Supplementary Material (ESI) for Soft Matter

Electronic Supplementary Information

Alternative pathway for constructing shell cross-linked nanoparticles: understanding of factors associated with the cross-linking efficiency

Guorong Sun,[a] Nam S. Lee,[a] William L. Neumann,*[b,c] John N. Freskos,[b] Jeng J. Shieh,[b] Richard B. Dorshow,*[b] and Karen L. Wooley*[a,d]

[a] *Department of Chemistry and Department of Radiology, Washington University in Saint Louis, Saint Louis, MO 63130, USA.*

[b] *Covidien Imaging Solutions R&D, Hazelwood, MO 63042, USA. E-mail: richard.dorshow@covidien.com*

[c] *Department of Pharmaceutical Sciences, School of Pharmacy, Southern Illinois University Edwardsville, Edwardsville, IL 62026, USA. E-mail: wneuman@siue.edu*

[d] *Department of Chemistry, Texas A&M University, College Station, TX 77842, USA. wooley@mail.chem.tamu.edu*

* To whom correspondence or reprint requests should be addressed:

Email: wneuman@siue.edu, richard.dorshow@covidien.com, wooley@mail.chem.tamu.edu

Experimental

Materials. The mono-methoxy terminated mono-hydroxy poly(ethylene glycol) (mPEG2k, *MW* = 2,000 Da, PDI = 1.06) was purchased from Intezyne Technologies (Tampa, FL) and was used for the synthesis of macro-CTA[1] without further purification. The cross-linker 1-3 were synthesized according to previous report.[2] Other chemicals were purchased from Aldrich and Acrose were used without further purification unless otherwise noted. Prior to use, styrene (99%), purchased from Aldrich, were distilled over calcium hydride and stored under N_2. The Supor 25 mm 0.1 μm Spectra/Por Membrane tubes (molecular weight cut-off (MWCO) 6-8 kDa), used for dialysis, were purchased from Spectrum Medical Industries Inc.. Nanopure water (18 mΩ•cm) was acquired by means of a Milli-Q water filtration system (Millipore Corp.; Bedford, MA).

Measurements. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 600 MHz spectrometer interfaced to a UNIX computer using Mercury software. Chemical shifts are referred to the solvent proton resonance. Infrared spectra were obtained on a Perkin-Elmer Spectrum BX FT-IR system using diffuse reflectance sampling accessories with FT-IR Spectrum v2.00 software.

The molecular weight distribution was determined by Gel Permeation Chromatography (GPC). The *N*,*N*-dimethylformamide (DMF) GPC was conducted on a Waters Chromatography, Inc. (Milford, MA) system equipped with an isocratic pump model 1515, a differential refractometer model 2414, and a two-column set of Styragel HR 4 and HR 4E 5 μm DMF 7.8 × 300 mm columns. The system was equilibrated at 70 °C in pre-filtered DMF containing 0.05 M LiBr, which served as polymer solvent and eluent (flow rate set to 1.00 mL/min). Polymer solutions were prepared at a concentration of *ca.* 3 mg/mL and an injection volume of 200 μL

was used. Data collection and analysis was performed with Empower Pro software (Waters, Inc.). The system was calibrated with poly(ethylene glycol) standards (Polymer Laboratories, Amherst, MA) ranging from 615 to 442,800 Da.

The atomic force microscopy (AFM) characterization of micelles was performed by tapping-mode AFM under ambient conditions in air. The AFM instrumentation consisted of a Nanoscope III BioScope system (Digital Instruments, Veeco Metrology Group; Santa Barbara, CA) and standard silicon tips (type, OTESPA-70; L, 160 μm; normal spring constant, 50 N/m; resonance frequency, 246-282 kHz). Samples for AFM imaging analysis were prepared through spin-coating *ca*. 1.0 μL of the micelle solution (0.5 mg/mL) onto freshly cleaved mica plates (Ruby clear mica, New York Mica Co.) and allowed to dry freely in air.

Samples for Transmission Electron Microscopy (TEM) measurements were diluted with a 1 % phosphotungstic acid (PTA) stain (v/v, 1:1). Carbon grids were exposed to oxygen plasma treatment to increase the surface hydrophilicity. Micrographs were collected at 100,000× magnification and calibrated using a 41 nm polyacrylamide bead from NIST. The number average particle diameters (D_{av}) and standard deviations were generated from the analysis of a minimum of 150 particles from at least three different micrographs.

