Supporting Information

Design of Asymmetric Particles Containing a Charged Interior and a Neutral Surface Charge: Comparative Study on in vivo Circulation of Polyelectrolyte Microgels

Kai Chen, Jing Xu, J. Christopher Luft, Shaomin Tian, Jay S. Raval, Joseph M. DeSimone

1Department of Chemistry, 2Lineberger Comprehensive Cancer Center, 3Institute for Nanomedicine, 4School of Pharmacy, 5Department of Pathology and Laboratory Medicine, 6Institute for Advanced Materials, University of North Carolina, Chapel Hill, North Carolina 27599, United States

7Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina 27695, United States

8Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, United States
**Materials**

Triethylene glycol acrylate was synthesized according to previously published protocols\textsuperscript{1,2}. Pyridinium p-toluenesulfonate (PPTS), 2-hydroxyethyl acrylate (HEA), 2-methoxypropene, 2,3-dihydrofuran, t-butyldimethylchlorosilane, diethoxyacetophenone (DEAP), 2-aminoethyl acrylate (AEM), succinic anhydride, Dulbecco’s phosphate buffer saline (PBS) were all received from Sigma-Aldrich. Polyethylene glycol (MW=4,000 g/mol) diacrylate (PEG\textsubscript{4k}DA), methacryloxyethylthiocarbamoyl rhodamine B (PolyFluor\textsuperscript{®} 570) were from Polysciences, Inc. Sulfo-N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Fluorescein-NHS, and 2-(N-morpholino) ethanesulfonic acid (MES) buffer, were all received from Thermo Scientific. DyLight 680 was from Piercenet. Methoxy-PEG-NHS with MW of 2k, 5k, 30k were received from vendors including Creative PEGworks, Nanocs Inc. and Laysan Bio. (three mPEG-NHS from each company); fluorescein-PEG\textsubscript{2k}-NHS was received from Nanocs Inc, and all the PEG reagents were purified before use with procedures described later. All organic solvents and buffers were received from Fisher Scientific unless otherwise noted. Perfluropolyether molds were received from Liquidia Technologies.
Synthesis of ADA and 1

ADA and 1 were synthesized through the same reaction and purified from each other. In a typical procedure, 15 g (129 mmol) HEA with 1.5 g (6 mmol) pyridinium p-toluenesulfonate was dissolved in 50 mL anhydrous dichloromethane (DCM) in a 3-neck flask charged with nitrogen purge and a stir bar. The flask was kept submerged in an ice bath. Then 5 g (69.4 mmol) 2-methoxylpropene dissolved in another 50 mL anhydrous DCM was gradually added to the flask from an addition funnel during a 0.5 h course. The reaction was allowed for 4h. After the reaction, the DCM solution was washed with 6 wt% NaHCO3 solution 3 times (100 mL each time). To the washed DCM phase, anhydrous Na2SO4 was added to dehydrate. The solids were filtered, and then DCM was removed on a rotovap in vacuo with 100 ppm hydroquinone to obtain colorless oil (10.5 g). ADA was separated from the other product 1 in the oil using a silica column with elution comprising of 5 : 1 : 0.03 of hexane/ethyl acetate/triethyl amine. Triethyl amine was used to avoid degradation of acetal in the column because silica gel is slightly acidic.

When ADA was synthesized through reacting HEA with 2,2-dimethoxypropanone at elevated temperature as in previous work by others,3–5 we found that trace amount of ethylene glycol diacrylate (EGDA) always formed as a byproduct through transesterification of HEA.6 It was extremely difficult to separate EGDA from ADA since both molecules had very similar polarity. Even very low amount of EGDA present in the ADA product could act as permanent cross-linker to make the particles unable to
swell to desired size as we had tested; therefore this reaction was always carried out in low temperature to avoid formation of EGDA at elevated temperature.

The $^1$H and $^{13}$C NMR of ADA were shown in Figure S1. ESI-MS: m/z calculated for [M+Na]$^+$ : 295.1158, observed: 295.1154.

The $^1$H and $^{13}$C NMR of 1 were shown in Figure S2. ESI-MS: m/z calculated for [M+H]$^+$ : 189.1127, observed: 189.1116; m/z calculated for [M+Na]$^+$ : 211.0947, observed: 211.0932.
Figure S1. NMR of precursor of ADA in CDCl$_3$ measured at 25°C. (A) $^1$H NMR, (B) $^{13}$C NMR.
Figure S2. NMR of precursor of HEA 1 in CDCl₃ measured at 25 °C. (A) ¹H NMR, (B) ¹³C NMR.
**Synthesis of 2**

![Chemical Reaction](image)

