COMMUNICATION

Supporting Information

A Novel Method for PEGylation of Chitosan Nanoparticles through Photopolymerization

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EXPERIMENTAL SECTION

1. Materials

Low molecular weight chitosan, dialysis membrane (14kDa cut-off), sodium tripolyphosphate (TPP) were provided from Sigma Aldrich (St. Louis. MO). Hydrochloric acid and sodium hydroxide were obtained from Merck. Glacial acetic acid (99.7+%) was purchased from Alfa Aeasar (Ward Hill, MA). Lithium phenyl- 2, 4, 6-trimethylbenzoylphosphinate (LAP) was supplied by Tokyo Chemical Industry Co. Ltd. Methacrylic Anhydride was provided from Sigma Aldrich (St. Louis. MO). Acrylate polyethylene glycol (Acr-PEG) with molecular weight of 5 kDa and 10 kDa was purchased from Laysan Bio Inc. (Arab, AL). 2, 4, 6- trinitrobenzenesulfonic acid (TNBS) was supplied Termo Fisher Scientific. DMEM (41966). Ultrapure water was obtained and used in all experiments (Milli-Q system water- 18.2 MΩ).

2. Preparation of Methacrylamide Chitosan (CSMA)

Low molecular weight chitosan (3% w/v) was dissolved in acetic acid (3% v/v). The chitosan solution was stirred at 65 °C and 1100 rpm for two days. After two days, 10 mL of chitosan solution was mixed with 50 μL of methacrylic anhydride for 3 hours using vortex mixer. The resulting solution was dialyzed against DI water using 14 kDa cut-off dialysis membrane for 5 days. After dialysis, purified CSMA solution was freeze-dried. The freeze-dried CSMA was stored at -20 ° C for further use.

The degree of methacrylation in CSMA was characterized with TNBS assay. 80 μL of 0.5 mg/mL CSMA solution (in 1 mg/mL acetic acid) and 0.5 mg/mL unmodified chitosan solution (in 1 mg/mL acetic acid) were transferred to adjacent wells of the transparent 96-Well UV Microplate as triplets. The samples in the plate were incubated with 40 μL of 2% (w/v) $NaHCO_3$ and 60 μL of 0.1% (v/v) TNBS solution at 37° C for an hour. 60 μL of 1 N HCl solution was added to each well to terminate the reaction after incubation and the absorbance of the samples were

measured via a plate reader (Biotek, Synergy HI) at 345 nm. The 1 mg/mL acetic acid solution was utilized as a control group and the methacrylation degree was of the CSMA was calculated using the following relation:

$$Degree\ of\ Methacrylation\ \% = 100 - \frac{Absorbance\ of\ Sample - Absorbance\ of\ Acetic\ Acid}{Absorbance\ of Unmodified\ Chitosan - Absorbance\ of\ Acetic\ Acid} x\ 100 \quad (1)$$

3. Synthesis of PEGylated Chitosan Nanoparticles

0.5 mg/mL CSMA was dissolved in 1 mg/mL acetic acid solution. The pH of this solution was adjusted to 4.8 by adding 6 M of NaOH. 0.5 mg/mL TPP solution was prepared in DI water. The pH of the TPP solution was decreased to 2.5 by adding 6 M HCl. The CSMA solution was heated to 37 °C and the TPP solution was cooled to 4 °C. 2.5 mL of TPP solution and different amount of LAP solutions (Table S1) were added simultaneously into 10 mL of CSMA solution. Reaction mixture was stirred at 1100 rpm for 45 minutes. 20 mg (4 μmole), 35 mg (7 μmole), 70 mg (10 μmole) of PEG with 5 kDa molecular weight and 40 mg (4 μmole), 70 mg (7 μmole), 100 mg (10 μmole) of PEG with 10 kDa molecular weight were added into these mixtures respectively and mixed for 5 minutes using a vortex mixer. After mixing, all samples were treated with 365 nm UV light which had 36 W total power for certain time periods (Table S1).

