Supporting Information

Rendering Protein-based Particles Transiently Insoluble for Therapeutic Applications

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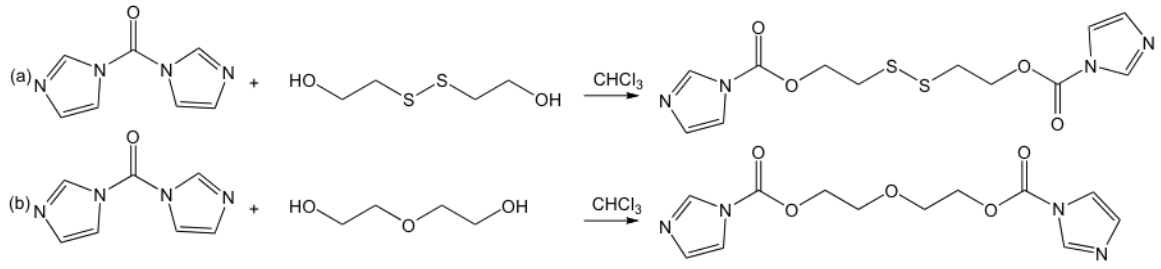
Materials. Bovine serum albumin was purchased from Calbiochem. Tyramine and 1'-Carbonyldiimidazole were purchased from Sigma Aldrich. DSP (Dithiobis[succinimidyl propionate]) was purchased from Thermo Scientific. Alexa fluor 555® labeled Bovine

serum albumin was purchased from Invitrogen. Bovine albumin ELISA quantitation set was purchased from Bethyl Laboratories, Inc. α-D-Lactose, glycerol, 2-hydroxyethyl disulfide and bis(2-hydroxyethyl)ether were purchased from Acros.

Cross-linker Synthesis. A solution of 2-hydroxyethyl disulfide (1g, 6.48mmol) in chloroform (50 mL) was added dropwise to a solution of 1,1'-Carbonyldiimidazole (10g, 61.67 mmol) in chloroform (300 mL) under reflux (Scheme S1a). The reaction mixture was stirred for 24 hours. The mixture was washed with cold water three times and the organic layer was dried with magnesium sulfate, filtered, concentrated and purified by column chromatography (EtOAc/chloroform=95:5) to give DIC (0.85g, yield 38%) as clear oil, which turned to white solid upon cooling. The reaction of Bis(2-hydroxyethyl)ether (0.69g, 6.48mmol) with 1,1'-Carbonyldiimidazole (10g, 61.67mmol) gave OEDIC (0.73g, yield 39%) as clear oil, which turned to white solid upon cooling (Scheme S1b). The synthesis and purification followed procedures described above for DIC.



Scheme S1. Synthesis of cross-linker DIC (a) and OEDIC (b)

Preparation of protein based particles. The bovine serum albumin (BSA) PRINT particles were derived from a mixture composed of 37.5 wt % of BSA, 37.5 wt % of D-

lactose and 25wt % of glycerol (Figure S1). A 7.8wt% solution of this mixture in water was prepared and then cast a film onto a poly(ethylene terephthalate) (PET) sheet. Water was removed with a heat gun moving back and forth. The film should be transparent and was laminated onto a piece of fluorocur patterned mold (Provided by Liquidia Technologies, 4 × 4 inch, cylindrical, d =1μm, h = 1μm), forming a sandwich structure with the film in the middle. The mold was delaminated by passing the mold and the PET through a heated laminator with a temperature of 60 °C on the top roller and a pressure of 80 psi between the rollers. The filled mold was re-laminated onto a sheet of plasdone covered PET. The laminated mold and PET were passed through the heated laminator again. After the particle cooled down, the mold and the PET were separated gently and all the PRINT particles were transferred from the mold to the plasdone film. The particles were harvested from the PET by dissolving plasdone with isopropanol. The harvested particles were washed with isopropanol for three times by centrifugation to remove plasdone. The particles were finally dispersed in isopropanol and the particle concentration was determined by Thermal Gravimetric Analysis (TGA) (TA Q5000).

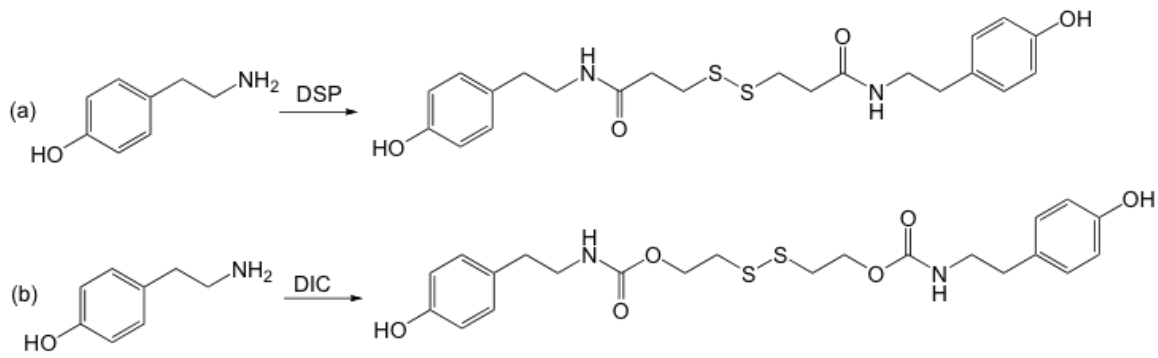
The Alexa fluor 555® labeled BSA particles were derived from a mixture composed of 37.0 wt% of BSA, 37.0 wt% of lactose, 25.0 wt% of glycerol and 1.0 wt% of Alexa fluor 555 labeled BSA.

Quantification of BSA, lactose and glycerol in particles prior to the crosslinking reaction: Particles were dispersed in water. The amount of BSA was measured using HPLC (Agilent Technologies 1260) with a C18 rapid resolution column (Zorbax Eclipse plus, 4.6 × 100 mm, 3.5 micron). A mobile phase of water and acetonitrile on a gradient

from 85% of water to pure acetonitrile over 15 minutes with a flow rate of 0.6 mL/min was employed with a detection temperature of 50 °C on the ELSD (Agilent Technologies 1260). The BSA peak appeared at 8.2 minutes and the peak area was compared to the BSA standard curve to determine the concentration of BSA in the particle solution. The particle solution was filtered with centrifugal filters (Amicon ultra, 0.5 mL, MWCO 30K) to remove BSA. The filtered solution was analyzed using HPLC with a Hi-plex Ca column (Agilent, 300 × 7.7 mm, 8 micron). A mobile phase of pure water over 25 minutes with a flow rate of 0.6 mL/min was employed with a detection temperature of 26 °C on the ELSD. The lactose peak appeared at 9.3 minutes and the glycerol peak appeared at 16.3 minutes. The concentrations of lactose and glycerol in the particle solution were calculated based on lactose and glycerol standard curves.

A Fully Reversible Disulfide Crosslinker.

Tyramine (0.24g, 1.75mmol) was added to a solution of DIC (0.12g, 0.35mmol) in isopropanol (15mL) (Scheme S2). The reaction mixture was stirred for 24 h at 40°C. The mixture was concentrated and purified by column chromatography (EtOAc) to give tyramine-DIC (0.10g, yield 59%) as light yellow solid. Tyramine (0.24g, 1.75mmol) was added to a solution of DSP (0.14g, 0.35mmol) in DMF (4mL). The reaction mixture was stirred for 24 h at 40°C. The reaction was stopped by adding water (15 mL) to the reaction mixture. Then the product was filtered and washed with water (10 mL) three times. The product tyramine-DSP (light yellow solid) was then dried and weighed (0.11g yield 70%).



Scheme S2. Synthesis of tyramine-DSP (a) and tyramine-DIC (b)

The products tyramine-DSP and tyramine-DIC were added to dithiothreitol solution (50 mM, PBS) at 37 °C and stirred for 24 h. Then the solutions were lyophilized. Isopropanol (1 mL) was added to the powder acquired and bath sonicated for 15 min. The supernatants from the solutions were collected and analyzed by gas chromatography-mass spectrometry (Agilent Technologies 5975 series MSD, 7820A GC system) and untreated tyramine was used as standard.

Tyramine generated from tyramine-DIC was purified through thin layer chromatography (TLC) (EtOAc 90%, methanol 10%). ^{1}H NMR (bruker Avance 400WB) and mass spectrometry (Agilent technologies 6210 LC-TOF) were used to confirm the structure of the compound.

Particle cross-linking reaction. Based on the TGA results, an appropriate amount of isopropanol was added to the particle dispersion to achieve a particle concentration of 1mg/mL. To 850 μL of particle dispersion, 1.275 mg of DIC was added to achieve a 4.4 mM DIC concentration. The resulting dispersion was shaken on a vortex machine for 24 h at 40 °C. The reaction was terminated by centrifuging particles down for 3 minutes, followed by removal of the supernatant containing the cross-linker and adding 850μL of

isopropanol. The particles were washed three times with isopropanol by centrifugation to remove the excess cross-linkers and then resuspended in water.

Physical Characterization of the PRINT Protein Particles. The PRINT particles were imaged by a scanning electron microscopy (Hitachi model S-4700) and the hydrodynamic diameters of the PRINT particles were measured by dynamic light scattering (Brookhaven Instruments Inc., 90Plus). For zeta potential measurements, the particles were dispersed in 1 mM potassium chloride at a concentration of 10 μg/ml and tested by a Zetasizer Nano Analyzer (Malvern Instruments Inc., Nano Zetasizer).