Briefly, 5 g (43 mmol) HEA with 1.08 g (4.3 mmol) pyridinium p-toluenesulfonate was dissolved in 50 mL anhydrous dichloromethane (DCM) in a 3-neck flask charged with nitrogen purge and a stir bar. The flask was kept submerged in an ice bath. Then 4.82 g (68.8 mmol) 2, 3-dihydrofuran dissolved in another 30 mL anhydrous DCM was gradually added to the flask from an addition funnel during a 0.5 h course. The reaction was allowed for 4h. After the reaction, the DCM solution was washed with 5 wt% NaHCO$_3$ solution 3 times (100 mL each time). To the washed DCM phase, anhydrous Na$_2$SO$_4$ was added to dehydrate. The solids were filtered, and then DCM was removed on a rotovap *in vacuo* to obtain colorless oil (92 % yield). NMR of 2 is shown in Figure S3.

ESI-MS: m/z calculated for [M+Na]$^+$: 209.0790, observed: 209.0776.
Figure S3. NMR of precursor of HEA 2 in CDCl$_3$ measured at 25 °C. (A) $^1$H NMR, (B) $^{13}$C NMR.
Synthesis of $3^7$

Briefly, 5.8 g (50 mmol) HEA was dissolved in 200 mL THF in a 3-neck flask charged with nitrogen purge and a stir bar. Two equivalents of imidazole (6.8 g, 100 mmol) was added to the solution. After cooling to 0 °C by submerging the flask in an ice bath, t-butyldimethyldichlorosilane (7.53 g, 50 mmol) dissolved in 150 mL DCM was added slowly over a 50 min course. Reaction was allowed to proceed for 12 h. After reaction, the white salt of imidazole chloride was filtered (under pressure to remove salt quickly which is highly hygroscopic). The organic phase was collected and removed on a rotovap in vacuo to obtain crude oil. The product was separated on a silica column using the elution of 1 : 3 of ethyl acetate/petroleum ether followed by removal of solvents. Final yield of 86 %. NMR of $3$ shown in Figure S4.

ESI-MS: m/z calculated for [M+Na]$^+$: 253.1236, observed: 253.1238.
Figure S4. NMR of precursor of HEA 3 in CDCl$_3$ measured at 25 °C. (A) $^1$H NMR, (B) $^{13}$C NMR.
Synthesis of 4, 5, 6

Please refer to previous publication from our group for the details of synthesis and characterization.\(^8\)

Fabrication of Particle A using PRINT.

About 50 µL of the pre-particle formulation in Table S1 was applied to a PRINT mold using a pipette tip. A polyethylene terephthalate (PET) sheet was applied on top of the mold, and this assembly was passed through a small, non-heated laminator with pressure of 50 psi to wet the mold completely. After passing through, the assembly was subjected to another hot laminator set at 60 °C, 40 psi, and the PET sheet was peeled away at the nip point of the laminator, leaving the cavities filled while wicking away excess solution. The filled mold was immediately transferred into a nitrogen purged UV oven and cured with UV light (\(\lambda = 365\) nm, power = 20 mW/cm\(^2\)) for 3 min. After curing, the filled mold was placed face down on a thin film of 0.1% Plasdone (GAF) in water on top of another PET sheet. This assembly was placed in a cooler half-filled with dry ice, allowing the water to freeze and adhere to the particles. After freezing, the mold was peeled away from the particles trapped in the ice layer, and the ice was allowed to thaw. Particles and water were collected, then washed and concentrated via repeated centrifugation at 10,000 rpm (4×3 min each time) in PBS. The concentration of particle suspensions was determined by thermogravimetric analysis (TGA) on a Perkin Elmer Pyris TGA. Both the suspension and the supernatant after spinning down the particles were subjected to TGA at the same volume (20 µL) to obtain the dry weight. The difference in the weight divided by the volume was the concentration of that particle suspension.
Table S1. Formulation for Particle A in the PRINT process

| Component                              | Weight percent |
|----------------------------------------|----------------|
| Triethylene glycol acrylate            | 26-26.9        |
| 2-aminoethyl acrylate (AEM)            | 20             |
| PEG diacrylate (MW = 4000 Da)          | 2              |
| ADA                                    | 50             |
| 2,2-Diethoxyacetophenone               | 1              |
| Fluorescent Dye                        | 0.1-1          |
| Total                                  | 100            |

Mesh size Calculation

The mesh size in particle I is calculated this way:

According to Peppas’ theory, the rubber elasticity of hydrogels can be expressed as

$$
\tau = \frac{\rho RT}{M_e} \left(1 - \frac{2M_e}{M_n}\right)\left(\alpha - \frac{1}{\alpha^2}\right)\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3}
$$

(Equation 1)$^9$

In this expression, $\tau$ is the stress, $\rho$ is the density of the polymer (1.15 g/cm$^3$ as measured), $M_n$ ($= 233$ kDa according to result in our previous publication$^2$) is the molecular weight of linear polymer chains prepared using the same conditions in the absence of a crosslinking agent, $\alpha$ is the elongation ratio defined as the elongated length over the initial length of the sample and $v_{2,r}$ (approximately 1) is the volume fraction of the polymer in the dry state, while $v_{2,s}$ ($= 0.04$ as calculated from the swelling ratio of 30.4) is the volume fraction of the polymer in swollen state.
Figure S5. Stress-strain curve for hydrogel of I, and the replot of the curve into stress versus elongation factor.