Hydrodynamic diameters (D_h) and size distributions for the vesicles in aqueous solutions were determined by dynamic light scattering (DLS). The DLS instrumentation consisted of a Brookhaven Instruments Limited (Worcestershire, U.K.) system, including a model BI-200SM goniometer, a model BI-9000AT digital correlator, a model EMI-9865 photomultiplier, and a model 95-2 Ar ion laser (Lexel Corp.) operated at 514.5 nm. Measurements were made at 25 ± 1 °C. Scattered light was collected at a fixed angle of 90°. The digital correlator was operated with 522 ratio spaced channels, and initial delay of 5 μs, a final delay of 100 ms, and a duration

of 6 minutes. A photomulitplier aperture of 100 μm was used, and the incident laser intensity was adjusted to obtain a photon counting of between, 200 and 300 kcps. Only measurements in which the measured and calculated baselines of the intensity autocorrelation function agreed to within 0.1 % were used to calculate particle size. The calculations of the particle size distributions and distribution averages were performed with the ISDA software package (Brookhaven Instruments Company), which employed single-exponential fitting, cumulants analysis, and CONTIN particle size distribution analysis routines. All determinations were repeated 5 times.

The UV-vis absorption spectra of SCKs were collected at room temperature using a Varian Cary 100 Bio UV-visible spectrophotometer and plastic cuvettes with 10 mm of light path. For each SCK absorption spectroscopy measurement, the pH 7.2 PBS (5 mM with 5 mM of NaCl) buffer solution outside the dialysis tubing was used as control.

The fluorescence spectra of SCKs were obtained at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer. All fluorescence spectra from SCK solutions were measured at optical densities at the excitation wavelength. If not specially mentioned otherwise, an excitation wavelength of the observed maximum absorption peak was used. Each fluorescence spectrum was normalized with respect to the absorbed light intensity at the excitation wavelength.

Synthesis of PEO_{45}-*b*-$PNAS_{95}$. To a 25 mL Schlenk flask equipped with a magnetic stir bar dried with flame under N_2 atmosphere, was added the mPEG2k macro-CTA (0.24 g, 0.10 mmol) and 1,4-dioxane (10 mL). The reaction mixture was stirred 0.5 h at rt to obtain a homogeneous solution. To this solution was added NAS (1.7 g, 10 mmol) and AIBN (0.8 mg, 5 μmol). The reaction flask was sealed and stirred 10 min at rt. The reaction mixture was

degassed through several cycles of freeze-pump-thaw. After the last cycle, the reaction mixture was stirred for 10 min at rt before being immersed into a pre-heated oil bath at 60 °C to start the polymerization. After 1.5 h, the monomer conversion reached *ca.* 90% by analyzing aliquots collected through ^{1}H-NMR spectroscopy. The polymerization was quenched by cooling the reaction flask with liquid N_2. The solution was diluted with 20 mL of DMSO and precipitated into 600 mL of cold diethyl ether at 0 °C twice. The precipitants were collected, washed with 100 mL of cold ether, and dried under vacuum overnight to afford the PEO_{45}-*b*-$PNAS_{95}$ block copolymer precursor as a yellow solid (1.2 g, 65% yield based upon monomer conversion). ^{1}H NMR (600 MHz, DMSO-d_6, ppm): δ 0.81 (t, J = 6 Hz, 3H, dodecyl *CH*$_3$), 1.09 (br, 5H, *CH*$_3$ and dodecyl *CH*$_2$), 1.20 (br, 19H, *CH*$_3$ and dodecyl *CH*$_2$*s*), 1.30 (br, 2H, dodecyl *CH*$_2$), 1.60 (t, J = 6 Hz, 2H, dodecyl *CH*$_2$), 2.01 (br, PNAS backbone protons), 2.75 (NAS *CH*$_2$*CH*$_2$*s*), 3.09 (br, PNAS backbone protons), 3.20 (s, mPEG terminal *OCH*$_3$), 3.47 (m, *OCH*$_2$*CH*$_2$*O* from the PEG backbone), 4.07 (br, 2H from the PEO backbone terminus connected to the ester linkage); ^{13}C NMR (150 MHz, DMSO-d_6, ppm): δ 172.8, 69.8, 41.2, 25.2; IR (NaCl, cm^{-1}): 2925, 1811, 1780, 1735, 1361, 1206, 1070, 649.

