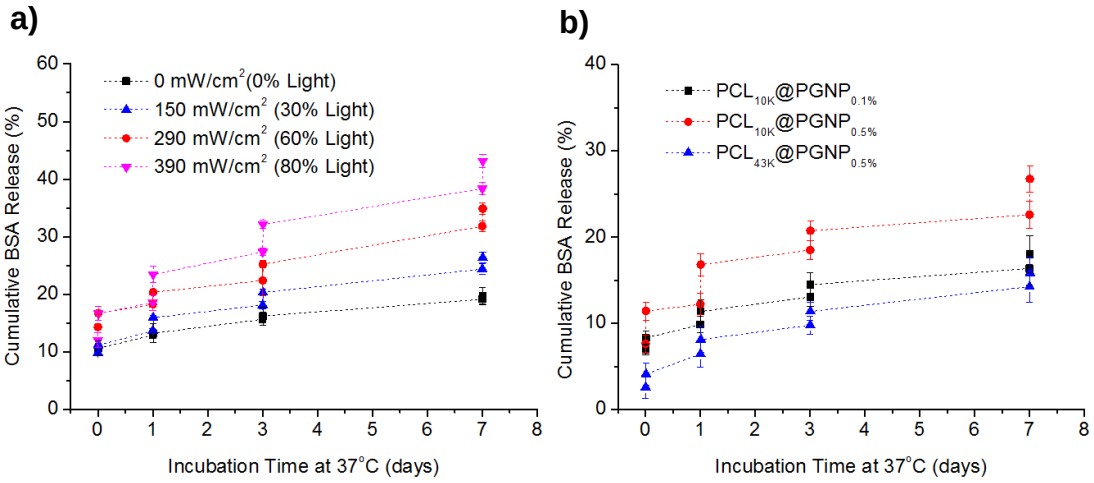

Figure S10. Controlled BSA release profile (a) from PCL_{2k}@$PGNP_{0.1\%}$ with different visible light intensity (400-500 nm). (b) Comparison of PCL_{10K} and PCL_{43K} containing the same 0.5% PGNP. The concentration of released BSA was measured before and after light exposure (10 minutes) at the start, 1st, 3rd and 7th day of incubation (37ºC). Each concentration measurement was performed in triplicate and the standard deviation plotted as error bars.