At short deformations, a plot of stress versus elongation factor \((\alpha^{-1} / \alpha^2)\) will yield a straight line where the slope is inversely proportional to the molecular weight between crosslinks in the polymer network. We plotted the stress against the elongation factor for the hydrogel from the stress-strain curve obtained before where we measured the modulus of the hydrogel bulk sample (Figure S5), and calculated the \(M_c\) to be 68 kDa.

Based on values for the crosslinking density or molecular weight between crosslinks, the network pore size can be determined by calculating end-to-end distance of the swollen polymer chains between crosslinking points defined in the following equation.
\[ \xi = \alpha \left( \vec{r}_o \right)^{1/2} \]  \hspace{1cm} \text{(Equation 2)}

In this expression, \( \alpha \) is the elongation of the polymer chains in any direction and \( \left( \vec{r}_o \right)^{1/2} \) is the unperturbed end-to-end distance of the polymer chains between crosslinking points. Assuming isotropic swelling of the gels, and using the Flory characteristic ratio, \( C_n \), for calculation of the end-to-end distance, the pore size of a swollen polymeric network can be calculated using the following equation:

\[ \xi = \left( \frac{2 C_n \bar{M}_c}{M_o} \right)^{1/2} l \nu_{2,n}^{-1/3} \]  \hspace{1cm} \text{(Equation 3)}

Where, \( l \) is the length of the bond along the backbone chain (1.54 Å for vinyl polymers). Since the value of \( \bar{M}_c \) was calculated to be 68 kDa, \( M_o \) is the molecular weight of a repeating unit as 204 Da, we could calculated the mesh size to be 26.6 nm.

The mesh size of Particle A can be calculated using Equation 3 without need to calculate the molecular weight between crosslinks (\( \bar{M}_c \)), because the particles are highly crosslinked by the crosslinker ADA. Therefore, \( \bar{M}_c \) can be approximated as the molecular weight of ADA (272 Da). Based on the swelling ratio \( Q \) of 1.5, the average mesh size for Particle A can be calculated to be only 1.5 nm.

**Calculation of PEG size in aqueous solution**

Water is a good solvent for PEG, therefore the size of PEG can be calculated using:

\[ D = b N^{1/2} \]  \hspace{1cm} \text{(Equation 4)}^{10}

Where \( b \) is the Kuhn length (1.1 nm) for PEG and \( N \) is the Kuhn monomer number (each Kuhn monomer contains 3 repeating unit of ethylene glycol).

For PEG with MW of 30 kDa, \( N = 30k/44/3 = 227 \)

Therefore, the size is \( 1.1 \text{ nm} \times 227^{1/2} = 16.6 \text{ nm} \)

Similarly, the hydrodynamic size of PEG\(_{2k}\) is 4.3 nm.
Particle characterization

Particle dimensions were determined by analysis of images taken from a microscope mounted camera (Zeiss AxioCam MRm) using a 100× objective (Zeiss Axio Imager D.1M). Over 50 fully hydrated particles in PBS were measured for each case. The ζ-potentials for particles were measured on a Malvern nano-ZS zetasizer. The particle suspension was spun down and resuspended into 0.1X PBS for the measurement at a concentration of around 0.3 mg/mL. All the experiments were performed at 25 °C.

Particle Degradation

![Figure S6. FTIR of dried particle from as prepared Particle A and degraded A for 1, 2 and 24 h. The peaks at 1211 and 1098 cm⁻¹ are characteristic peak for acetal group.](image)

**Purification of mPEG-NHS**

Most commercial mPEG-NHS are synthesized by activation the carboxyl of mPEG-COOH using combination of DCC and NHS, or other similar activators. The problem of using DCC and NHS is that excess DCC and NHS can form a byproduct β-alanine. As tested in particle A, after reaction with unpurified mPEG-NHS, the particles could not
swell to the right size even after pH=3 degradation for a long time (Figure S7). This is probably due to presence of β-alanine in the commercial product, which acted as a short-chain crosslinker to tighten the particles. The same problem existed even for mPEG-NHS from other vendors (including Sigma, Polysciences, Nanocs). Once purified using our procedure, the problem was solved.