Dissolution Studies. The GSH concentration in cytoplasm of cells ranges from 1 to 15 mM. In this study, PBS containing 5 mM GSH and PBS only were used to simulate intracellular and extracellular environment, respectively. In order to monitor the degradation of albumin particles, 1 wt% of BSA Alexa Fluor® 555 conjugate was incorporated into the particles and the amount of this dye-conjugated protein released from particles upon particle dissolution was measured using fluorescence spectroscopy (Figure 1). Bovine serum (BSA), Alexa Fluor® 555 conjugate was incorporated into the particles and the release of this dye-conjugated protein was used to characterize the dissolution rate of the particles. Typically, particles were fabricated from a mixture of 37 wt% of BSA, 1wt% of albumin from bovine serum (BSA), Alexa Fluor® 555 conjugate, 37 wt % of D-lactose and 25wt % of glycerol. The particles were crosslinked and then resuspended in water to achieve a particle concentration of 1.33 mg/mL following the procedures described above. To each mini dialysis unit (purchased from Fisher Scientific,

MWCO 20K), 75 μL of particle solution was added. Typically, 24 units were dialyzed against 1 L of Phosphate Buffers Saline solution (PBS) containing 5 mM glutathione with a magnetic bar stirring gently at the bottom of the beaker. Another 24 units were dialyzed against 1 L of PBS buffer without glutathione as controls. The dialysis process was carried out in a 37 °C incubator. At different time points (0 h, 1.5 h, 3 h, 5 h, 12 h, 24 h, 48 h), one unit was withdrawn from each bath. The particle solution was recovered from the units and each unit was washed with 75 μL of PBS. The wash was combined with recovered particle solution and appropriate amount of PBS was added to achieve a total mass of 200 mg. The solution was centrifuged at 14000 rpm for 10 min. The supernatant was measured for fluorescence (excitation 545 nm, emission 575 nm) by a SpectraMax M5 plate reader (Molecular Devices). The fluorescence from PBS was used as background and the fluorescence from un-cross-linked particles (0.5 mg/mL in PBS) was used as a 100% control.

A plot of protein release versus time for particles cross-linked with DIC at 4.4 mM showed an accelerated rate of dissolution when dispersed in PBS with 5 mM GSH. The same particles dispersed in PBS only showed minimal dissolution at 48 h. Under identical conditions, particles cross-linked with OEDIC showed no noticeable difference in PBS with and without GSH. Particles cross-linked with the DIC at 6.6 mM also dissolved preferentially in PBS with GSH, but the rate was noticeably slower than particles cross-linked using 4.4 mM of DIC. When particles were cross-linked with the DIC cross-linker at 9.9 mM, very minimal dissolution of particles was observed both in PBS with GSH and PBS only during a 48-h time frame. Fluorescence microscopy was also used to further investigate the integrity of particles cross-linked with DIC and

OEDIC at 4.4 mM of cross-linker concentration (Figure S5).

Preparation of self-replicating RNA

Replicon Plasmid Construction: Plasmids containing the reporter genes chloramphenicol acetyltransferase (CAT) was purchased from Promega (CAT # E187A). These genes were PCR amplified using iProof™ HF Master Mix Taq polymerase (BioRad), and gene specific primers containing AscI and PacI restrictions sites for sub-cloning. The PCR products were cloned into an intermediate vector for nucleotide sequence verification, pCR®4 Blunt-TOPO (Life Technologies # K2875-40). Upon sequence confirmation the PCR fragments were sub-cloned into an DNA plasmid constructed to contain the following elements: a T7 RNA polymerase promoter for in vitro RNA transcription; the 5' untranslated region, nonstructural protein genes, 26S promoter followed by sites for Asc I and Pac I restriction enzymes, and the 3' untranslated region of Venezuelan Equine Encephalitis Virus (VEE) immediately followed by a site for Not I restriction. Specifically the PCR products and the replicon plasmid base vector were digested with AscI and PacI. The PCR products were ligated using T4 DNA Ligase into the AscI and PacI restriction enzyme sites contained in the multiple cloning site of the replicon plasmid.

RNA production: the replicon plasmid was linearized with Not I restriction enzyme and used as template for in vitro transcription of capped self-replicating RNA utilizing the T7 RiboMAX™ Express Large Scale RNA Production System (Promega #P1320) supplemented with 7.5 mm methyl G CAP analog (Promega #P1711). RNA was purified using the SV Total RNA Isolation System (Promega #Z3101) as specified by the manufacturer.

Preparation of self-replicating RNA loaded BSA-based particles and quantification of RNA in the particles.

The bovine serum albumin (BSA) PRINT particles were derived from a mixture composed of 36.7 wt% of BSA, 37.0 wt% of lactose, 25.0 wt% of glycerol, 1.0 wt% of RNA (CAT) and 0.3 wt% of Alexa fluro 488 labeled BSA. Uncross-linked particles were vortexed for 24 h at 40°C in IPA and centrifuged down. IPA was removed and particles were dissolved in water. Quant-iT ^{TM} RNA assay kit (Invitrogen) was used to quantify the amount of RNA in the solution. The assays were performed according to vendor's instructions and each assay was done in duplicate and three independent samples were measured. According to the results, particles contain 1.5 wt% of RNA after partial removal of lactose and glycerol. The particles were then cross-linked with DIC at 4.4 mM of cross-linker concentration as previously described.

Analysis of CAT expression

Vero cells were maintained at 37 °C in an atmosphere containing 5% CO2. The cells were grown in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT), MEM non-essential amino acid solution (Invitrogen) and antibiotic–antimycotic (Invitrogen).

The expression of CAT protein from CAT RNA or 1 μm BSA PRINT particles containing 1wt% CAT RNA as cargo was compared. Typically, 2×10^4 Vero cells were plated into 24 well tissue cultured treated plates 18-24 h prior to assay. Vero cells were transfected with CAT RNA or PRINT BSA particles containing 1wt% CAT RNA

utilizing the TransIT® mRNA transfection kit (Mirus Bio, Madison, WI) following the manufacturer's protocol. Briefly, to 100 μL of Opti-MEM® I Reduced-Serum Medium, 2 μg of particles, 2 μL of TransIT and 1 μL of boost were added and mixed through pipetting. The mixture was subsequently incubated with Vero cells for 4 h at 37 °C and the non-internalized particles were removed. The cells were further incubated for another 48 h at 37 °C to allow CAT protein to express. Cell lysates were prepared 48h post-transfection and CAT ELISA (Roche, Indianapolis) analysis was carried out according to the manufacturer's instructions. The amount of CAT protein generated was calculated based on a standard curve from 2, 1, 0.5, 0.25, 0.125 and 0 ng/mL of CAT protein.

Cytotoxcity study of self-replicating RNA loaded BSA-based particles

Typically, 5×10^3 Vero cells were plated into 96 well tissue cultured treated plates 18-24 h prior to assay. The samples were prepared as described in section of analysis of CAT expression and dosed to cells. The mixture was subsequently incubated with Vero cells for 4 h at 37 °C and the non-internalized particles were removed. The cells were further incubated for another 48 h at 37 °C before ATP was quantitated using the CellTiter-G10 Luminescent Cell Viability Assay (Promega) following vendor's protocol.

Immunofluorescence Microscopy of self-replicating RNA loaded BSA-based particles

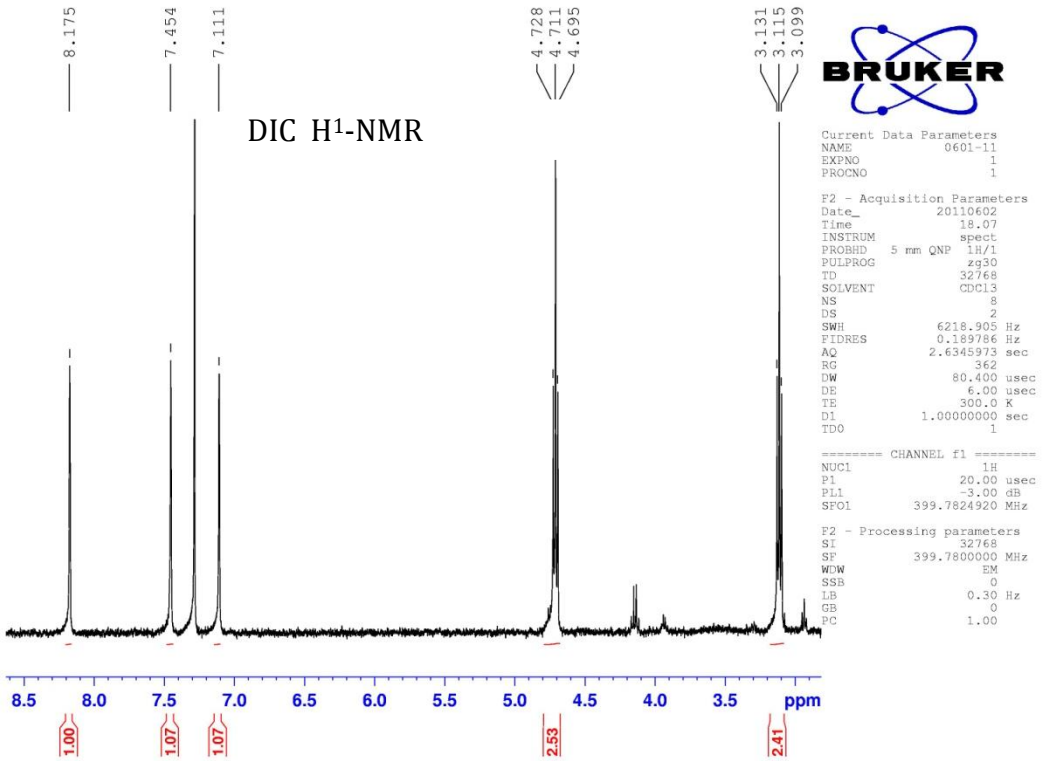
Vero cells plated at on cover slips in 6-well dishes and grown for 24 hours. Cells were treated with particles/TransIT for 48 h. Cells were then washed with 3X with cold PBS and fixed/permeablized with ice cold acetone:methanol mixture (1:1) for 3 min at 4°C. Samples were washed 3 times with ice cold PBS. Samples were then incubated in primary antibody abcam (CAT#ab50151) diluted 1:400 in a 1:1 mixture of PBS:FBS for