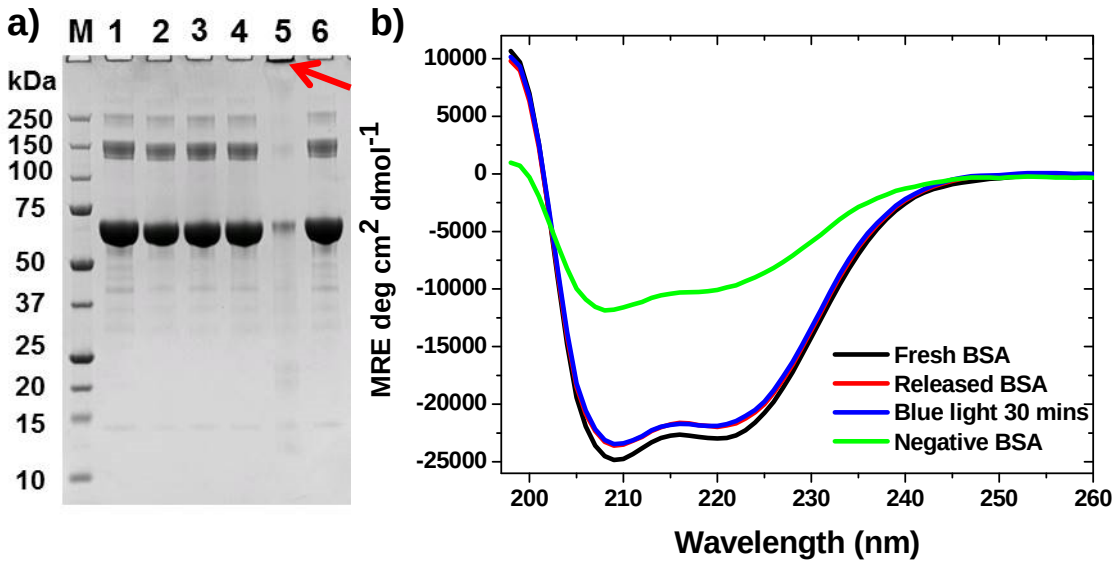

Figure S11. (a) SDS–PAGE of BSA of released (lanes 2 and 3). Lane 1: fresh standard BSA; lane 2: BSA released from PCL_{2k}@$PGNP_{0.1\%}$ microspheres; lane 3: BSA released from PCL_{2k}@$PGNP_{0.5\%}$ microspheres; lane 4: standard BSA with light 30 mins under intensity 60%; lane 5: standard BSA heated for 60 mins at 100 °C; lane 6: standard BSA heated for 60 mins at 50°C; (c) CD spectra of fresh BSA and released BSA of PCL_{2k}@$PGNP_{0.5\%}$ microspheres.

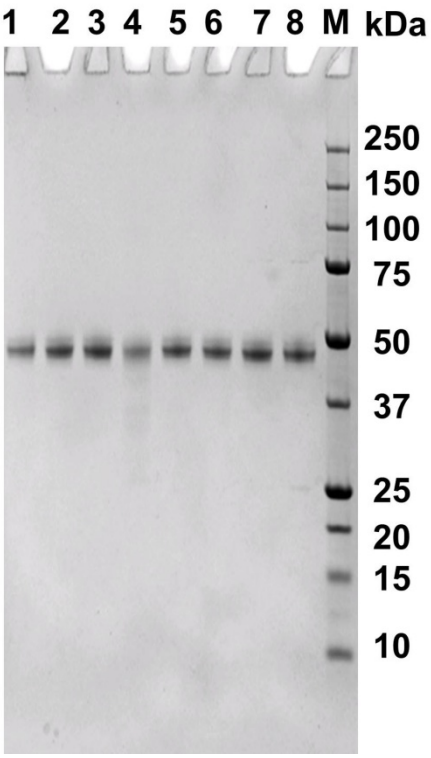

Figure S12. SDS–PAGE of HRP with different treatments: Lane 1: Fresh HRP; Lane 2: Fresh HRP with sonicator 20 s; Lane 3: Fresh HRP with 50˚C for 10 mins; Lane 4: Fresh HRP with 100˚C for 60 mins; Lane 5: Fresh HRP with same blue light as drug released; Lane 6: Fresh HRP with 37˚C for 30 mins; Lane 7: Released HRP from PCL_{2k} microspheres; Lane 8: Released HRP from PCL_{2k}@$PGNP_{0.1\%}$ microspheres.

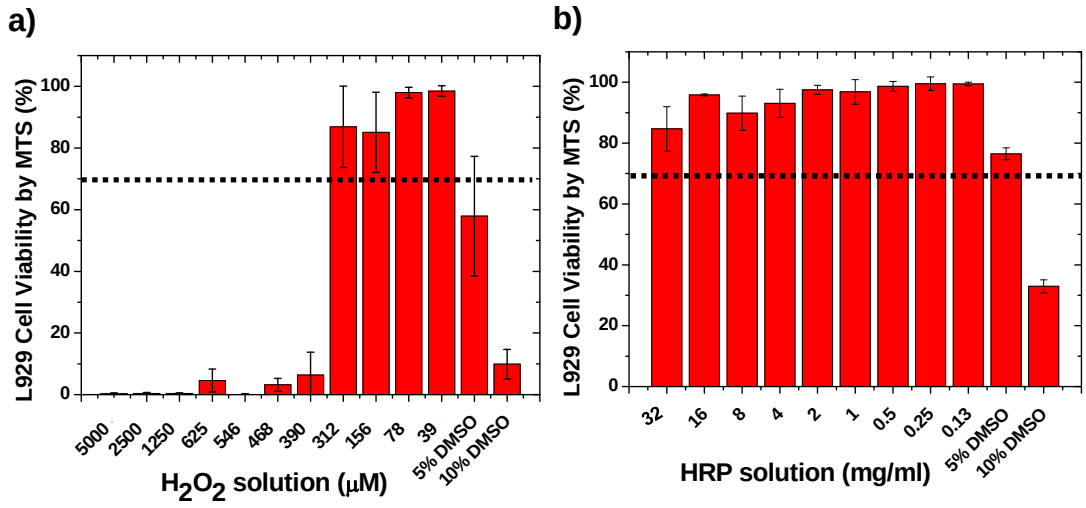

Figure S13. Cell viability of L929 cells co-incubated for 24 h with (a) H_2O_2 and (b) HRP at different concentrations. 5% DMSO and 10% DMSO were used as controls and the cells viabilities were normalised against medium alone as the 100% control.