4. Characterization of PEGylated Chitosan Nanoparticles

The size and zeta potential of the particles were measured by dynamic light scattering (DLS) method (Zetasizer, Malvern ZS). Size and zeta potentials were measured both before and after adjusting the pH of the particle solutions to 7.4 using 1 M NaOH. The z-average diameter and zeta potentials were noted with three measurements and recorded as mean ± standard deviation. Additionally, the shape of the CS-PEG NPs was determined using scanning electron microscopy (SEM) (Zeiss Ultra Plus, Bruker, MA). To do so, the solutions were dialyzed against ultrapure water inside a 14 kDa cut-off dialysis membrane for two days. The dialyzed solutions were dropped onto

silicon wafers and dried under vacuum overnight. The SEM images were obtained using different magnification degrees. PEGylation of chitosan nanoparticles was also confirmed with FTIR analysis. The FTIR spectra of chitosan, Acr-PEG and PEGylated chitosan were recorded in the region of 650-4000 cm^{-1} with Thermo Scientific iS10 FTIR. Samples were freeze dried before FT-IR experiments.

5. Cell Viability and Cytotoxicity Assay for PEGylated CSNP

Cytotoxicity of PEGylated chitosan nanoparticles was evaluated on HEK293-T cell line using CellTiter-Glo Luminescent Cell Viability Assay (Promega). HEK293-T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an incubator with 5% CO2 and 37 °C. For cell viability assay, cells (1x104 cells per well) were seeded on 96-well plates in triplicates and incubated for 24 h at 37 °C. Next, cells were exposed to varied concentrations of PEGylated chitosan nanoparticles (25, 50, 75 μg/mL) for 24 h and 48 h. After the defined time intervals, cells were treated with CellTiter-Glo reagent and stirred at 100 rpm for 10 min at 25 °C. Luminescence was measured using a plate reader (BioTek's Synergy H1, Winooski, VT, USA).

PEGYLATION OPTIMIZATION

Table S1. Optimization Experiments

Methacrylic Anhydride (µL) *	Sample No	LAP (µL)**	PEG (µmole)**/***	UV Exposure (min)	pH	Particle
100	1	0	0	0	4.7	Agg.
	2	69	0	0	4.7	Agg.
50	3	69	0	1	4.7	Agg.
	4	6.9	0	1	4.7	Yes
	5	6.9	7	0	7.4	Agg.
	6	6.9	7	1	7.4	Agg.
	7	6.9	7	5	7.4	Agg.
	8	6.9	7	10	7.4	Yes
	9	6.9	7	20	7.4	Yes
	10	1	0	10	4.7	Yes
	11	1	7	10	7.4	Agg.
	12	0	7	10	7.4	Agg.

*Volume of Methacrylic Anhydride per 10 mL of 3% (w/v) chitosan solution

**Amount in 5 mg CSMA solution (10 mL of 0.5 mg/mL CSMA solution)

*** PEG MW= 5 kDa

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	101.4	Peak 1:	121.7	99.5	53.93
PdI:	0.198	Peak 2:	4722	0.5	765.6
Intercept:	0.936	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

