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Tuning the Elasticity of Nanogels Improves Their Circulation Time by Evading Immune Cells

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MATERIALS

Hydroxy terminated, six arm, star-shaped poly (ethylene oxide-stat-propylene oxide) sPEG with a backbone consisting of 80 % ethylene oxide and 20 % propylene oxide (\( M_n = 3000\) g/mole & 12000 g/mole) was obtained from Dow Chemicals (Terneuzen, NL). sPEG was purified before further use by dissolving in tetrahydrofuran followed by precipitation in cold diethyl ether. 2,2′,2″-(10-(2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) ethyl) amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (maleimide-DOTA) was purchased from CheMatech. 4-(Dimethylamino)pyridine (DMAP) (TCI), Dithiothreitol (DTT) (abcr), Alexa FluorTM 488 C5 maleimide (3,6-diamino-9-[2-carboxy-4(or 5)-[[[5-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl) pentyl]amino]carbonyl]phenyl]-4,5-disulfo-, inner salt, monosodium salt) (Invitrogen) were used as received. Dialysis membranes with a molecular weight cut-off of 2 kDa and 50 kDa were purchased from Spectrum Laboratories, Inc. The \(^{68}\)Ge/\(^{68}\)Ga-generator was obtained from iThemba, South Africa using 0.6 M HCl as eluent, where the \(^{68}\)Ge is fixed on a modified tin dioxide column. The human monocyte cell line THP-1 was a kind gift from Dr. Inga Wessels (Institute for Immunology, Uniklinik Aachen). The rat pancreatic tumor cell line AR42J was obtained from ECACC. The cell culture medium RPMI 1640 and \( \beta \)-Mercaptoethanol 50 mM were purchased from Pan Biotech. Fetal bovine serum (FBS) was obtained from Biowest. Penicillin/Streptomycin and trypsin were obtained from Thermo Fischer Scientific. The any kD TGX gels, dual color protein marker and native sample buffer were purchased from Biorad. Amicon Ultra-0.5 Centrifugal Filter Unit with a molecular weight cut off of 50 kDa was purchased from Sigma Aldrich. Other chemicals and solvents of analytical grade were procured from Sigma and VWR chemicals, respectively, and used as received.

METHODS

**Synthesis of Thiol functionalized sPEG**

Thiol functionalized sPEG (sPEG-SH) was prepared in two steps using the previously established protocol for both the chosen molecular weights.\[^{1}\] Briefly, in the first step, sPEG was cross-linked with a disulfide cross-linker 3,3′-dithiodipropionic acid via Steglich esterification. In the second step, disulfide bonds were reduced to thiols using DTT followed by dialysis (MWCO 2 kDa; reg. cellulose) against water purged with nitrogen. The obtained pre-polymer was lyophilized and stored at -20°C under nitrogen.

**Functionalization of sPEG-SH with Alexa Fluor 488**

sPEG-SH (0.800 g, 0.066 mmol) and Alexa Fluor 488 maleimide (0.1 mg, 1.38 x 10⁻⁴ mmol) were reacted
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in a 5 mL degassed PBS (0.01M, pH 7.2) in dark for 2 h under nitrogen. The final product was obtained after dialysis (MWCO: 2 kDa reg. cellulose) against water purged with nitrogen and freeze-drying.

sPEG-SH (0.800 g, 0.266 mmol) was functionalized with Alexa Fluor 488 maleimide (0.4 mg, 5 x 10^{-4} mmol) according to the same procedure.

Synthesis of elastic Nanogels

NG was prepared via inverse miniemulsion technique.\cite{1} For the preparation of miniemulsion, 300 mg of surfactant in a 3:1 weight ratio of Span 80 and Tween 80 was dissolved in 10 mL of n-hexane. The aqueous phase consisted of 60 mg (5 x 10^{-3} mmol) of Alexa fluor 488 functionalized sPEG-SH in 140 µl 0.04 M PBS (pH 7.2). The organic phase was added to the aqueous phase in the ratio 10:1 and pre-emulsified by stirring for 10 min. After stirring, the system was ultrasonicated using a Branson sonifier (W250), at an amplitude of 10% for 120 s (Pulse time: 3s, Pause time: 0.2s) under the ice. Crosslinking of the NGs was initiated by the subsequent addition of 25 µl of 1M H_2O_2 in PBS followed by further sonication for 60 s. The reaction was allowed to proceed under constant stirring for 15-20 min at RT, followed by quenching of unreacted thiol groups by hydroxyl ethyl acrylate (HEA). Any further oxidation was stopped by the addition of acidic water (pH = 3). Separation of the NGs was achieved by centrifugation at 10,000 rpm for 10 min and washing the NG pellet with n-hexane twice and once with THF to remove surfactants and unreacted pre-polymers. The remaining organic solvents were removed by dialysis (MWCO: 50 kDa; reg. cellulose). Purified NG was stored in ultra-pure water at -20°C until further use. The above NG was labeled as Soft Reducible NGs. Hard Reducible NG (60 mg, 0.02 mmol) was synthesized according to the same procedure. Soft Non-reducible NGs were prepared according to the same procedure, except in the cross-linking step, PEG diacrylate (0.0072 g, 0.01 mmol) was used as a cross-linker.

