Supplementary Information

1. Materials

Tissue culture dishes (6, 96 well plates) were purchased from Becton Dickinson (Catalog No. 35-3078) and used as received. 2,3-dimercapto-1-propanol tributyrate, 5,5′-dithiobis(2-nitrobenzoic acid), lipase from Candida rugosa (Type VII 700≥ unit/mg), peroxidase from horseradish (Type II, 44 kD), SIGMAFAST OPD (substrate for HRP activity assay catalog no P9187), insulin human (27.5≥ units/mg), phosphatase substrate (alkaline phosphatase reagent), glycine, sodium bicarbonate, zinc chloride, dioctyl sulfosuccinate (AOT), D-(+)-trehalose dehydrate (trehalose), tween-20, Dulbecco's Modified Eagle's Medium (high glucose), and ammonium bicarbonate salts were purchased from Sigma (St. Louis, MO). PCL (poly (ε-caprolactone)) (80 kDa), molecular sieves, 4A and poly(ethylene oxide) (100 kDaltons) were purchased form Sigma (St. Louis, MO). All solvent (except hexafluoroisopropanol; HFIP) i.e., 2,2,4-trimethylpentane (isooctane), toluene, dichlormethane, tetrahydrofuran, chloroform, acetone, methanol and dimethylformamide used in this studies were HPLC grade and also obtained from Sigma (St. Louis, MO). HFIP was purchased from FREON Fluorocarbons, USA. Rat L6 myoblast, mouse stromal cell line W-20-17, Dulbecco's Modified Eagle's Medium (DMEM) were purchased from American Type Culture Collection (ATTC, Rockville, MD). The reagents for ELISA detection of Phospho-Akt1 and total Akt1 i.e., PathScan Phospho-Akt1 (Ser473) and PathScan Total Akt1, STOP Solution TMB, Substrate Cell Lysis Buffer (10X), Phosphate Buffered Saline were procured from Cell Signaling Technology (Danvers, MA). Costar 96-Well EIA/RIA Plate (Coring catalog no 2592) was purchased from Immunochemistry Tech, (Bloomington, MN). Recombinant human BMP-2 and human/mouse/rat BMP-2 Quantikine ELISA Kit were obtained from R & D System, USA. Insulin EIA (96 Wells), "IVD"

ELISA (enzyme-linked immunosorbent assay) kit and human insulin Zero Standard were purchased from ALPCO (Salem, NH). Fetal bovine serum and antimycotic solution were purchased from Invitrogen Inc. (Carlsbad, CA).

2. Methods

2.1. Enzymatic assays of HRP and lipase

2.1.1. HRP activity

HRP catalyzes the oxidative coupling reaction of *o*-phenylenediamine (OPD) to 2,3-diaminophenazine (DAP), which is measured by colorimetry at 430 nm. The protocol reported by Bovaird at al. [33] was followed with some modification for the activity assay. 0.050 mL of HRP sample was added to 0.150 mL of substrate solution in each well of 96 well plates to initiate the reaction. After incubation for 30 min at room temperature (25 °C), the absorbance of samples was recorded at 430 nm using SpectraMax 5 microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance of substrate solution at 430 nm was used as blank correction. At least three replicate samples were used in order to establish error bars. The percentage (%) of HRP activity retained after solvent treatment was calculated using HRP sample without any solvent treatment as the reference - i.e. HRP from supplier reconstituted in Tris-HCL buffer, pH 6 for HRP-buffer samples or HRP in SGnP for HRP-SGnP samples. If necessary, proper dilutions of HRP samples were made to attain the concentration within the linear range of HRP activity assay (0.1 nmole/L to 2nmole/L). We generated a standard curve using (0.1 nmole/L to 4 nmole/L) of HRP in 50 mmole/L Tris-HCl buffer, pH6 and OPD of 3.7 mmole/L and 3.2 mmole/L H_2O_2 in 50 mmole/L Tris-HCl pH 5.6 (see Figure SI 11).

2.1.2. Lipase activity

Lipase samples in three forms were examined; these were lyophilized powder after solvent treatment, pure lyophilized protein, dried SGP-lipase particles. These were always reconstituted or

diluted with buffer consisting of 10 mmole/L KCl and 10mmole/L Tris-HCl at pH 7.5. This assay was performed according the protocol already reported by Choi et al. [34] In brief, a 40 mmole/L stock solution of 5,5-dithiobis(2-nitro benzoic acid) (DTNB) was prepared by adding 0.1584 g of DTNB in 10 mL of ethyl alcohol. A 10 mmole/L stock solution of 2,3-Dimercapto-1-propanol tributyrate (DMPTB) was made in 6 % Triton X-100, 50 mmole/L Tris-HCl, pH 7.2. The two stock solutions were stored at -20 °C between usages. The standard substrate solution contained 0.2 mmole/L DMPTB, 0.8 mmole/L DTNB, 1 mmole/L EDTA, and 0.05 % Triton X-100, and 50 mmole/L Tris-HCl, pH 7.5. It was prepared by addition of 20 μL of 10 mmole/L DMPTB, 20 μL of 40 mmole/L DTNB, 2 μL of 0.5 M EDTA, 5 μL of 10% Triton X-100, and 50 μL of 1 mole/L Tris-HCl at pH 7.5 in a centrifuge tube and 803 μL of deionized water was added to generate a final volume of 900 μL. For assay, 0.025 mL of lipase solution was taken in each well of 96-well plate and180 μL of the substrate solution was added in each well. The plate was incubated for 30 min at 37 °C and the absorbance at 405 nm was measured in SpectraMax 5 microplate reader. A Standard activity plot was generated under the same conditions using lipase concentration (0 to 150) IU/ml (see Figure SI 12). The percentage of lipase activity retained after solvent treatment was calculated using activity of lipase without any solvent treatment as a reference. Lipase samples were diluted as necessary to attain the concentration within the linear range of the lipase activity assay (≤ 120 IU/ml for 30 minute incubation at 37 °C)

2.2. Bioassay of insulin

2.2.1. Insulin activity loss in presence of different organic solvents

The effect of solvent exposure on the activity of insulin was determined as described previously in the general experimental procedure. The insulin samples were prepared by dissolving freeze-dried insulin in sterile 30 mmole/L HCl to make a standard stock solution of 1 mg/mL. For solvent exposure studies for insulin in buffer, 0.1 mL insulin in PBS (7 μg/mL) was added to 0.5 mL of the

various different solvents (separately) in 1.5 mL centrifuge tubes. For solvent exposure to insulin in SGnP, 0.04 mL isooctane suspension of insulin-SGnP was added to 0.5 mL of the various solvents. After the solvent exposure experiments, solvent was removed by lyophilization. The lyophilized samples were reconstituted using DMEM media such that the concentration of insulin in the solution was in the range (0.5 to 0.7) μg/mL. The reconstituted solution was then used for bioactivity assay. The solvent experiments and reconstitution of lyophilized samples were performed under sterile conditions.

2.2.2. Cell culture and assay for insulin assay

In vitro bioactivity assay of insulin samples was performed using the rat myoblast L6 cell line. This assay is based on the fact that the insulin induces phosphorylation of Akt in L6 cells (i.e., production of pAkt, a downstream effectors of the insulin signaling pathway) in a dose dependent manner.[35] The pAkt and Akt were quantified using ELISA techniques. L6 cells were cultured and maintained according to the supplier (ATCC) protocol. In brief, L6 was cultured in a 75 cm^2 culture flask with DMEM supplemented with 10 % fetal bovine serum (Gibco, USA) and 1% antimycotic solution. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Sub-confluent monolayers were dissociated with 0.25 % trypsin/1mM EDTA solution, re-suspended in cell culture media, and then plated on 6-well plate with 0.5x 10^6 cells per well.

Cells in 6-well plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Media was removed and each well washed twice with DMEM media. Finally 2 mL of DMEM media was added into each well and incubated (37 °C and 5 % CO_2) for 1 h before stimulation by insulin. Media from each well was removed and replaced by 1 mL of DMEM containing different concentrations of insulin. The plate was immediately transferred into the incubator and kept for 10 min. After 10 min the insulin media was replaced by ice cold PBS and washed twice with PBS

carefully. Cells were lysed by adding 0.4 mL to 0.5 mL of cell lysis buffer (Cell Signaling Technology, USA) supplemented by 1 mmole/L Phenylmethanesulfonylfluoride (PMSF) to each well, followed by incubation for 5 min on ice. Cells were scrapped from each well and lysis solution with cell debris transferred into 1.5 mL centrifuge tube. This vial was centrifuged at 14000 g (Eppendorf, Model 5810 R) for 10 min at (0 to 1) °C. The supernatant was removed and stored at -80 °C till the further use for ELISA detection of Akt and pAkt. Various stimulation times (1 min to 30 min) and insulin concentrations were used to optimize the bioactivity assay conditions for exposure time and insulin concentration. To determine the linear range of insulin dose on the response of L6 cells (i.e., production of pAkt), a series of different known concentration of insulin solution (0 to 5) µg/mL were used to stimulate the cell under the same assay condition (see Figure SI 3).