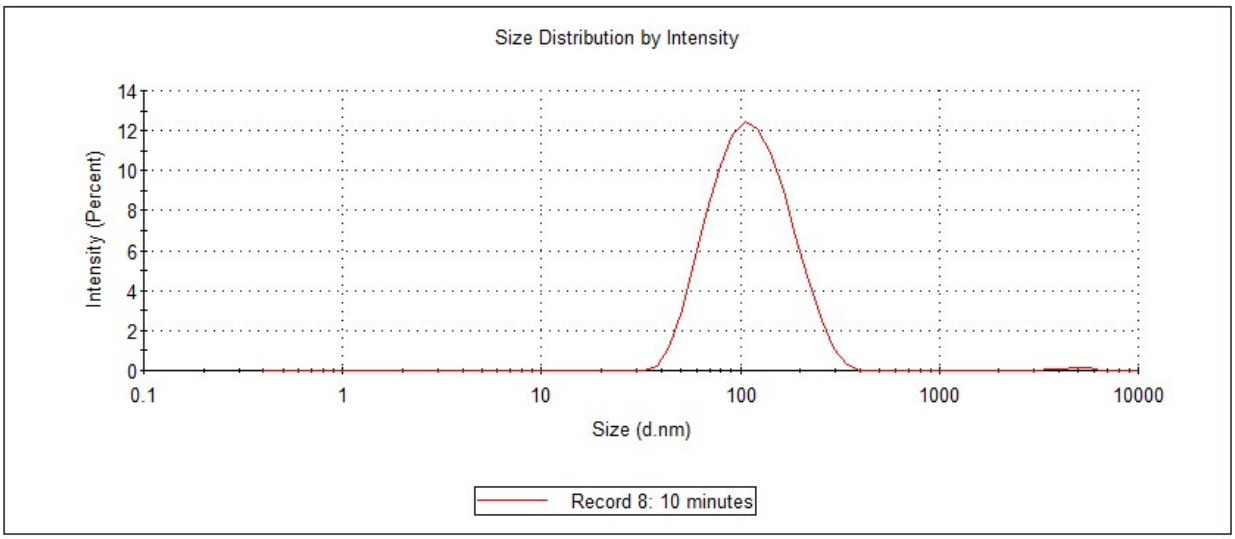


Figure S1. DLS data of Sample 8 with 10 min UV Exposure

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 133.1	Peak 1:	231.8	98.9	189.7
PdI: 0.435	Peak 2:	4932	1.1	653.6
Intercept: 0.727	Peak 3:	0.000	0.0	0.000
Result quality : Good				