Figure S7. Degraded Particle A after PEGylation with unpurified (A) and purified (B) mPEG<sub>5k</sub>-SCM.

Herein, the byproduct was removed through selective precipitation of mPEG-NHS out of solution. For PEG of 5k Da and larger MW, about 100 mg mPEG-NHS was dissolved in 50 µL anhydrous acetonitrile, and then added with 1 mL anhydrous isopropanol (IPA). After 5 min, mPEG-NHS would precipitate out. For mPEG-NHS with MW of 2k Da, the PEG (∼ 100 mg) was dissolved in ∼ 500 µL IPA, and ultra-sonicated to dissolve completely. The solution was stored at -20 °C for 3 min and mPEG-NHS would precipitate out. The mPEG-NHS solids were spun down and the process was repeated another 3 times. IPA was later removed through solvent exchange with diethyl ether by centrifugation for 2 times. Finally, the solids were collected by decanting the ether and dried in vacuo to remove residual ether overnight. The purified mPEG-NHS was stored in a vial top-filled with nitrogen for future use if not used immediately.
**Quenching of surface charge (Particle A to B)**

Typically, 1 mg of particle A was suspended in 500 µL PBS. Then 6 mg of mPEG$_{2k}$-NHS in 50 µL acetonitrile was added to the particle suspension and reacted for 4 h on a shaker set at 1000 rpm at room temperature. After reaction, the particles were purified by repeated circles of centrifugation/washing 3 times with PBS.

**Swelling of particles through acetal degradation**

ADA cross-linked particles were spun down and re-suspended in acidic buffers with different pHs at a concentration of around 0.8 mg/mL. The suspensions were shaken at RT with aliquots taken out at different time and re-suspended in PBS for size measurement under microscope.

**Succinylation**

Particle C or E in 1X PBS was spun down and re-suspended in 10X PBS (to ensure constant pH even after hydrolysis of excess succinic anhydride, SA) to a concentration around 1 mg/mL. To 1 ml of the suspension, 4 mg SA dissolved in 50 µL acetonitrile was added and reacted for 2 h by shaking at RT. After that, the particles were spun down and re-suspended in 10X PBS, followed by addition of another 4 mg of SA in 50 µL acetonitrile and reaction of 1 h. Finally particles were purified by repeated centrifugation/washing circles for 4 times by 1X PBS.
Reason for quenching at tight particle state rather than directly quenching PRINT swollen particles

We initially tried to directly quench the surface charges of soft particles made from PRINT. For example, particle I, carboxyl-containing particles as we studied before,1,2 were tried to be PEGylated with mPEG$_{30k}$-amine using EDC/NHS chemistry; 30k Da is the largest MW available for commercial PEGs. However, even with this large MW, the PEG molecules could still diffuse into the interior of particles, as can be seen in Figure S8, the particles swelled even more from about 6 µm to 7.5 µm after PEGylation. Similarly for AEM containing soft particles directly made from PRINT, PEGylation also happened within interiors as the particles swelled more after PEGylation.

Figure S8. Fluorescent images of particle I before (A) and after PEGylation (B) with mPEG$_{30k}$-amine.
Reason for starting with AEM-containing tight particles rather than CEA-containing tight particles

When 2-carboxylethyl acrylate (CEA) was used instead of AEM in the formulation as in Table S1, we made tight particles containing carboxyls (Figure S9A). The particles could swell to about 5.5 µm after pH=3 treatment for 3h (Acidic buffer would deprotonate carboxyl, thus making particles even tighter at the very beginning; therefore it took longer time to completely swell for carboxyl-containing particles than AEM-containing particles). However, once first PEGylated by using EDC/NHS to conjugate mPEG-amine, then degraded, no matter for how long, the particles could not swell to the right size (Figure S9B). The reason is because of the many side reactions during activation of carboxyls. We even tried many other carboxyl activators including: TSTU, HATU, DMT-MM, EDC alone, with different polar solvents (DMF, ACN), however the problem remained. For carbodiimides, activated carboxyl could undergo the [O→N]-Acyl migration reaction to generate o-Acylisoureas, thus decreasing the available carboxyls because all these activators are small molecules and could migrate to the particle interior even cross-linked by ADA. Moreover, within the interior of particles, due to absence of PEG-amine because of the size exclusion, activated carboxyls could react with hydroxys found in triethylene glycol acrylate. Even when we tried to replace triethylene glycol acrylate by other non-hydroxyl monomers, this problem could still happen due to trace amount of hydrolyzed ADA, thus tightening the particles. Therefore, As long as carboxyls are present within particles, all the activation toward conjugation with amine-PEG would probably affect the final size, thus deformability due to these side reactions.
Figure S9. ADA crosslinked CEA containing particles at different stages. (A) as prepared; (B) after pH=3 treatment for 3 h; (C) PEGylated by mPEG$_{5k}$-amine using EDC/NHS; (D) pH=3 treatment for 18 h after PEGylation.