Akt and pAkt were quantified by ELISA techniques using PathScan Total Akt1 and PathScan Total Phospho-Akt1 (Ser473) sandwich ELISA antibody pair respectively from Cell Signaling Technology, USA. We followed the ELISA protocol provided by the company. In brief, 96-well plate was coated by capture antibody (pAkt or Akt) for (15 to17) h at 4 °C followed by washing with washing buffer and blocked by blocking buffer. 0.1 mL of cell lysate was added into each well and incubated for 2 h at 37 °C. After washing, detection antibody was added into each well followed by washing, addition of secondary antibody (anti-mouse-HRP), incubation at 37 °C and a third washing. TMB substrate (0.1 mL) was added to each well and incubated for 10 min at 37 °C. After incubation, stop solution (0.1 mL) was added into each well and absorbance was recorded at 450 nm using SpectraMax5 at room temperature Blocking buffer was used as a control sample and its absorbance applied for blank correction.

2.2.3. Bioactivity calculation

The amount of pAkt was considered as proportional to the amount of bioactive insulin in the sample. For all samples total absorbance of pAkt was normalized with total absorbance of Akt for the same sample. The normalized value of pAkt was used to calculate the insulin bioactivity of the samples. The percentage of insulin bioactivity retained after solvent treatment was calculated with respect to control insulin samples without any solvent treatment (100 % activity). Controls were performed by stimulating L6 cells with trehalose solution (0.2 mg/mL to 5 mg/mL) and sugar glass nano-particles (SGnPs) without insulin to rule out possible contribution in the phosphorylation of Akt from SGnPs or SGnP-related excipients (see Figure SI 13). DMEM media without and with insulin (0.3 μg/ml) was used as negative and positive control for this assay.

2.2.4. Insulin quantization

Insulin solutions for testing were diluted with Zero Standard solution from ALPCO Diagnostics to achieve the insulin concentration within the assay limit in the present assay condition (5 ng/mL) and quantified by ELISA technique (ALPCO Diagnostics, USA). A standard curve was generated using a series insulin solution of known concentration (0.1 ng/mL to 9 ng/mL). The insulin concentration in the unknown samples was calculated by reference to the standard plot. We followed the manufacture protocol for this assay. In brief, 0.025 mL of insulin samples in Zero Standard solution and enzyme conjugate was added into the each well of 96-well ELISA plate. The plate was incubated at room temperature for 1 h while shaking on an orbital shaker and washed 6 times with washing buffer. Substrate solution was added to each well and incubated for 15 min at room temperature. After incubation, stop solution was added and absorbance was recorded at 450 nm with a reference wavelength of 650 nm.

2.3. Bioassay of BMP-2

2.3.1. BMP-2 activity loss in presence of different organic solvents

A BMP-2 stock solution was prepared by reconstituting BMP-2 lyophilized powder from BD bioscience to100 μg/mL in 4 mmole/L sterile HCl. Solvent exposure studies were performed on BMP-2 from buffer diluting the BMP-2 stock solution to 600 ng/mL BMP-2 in 4 mmole/L HCl, and adding 0.1 mL of this to 0.5 mL of solvent in a 1.5 mL centrifuge tube. Solvent exposure studies were performed on BMP-2 protected in SGnPs by adding 0.020 mL isooctane suspension of BMP-2-SGnP to 0.5 mL solvent. After solvent exposure, the solvent was removed by lyophilization. The lyophilized samples were reconstituted by DMEM with high glucose to achieve concentration of BMP-2 between (20 to 60) ng/mL and this was used for bioactivity assay.

2.3.2. Cell culture and assay for BMP-2

Bioactivity of BMP-2 samples was assayed by determining its ability to induce an increase of alkaline phosphatase (AP) activity in a mouse stromal cell line W-20-17. The W-20-17 cells response to BMP-2 with an increasing of alkaline phosphatase concentration in dose-dependent manner. Optical density at 405 nm of p-nitrophenol generated from the alkaline phosphatase substrate was used as a measure of alkaline phosphatase enzyme level in cells. We have followed the slide modified ASTM International protocol (Designation: F2131-02) for BMP-2 activity assay and performed the assay in a 96-well plate format. Before using the cells for assay, the W-20-17 cells were expanded. The cells were cultured in DMEM containing 4.5 % glucose, 10 % fetal bovine serum and 1 % antimycotic. Cultures were maintained at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2 Sub-confluent monolayers were dissociated with 0.25 % trypsin+1mmole/L EDTA solution, re-suspended in cell culture media, pooled to create a homogeneous mixture, and cryopreserved in multiple aliquots of 1x 10^6 cell/mL per vial. For each activity assay an aliquot was thawed, expanded for 3 d and re-plated in 96-well plates at 10,000 cells/well (passage 26 upon use).

After 24 h of incubation, the medium was replaced with 0.10 mL/well of fresh media followed by addition of 0.10 mL of media with or without BMP-2. The plate was then incubated for another 24 h ± 4 h before use for AP determination. After incubation, the culture media was discarded and 0.20 mL of 1xPBS (without Ca^{+2}/Mg^{+2}, preheated at 37 °C) was added. The plates were carefully washed twice using PBS. 0.025 mL of sterile water was added per well and stored at -80 °C until assayed. The assay mixture was prepared by mixing 0.34 % of p-nitrophenol phosphate in glycine buffer, containing 0.75 % mass fraction glycine, 0.8 % by volume of 12.5 % Triton X-100, and 0.13 % mass fraction $MgCl_2.6H_2O$ in sterile water at pH 10.3 ± 0.1. Just before assay, the plate was removed from -80 °C and thawed at room temperature. After repeated freezing and thawing cycle 3 times, the plate was allowed to reach to the room temperature and then 0.1 mL of assay mixture was added to each well and placed on an orbital shaker. After 40 min of room temperature incubation 0.10 mL of 0.2 N NaOH was added to each well and absorbance was recorded at 405 nm. Media only (without BMP-2) was used to determine the basal AP activity of W-20-17 cells. To generate the standard dose response curve BMP-2 activity, cells were treated with culture media containing (0 to150) ng BMP2/mL (BMP-2 source was reconstituted lyophilized powder and BMP2-SGnP).

2.3.3. Bioactivity calculation

Activity of BMP-2 was obtained through optical density of samples at 405 nm, referenced to negative control samples containing media only. Each sample was diluted to at least two different concentrations of BMP-2 within in the linear concentration range i.e., (0 to 80) ng/mL. The BMP-2 bioactivity in the samples was calculated with respect to positive controls, which were fresh BMP-2 from buffer or BMP-2 from SGnP without any solvent treatment. In the calculation of BMP-2 activities of different BMP-2 samples, AP the absorbance value of a sample (well) was normalized with the total protein contents of the cell lysate (of same well) measured by the bicinchoninic acid

assay (BCA Protein Assay Kit, Pierce). Trehalose solution (0.2 to 5) mg/mL and sugar-glass particles alone without BMP-2 were also assayed to rule out possible contribution in the induction of alkaline phoshpatase.

2.3.4. BMP-2 quantization

The amount of BMP-2 in samples was quantified by ELISA (BD Bioscience, USA) according to the manufacturer's protocol. Concentrations of BMP-2 in the samples were calculated from the standard curve ranged from (62.5 to 4000) pg/mL.

2.4. Scaffold fabrication

Scaffolds with two distinct structures made of PCL or PEO were prepared using electrospinning[36] or gas-foaming.[37] PCL scaffolds were fabricated mainly to study the release profile of protein, whereas PEO based scaffolds were prepared to study activity loss of protein during scaffold preparation and storage. The advantage of PEO over PCL is that, PEO scaffolds could be dissolved immediately in the buffer and eliminating the use of organic solvent and the possibility of deactivation of protein molecules by the organic solvent at this stage. On the other hand, PCL required organic solvent to dissolve the scaffold to estimate the protein within the scaffold, which may lead to the deactivation of protein at this stage.