Synthesis of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60}. To a 10 mL Schlenk flask equipped with a magnetic stir bar dried with flame under N_2 atmosphere, was added the PEO_{45}-*b*-$PNAS_{95}$ macro-CTA (0.55 g, 30 μmol), 1,4-dioxane (2.2 mL), and DMF (2.2 mL). The reaction mixture was stirred 0.5 h at rt to obtain a homogeneous solution. To this solution was added styrene (0.94 g, 9.0 mmol) and AIBN (0.24 mg, 1.5 μmol). The reaction flask was sealed and stirred 10 min at rt. The reaction mixture was degassed through several cycles of freeze-pump-thaw. After the last cycle, the reaction mixture was stirred for 10 min at rt before being immersed into a pre-heated oil bath at 60 °C to start the polymerization. After 16.5 h, the monomer conversion reached *ca.*

19% by analyzing aliquots collected through ^{1}H-NMR spectroscopy. The polymerization was quenched by cooling the reaction flask with liquid N_2. The polymer was purified by precipitation into 250 mL of cold diethyl ether at 0 °C twice. The precipitants were collected and dried under vacuum overnight to afford the block copolymer precursor as a yellow solid (0.58 g, 77% yield based upon monomer conversion). ^{1}H NMR (600 MHz, CD_2Cl_2, ppm): δ 0.81 (br, dodecyl *CH_3*), 1.10-2.40 (br, dodecyl Hs, PNAS, and PS backbone protons), 2.75 (NAS *CH_2CH_2s*), 3.15 (br, PNAS backbone protons), 3.28 (s, mPEG terminal *OCH_3*), 3.60 (m, *OCH_2CH_2O* from the PEG backbone), 6.20-7.30 (br, Ar Hs); ^{13}C NMR (150 MHz, DMSO-d_6, ppm): δ 172.8, 145.2, 128.0, 125.7, 69.8, 41.6, 25.2; IR (NaCl, cm^{-1}): 2925, 1810, 1779, 1732, 1452, 1362, 1208, 1070, 813, 699, 648.

General Procedure for Micellization of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60}. To a solution of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} block copolymer in DMF (*ca.* 1.0 mg/mL), was added dropwise an equal volume of nano-pure H_2O *via* a syringe pump at a rate of 15.0 mL/h, and the mixture was further stirred for 1 h at rt before using for characterizations and cross-linking reactions.

General Procedure for Cross-linking of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} Micelles. To a solution of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} micelles (15.0 mg of block copolymer precursor, 57.7 μmol of NAS residues) in 30.0 mL of DMF/H_2O (v:v = 1:1) at rt, was added dropwise over 10 min, a solution of cross-linker (5.8 μmol for nominal 20% of cross-linking, 14.5 μmol for nominal 50% of cross-linking, and 29.0 μmol for nominal 100% of cross-linking, respectively) in nano-pure H_2O. The reaction mixture was allowed to stir for 48 h at rt. For reactions involving cross-linker **1**, **2**, and **4**, the mixture was transferred to pre-soaked dialysis tubing (MWCO 6,000-8,000 Da) and dialyzed against 5.0 mM PBS (pH 7.2, with 5.0 mM NaCl) for 7 days to remove DMF, un-reacted cross-linker, and the small molecule by-products and afford an aqueous solution of cross-

linked nanoparticles. For reactions involving cross-linker **3**, the mixture was transferred to pre-soaked dialysis tubing (MWCO 6,000-8,000 Da) and sequentially dialyzed against 5.0 mM PBS (pH 7.2, with 5.0 mM NaCl) for 3 days, 5.0 mM PBS (pH 7.2, with 150 mM NaCl) for 2 days, and 5.0 mM PBS (pH 7.2, with 5.0 mM NaCl) for 2 days to remove DMF, un-reacted cross-linker, and the small molecule by-products and afford an aqueous solution of cross-linked nanoparticles.