For the radiolabeling purpose, the pre-polymer was pre-mixed with maleimide- DOTA in 0.04 M PBS (pH 7.2) for 15 min. Following this, the NG was prepared according to the above procedure. Three different NG were prepared in this study and were labeled as follows: Hard Reducible NG, Soft Reducible NG, and Soft Non-Reducible NG. Two sets of each were prepared – One set labeled with Alexa fluor 488 for in vitro studies and the other set with chelator for radiolabeling and in vivo studies.

The NG concentration was determined by freeze-drying a known NG solution volume and finally measuring its final freeze-dried mass.
Characterization of elastic NGs

Hydrogel storage modulus

Rheological measurements were conducted with a Discovery Hybrid Rheometer HR 3 (TA Instruments, New Castle, USA) using a 20 mm cone plate with a cone angle of 2°. A solvent trap was used to prevent solvent evaporation. The components of (3 kDa and 12 kDa sPEG-SH with different polymer volume fractions (15, 25, 35 and 45 wt%) and 1M H\textsubscript{2}O\textsubscript{2}) were mixed immediately prior to measurement. The measurements were performed in dependence on the time with a constant frequency of 1 Hz, an oscillation strain of 0.5 %, and a temperature of 37°C.

Raman spectroscopy

The NG synthesis was further confirmed by FT-Raman spectroscopy performed on a RFS 100/S FT-Raman spectrometer (Bruker Optik, Ettlingen, Germany). The laser used was Nd:YAG at 1064 nm wavelength at a power of 250 mW. On an average 1000 scans were taken at a resolution of 4 cm\textsuperscript{-1}. For sample holding aluminum pans of 2 mm bore was used.

Dynamic Light Scattering (DLS)

The hydrodynamic radius (R\textsubscript{h}) of the particles was determined in an ALV/CGS-3 compact goniometer system with ALV/LSE-5004 Tau Digital Correlator and a JDS Uniphase laser operating at 632.8 nm. Each measurement was repeated three times for an attenuation time of 180 sec. The NG solution was diluted in HPLC grade water and passed through a 5 µm poly (tetrafluoroethylene) membrane filter to remove dust. All measurements were made at an angle of 90°, at an attenuation time of 180s. Each measurement was run 3 times. For the Zeta potential measurements, a Malvern zeta sizer nano ZS was employed. The pre-polymer and NGs solution was diluted to a concentration of 1 mg.mL\textsuperscript{-1} in HPLC grade water (pH 5.5) and the measurements were carried out at 25°C. The measurements were performed for 3 runs (Maximum measurements of 100 for each run).

Cryo-Field Emission Scanning Electron Microscopy (Cryo-FESEM)

The morphology of NGs was investigated by FESEM with a HITACHI S-4800 (Hitachi, Tokyo, Japan) equipped with a Gatan Cryosystem Alto 2500 in a cryo mode. A drop of the NGs sample dispersed in water was fixed on a holder and rapidly frozen in liquid nitrogen and the NG samples were fractured in the cryo pre-chamber before transferring the sample to the FESEM. To remove the ice, the samples were sublimated at -90°C for 4 min.
Atomic force microscopy was measured with a Nano-Scope V from Veeco Instruments in tapping mode in the air at room temperature. ANCH-50 Pointprobe-Silicon SPM-Sensor from Nanoworld with a resonance frequency of 320 kHz and a force constant of 42 N/m was used as a cantilever. All data were analyzed with software Gwyddion 2.28. The average height and diameter of surface deposited particles was calculated extrapolating the height profiles of a pool of 20 particles for each sample. The profiles were then plotted using GraphPad Prism. For each NG characterized, 50 μL of a 0.1 mg.mL⁻¹ water dilution were spin coated at 2000 rpm, with an initial acceleration of 800 rpm, for 1 minute. We utilize Peak-Force and force-volume modalities to simultaneously map the NG morphology and probe the elastic modulus of the NG at the water/gold interface. The NG were immobilized on a gold-coated glass slide using the cysteine residues available in the hydrogel. Prior coating, each gold chip was treated with air plasma for 5 min at the pressure of 0.2 mbar for oxidation and stored in ethanol. Prior coating, each gold chip was dried using nitrogen flow. NG dilutions with a concentration of approximately 15 mg.mL⁻¹ were deposited by drop casting. The samples were incubated for 20 min, rinsed with MilliQ water, and dried by nitrogen flow.