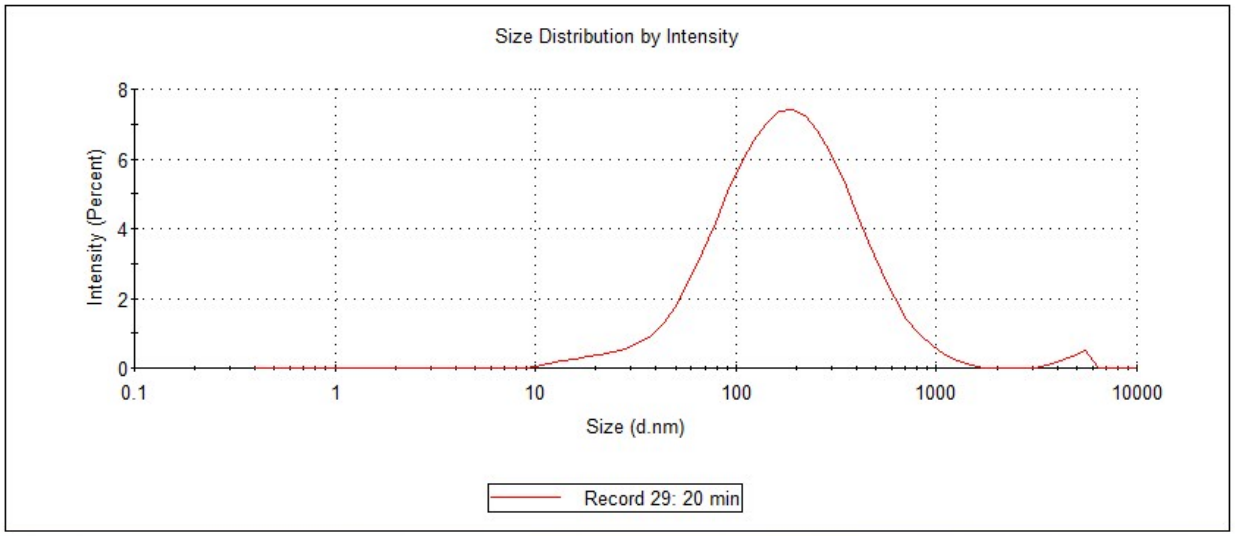


Figure S2. DLS data of Sample 9 with 20 min UV Exposure

CELL VIABILITY EXPERIMENTS

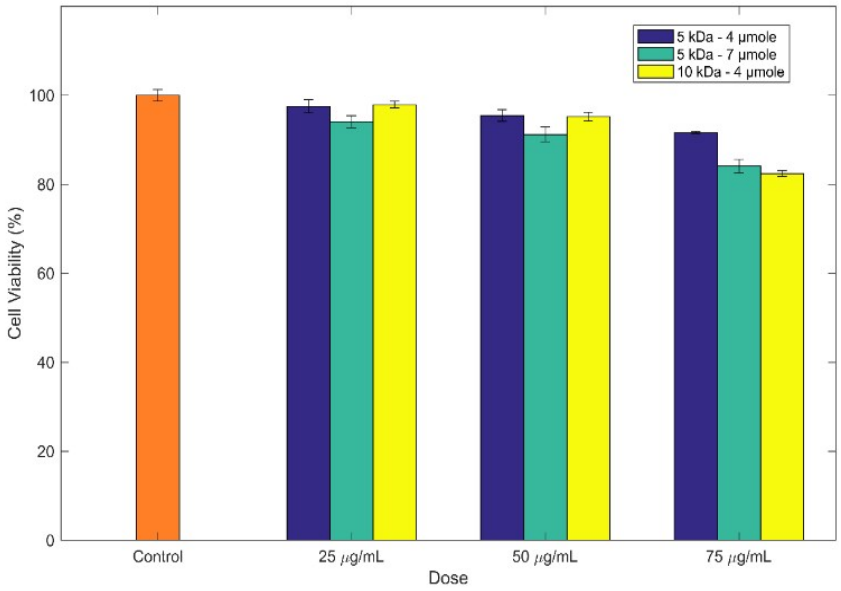


Figure S3. Cell viability experiments for the non-aggregated groups.