1 hr at 37°C. Following incubation, cells were then washed 3X with PBS and then incubated in secondary Alexa Fluor® 546 goat anti-rabbit IgG (H+L) (A11010, Invitrogen) diluted 1:300 in 1:1 mixture of PBS:FBS and incubated for 1hr at 37°C in dark. Washed twice in PBS and mounted with Fluorsave™ reagent. Confocal images were acquired using a Ziess 710 laser scanning confocal imaging system (Olympus) fluorescence microscope fitted with a PlanApo 40× objective (Olympus). The final composite images were created using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

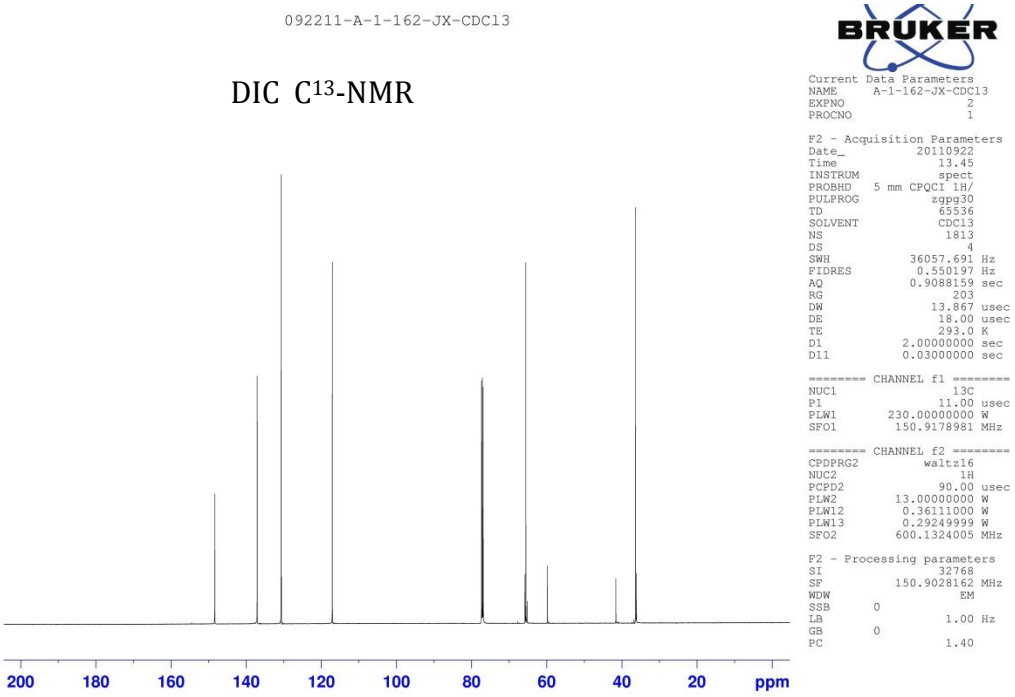
Compound Characterization:

Dithio-bis(ethyl 1 H-imidazole-1-carboxylate) DIC

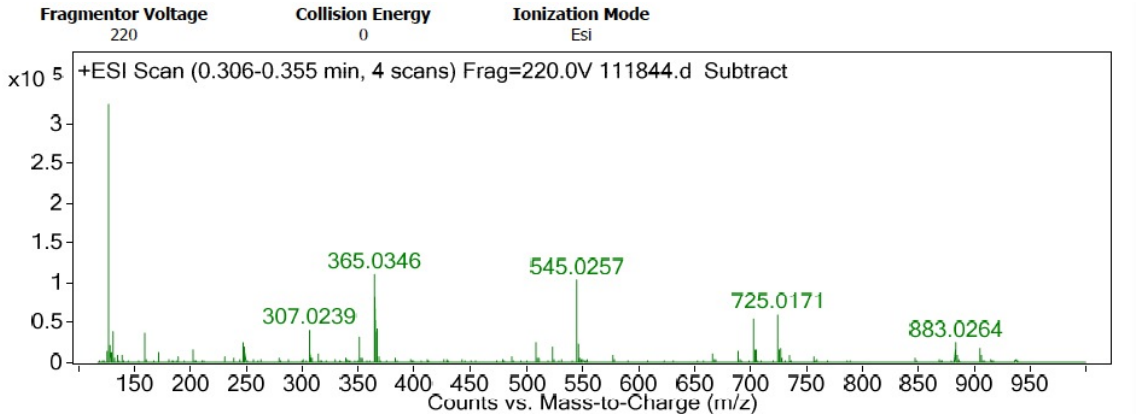
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 2H), 7.45 (s, 2H), 7.11 (s, 2H), 4.71 (t, J = 6.8Hz, 4H), 3.11 (t, J = 6.4Hz, 4H)