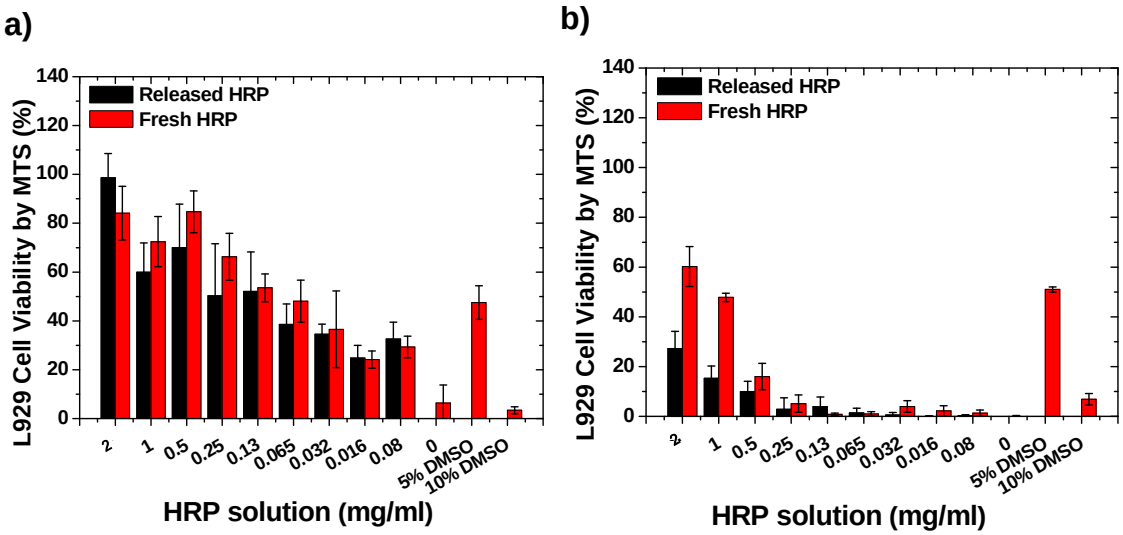

Figure S14. MTS Cell viabilities of L929 cells in (a) 390 µM H_2O_2 and (b) 546 µM H_2O_2 co-incubated with fresh and released HRP at different concentrations for 24 h. DMSO at 5% and 10% served as the positive control while the cell viabilities were normalised against the negative control (100%), medium alone.

Figure S15. The injectability of PCL_{2k}@$PGNP_{0.5\%}$ microspheres dispersed in an aqueous solution with 26 gauge needle.