**Advantage of ADA over other HEA-based acid labile cross-linkers (4, 5, 6)**

As discussed, 5 or 6- cross-linked particles took longer time to swell to desire size. But for 4, the particles demonstrated premature swelling before PEGylation was generally carried out.

Figure S10. Fluorescent image of 4-crosslinked particles just harvested (A) and stored for 6 h (B) in PBS
**PEGylation of model particles using fluorescein-PEG\textsubscript{2k}-NHS (F-PEG-NHS)**

Commercial Fluorescein-PEG-NHS was made by reacting FITC to amine-PEG-COOH followed by conversion of COOH to NHS ester. Therefore, there may be remaining FITC in the final product. When unpurified product (even possibly under 5%) was used to react with dyeless model particles, fluorescence was seen all over the particles because FITC could diffuse in to react with interior amines. To purify, 1 mg F-PEG-NHS was dissolved in 50 µL PBS. The solution was loaded to a small column (Diameter: 0.5 cm, length: 5 cm) packed with Sephadex G-25. Elution with PBS could separate F-PEG-NHS from FITC as PEG would come out first. The collected solution of F-PEG-NHS was added with another 5 mg purified normal mPEG-NHS to 1 mg model particles for PEGylation which lasted 4 h by shaking at RT. After reaction, the particles were thoroughly washed by PBS 4 times with centrifugation. In a control experiment, the same moles of fluorescein-NHS was used to replace F-PEG-NHS for the reaction.

**Quantifying amine content within particles**

For swollen Particles C and E, we used a fluorescent assay to quantify the amine content. Both C and E were prepared starting from 1 mg of Particle A. To suspensions of Particle C or E in 500 µL PBS, 10 mg fluorescein-PEG\textsubscript{2k}-NHS was added and allowed to react for 12 h followed by purification. The purified particles were re-suspended and diluted to measure fluorescence (excited at 492 nm, emission at 518 nm). A standard curve of fluorescein-PEG-NHS at different dilutions was prepared. Based on the standard curve of fluorescein-PEG-NHS, the amount conjugated to the particles could be fitted. Based on a
1:1 stoichiometry, the amine content was calculated. Herein, fluorescein-PEG$_{2k}$-NHS was used rather than the small molecule fluorescein-NHS because otherwise particle E would stick to reaction vials and could not be spun down because of quenching of amines on the surface. The trace amount of possible FITC in fluorescein-PEG-NHS is unnecessary to remove because of the low percentage that would not affect the final result too much.

Figure S11. Standard curve for fluorescein-PEG$_{2k}$-NHS regarding fluorescence excited at 492 nm and emitted at 518 nm (3 readings averaged for each concentration). Starting from 1 mg dyeless particle A, the same numbers ($5 \times 10^{11}$) of particle C and E were prepared respectively. Both C and E were reacted with 10 mg fluorescein-PEG$_{2k}$-NHS for 12 h followed by thorough, repeated washing/centrifugation circles. The obtained particle suspensions were re-suspended in PBS to obtain concentration of $1 \times 10^{14} \text{particles/mL}$ for both particles. Then starting from this concentration, defined dilutions were made to fit into the linear range of the standard curve. Particle E reacted with mPEG$_{2k}$-NHS for 6 h (needed time to acquire near-neutral zeta-potential) was also measured for amine content using this method.
Normalizing particle numbers for macrophage uptake and \textit{in vivo} experiment

To better compare the performance of all the particles regarding their interaction with macrophages, and \textit{in vivo} circulation, we decided to dose the same numbers of particles rather than the same weight because the soft particles had similar shape, size, yet their individual weight vary more.

First of all, we could estimate the weight of a single particle A, knowing the dimensions of PRINT mold and the pre-mix density,

$$m_A = \rho \pi r^2 = \frac{1.15g}{cm^3} \times 0.6 \mu m \times 3.14 \times 1 \mu m^2 = 2.1 \mu g$$

Then, for a single well with $1 \times 10^5$ macrophages, the dose would be 400 particles per cell, as in weight of $2.1 \mu g \times 400 \times 10^5 = 80 \mu g$ for total Particle A dosed.

For the other particles, to ensure same numbers as 80 \mu g A, we could start with 8 mg Particle A to fabricate different particles and divide the final volume of each particle 100 times to obtain the same numbers of particles.