^{13}C NMR (150 MHz, $CDCl_3$) δ 36.3, 65.6, 117.2, 130.8, 137.1, 148.4

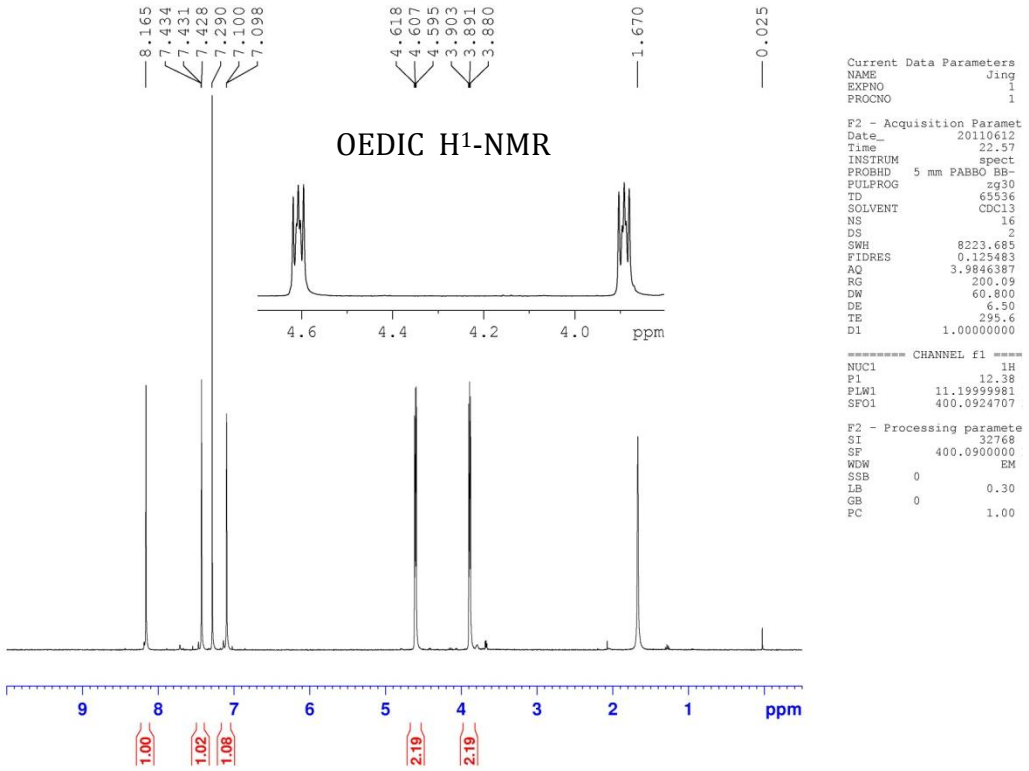


MS (LC-TOF) m/z calculated for $[M+Na]^+$=365.0354, found $[M+Na]^+$=365.0346

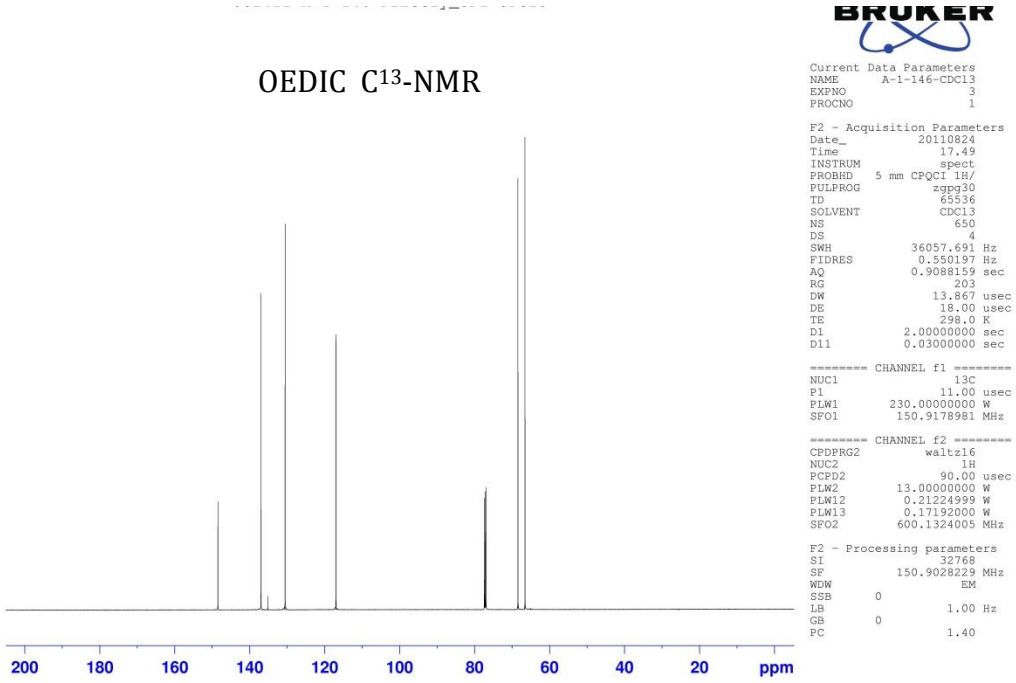


2,2'-Oxybis(ethane-2,1-diyl) bis(1H-imidazole-1-carboxylate) OEDIC

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.17 (s, 2H), 7.43 (s, 2H), 7.10 (s, 2H), 4.61 (t, J = 6.6Hz, 4H), 3.89 (t, J = 7.2Hz, 4H)



^{13}C NMR (150 MHz, $CDCl_3$) δ 66.5, 68.4, 115.9, 130.5, 137.0, 148.4



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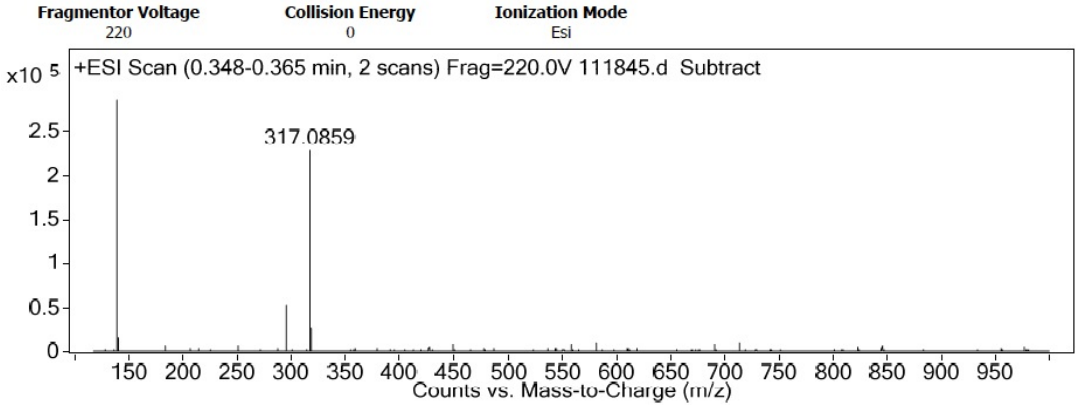
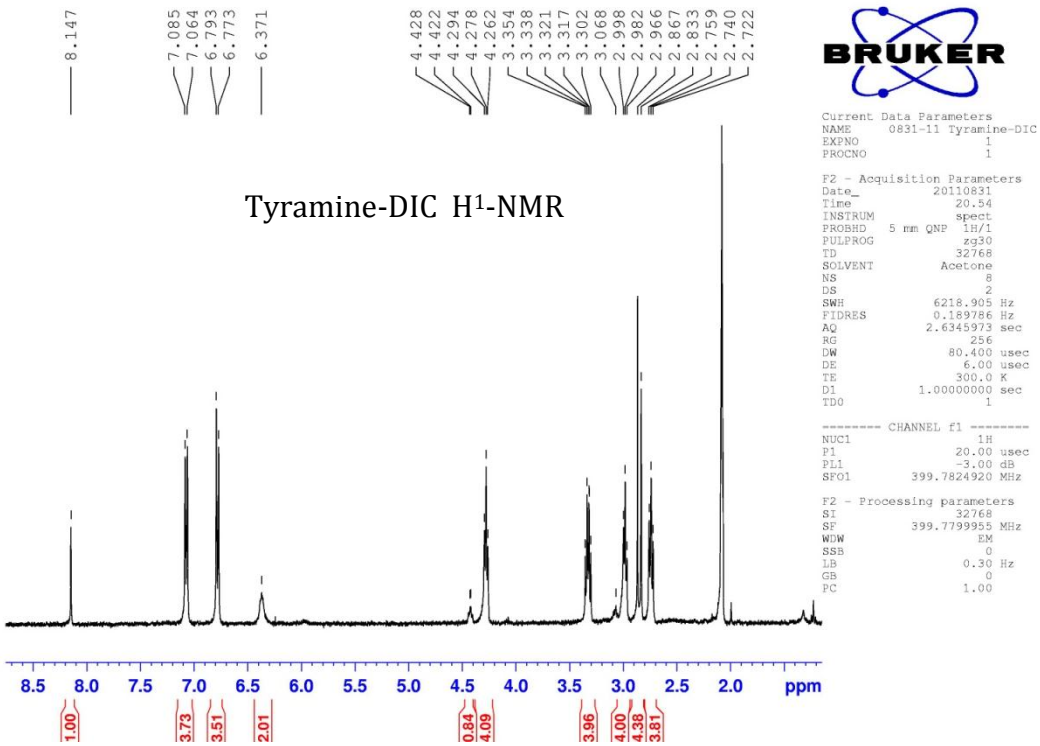
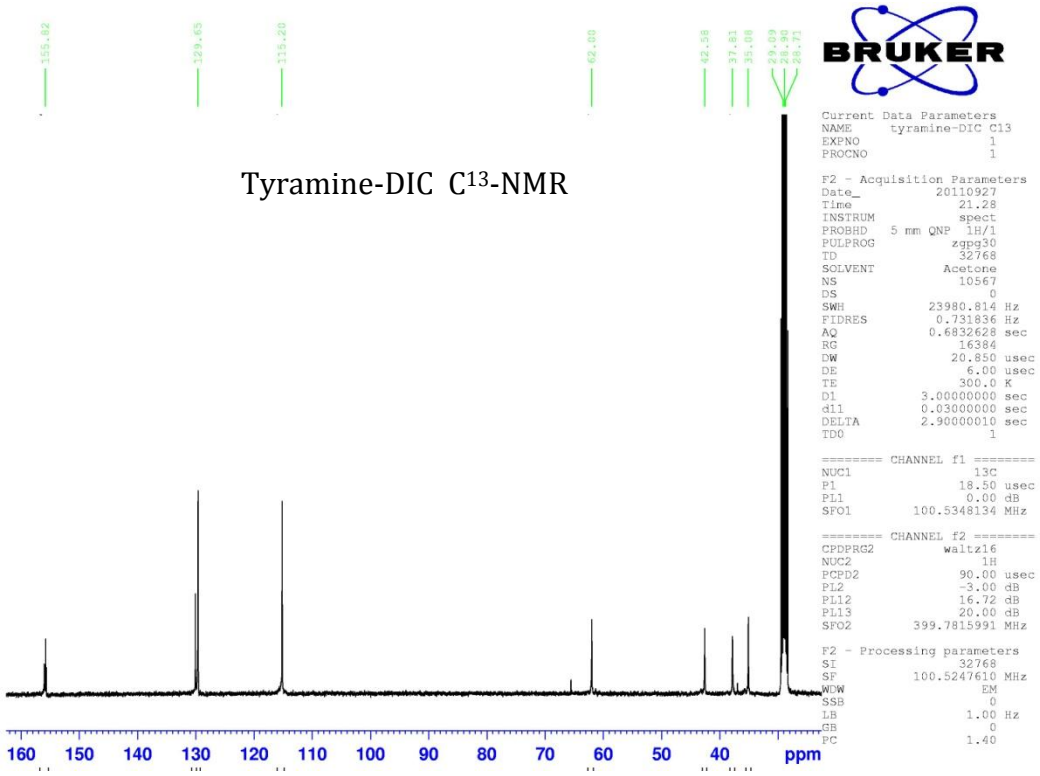


Tyramine-DIC

^{1}H NMR (400 MHz, actone-D6) δ 7.07 (d, J=8.4Hz, 4H), 6.78 (d, J=8Hz, 4H), 4.28(t, J=7.6Hz, 4H), 3.33 (q, J = 6.4Hz, 4H), 2.98 (t, J = 6.4Hz, 4H), 2.74(t, J=7.2Hz, 4H)



^{13}C NMR (100 MHz, actone-D6) δ 155.8, 130.1, 129.6, 115.2, 115.1, 62.0, 42.6, 37.8, 35.1