Synthesis of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60}. A 25 mL round bottom flask equipped with a stir bar was charged with PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} (100 mg, 4.05 μmol), 10 mL of CH_2Cl_2 and 1 mL of trifluoroacetic acid (TFA). After adding 0.1 mL of water, the reaction mixture was stirred vigorously for 24 h at rt and then, was concentrated under vacuum. The crude product was dissolved into 10 mL of DMF, transferred into pre-soaked dialysis tubing (MWCO 6,000–8,000 Da) and dialyzed against nano-pure H_2O (18.0 MΩ•cm) for 4 days to remove small molecule impurities. The aqueous solution was lyophilized to afford the product as slightly yellow solid (60 mg, 95 % yield). ^{1}H-NMR (600 MHz, DMSO-d_6, ppm): δ 0.81-2.40 (br, docecyl Hs and polymer backbone Hs), 3.37 (s, 3H, mPEG terminal OCH_3), 3.42-3.82 (br, mPEG backbone -OCH_2CH_2O- Hs), 6.22-7.30 (br, Ar Hs), 12.25 (br, -COO*H*); ^{13}C NMR (150 MHz, DMSO-d_6, ppm): δ 175.8, 145.2, 128.0, 125.7, 69.8, 41.6; IR (NaCl, cm^{-1}): 3450-2900, 2925, 1718, 1458, 1258, 1183, 1103, 954, 794, 699.

Micellization of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60}. To a solution of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60} (30 mg) in 30 mL of DMF, was added dropwise 30 mL of nano-pure H_2O *via* a syringe pump at a rate of 15.0 mL/h, and the mixture was further stirred for 16 h at rt. Finally, the mixture was transferred into pre-soaked dialysis tubing (MWCO 6,000-8,000 Da) and dialyzed against nano-pure water for 5 days to afford the micelle solution.

Cross-linking of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60} Micelle with 3 through "Conventional" Amidation. To a solution of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60} micelle (10 mg of PEO_{45}-*b*-PAA_{95}-*b*-PS_{60} block copolymer precursor, 62 μmol of AA residues) in 40 mL of nano-pure water, was added dropwise over 30 min, a solution of **3** (3.7 mg, 6.2 μmol for **SCK10** and 9.2 mg, 15 μmol for **SCK11**, respectively) in nano-pure water. The mixture was stirred 2 h at rt and a freshly prepared EDCI solution (3.8 mg, 13 μmol for **SCK10** and 9.2 mg, 31 μmol for **SCK11**, respectively) in nano-pure water was then added over 1 h. The reaction mixture was further stirred 48 h at rt before transferring into a pre-soaked dialysis tubing (MWCO 6,000-8,000 Da) and sequentially dialyzing against 5 mM pH 7.2 PBS (with 5 mM NaCl) for 3 days, 5 mM pH 7.2 PBS (with 150 mM NaCl) for 2 days, and 5 mM pH 7.2 PBS (with 5 mM NaCl) for 2 days to afford an aqueous solution of the cross-linked nanoparticles.