2.4.1. Electrospun nanofiberous scaffold fabrication

Electrospinning was performed using optimized concentration of PCL (25 % mass fraction) and PEO (15 % mass fraction) in chloroform. Polymers were initially dissolved in chloroform at 1.5X higher concentration of their final desired concentration (e.g. 35 % mass fraction for PCL and 20 % mass fraction for PEO). The protein was added into the scaffold in the form of protein- buffer (conventional technique as an emulsion of protein in organic solvent) or protein in the form of protein-SGnP. For conventional protein emulsion techniques, dry protein was weighed and

dissolved in aqueous buffer then added with polymer solution to generate a weight ratio of polymer: protein = 100:1 and 1000:1 (i.e., proteins concentration in the scaffold were 0.1 % and 1 %). Total volume of protein solution used was (5 to 10) % by volume of the polymer solution. After addition of protein solution in the polymer solution, the mixture was vigorously vortex for (10 to 15) min to get a homogeneous dispersion of protein.

Protein in the form of protein-SGnP was added to polymer solution by adding an appropriate volume of isooctane suspension to obtain (0.5 to 5) % by mass loading of SGnP in the scaffold. Protein-SGnP suspension in isooctane was centrifuged at 300g to precipitate out particles before it was added to the polymer solution. After decanting the supernatant isooctane, chloroform was added and the suspension was vortexed for 1 min to get uniform dispersion. A volume of chloroform was added to the protein-SGnP such that after addition of the SGsP-chloroform suspension to the polymer solution, the optimum polymer concentrations (i.e., 25 % for PCL and 15 % for PEO) for electrospinning were attained. The sample was then vortexed for (2 to 3) min to get uniform suspension of protein-SGnP in the polymer solution. The freshly prepared solution was loaded into a syringe and appropriate flow rate (0.3 ml/h for PCL and 2.5 ml/h for PEO) was set using a syringe pump. An aluminum sheet was used as collector for electrospinning. The distance between needle and collector was set at 15 cm for PCL and 17.5 cm for PEO. A fixed voltage of 12 kV and 15 kV was used for PCL and PEO fiber respectively. Nanofibers of PCL or PEO were collected on aluminum sheet for a maximum of 1h. The fiber scaffolds were kept overnight in vacuum oven to remove trace amounts of solvent and stored in desiccators until used.

2.4.2. Gas-foamed scaffold fabrication

Polymer / protein solution or suspension was prepared as indicated under the heading "electrospun nanofiberous scaffold fabrication." The polymer + protein solution in chloroform was

mixed thoroughly with sieved NH_4HCO_3 crystals, (250 to 425) μm in size, to make a homogeneous gel paste mixture of polymer/salt/solvent. The weight ratio of NH_4HCO_3 to PCL was adjusted to 7:1. The polymer/salt/solvent paste mixture was quickly casted into a disk shaped Teflon mold (12 mm diameter, 4 mm thickness). The paste mixture within the Teflon mold was air dried for 2 h at room temperature. Dried, solid polymer/salt scaffolds were immersed into an excess amount of warm DI-water (40 °C) for about 2 h until gas bubbles are no longer generated. Afterwards the foamed scaffolds were washed in cold DI-water 3 to 4 times to remove residual NH_4HCO_3. Washed scaffolds were dried over night in vacuum and stored in desiccators until used.

2.5. Activity test during processing of scaffold

We monitored the activity loss of protein at different stages of processing in order to compare activity loss of protein during encapsulation by conventional protein-buffer emulsion and by the protein-SGnP method. After addition of protein (HRP or insulin) in the form of protein-buffer or protein-SGnP (as described above) to PEO polymer (10 mL), the mixture was subjected to vortex for (2 to15) min to get uniform dispersion. Approximately one half (5 mL) of this polymer solution was incubated for more time whereas other half was used for scaffold preparation (electrospinning or gas-foaming). Of the 5 mL solution set aside, 0.2 mL to 0.3 mL was taken out and cast as thin film on glass slide at predetermined interval of incubation (30 min, 60 min and 120 min) and immediately placed under vacuum to evaporate solvent. The films were cut into small squares (5 nm x 5 mm) and dissolved in 1 mL suitable buffer in a centrifuge tube for activity assay. The scaffold was cut into disk or pieces and dissolved in buffer for activity assay as described above. The protein activity at different stages of processing was determined and presented as residual activity in the different stages of processing using lyophilized protein from the manufacturer as the reference.

2.6. Analysis of activity as a function of storage at different temperature

Disks of (5 to 7) mm diameter were cut randomly from the as prepared protein encapsulated nanofiber scaffold sheets prepared by either conventional encapsulation method (i.e., protein-buffer) or using protein-SGnP and stored up to 150 d at different temperature (25 °C and 37 °C) in desiccators. Protein activity in the scaffold was measured immediately after scaffold preparation to establish the baseline activity (time 0) for the various storage conditions. Four samples (n=4) were used for each analysis time point. HRP encapsulated scaffolds were immersed into 1 mL of Tris-HCl buffer, pH 6 (with 0.1 % BSA) in a centrifuge tube and incubated with vortex to dissolve the scaffold. The solution was then subjected to suitable dilution followed by protein activity measurement as described above. Scaffold with no protein loaded were used as controls samples for protein activity in the scaffold. The protein activity determined was then compared to that originally added to the scaffold (lyophilized protein powder from manufacture) and result was presented as residual % activity in the scaffold. The protein activity change after storage was presented as % of residual activity compared to the activity just after casting of scaffold.

2.7. *In vitro* release study

HRP and insulin were used as model proteins to study release profiles from PCL electrospun or gas-foamed scaffolds. For electrospun scaffolds. (5 to10) mm diameter circular disk of weight about (20 to 30) mg nanofiber scaffold were cut from various parts of the fiber sheet and used for release studies. For gas-foamed scaffolds, approximately (20 to 30) mg of scaffold was cut (12 mm diameter, 4 mm thickness) and used for release studies. For HRP release studies, 1.5 mL of Tris-HCl buffer pH 6.0 + 0.01% BSA was added into a 2 mL of centrifuge tube containing a scaffold sample and placed on a microplate shaker. At predetermined intervals, the release media was completely removed and replaced with fresh media. The released active HRP was quantified in the release

media by HRP activity assay as described before. Concentrations of active HRP were determined from the standard curve ranged from 0.2 ng/ml to 4 ng/ml.

For insulin release studies, 2 mL of PBS/Zero standard media (pH 7.4) from ALPCO Diagnostic, USA was added to a scaffold sample in a 12 mL centrifuge tube and placed on the shaker. At predetermined intervals, 0.150 mL of the release media removed and replaced with fresh media. The amount of insulin in release media was quantified using ELISA as describe previously. Insulin solution (100 μIU/mL) was incubated at room temperature to generate the ELISA activity decay curve of insulin due to room temperature incubation. Concentrations of insulin in the samples (release media or scaffold) were determined from the standard curve (0.1 ng/mL to 9 ng/mL). The correction for room temperature ELISA activity decay of insulin was incorporated in the calculation of insulin concentration in the release media.

3. Characterization techniques

3.1. Scanning electron microscopy

Scaffolds were freeze-fractured in liquid nitrogen and sectioned with a scalpel blade. After sputter coating scaffold sections with gold for 90 s, scaffold morphology was viewed using scanning electron microscopy (SEM, 15 kV, Hitachi s-4700-II FE-SEM).

3.2. Transmission electron microscopy

For this study, we added OsO_4 solution to aqueous protein solutions and processed them to obtain SGnPs with enhanced the TEM contrast. Diluted suspensions of SGnPs in chloroform solvent and isooctane were prepared. One drop was deposited onto a copper grid coated with carbon film and dried. The chloroform or isooctane dispersion was centrifuged @ 100 x g for 3 min to remove agglomerated particles if present. The samples were analyzed by Philips EM400T, USA.

3.3. Confocal laser scanning microscopic analysis and image processing.

Fluorescence-SGnP (FITC) encapsulated electrospun fiber were examined with Leica AF using 60 oil immersion objective. FITC encapsulated fibers were analyzed using an argon laser (excitation 488 nm; emission 505 nm to 550 nm). Images and a gallery of 74 optical sections (0.1 μm) through the *z* plane were collected and processed using ImageJ software (NIH, USA).

4. Statistical analysis

Statistical analysis of the assay measurements was performed in OriginPro7 Software using a one way analysis of variances test with a tukey comparison and t-test. The level of significance was set at 0.05.