FT-IR ANALYSIS FOR PEGYLATED CHITOSAN NANOPARTICLES

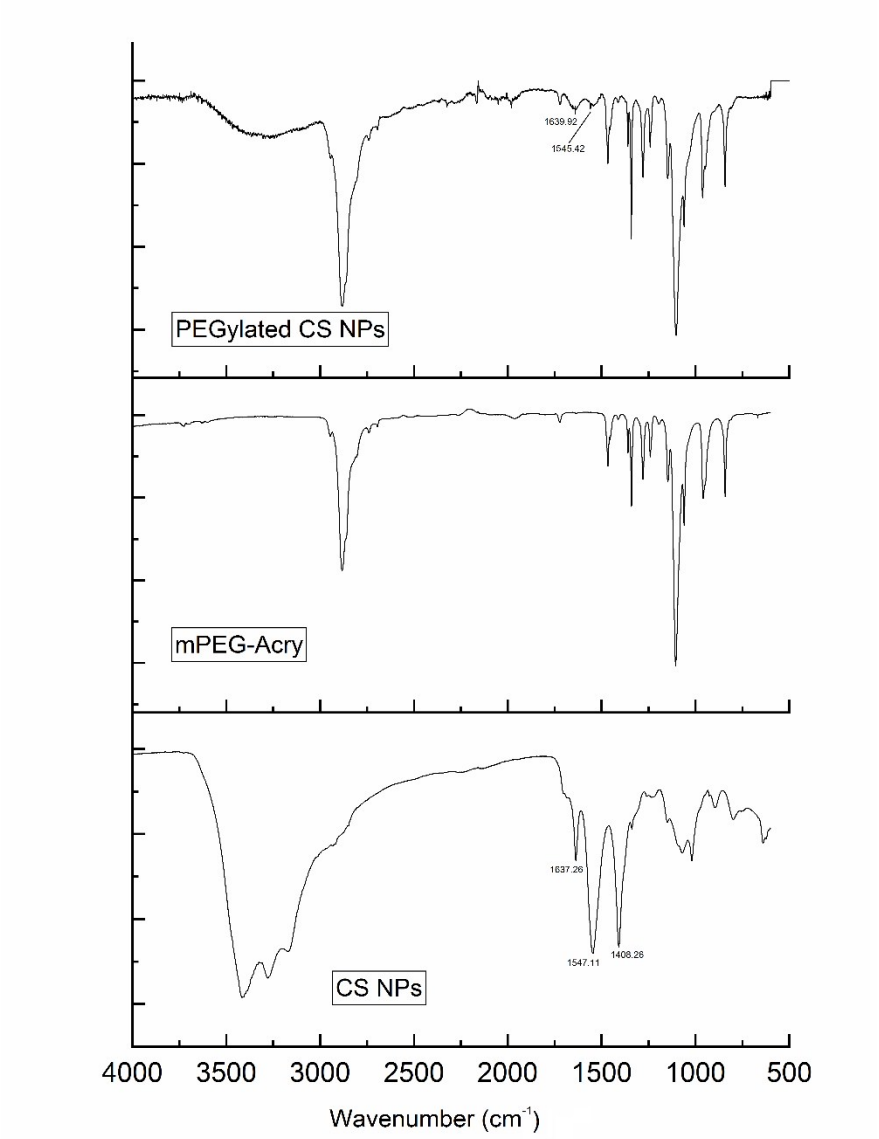


Figure S4. FTIR spectra of PEGylated chitosan.

After intense dialysis, PEGylated chitosan nanoparticles still retained characteristic PEG peaks such as C-H stretch around 2800 cm^{-1}. The both unmodified and PEGylated particles have characteristic amine stretch peaks around 1639 cm^{-1}.