For animal studies, $10^7 \text{particles/g}$ mouse weight was dosed for the RBC-sized particles. The dose, in numbers of particles, equals to 0.4 mg of starting particle A for mouse of 20 g body weight.
**Cytotoxicity study**

HUVECs were seeded in 200 µL of media (HuMEC containing HuMEC supplement and bovine pituitary extract or MEM containing Earle’s salts and both supplemented with 10% fetal bovine serum) at a density of 5000 cells per well into a 96-well microtiter plate. Cells were allowed to adhere for 24 h and subsequently incubated with different particles at concentrations ranging from 1000 to 31.25 µg/mL for 24 h at 37 °C in a humidified 5 % CO₂ atmosphere. After the incubation period, all medium/particles were aspirated off cells. Cell viability was evaluated using the MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay according to the manufacturer’s instructions. The viability of the cells exposed to particles was expressed as a percentage of the viability of cells grown in the absence of particles.

**Macrophage association assay**

The Raw254.7 cells were cultured with Dulbecco Eagle media which was supplemented with 1% penicillin/streptavidin and 10% fetal bovine serum. Cells were grown in standard culture conditions (37 °C and 5 % CO2). The cells were seeded in 24 well plates at a concentration of $1 \times 10^5$ per well and left overnight in the incubator. On the next day, the media was replaced with fresh media warmed to 37 °C just prior to the experiment. The particle samples (80 µg in 1 mL water) were incubated with the cells for 4 and 24 h. Cells grown without any particles were used as control. At the set time points, cells were washed three times with 500 µL 1X PBS and detached by the addition of 1X trypsin/EDTA (300 µL) to each well. Following a 5 minute incubation (37 °C), 1X
DBPS/10 % FBS (500 µL) was added to each well and was mixed vigorously. This final solution was then transferred to a polypropylene tube and analyzed using flow cytometer.

**Intravital Microscopy**

Animal studies were carried out in accordance with an animal use protocol approved by the University of North Carolina Animal Care and Use Committee. The experiment was performed using an IV 100 laser scanning microscope (Olympus) on female Balb/c mice of 18 to 26-g body weight (Jackson Lab). Hair was removed from the ear and a tail vein catheter was applied. The mice were anesthetized with isofluorane and placed onto a heated stage (37 °C) in a prone position, with an ear immobilized by taping to an aluminum block (Figure S13). Vasculature was located by injection with a solution of 5 mg/mL rhodamine-B labeled 70k Da dextran (Invitrogen) in PBS and visualized by excitation with a 568 nm laser. A suspension of particles in PBS was then injected and visualized using a 633 nm laser. Imaging scans proceeded for 2 h, with an image taken every 5 s. We analyzed the region of interest containing vasculature for fluorescent signal in each scan and corrected for variation in laser intensity or autofluorescence by background correcting each image with the signal from a region of the scan that was free of vasculature. Harvested tissues including liver, lung, spleen, kidneys, heart were weighed, then imaged using an IVIS Kinetic fluorescence imaging system (Caliper Life Sciences) with excitation at 675 nm and emission measured at 720 nm (NIR dye). Blood was harvested via cardiac puncture and pipetted in 100 µL aliquots to black 96-well plates for analysis on the imager. The fluorescent signal for each tissue sample was background corrected by subtracting the signal from control tissues. Biodistribution
profiles for the particles at 2 h postdose were determined by percent of recovered fluorescence in the above tissues.

Figure S12. Cytotoxicity of different particles dosed to Raw264.7 cell line at 4 and 24 h.

Figure S13. Set-up for the IVM experiment.
**PEGylation density:**

Since Particle I published in our previous work\(^1\) could circulate a very long time even without PEGylation, we could conclude that a PEG surface is not necessary for highly deformable hydrogel particles as long as the zeta-potential is not too high. For our intended applications, more charged monomers in the particles were desired, which however would make the particles not circulating as long as seen for the control particles (Particle E, F). Therefore, we used the strategy reported herein to quench the surface charge of particles in order to obtain near zero zeta-potential while still retaining most of the internal charged groups.

Figure S14. Biodistribution of Particle I to major organs of mice (n=3).
Starting from 4.0 mg Particle A, the weight of Particle B was measured by TGA to be 5.2 mg. Therefore, 1.2 mg mPEG$_{2k}$ was reacted to 4.0 mg of Particle A. Our confocal study in Figure 3a showed that mPEG$_{2k}$ was confined close to the particle surface, and there was a diffusion depth of 260 nm measured from the linear scan. Because the calculated mesh size of Particle A was just an average size, there would be possibility of PEG$_{2k}$ infiltration (especially for lower MW PEG since 2k Da is just the number average MW). This diffusion depth could help ensure that charged groups close to the surface were efficiently quenched to give rise to near neutral zeta-potential.