Supplementary Information: Figures

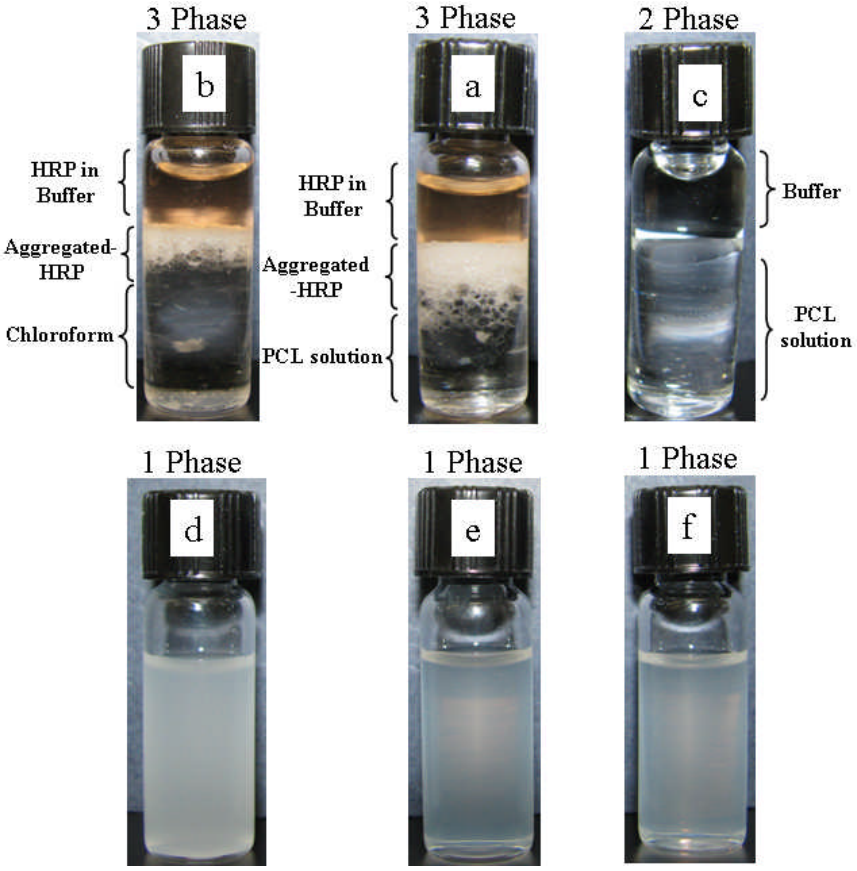

Figure SI 1. Protein (HRP) is aggregated when protein-in-buffer were mixed with organic solvent-chloroform or PCL-chloroform solution [10% volume fraction of protein-buffer in chloroform/PCL-solution] for 30 min. Aggregated protein phase separated from buffer and formed 3 phases after incubation with (a) chloroform and (b) PCL-chloroform solution (see SI for experimental procedure). (c) Two phases, buffer and PCL solution with clear interface were observed when same experiment was performed in absence of protein (HRP) [i.e. with only 10% (v/v) buffer in chloroform/PCL-solution]. Conversely, when protein were encapsulated into sugar-glass (i.e., HRP-SGnP) and mixed with isooctane, chloroform and PCL-chloroform solution for 30 min, no precipitation or aggregation of protein was observed we retained a single-phase homogeneous

dispersion of HRP-SGnP in (d) isooctane, (e) chloroform, and (f) PCL-chloroform solution.

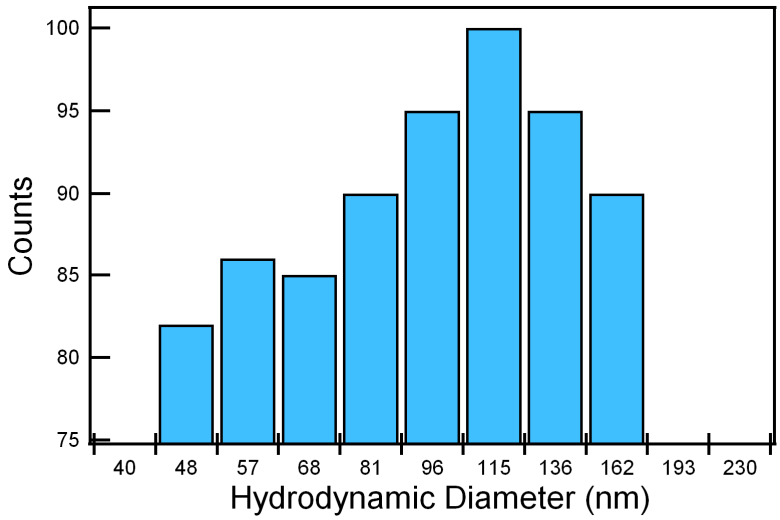

Figure SI 2. Hydrodynamic size distribution of HRP-SGnP dispersed in chloroform as measured by dynamic light scattering (DLS).

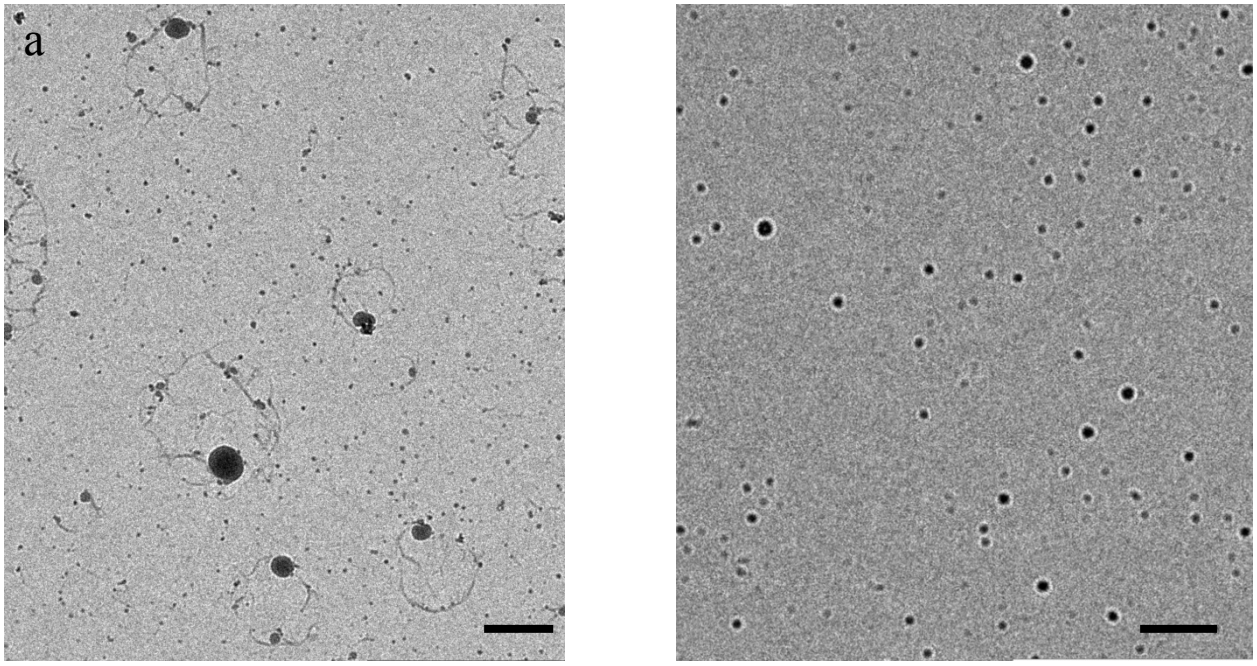

Figure SI 3. Typical TEM micrograph of HRP-SGnP dispersed in isooctane; (a) As prepared SGnP contain few bigger particles ≈ 500 nm, which could be removed by centrifugation at 100 g for 3 min. (b) SGnPs after centrifugation. Here scale bars are 1000 nm.