SEM EXPERIMENTS

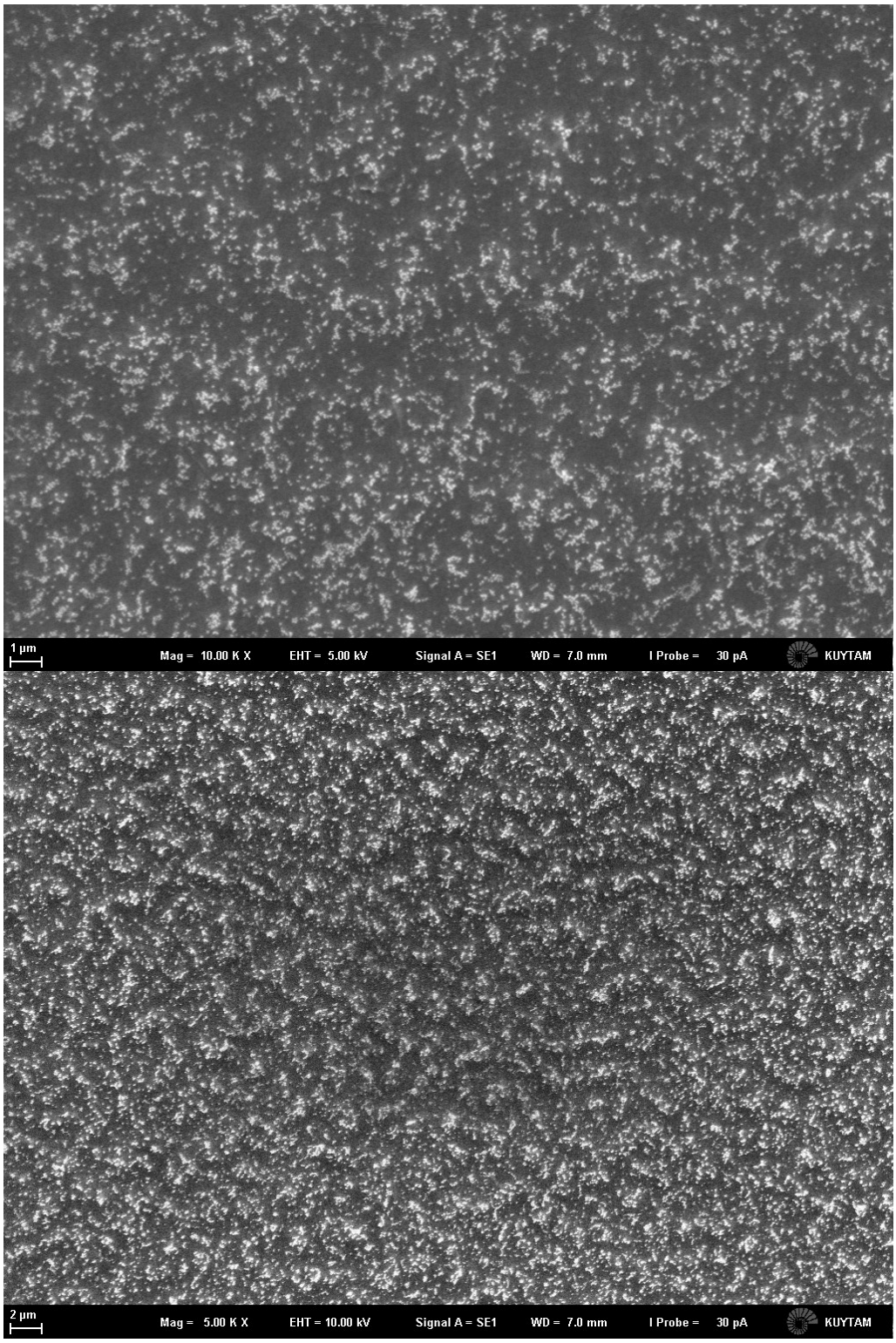
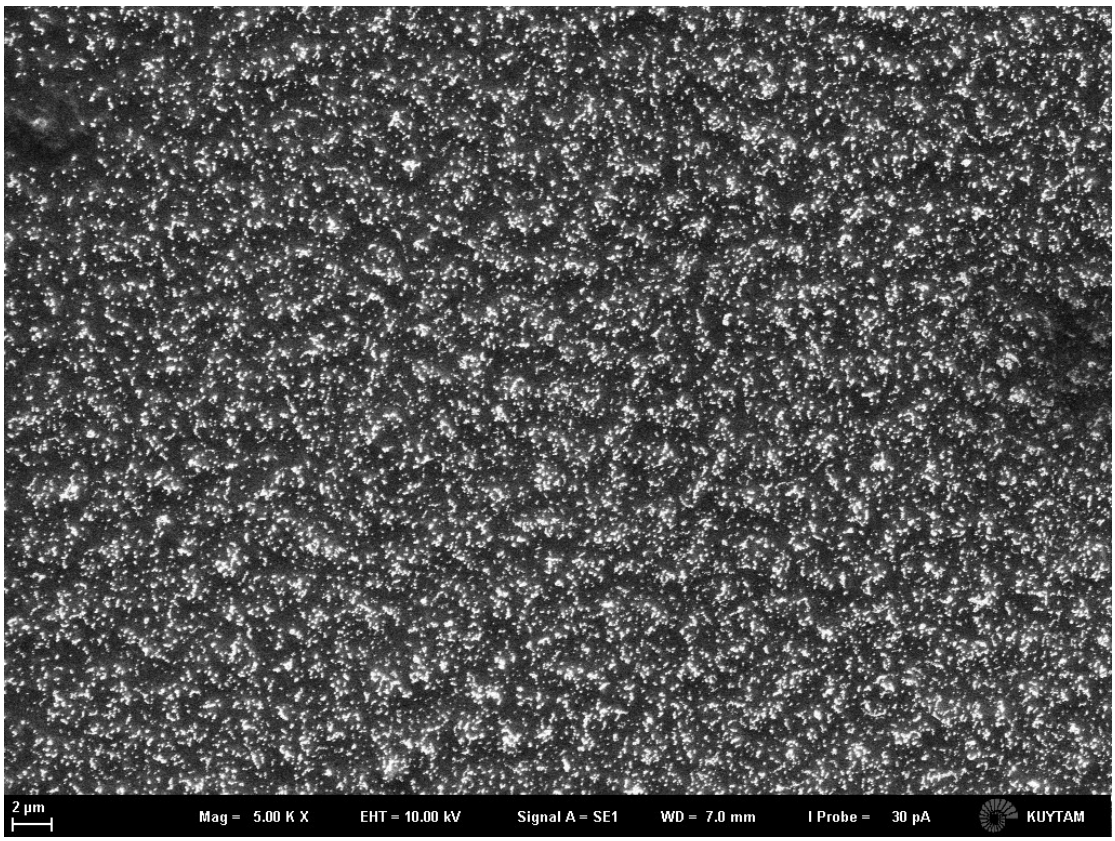