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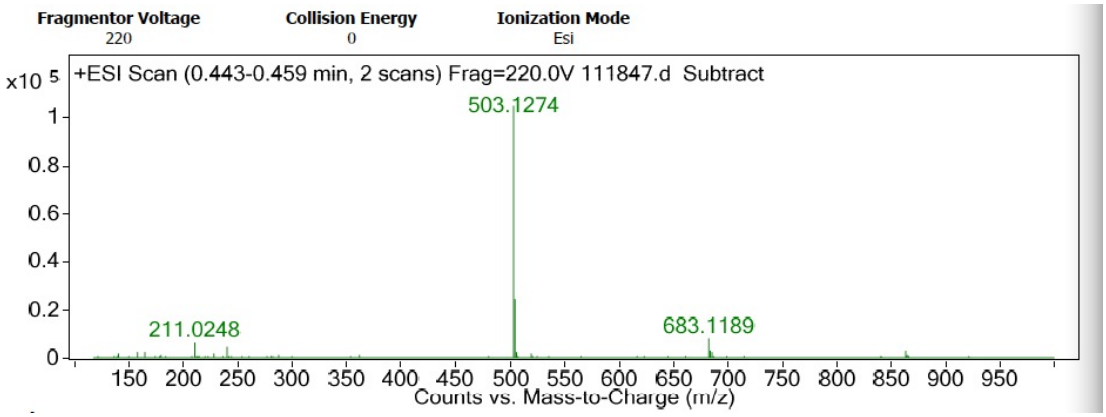
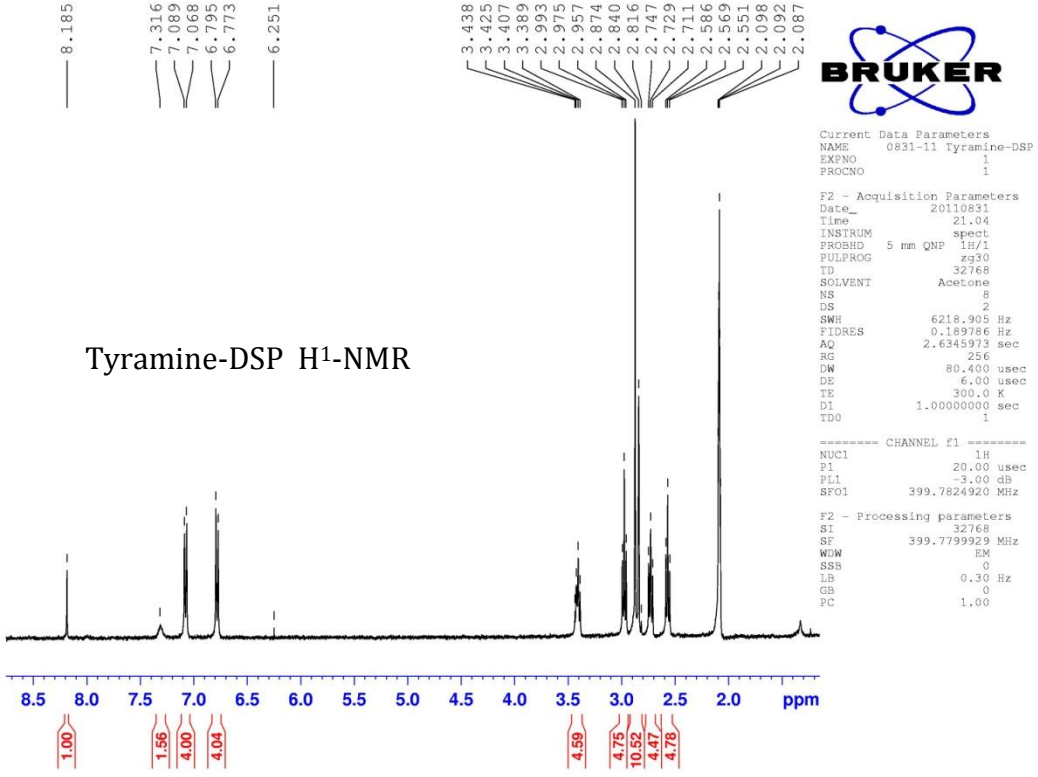
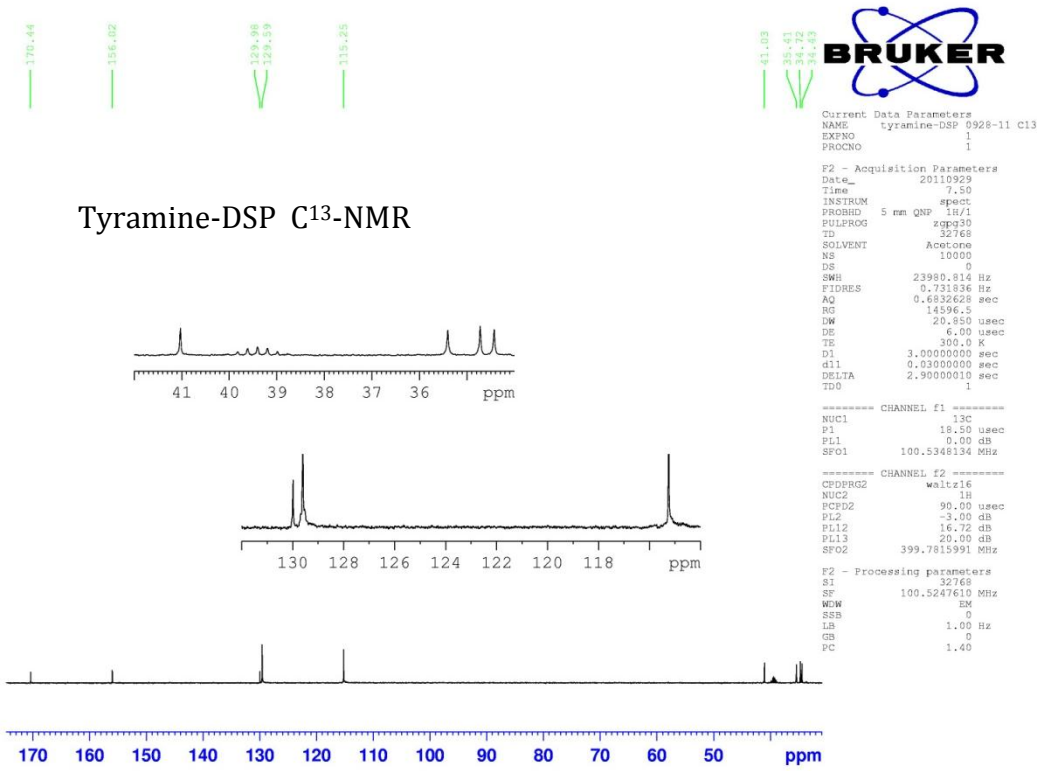


Tyramine-DSP

^{1}H NMR (400 MHz, actone-D6) δ 7.08 (d, J=8.4Hz, 4H), 6.78 (d, J=8.8Hz, 4H), 3.41(q, J=7.6Hz, 4H), 2.98 (t, J = 7.2Hz, 4H), 2.73(t, J=7.2Hz, 4H), 2.57(t, J=6.8Hz, 4H)



^{13}C NMR (100 MHz, actone-D6) δ170.4, 156.0, 130.0, 129.6, 115.3, 41.0, 35.4, 34.7, 34.4



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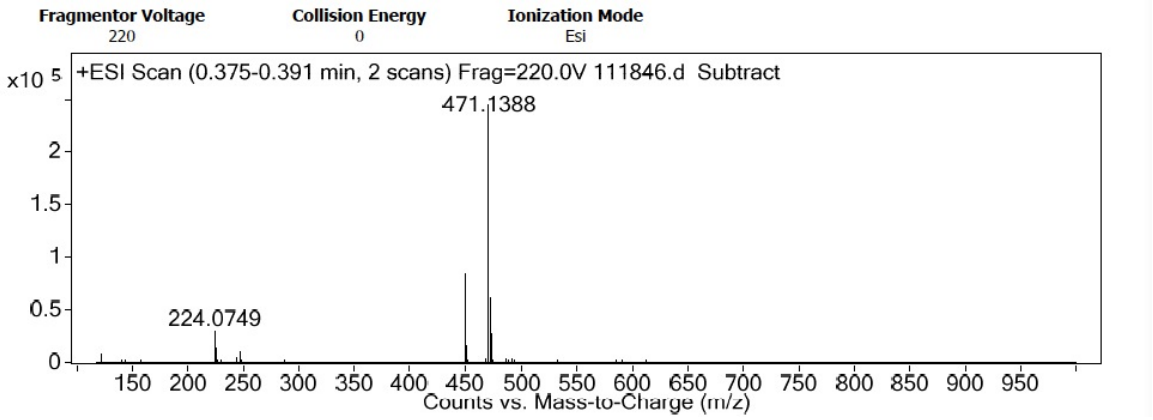
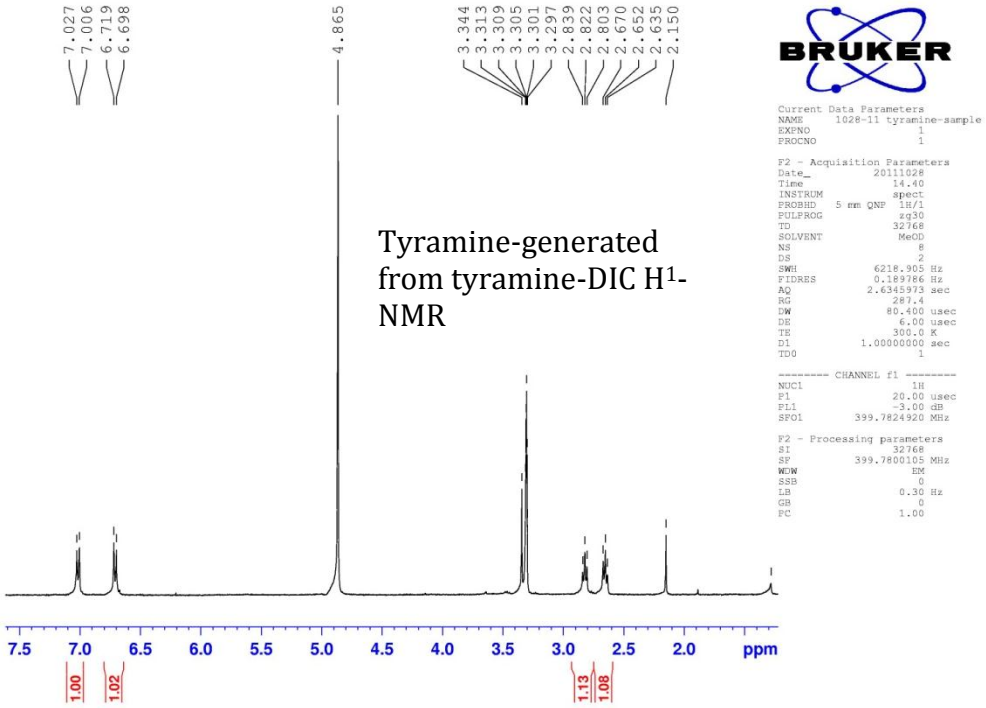