AFM measurements were performed on a Dimension Icon AFM with a closed loop (Veeco Instruments Inc., software Nanoscope 9.4 (Bruker Corporation). Samples were allocated in a liquid cell filled with MilliQ water and characterized at Room Temperature. MSCT tips (Bruker Corporation) were activated by oxygen plasma treatment (PVA TePla plasma system 100) to increase their hydrophilicity and prevent the formation of air bubbles in their proximity. The E probe, with a nominal resonance frequency of 38 kHz in air and a nominal spring constant of 0.1 N/m of the cantilever, was used after calibration (k= 0.1 N/nm).

Effect of elastic nanogels \textit{in vitro}

The THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2.0 g.mL⁻¹ of Sodium bicarbonate and L-Glutamine. Complete culture medium was prepared by adding 10% fetal bovine serum (FBS), 100 U.mL⁻¹ penicillin, and 0.1 mg.mL⁻¹ streptomycin. Additionally, 0.05 mM β-mercaptoethanol was added to culture THP-1 cells. Stimulated THP-1 cells were obtained by treating the THP-1 cells with PMA in a complete culture medium. Cells were incubated at 37°C in a 5% CO₂ atmosphere.

\textit{Cell uptake by THP-1 and stimulated THP-1 cells}

Cellular uptake of NGs in THP-1 and stimulated THP-1 cells was investigated using flow cytometry. For the stimulated THP-1 cells, 1 x 10⁵ cells in 12 well-plate were treated with 100 ng.ml⁻¹ of PMA for 72h in
a complete growth medium. After 72h, the PMA containing media was removed, cells were washed twice with PBS, and allowed to recover for 24h in complete medium before NG treatment. Alexa fluor labeled NGs (0.5 mg.mL$^{-1}$) were added to the cells and incubated for 4h and 24h. At the end of the incubation period, the cells were washed twice with PBS, trypsinized, and analyzed by flow cytometry in the FITC channel (excitation: 488 nm, emission: 516 nm). FlowJo Version 10.7.1 was used to plot the histograms and determine the median fluorescence intensity (MFI).

To investigate the effect of actin polymerization on the uptake of hard and soft NGs, the stimulated THP-1 cells were pre-treated with 2 µg.mL$^{-1}$ CytD for 90 min, after which 0.5mg.mL$^{-1}$ NGs were added to the culture medium with or without CytD and incubated for 4h. NGs treated in the absence of CytD were considered as a positive control. Cells only treated with and without CytD were considered as a negative control. Following NG incubation, flow cytometry was performed according to the procedure mentioned above.

Transmission Electron Microscopy (TEM)

The internalized NGs (after 4h incubation) by stimulated THP-1 cells were additionally investigated using TEM. Samples were fixed in 3% glutaraldehyde (Science Services, Munich, Germany) for at least 4h, washed in 0.1 M Soerensen's phosphate buffer (Merck, Darmstadt, Germany), and post-fixed in 1% OsO$_4$ in 17% sucrose buffer. After fixation, the samples were rinsed in 17% sucrose buffer and deionized water and dehydrated by an ethanol series (30, 50, 70, 90 and 100%) for 10 min each and the last step thrice. The dehydrated specimens were incubated in propylene oxide (Science Services, Munich, Germany) for 30 min, in a mixture of Epon resin (Serva, Heidelberg, Germany) and propylene oxide (1:1) for 1h and finally in pure Epon for 1h. Epon polymerization was performed at 90°C for 2h. Ultrathin sections (70-100 nm) were cut with a diamond knife and picked up on Cu/Rh grids. Negative staining by uranyl acetate and lead citrate (all EMS, Munich, Germany) was performed to enhance TEM contrast. The specimens were viewed using a Hitachi TEM HT7800 electron microscope, operated at 100 kV.