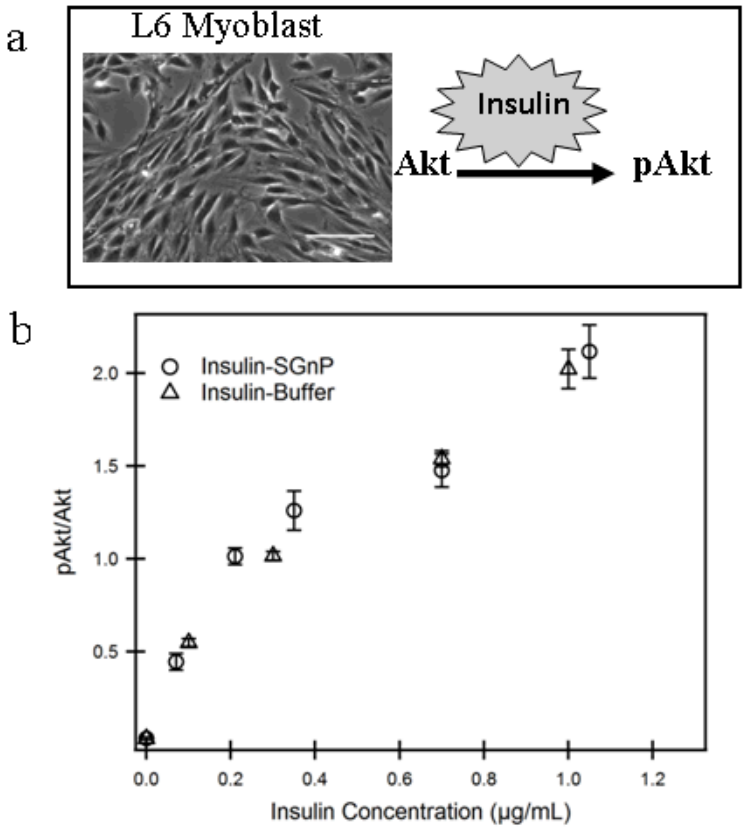

Figure SI 4. (a) Optical image of L6 myoblast cell and schematic presentation of principle of insulin activity assay where L6 myoblast cells show dose dependent induction of Akt phospholylation i.e. production of pAkt. (b) Typical insulin dose dependent plot of pAkt production when L6 myoblast cells were treated with different concentrations of insulin (added into the media) in the form of insulin-SGnP and insulin-buffer. The linear range of dose response plot was used as calibration curve for active concentration determination. The total pAkt was normalized with total Akt, which is proportional to number of cells. The intensity ratio of pAkt/Akt was used to express the extent of induction of phosphorylation of Akt. This plot also shows no loss of insulin activity after encapsulation into the SGnP system as the pAkt production are comparable by L6-myoblast cell when treated with any specific dose of insulin from two sources, insulin-buffer and encapsulated

insulin-SGnP. Values are average ± one standard deviation of n=4 samples for each concentration.

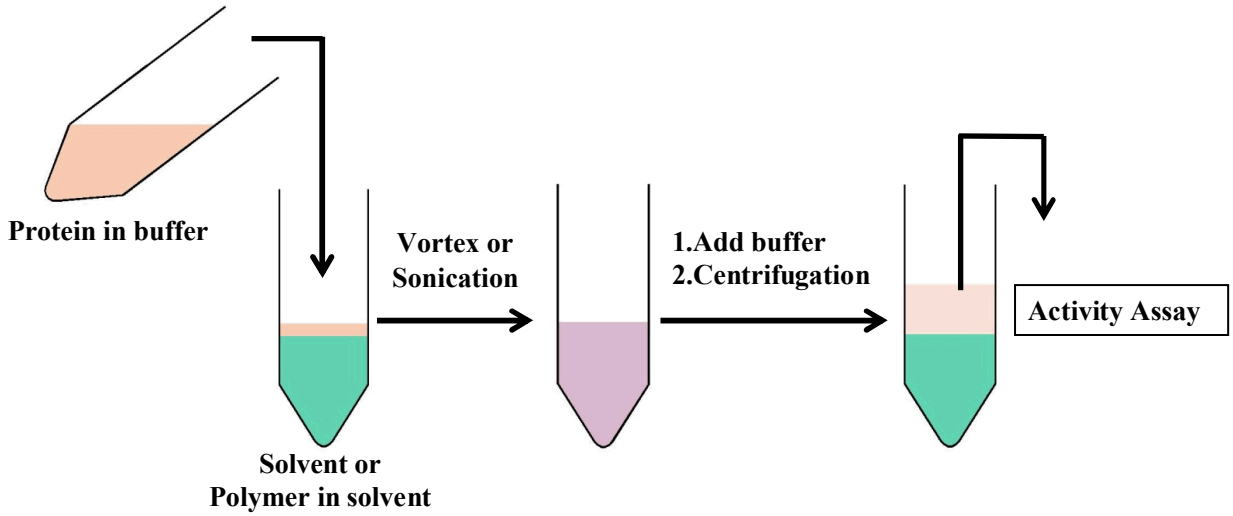

Figure SI 5. Schematic presentation of experimental procedure to study the effect of solvents or polymer-solvent mixture on the protein activity. Here protein in the 'protein-buffer' form was added into the solvent or polymer-solvent solution in a way similar to that used commonly in a conventional protein encapsulation technique. After incubation for 30 min, protein was extracted and activity assay was performed.

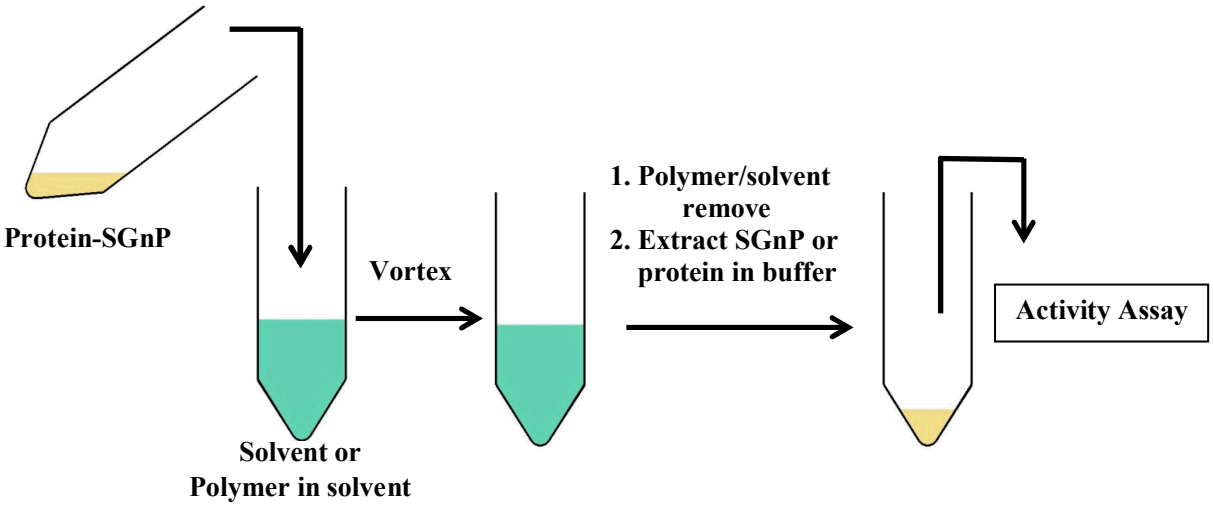

Figure SI 6. Schematic presentation of experimental procedure to study the protective capacity of the SGnP system for the protein when mixed with the solvents or polymer-solvent solution. Here protein in the 'protein-SGnP' form was added into the solvent or polymer-solvent solution and incubated for 30 min. After incubation protein was extracted and an activity assay was performed.