Tyramine generated from tyramine-DIC:

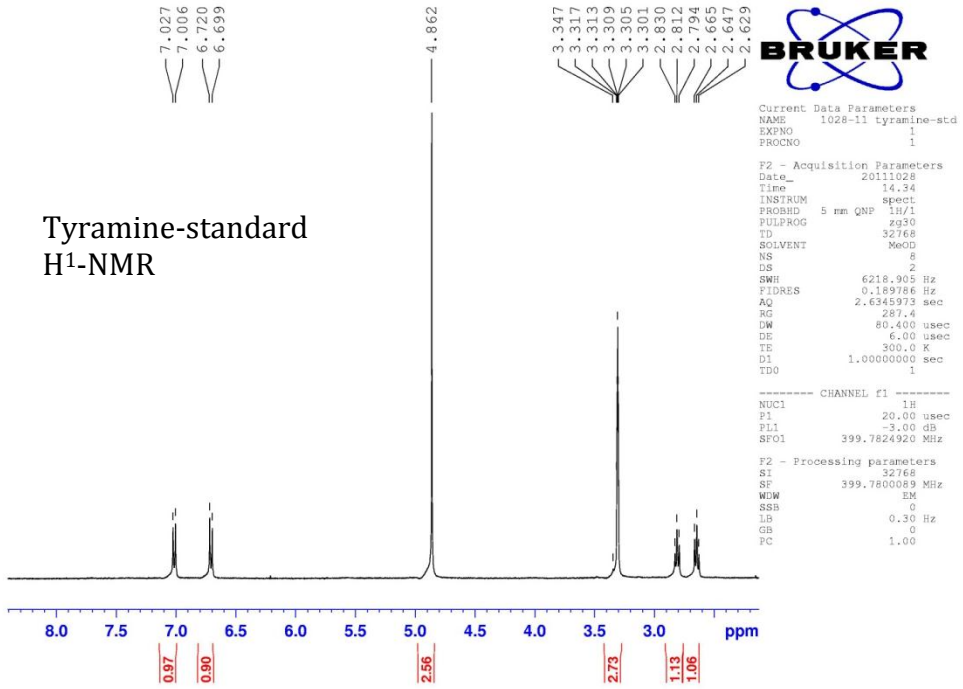
^{1}H NMR (400 MHz, MeOD) δ 7.02 (d, J=4.2 Hz, 2H), 6.71 (d, J=4.2 Hz, 2H), 2.82 (t, J = 7.2 Hz, 2H), 2.65(t, J=7.2 Hz, 2H)



MS (LC-TOF) m/z calculated for $[M+H]^+$=138.0919, found $[M+H]^+$= 138.0916

Tyramine standard:

^{1}H NMR (400 MHz, MeOD) δ 7.02 (d, J=4.2 Hz, 2H), 6.71 (d, J=4.2 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 2.65(t, J=7.2 Hz, 2H)



MS (LC-TOF) m/z found $[M+H]^+$=138.0914

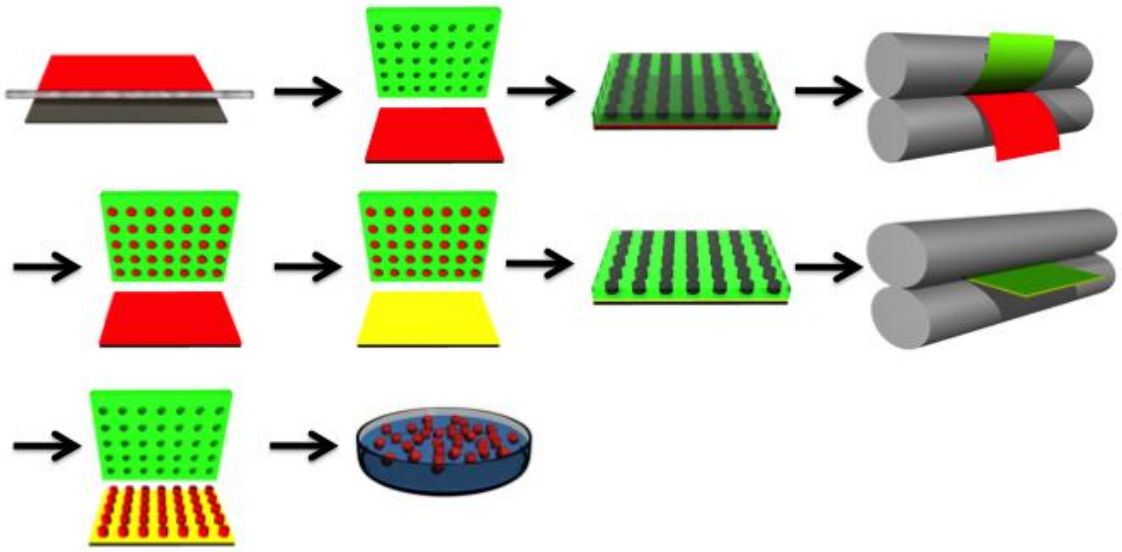


Figure S1. The fabrication process for protein-based PRINT particles. BSA, lactose and glycerol were dissolved in water to create a solution (red). A mayer rod is then used to draw a film from this solution on a high surface energy polyethylene terephthatlate (PET)

sheet. Water is removed and a solid film is generated. A PFPE mold (green, from Liquidia Technologies) is laminated onto the film, passed through a heated pressured nip (grey) and split. The cavities of the mold are filled. The filled mold is laminated onto a sacrificial adhesive layer (yellow) and passed through the nip again without splitting. After the mold cools down, the mold is peeled off and particles are transferred to the adhesive layer, which is then dissolved in isopropanol to free the particles.

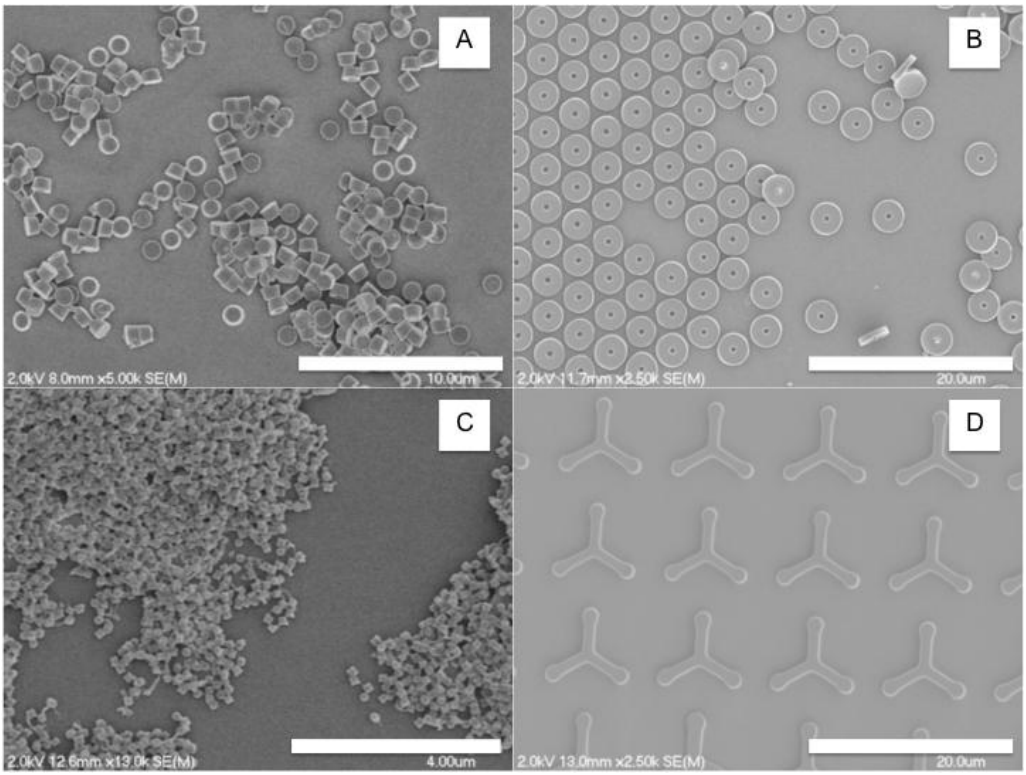


Figure S2. Scanning microscopy image (SEM) of BSA nano and micro-sized particles fabricated using PRINT. A: 1 μm × 1 μm cylinders, scale bar represents 10 μm, B: 3 μm × 1 μm donut, scale bar represents 20 μm, C: 200 nm × 200 nm cylinders, scale bar represents 4 μm, D: 3 μm × 1 μm helicopters, scale bar represents 20 μm.