Suppose all the PEG molecules were on the outer-most surface of particle B, we could calculate the PEG density as follows:

The weight of a single Particle A is 2.1 pg as calculated above (Page S23), with a surface area of

$$A = 2\pi r^2 + 2\pi rh = 2\pi \left(\frac{2.3 \ \mu m}{2}\right)^2 + 2\pi \times \frac{2.3 \ \mu m}{2} \times 0.8 \mu m = 14 \ \mu m^2$$

Therefore, the density of PEG on the surface of Particle B is:

$$\sigma = \frac{N \times \frac{1.2 \ mg}{2 \times 10^3 \ g/mol}}{\frac{4.0 \ mg}{2.1 \ pg} \times A} = \frac{6.02 \times 10^{23} / \ mol \times \frac{1.2 \ mg}{2 \times 10^3 \ g/mol}}{\frac{4.0 \ mg}{2.1 \ pg} \times 13.3 \ \mu m^2} = 12.2 \ \text{PEG/nm}^2 \ \ (N \ \text{is \ the \ Avogadro \ constant})$$

This PEG density is definitely way above the theoretical limit, also indicating PEG molecules were not just attached to the outer-most surface.

To estimate the real density of PEG on the surface of Particle B, we could assume that there were different layers of PEG brushes within the diffusion depth (D) of 260 nm, and each layer had the same thickness as the brush length.

The brush length would be

$$L = n\sigma^{1/3}b^{5/3}$$
In which \( n \) is the Kuhn monomer number in PEG\(_{2k}\), and \( n = \frac{2000 \text{ g/mol}}{44 \text{ g/mol} \times 3} = 15; \sigma \) is the PEG density, and \( b \) is the Kuhn monomer length of 1.1 nm.

At a diffusion depth of \( x \), the total surface area of the segmented layer would be:

\[
2\pi (r - x)^2 + 2\pi (r - x)(h - 2x)
\]

In which \( r \) is the radius of Particle B (disk-shape), as 1.07 \( \mu \)m; while \( h \) is the height of the disk, 0.8 \( \mu \)m.

Because \( r > h > x \), we could approximate the surface area by \( 2\pi r^2 + 2\pi rh \).

The number of such segmented layers would be: \( \frac{D}{L} \)

So, the total number of PEG chains in a single particle could be:

\[
\frac{D}{L} \cdot (2\pi r^2 + 2\pi rh) \cdot \sigma
\]

Which, equals \( \frac{1.2 \text{ mg}}{4.0 \text{ mg}} \cdot \frac{2 \times 10^3 \text{ g/mol}}{2 \times 10^2 \text{ g/mol}} = 1.8 \times 10^8 \)

Therefore we have:

\[
\frac{D}{n\sigma^{1/3} b^{5/3}} \cdot (2\pi r^2 + 2\pi rh) \cdot \sigma = \frac{6.02 \times 10^{23} \text{ mol} \times \frac{1.2 \text{ mg}}{4.0 \text{ mg}} \times \frac{2 \times 10^3 \text{ g/mol}}{2 \times 10^2 \text{ g/mol}}}{2.1 \text{ pg}} = 1.8 \times 10^8
\]

With the known values of \( n = 15 \), \( r = 1.07 \mu \)m, \( h = 0.8 \mu \)m, \( b = 1.1 \) nm, \( D = 260 \) nm, we could solve that the PEG density on surface of Particle B was

\[
\sigma = 0.31 \text{ PEG/nm}^2
\]

Because PEG diffused radially into the interior gradually, the PEG density would probably be even higher than this number.

In particle C, the total surface area increased from 14 \( \mu \)m\(^2\) to 75.7 \( \mu \)m\(^2\) because of swelling.

Therefore, the PEG density on Particle C would be \( \geq \frac{0.31}{75.7/14} = 0.057 \text{ PEG/nm}^2 \)

Similar PEG density would be present on Particle D.
For Particle A, suppose all the amine groups were reacted with mPEG_{2k}-NHS, the weight gain for 4 mg of Particle A would be:

\[
\frac{4.0 \text{ mg} \times 20\%}{166 \text{ g/mol}} \times 2 \times \frac{10^3 \text{ g}}{\text{mol}} = 9.6 \text{ mg}
\]

Therefore, theoretically we reserved most of the internal amines as \( 1 - \frac{1.2 \text{ mg}}{9.6 \text{ mg}} = 87.5 \% \), slightly higher than what we measured to be 72 %.