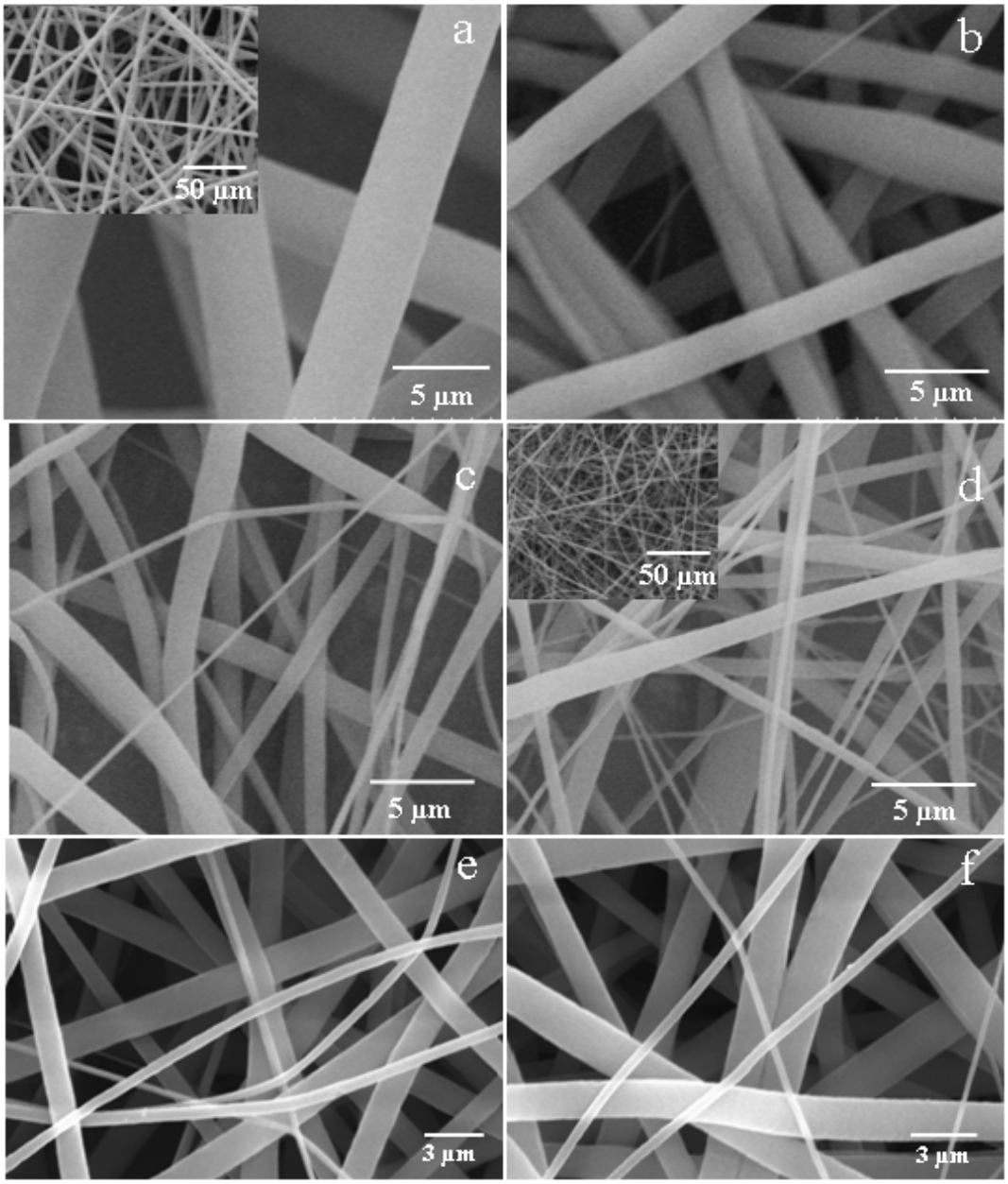

Figure SI 7. Typical SEM images of protein-SGnP loaded PCL nanofiber scaffold. (a) 0 % HRP-SGnP (mass fraction) loading, (b) 5 % HRP-SGnP (mass fraction) loading, (c) 20 % HRP-SGnP (mass fraction) loading, (d) 40 % HRP-SGnP (mass fraction) loading. (e) HRP-SGnP and (f) insulin-SGnP loaded fiber used for release study. Inserts show lower magnification images of the same scaffolds.

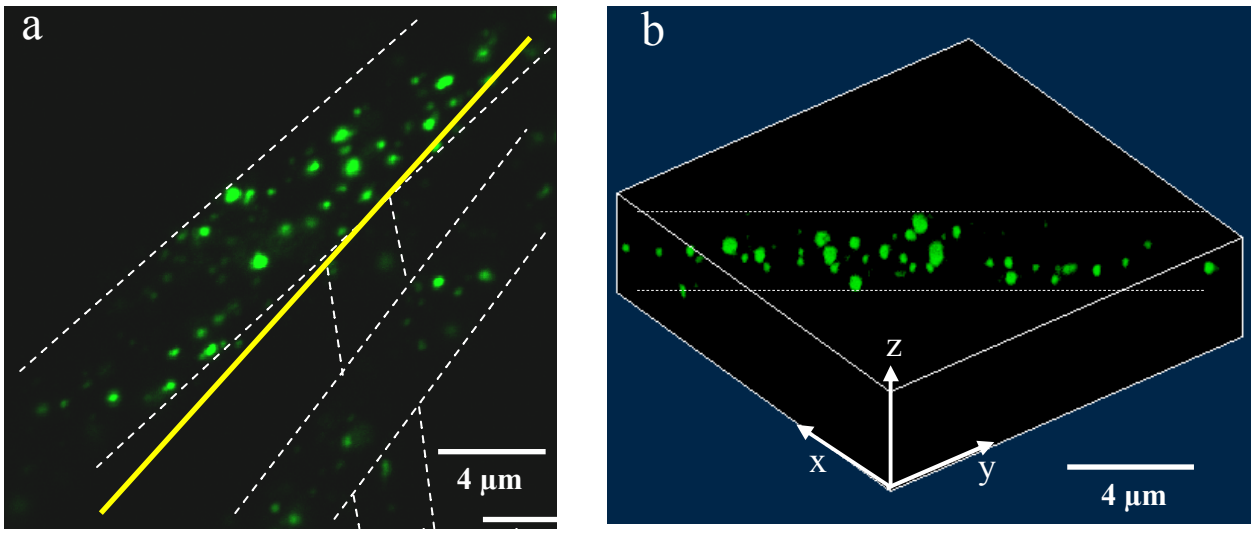

Figure SI 8. Confocal image of FITC-SGnP encapsulated PCL fiber. (a) Typical xy scan image at specific z plane. (b) 3D view of the fiber when cut through the z-plane as shown by yellow line in left Figure. White dashed lines show the boundary of the fiber. The particles are arranged through out the matrix of the fiber. Note that the smaller particles are beyond the resolution of the instrument with this objective (63X Oil immersion); also, the fluorescence spot size does not reflect the actual particle size because of optical aberration along z-axis.