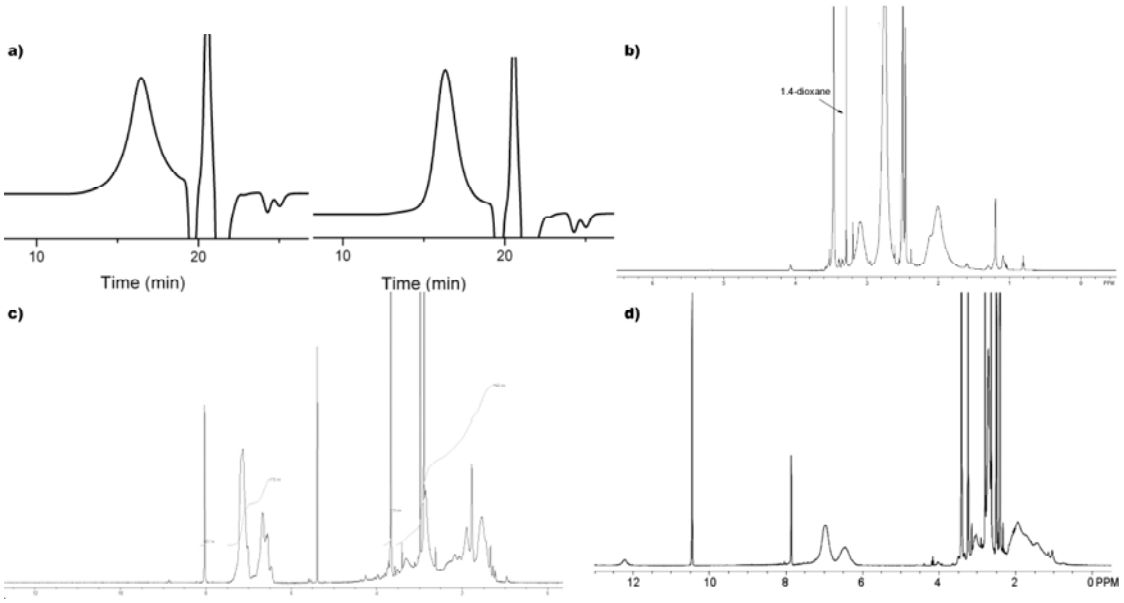

Fig. S1 Characterizations of PEO_{45}-*b*-$PNAS_{95}$ and PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} block copolymer precursors. a): DMF-GPC profiles of PEO_{45}-*b*-$PNAS_{95}$ (left) and PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} (right) block copolymers. b): ^{1}H NMR spectrum of PEO_{45}-*b*-$PNAS_{95}$. c): ^{1}H NMR spectrum of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60}. d): ^{1}H NMR spectrum of PEO_{45}-*b*-$PNAS_{95}$-*b*-PS_{60} after 7 month of storage.

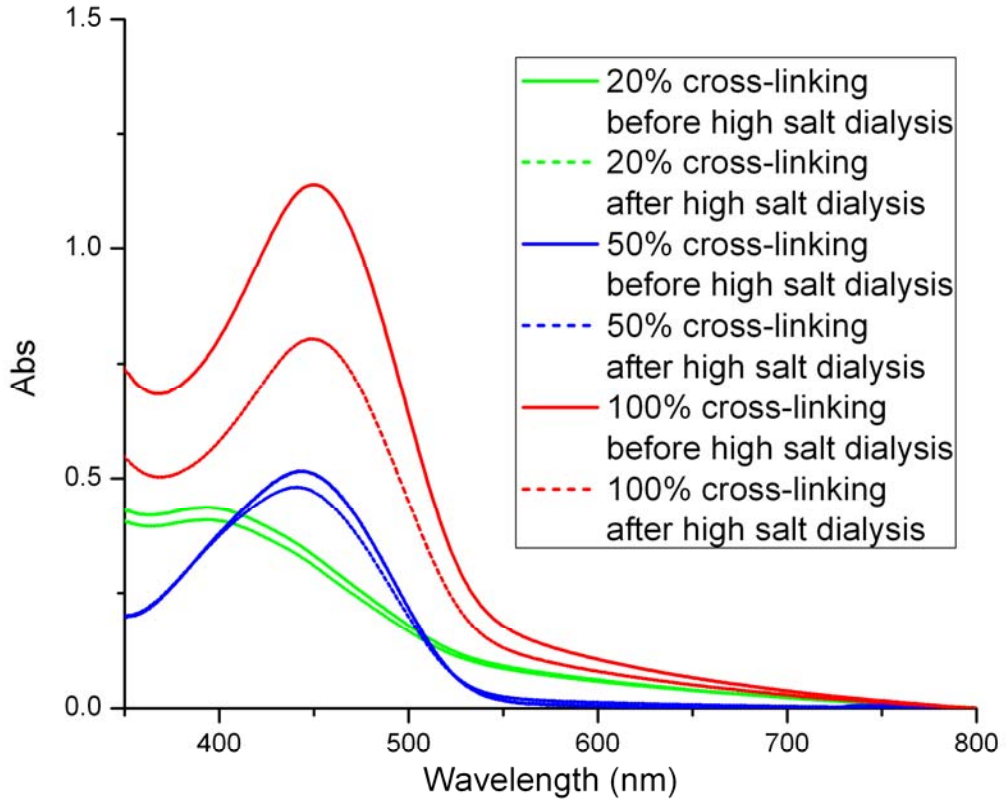

Fig. S2 UV-vis profiles of **SCK7** (green), **SCK8** (blue), and **SCK9** (red) before (solid line) and after (dashed line) dialysis against pH 7.2 5 mM PBS (with 150 mM of NaCl).