Figure S5. SEM images of 5 kDa PEG – 7 µmole nanoparticle - 1



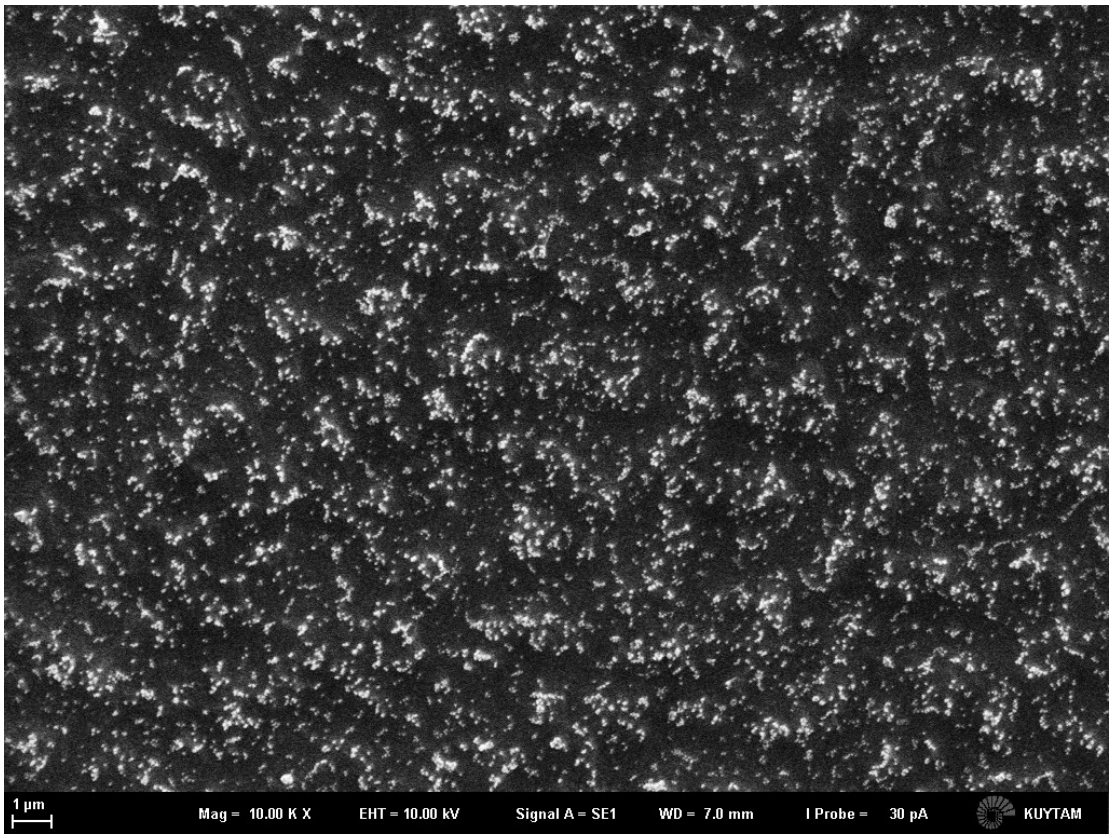


Figure S6. SEM images of 5 kDa