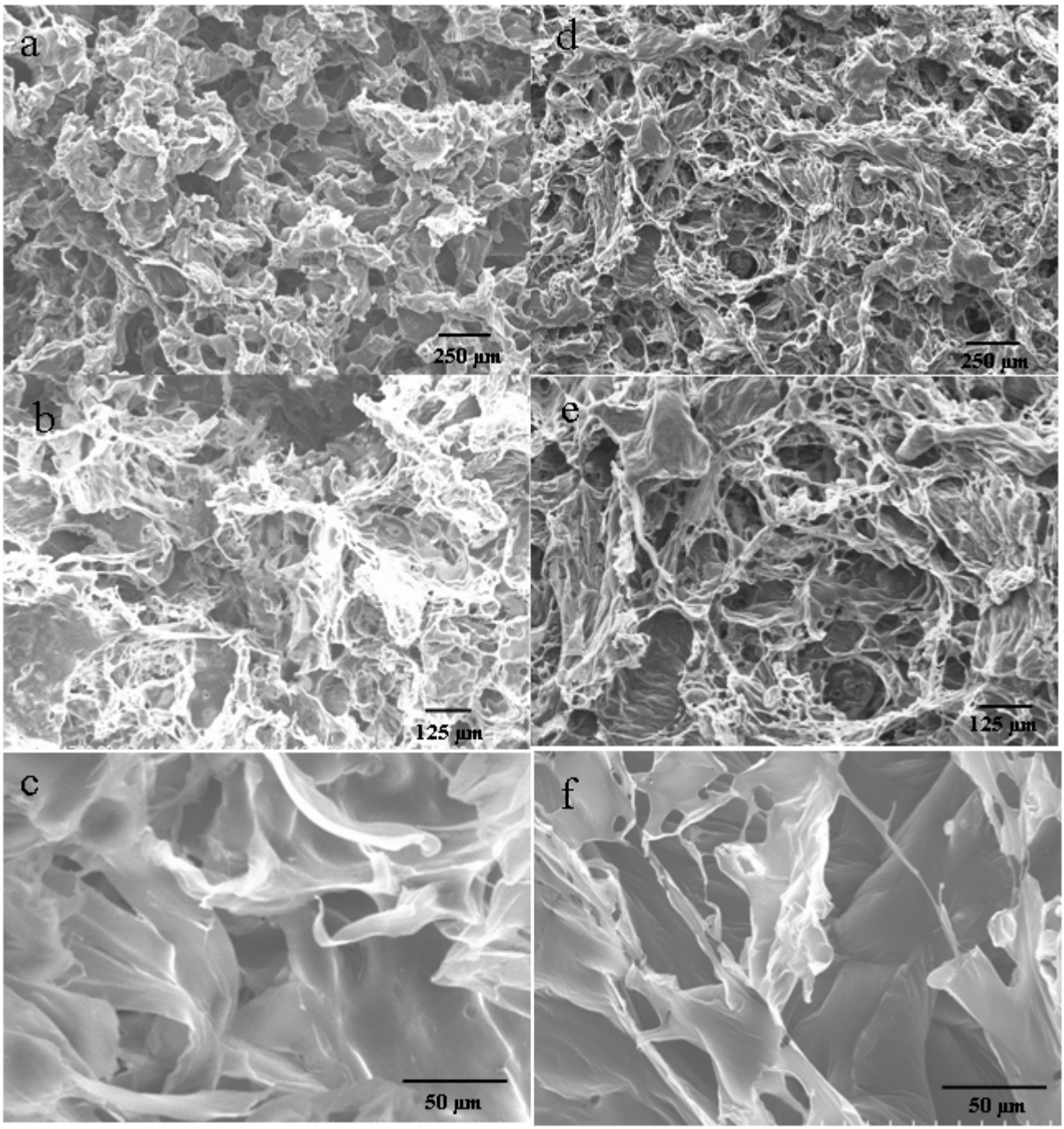

Figure SI 9. Typical SEM images of PCL gas-foamed scaffold in different magnification with (a to c) and without (d to f) loading of protein-SGnP [2% mass fraction]. (a) to (c) or (d) to (f) lowest to highest magnification.

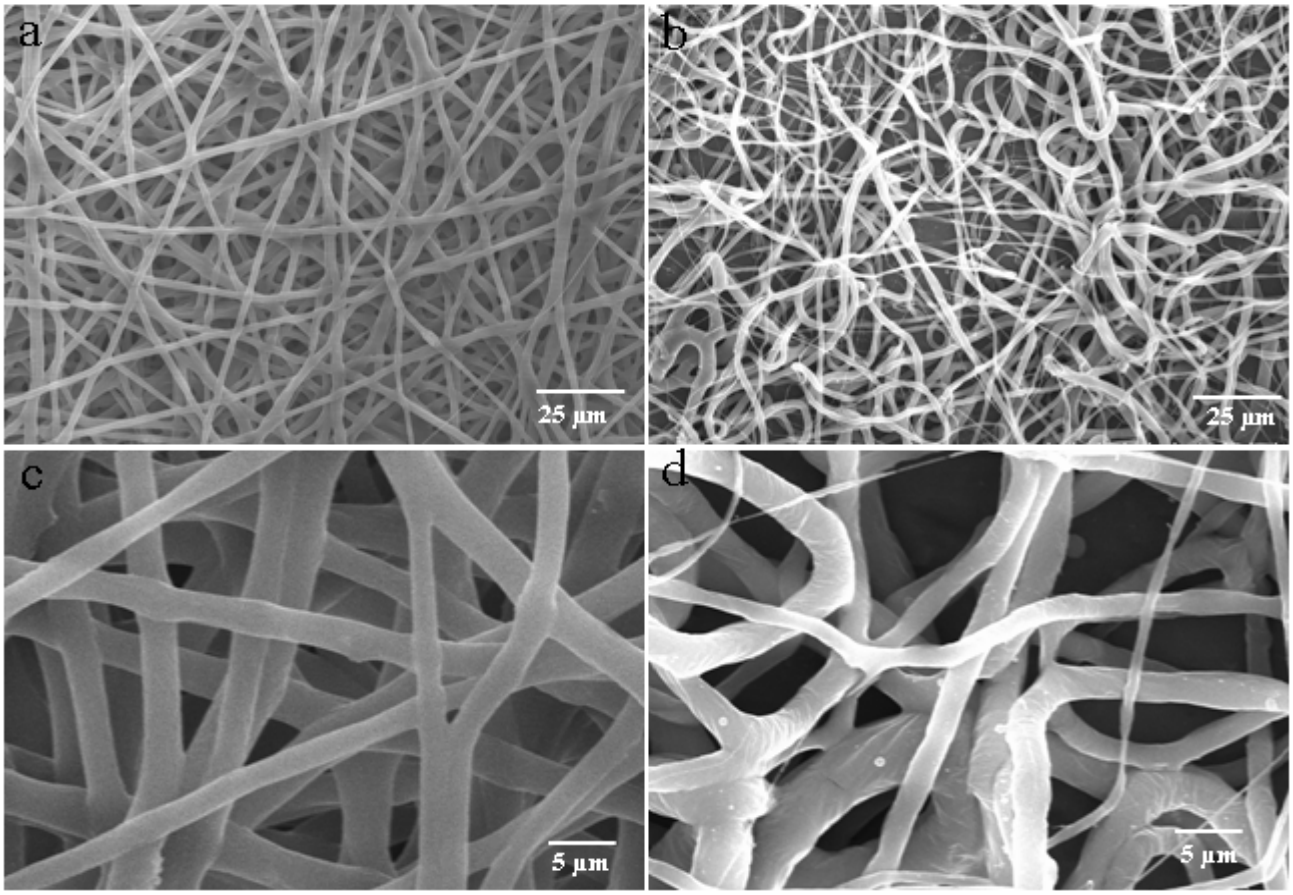

Figure SI 10. Typical SEM images of HRP loaded PEO nanofiber scaffold prepared by electrospinning used for storage study. HRP encapsulation into PEO fiber using SGnP system (a) & (b) and conventional HRP-buffer (c) & (d) at different magnifications.

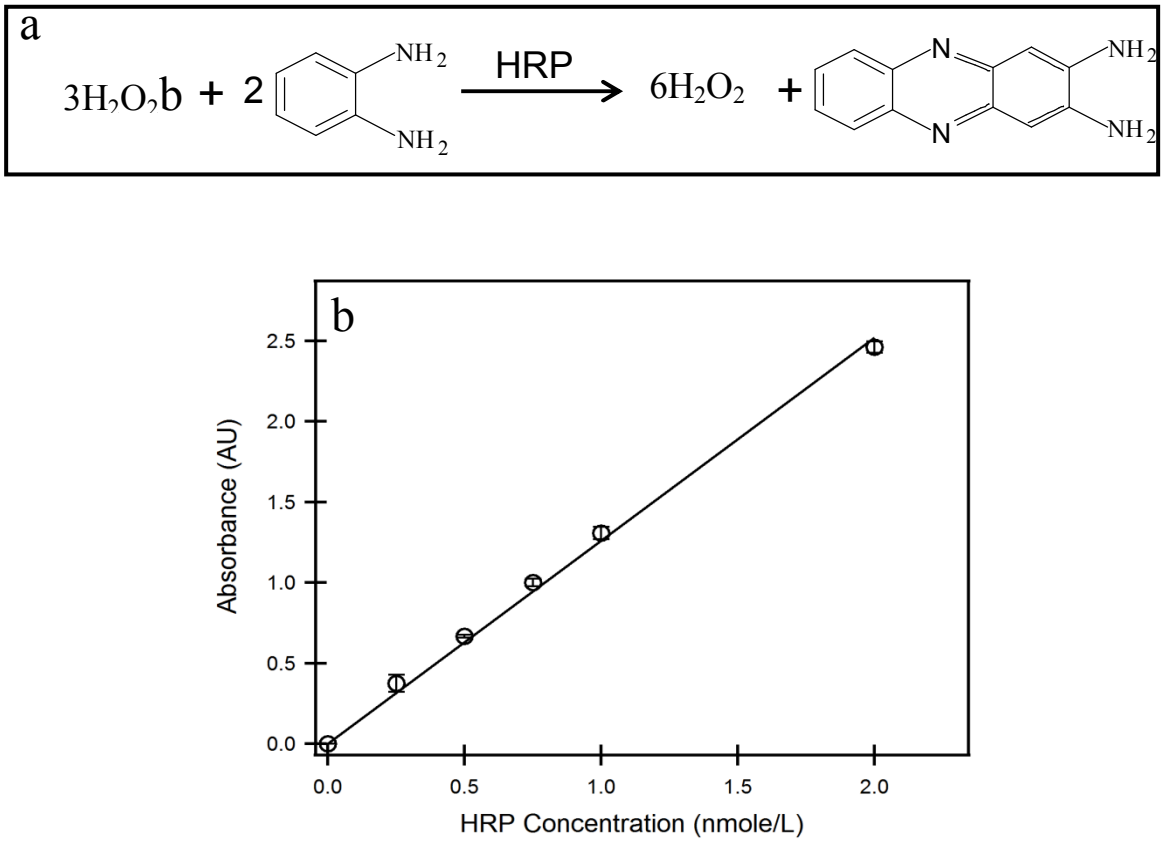

Figure SI 11. (a) Schematic of enzymatic reaction involves in HRP assay. (b) Typical HRP concentration dependent calibration plot of HRP activity assay. The upper limit of linear range of HRP concentration was 2 nmole/L. Values are average ± one standard deviation of n=3 samples for each concentration.