Table S1 Particle composition

	Charged Composition [a] (wt%)	Final Composition [b] (wt%)
BSA	37.5	86.7±1.1
Lactose	37.5	10.2±1.7
Glycerol	25.0	3.1±0.7

[a] The weight percentage of components charged into the pre-particle solution that was then drawn into a film on the PET sheet. [b] Final particle composition after harvest and purification step. The errors stand for standard deviation calculated from three experiments.

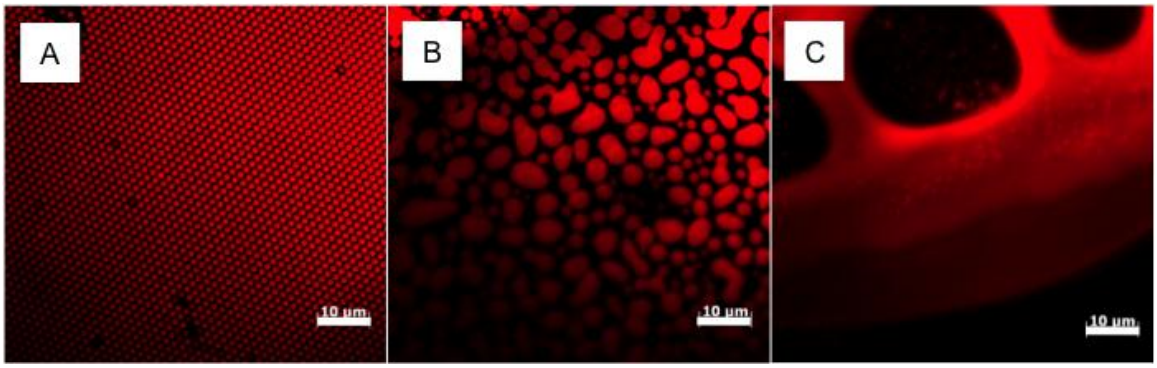


Figure S3. BSA particle dissolution utilizing fluorescent microscopy. To monitor dissolution of BSA particles in water using fluorescent microscopy, 1 wt% of Alexa Fluor 555® dye labeled BSA was added to particles. Images were taken of the particles on the sacrificial adhesive layer before and after addition of water. (A) Particles transferred on to plasdone PET sheet (B) Particles with water added after 10 s, (C) Particles with water added after 5 min.

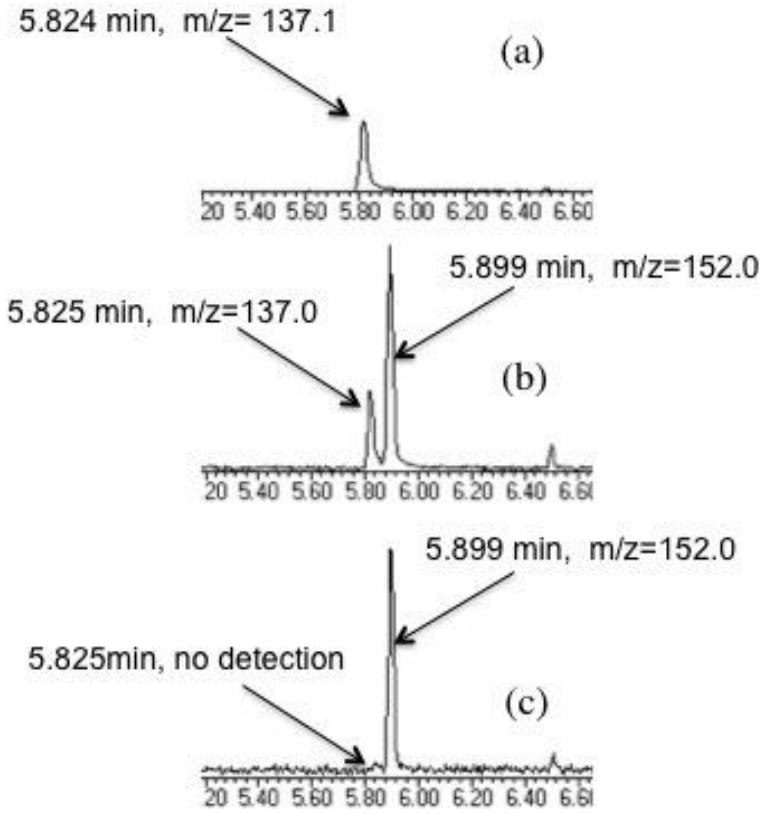


Figure S4. GC-MS characterization of tyramine-DIC and tyramine-DSP after treatment with DTT, (a) standardard tyramine, (b) tyramine-DIC, (c) tyramine-DSP. The peak at 5.899 min (m/z=152.0) in (b) and (c) represents oxidized DTT.

Table S2. Characterization of crosslinked BSA particles [a]

	Diameter, [b] nm	PDI [c]	ζ-Potential, [d] mV
DIC-4.4 mM	1201 ± 152	0.016	-13.6 ± 0.5
DIC-6.6 mM	1164 ± 393	0.114	-16.3 ± 1.0
DIC-9.9 mM	1069 ± 346	0.105	-23.1 ± 0.4
OEDIC-4.4 mM	1069 ± 362	0.114	-10.9 ± 0.3

[a] The particles fabricated for dissolution study. DIC-4.4 mM: particles cross-linked with DIC at 4.4 mM. DIC-6.6 mM: particles cross-linked with DIC at 6.6 mM. DIC-9.9 mM: particles crosslinked with DIC at 9.9 mM. OEDIC-4.4 mM: particles cross-linked with OEDIC at 4.4 mM. [b] Hydrodynamic diameter measured by dynamic light scattering. The average hydrodynamic diameters were obtained from three measurements. The error bars are the half-width of the effective diameters. [c] Polydispersity index from the dynamic light scattering measurements. [d] ζ-potential was measured in 1mM KCl by Zetasizer. The error bars are standard deviations from three measurements.

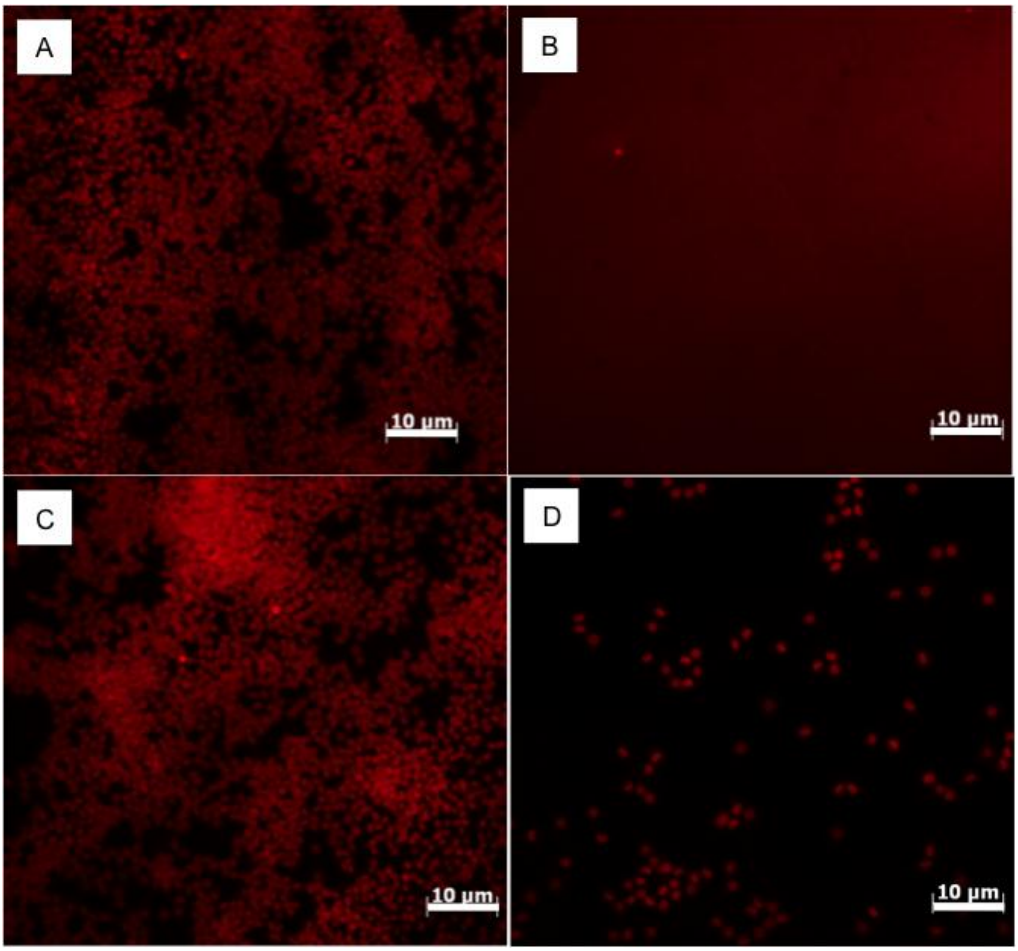


Figure S5. Particle dissolution by fluorescent microscopy at 5-h time point. (A) Particles cross-linked with 4.4 mM of DIC, in PBS, (B) Particles cross-linked with 4.4 mM of DIC, in PBS containing 5 mM of GSH, (C) Particles cross-linked with 4.4 mM of OEDIC, in PBS (D) Particles cross-linked with 4.4 mM of OEDIC, in PBS containing 5 mM of GSH