PEG – 7 µmole nanoparticle - 2

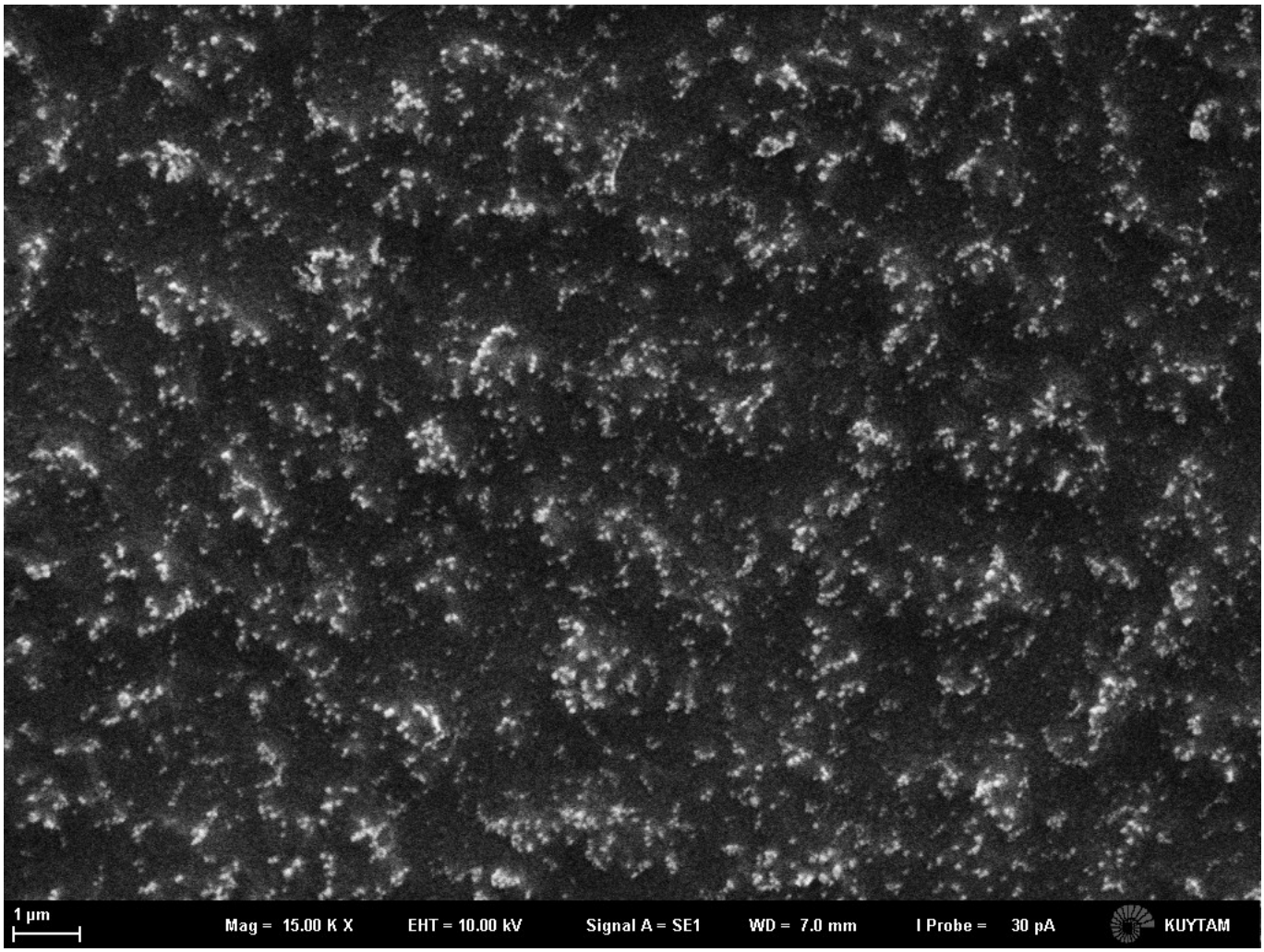


Figure S7. SEM images of 5 kDa PEG – 7 µmole nanoparticle - 3