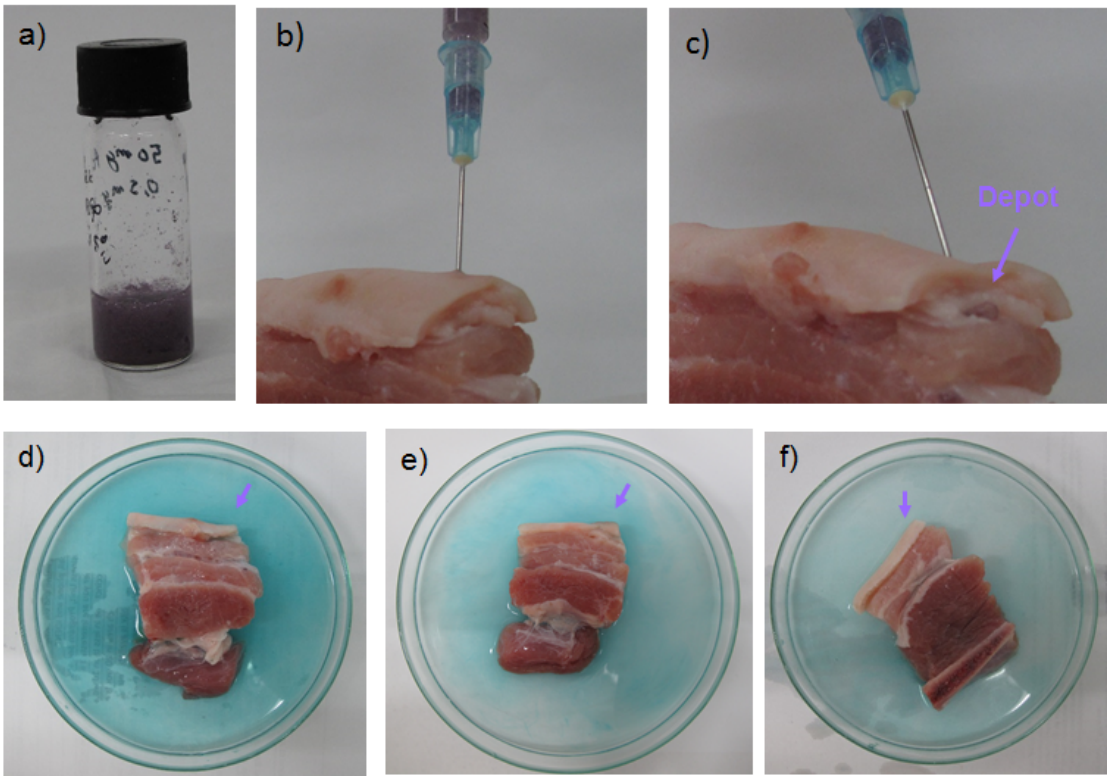

Figure S16. a) Encapsulation of PCL_{2k}@$PGNP_{0.5\%}$ microspheres (50 mg) in 1.5% w/w hyaluronic hydrogel (950 mg), followed by (b) 150 µL epidermal injection of the formulation to form a skin implanted HRP depot (c). After irradiation of the implant using visible light (400-500 nm, 390 mW/cm^2, 5 minutes) above the skin, the whole porcine tissue was incubated in a TMB aqueous solution (5 minutes, r.t.) to indicate the release of HRP from the implanted PCL_{2k}@$PGNP_{0.5\%}$ microspheres (d). Less intense blue colour (TMB oxidation) was also observed after the same treatment of porcine skin implanted with 1.5% w/w HA encapsulated PCL_{2k} microspheres (e) and blank 1.5% w/w HA hydrogel (f).

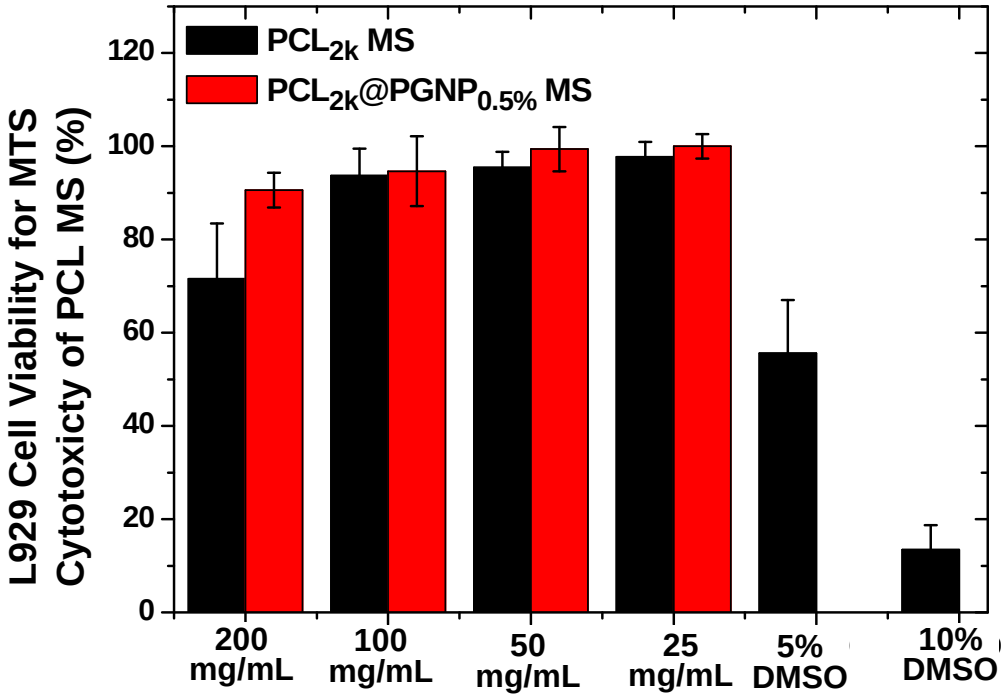

Figure S17. *In vitro* cytotoxicity of PCL_{2k} and PCL_{2k}@$PGNP_{0.5\%}$ extract solution on L929 cells co-incubated for 24 h.