**Nucleic Acid Binding Capacity of Particle C.**

We first compared the binding capacity of Particle C to the Control particle, which is directly PEGylated version of Particle E. When mPEG_{2k}-NHS was used to PEGylate particle E directly under identical conditions and molality as that for Particle B to C, we obtained Control particle with a zeta-potential of -0.23 mV. To 100 µL of Particle C or Control particles (containing the same numbers of particles corresponding to 0.05 mg starting Particle A) suspended in PBS, 10 µL solution of cy3-DNA (25 µL/mL) was added to each and incubated at 37 °C for 10 min. The cy3-DNA is a cy3 dye-labeled 20 base DNA (5’TCCATGACGTTCTGATGCT3’). Before spinning down, the suspension of both particles were reddish because of the cy3 dye as shown in Figure S15A. However,
when spinning down at a centrifuge, as shown in Figure S15B, Particle C efficiently binded with all the DNA since the supernatant is colorless while Control particles could not as the supernatant remained very reddish. The difference confirmed that direct PEGylation would scavenge internal amines to reduce the binding capacity of cationic particles, while our strategy can help solve this problem.

Figure S15. Particle C and control incubated with cy3-DNA before (A) and after spinning down (B). The cy3-DNA used was a cy3 dye labeled 20 base DNA. There is no dye in the particle formulation; therefore the red pellet volume indicates the amount of particle-DNA complex.

To quantify the binding capacity of Particle C, we mixed the particles with varying amount of a 100 bp DNA, and used heparin to release the binded DNA which was quantified by a gel, shown in Figure S16. Specifically: for 0.1 mg particles, 0.005, 0.01, 0.02, 0.05 mg DNA were added and allowed to bind in PBS at 37 °C for 10 min. Then particles were spun down and the supernatants were taken out and reserved. The pellets were added with heparin solution to release DNA out. Particles were spun down again and the extracts were taken out for gel electrophoresis.
We can see that 0.1 mg particles could effectively bind DNA up to 0.02 mg (Lane 6), while for 0.05 mg added DNA, there was unbound DNA in the supernatant (Lane 7). From the band intensity, it can be estimated that the maximal binding capacity of 0.1 mg particles was around 0.025 mg DNA.

When we tried to use 10X PBS to replace heparin, the bound DNA could not be completely released out, confirming the strong interaction between the particles and oligonucleotides.

Figure S16. DNA (100 bp) binding capacity of Particle C. Lanes 1-3 are DNA standards (0.01, 0.04, 0.1 mg). Lane 4-7 are the supernatant of particles mixed with increasing amount of DNA (0.005, 0.01, 0.02, 0.05 mg). Lane 8-11 are the extracts by heparin corresponding to lane 4-7 respectively.

References

(1) Merkel, T. J.; Chen, K.; Jones, S. W.; Napier, M. E.; Zamboni, W. E.; Desimone, J. M. J. Control. Release 2012, 162, 37-44.

(2) Chen, K.; Merkel, T. J.; Pandya, A.; Napier, M. E.; Luft, J. C.; Daniel, W.; Sheiko, S.; Desimone, J. M. Biomacromolecules 2012, 13, 2748-59.

(3) Palmieri, F.; Adams, J.; Long, B.; Heath, W.; Tsiartas, P.; Willson, C. G. ACS Nano 2007, 1, 307-312.

(4) Cohen, J. a; Beaudette, T. T.; Cohen, J. L.; Broaders, K. E.; Bachelder, E. M.; Fréchet, J. M. J. Adv. Mater. 2010, 22, 3593-7.
(5) Heath, W. H.; Palmieri, F.; Adams, J. R.; Long, B. K.; Chute, J.; Holcombe, T. W.; Zieren, S.; Truitt, M. J.; White, J. L.; Willson, C. G. *Macromolecules* **2008**, *41*, 719-726.

(6) Gibas, M.; Korytkowska-Walach, A. *Polymer Bulletin* **2003**, *51*, 17-22.

(7) Yin, M.; Habicher, W. D.; Voit, B. *Polymer* **2005**, *46*, 3215-3222.

(8) Parrott, M. C.; Luft, J. C.; Byrne, J. D.; Fain, J. H.; Napier, M. E.; Desimone, J. M. *J. Am. Chem. Soc.* **2010**, *132*, 17928-32.

(9) Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. *Adv. Mater.* **2006**, *18*, 1345-1360.

(10) Rubinstein, M.; Colby, R. H. *Polymer Physics*; OUP Oxford, 2003; p. 456.

(11) Sun, L.; Yang, Z.-G.; Li, X. *J. Appl. Polym. Sci.* **2008**, *107*, 1842-1849.

(12) Wilchek, M.; Miron, T. *Biochemistry* **1987**, *26*, 2155-61.

(13) Han, S.-Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447-2467.

(14) Valeur, E.; Bradley, M. *Chem. Soc. Rev.* **2009**, *38*, 606-31.