Table S3. Characterization of crosslinked BSA particles with and without TransIT [a]

	Diameter, nm	PDI	ζ-Potential, mV
Without TransIT	1214 ± 483	0.159	-15.4 ± 1.0
With TransIT	1179 ± 721	0.374	+ 0.8 ± 0.3

[a] The particles charged with 1 wt% of CAT RNA replicon. The particles (2 μg) were added into 100 μL (Opti-MEM® I Reduced-Serum Medium), and then 2 μL of TransIT and 1 μL of boost were added subsequently. The reaction went for 5 min before measurements were taken.

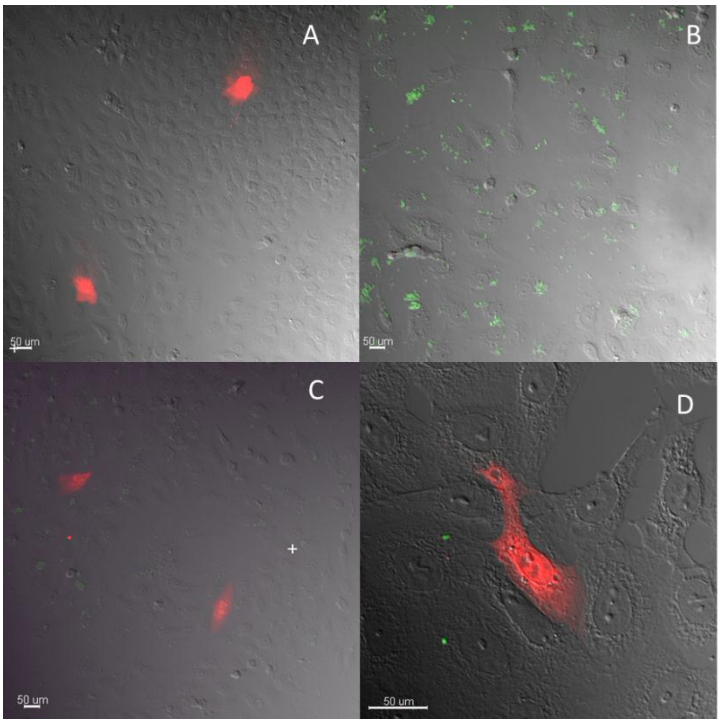


Figure S6. Confocal images of CAT protein. (A) 100 ng of CAT RNA with TransIT, (B) blank particles coated with TransIT. (C and D) BSA particles containing CAT RNA coated with TransIT. Scale bars represent 50μm.

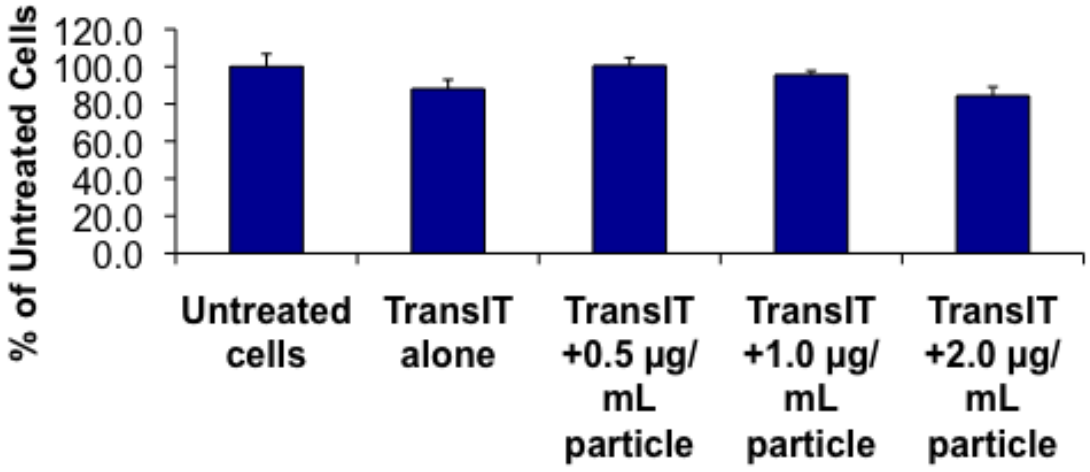
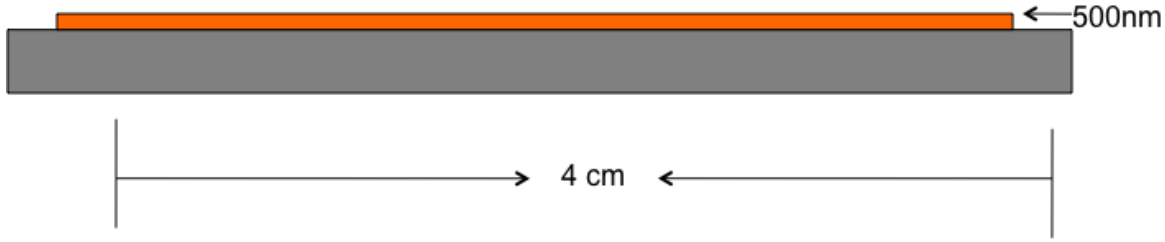


Figure S7. Cytotoxicty of particles coated with TransIT. Particles were dosed at 0, 0.5, 1.0 and 2.0 µg/mL of concentrations, ATP measured by the CellTiter-G10 Luminescent cell viability assay. Error bars represent standard deviation calculated from three wells.

Study of the depth of cross-linking reaction on particles

The cross-linking of the particles was performed after PRINT process. The goal of this study is to determine if the cross-linking also occurs in the center of the particles.

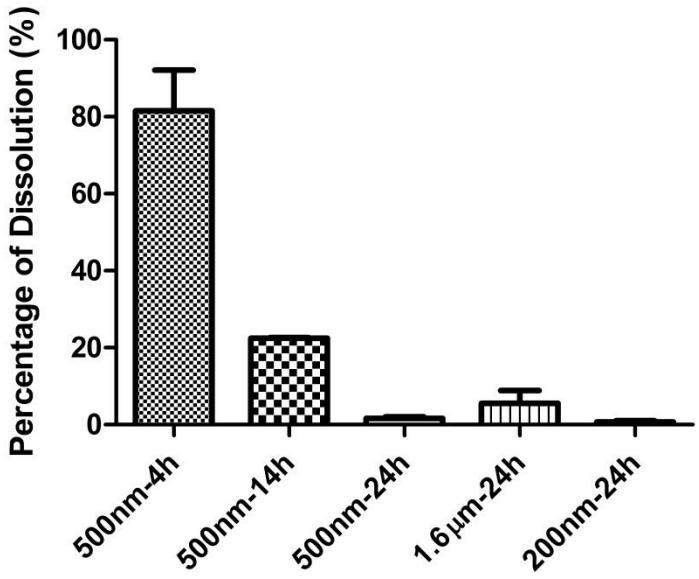


The above figure shows a macroscopic film that is prepared on PET sheet from 36.5 wt% of BSA, 1 wt% of Alexa fluor 555 labeled BSA, 37.5 wt% of lactose and 25 wt% of

glycerol, the same composition as the PRINT particles used in this study. The film is shown in orange and PET is shown in gray. The film was washed with isopropanol three times in order to mimic the particle fabrication process and then reacted with DIC at 4.4 mM of cross-linker concentration for 24 h at 40 °C. Then the film was collected with PBS using cell scraper. The solution was vortexed for 1 h and centrifuged down for 10 min at 14000 rpm to remove the cross-linked material. The supernatant was analyzed using fluorescence spectrometry for the amount of free protein and the percentage of free protein was calculated using un-cross-linked film as 100% control.

Based on this method, the cross-linking depth in one dimension can be determined. If the cross-linking occurs close to the bottom of the film, minimal of free protein can be released after the cross-linking reaction. If the cross-linking reaction only occurs at the surface of the film, the free protein in the center will instantaneously dissolve upon exposure to PBS.

Films with thicknesses of 200 nm, 500 nm and 1.6 μm after 24 h of cross-linking reaction and films with a thickness of 500 nm for 4 h and 14 h of cross-linking reaction were examined. Each experiment was done in triplicate.



This graph showed that after 24 hour of cross-linking reaction, the films with thicknesses from 200nm to 1.6 μm all showed minimal dissolution, indicating that the film was cross-linked at the bottom of the film and the cross-linking reaction can penetrate at least 1.6 μm in one dimension. At 4-h and 14-h, the 500nm film was only partially cross-linked. Based these results, we can come to the conclusion that the cross-linking reaction occurs at the center of the 1-micron cylindrical particles.