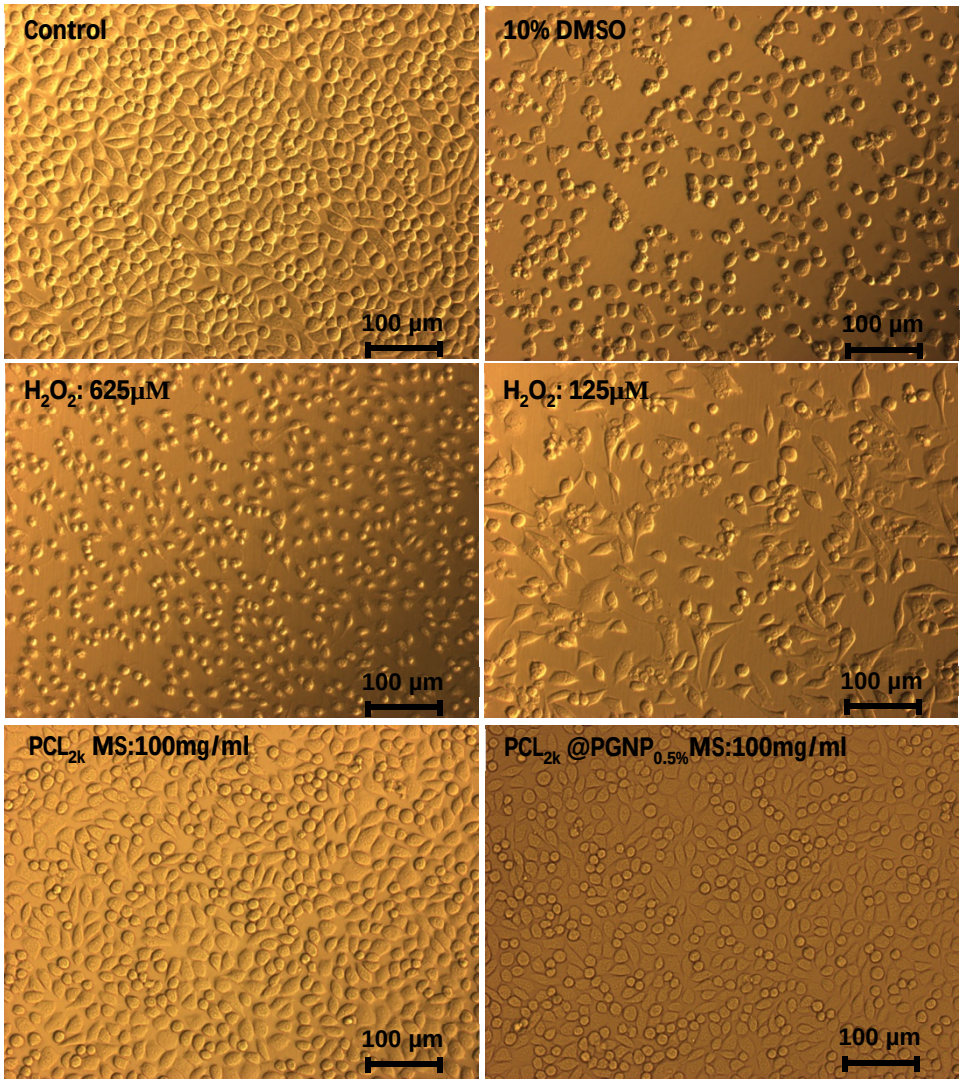

Figure S18. Microscope images of L929 cells after being co-incubated for 24h with extract solution of PCL_{2k} and PCL_{2k}@$PGNP_{0.5\%}$. The negative control was L929 cells treated with normal culture medium for 24 h, and the positive control was cells exposed to 10% v/v DMSO. Treatment of L929 cells with 625 µM and 125 µM H_2O_2 indicates the concentration-dependant cytotoxic effect of H_2O_2.

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