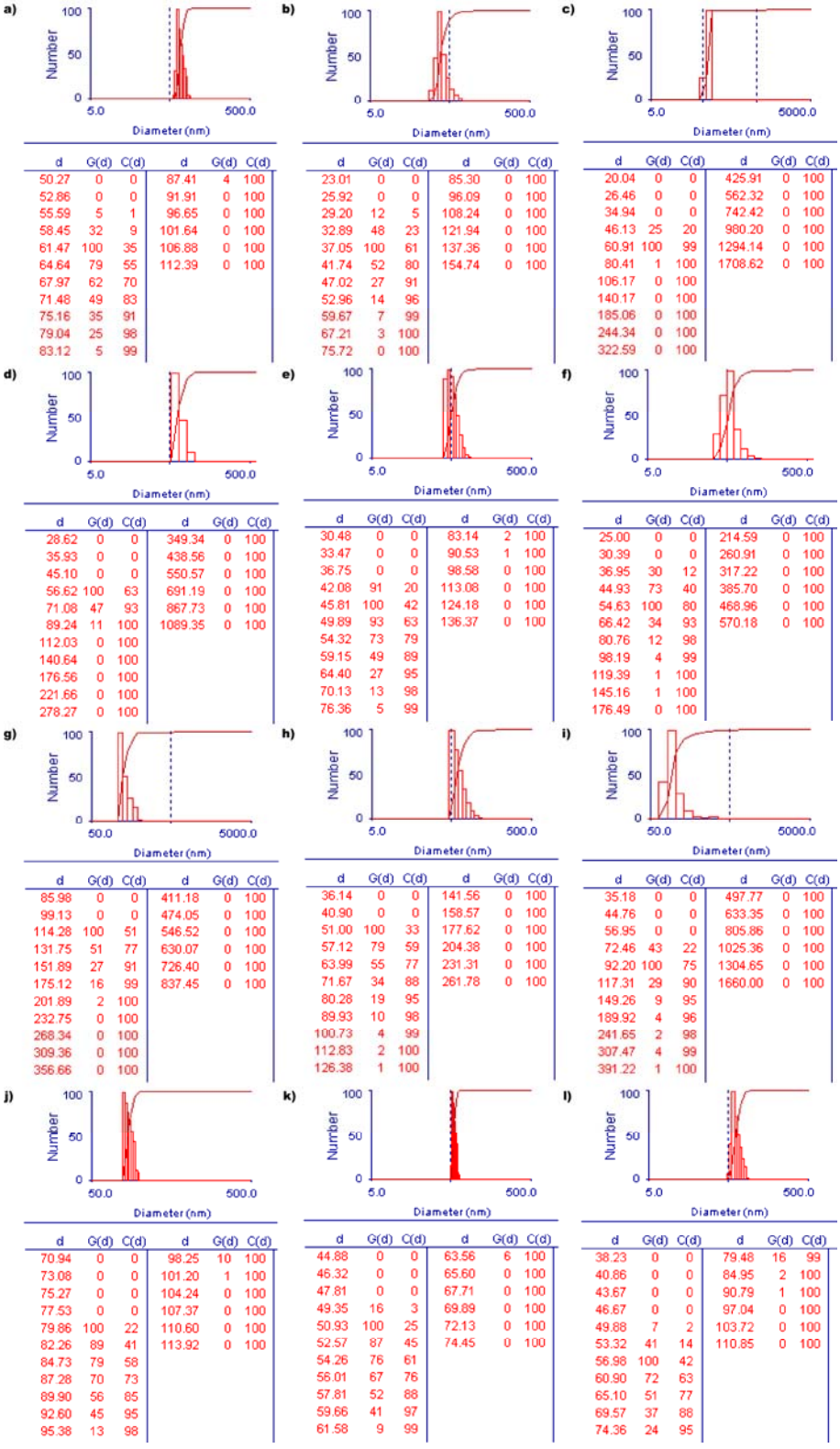

Fig. S3 Histograms of number-averaged hydrodynamic diameter distributions for **SCK1-12**.

References:

1. G. Sun, H. Fang, C. Cheng, P. Lu, K. Zhang, A. V. Walker, J. S. Taylor and K. L. Wooley, *ACS Nano*, 2009, **3**, 673-681.
2. W. L. Neumann, R. Rajagopalan, R. B. Dorshow, J. J. Shieh, J. N. Freskos, N. S. Lee and K. L. Wooley, US Provisional Patent #60/986,171, filed Nov. 7, **2007**.