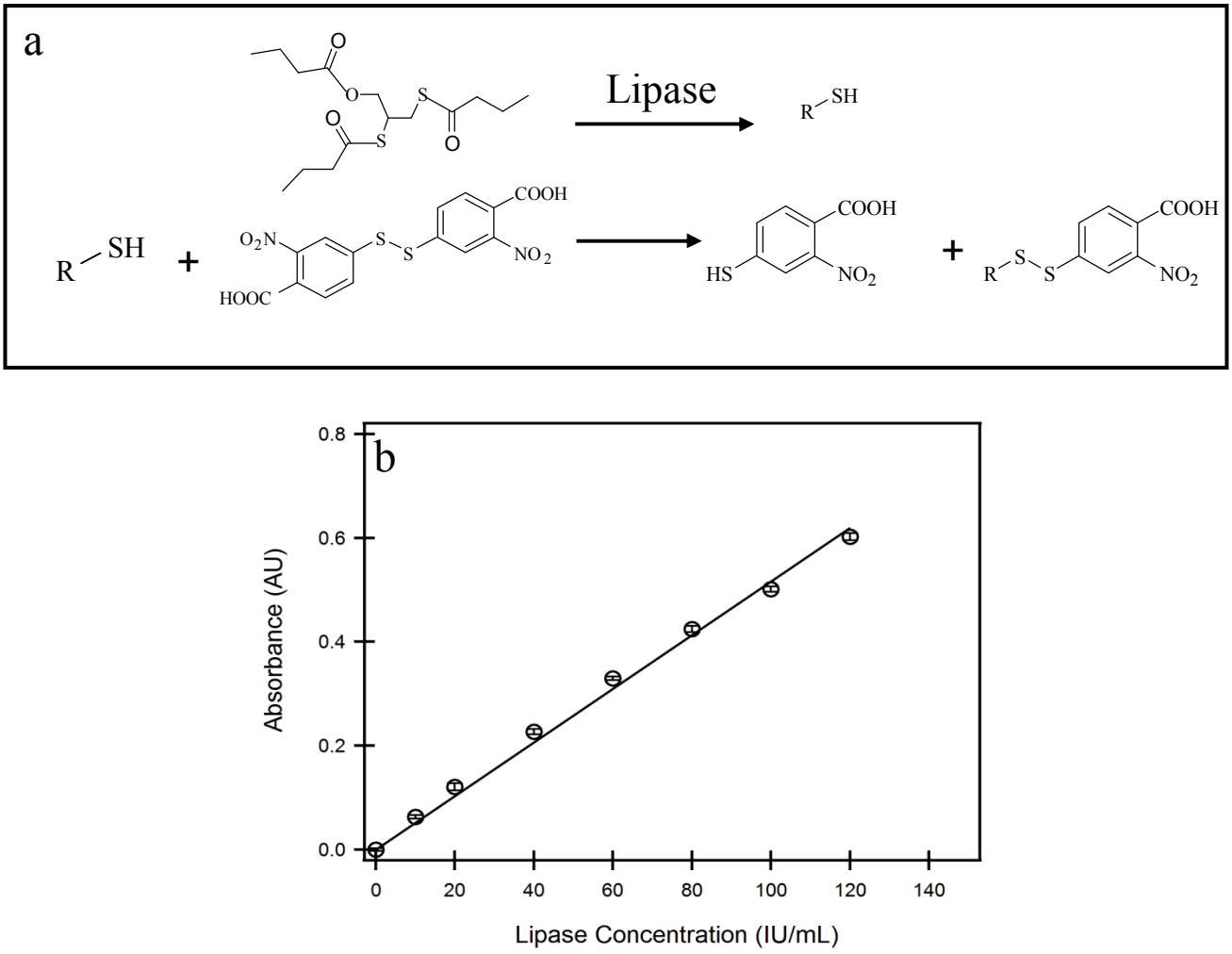

Figure SI 12. (a) Schematic of enzymatic reaction involves in lipase assay. (b) Typical lipase concentration dependent calibration plot of Lipase activity assay. In the present assay condition the upper limit of the linear range of lipase concentration was found 120 IU/mL. Values are average ± one standard deviation of n=3 samples for each concentration.

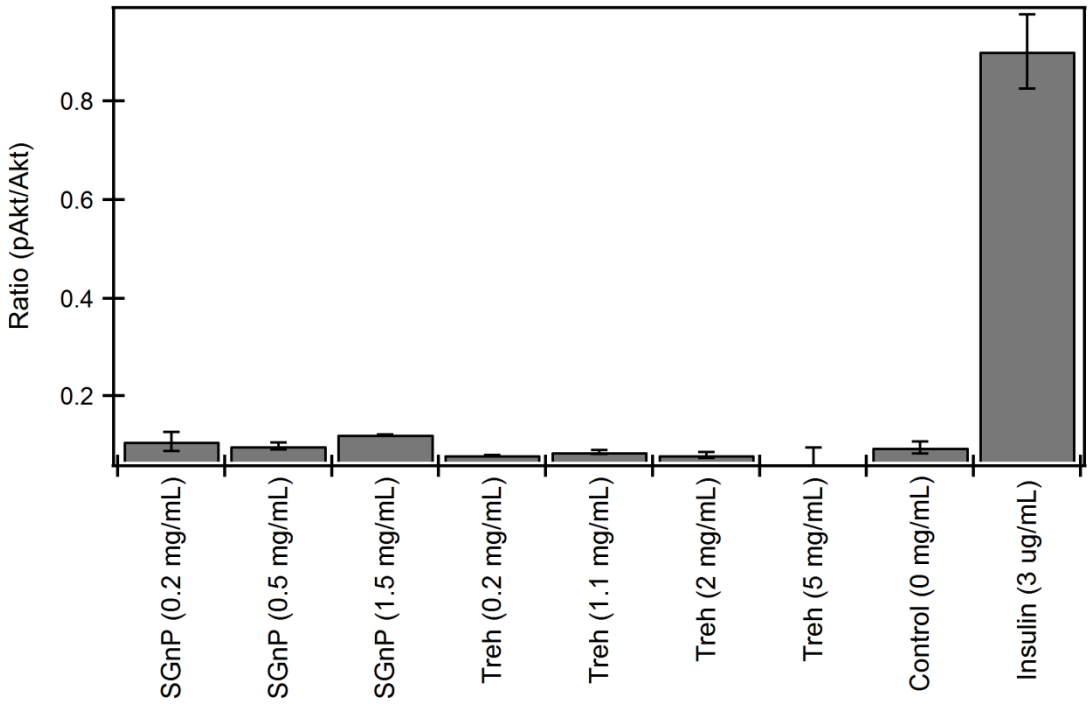

Figure SI 13. Induction of Akt phospholylation (i.e., production of pAkt) in L6 myoblast cells when treated with different materials (without insulin), typical constituents of nanoparticles, e.g. only SGnP, Trehalose. Media-only samples were taken as a negative control and insulin-containing samples as positive control. After induction of L6 cells using different concentration of these materials (as described in experimental section), the pAkt and Akt was quantified using ELISA. The total pAkt was normalized with total Akt and intensity ratio of pAkt/Akt was used to express the extent of induction of phosphorylation of Akt. Values are average ± one standard deviation of n=4 samples for each groups.

Table SI 1. Solvents used in this study and their properties[#]

Polarity Index	Solvents	Abbreviation Used	Solubility in Water (mass fraction)
0.1	Isooctane	**ISO**	0.01*
2.4	Toluene	**TOL**	0.051
3.1	Dichloromethane	**DCM**	1.6
4	Tetrahydrofuran	**THF**	100
4.1	Chloroform	**CHOL**	0.815
5.1	Acetone	**ACH**	100
***	Hexafluoroisopropanol	**HFIP**	100
5.1	Methanol	**ME**	100
6.4	Dimethylformamide	**DMF**	100

Take from: *http://www.erowid.org/archive/rhodium/pdf/solvent.miscibility.pdf*
** Considered equal to cyclohexane, *** Values not available*