Nanogel radiolabeling with Gallium-68 ($^{68}$Ga)

For radiolabeling, $^{68}$GaCl$_3$ was eluted with 0.6 M HCl in metal-free water from a $^{68}$Ge/$^{68}$Ga-generator. The purified NGs were labeled with $^{68}$Ga at T = 90°C at a pH of 5.0 by combining 0.5 mg NG in water with 3M ammonium acetate (1/2.5$^{th}$ the volume of $^{68}$GaCl$_3$). Finally, 120 MBq of $^{68}$GaCl$_3$ was added to the reaction mixture. After 10 min at T = 90°C, the mixture was cooled down to room temperature, and the radiolabeling efficiency was estimated using Radio-Thin Layer Chromatography (Radio-TLC, Elysa-Raytest GmbH, Germany). Briefly, the radiochemical yield was obtained with iTLC-silica gel strips.
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(instant TLC silica gel; Agilent, USA) as stationary phase and citrate buffer (pH 5.0) as mobile phase. The TLC was developed with a Radio-TLC-Scanner (mini-Gita) equipped with NaI-detector (Raytest). Unbound $^{68}$Ga was detected at the front, whereas $^{68}$Ga-NG remained at the origin. The radiolabeled NGs stability was studied in the presence of serum and PBS for up to 2h.

Native SDS PAGE/phosphor imager analysis

After radiolabeling of the nanogels, they were evaluated for purity by gel electrophoresis (native running conditions). The radiolabeled NGs were spun down using an Amicon Ultra-0.5 Centrifugal Filter Unit with a molecular weight cut-off of 50 kDa (reg. cellulose membrane). The concentrate and the filtrate were additionally evaluated for purity by gel electrophoresis. Here, the three radiolabeled samples (NGs before filtration, NGs after filtration, and the filtrate) were mixed (1:1 ratio) with a native sample buffer and loaded into any kD TGX gel (50 kBq). For visualization, the radioactively labeled samples were exposed for 14 h to phosphor screens and analyzed by Typhoon FLA 7000 phosphor imager (GE Healthcare). The urine samples collected from the mouse at the time of finalization was mixed directly in 1:1 ratio with a native sample buffer after which the procedure as stated above was followed.

In vivo PET/CT imaging of NG biodistribution

All animal experiments were approved by a German competent authority (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) for compliance with the Animal Protection Act, in conjunction with the regulation for the protection of animals used for experimental and other scientific purposes, Project Number 84-02.04.2016.A211. Female Balb/c CAnN.Cg-Foxn1nu/Crl mice were purchased from Charles River Laboratories and housed under a 12-h-light/12-h-dark cycle with free access to food and water. The room temperature and relative humidity were kept between 20–25°C and 45–65%, respectively.

The molecular imaging analyses were performed using a small animal PET/SPECT/CT system (i.e. Triumph® II, Northridge Tri-Modality Imaging, Inc., Chatsworth, CA, USA), however, only the PET and CT modalities were used for this study. The biodistribution of NGs was evaluated in a total of 9 mice (16-20 g). Under 1.5–2.5% isoflurane anesthesia in oxygen at 0.8 L/min, the lateral tail vein was injected with 120 µL of NaCl containing 10±3 MBq of $^{68}$Ga-labeled NGs (hard reducible NG, soft reducible NG, and soft non-reducible NG). At 1h and 4h after the tracer application, the mice were placed on the scanner bed and the CT scan was initiated. The exposure settings used were as follows: 130 µA, 75 kVp, 230 ms exposure time, and 360° rotation with 512 views. A dynamic 40 min PET scan was initiated at the end of the CT scan. The CT had an axial field of view of 91.1 mm and a PET one of 112 mm. During the...
scans, the isoflurane concentration was adapted to achieve a respiratory rate between 75–50 breaths per minute. At the end of the last scan, the mice were euthanized, and blood and the major organs (heart, lungs, liver, spleen, left kidney, colon, muscle) were harvested to quantify radioactivity accumulation. The organs were weighed to evaluate the radioactivity concentration in the gamma counter (PerkinElmer Inc.). The decay-corrected radioactivity was expressed as % of injected dose/gram tissue (% ID/g tissue), which was obtained by dividing tissue radioactivity by injected dose assuming tissue density as 1 g/cm³). The CT images were reconstructed using a Feldkamp filtered back-projection reconstruction process to a voxel size of 0.154 × 0.154 × 0.154 mm³ in a 592 × 592 × 560 matrix. Using vendor software, the CT values were converted into Hounsfield units (HU) using the following formula:

\[
HU = 1000 \times \left(\frac{\mu_t - \mu_w}{\mu_w}\right),
\]

where \(\mu_w\) is the linear attenuation coefficient of the water and \(\mu_t\) is the linear attenuation coefficient of the tissue. The PET data were reconstructed using a 3D ordered-subset expectation-maximization (i.e., OSEM-3D with three iterations and eight subsets) with a maximum a posteriori probability algorithm (30 iterations) into a 240 × 240 × 192 image matrix (resulting in final voxel dimensions of 0.25 × 0.25 × 0.597 mm³). PET normalization, CT attenuation correction, and CT scatter correction were applied to all of the PET reconstructions. The PET images were automatically aligned to the CT using a custom-made transformation in PMOD software package version 3.13 (PMOD Technologies LLC, Zürich, Switzerland) from a capillary phantom. The co-registered PET/CT images were further used for PET quantification.

For the heart, kidneys, liver, and bladder segmentation an initial mask was manually applied based on CT. Afterward, an automatic isocontour was generated using 2 kBq/cc as the minimal threshold. Upon completion of all volumes of interest, the average of all of the voxels (in kBq/cc) were recorded and further used for the quantification.

**Statistical analysis**

Statistics were computed with GraphPad Prism 9.1.0. If stated, t-test (Welch’s correction) and ANOVA were used to test for statistical significance between groups, with p values of <0.05, <0.01, <0.001 and <0.0001 denoted as *, **, *** , **** respectively. Values are represented as Means ± SD. If not stated, the statistics are not significant (n.s).
Dissipative particle dynamics simulations (DPD) were performed to study the interactions between the single NGs and a lipid bilayer. Such method has been widely used to study the uptake of nanoparticles of various compositions, forms and rigidity. Within the standard DPD framework, all the substances are represented in terms of spherical beads of equal mass $m$, whereas each bead usually comprises a group of atoms. The beads interact with each other by a pairwise additive force:

$$F_{ij} = \sum_{ij} \left( f_{R}^{ij} + f_{D}^{ij} + f_{c}^{ij} \right),$$  \hspace{1cm} (S2)

where $f_{c}^{ij}$ is a conservative force responsible for the repulsion via soft potential characterized by the parameter $a_{ij}$: the bigger the value of $a_{ij}$, the stronger the repulsion between the $i$th and $j$th beads. $f_{D}^{ij}$ and $f_{R}^{ij}$ are the dissipative and random forces, respectively, which serve as heat sink and source. The forces in the standard DPD framework are given by the following expressions:\[2b\]

$$f_{c}^{ij} = \begin{cases} a_{ij} (1 - r_{ij}^2) \bar{r}_{ij}, & r_{ij} < 1 \\ 0, & r_{ij} \geq 1 \end{cases}$$  \hspace{1cm} (S3)

$$f_{D}^{ij} = -\lambda \omega(r_{ij}) \bar{v}_{ij} \cdot \bar{r}_{ij}$$  \hspace{1cm} (S4)

$$f_{R}^{ij} = \sigma \omega(r_{ij}) \xi_{ij} \Delta t \bar{r}_{ij}$$  \hspace{1cm} (S5)

Here $\bar{r}_{ij} = (\bar{r}_{ij})_{/ij} \bar{r}_{ij}$ is the unit vector pointing from the $j$th to the $i$th bead, $\omega(r_{ij}) = (1 - r_{ij})$ is a weight function which turns to zero when $r_{ij} \geq 1$, $\bar{v}_{ij} = \bar{v}_i - \bar{v}_j$ is the relative velocity of the beads $i$ and $j$, $\xi_{ij}$ is a zero-mean normally distributed random variable and $\Delta t$ is a simulation timestep.

In the classic DPD approach, the momentum for each pair of beads is preserved. To satisfy the fluctuation-dissipation theorem, a relation $\sigma^2 = 2k_{B} T \lambda$ must be provided\[2b\] while the value of $\lambda$ is set to 4.5 for the decent rate of equilibration of the temperature. The evolution of the system is described by $N$ equations of motion expressed through the second Newton’s law, $m \frac{dv_i}{dt} = \bar{F}_i$. All quantities are measured in units of the mass of the bead, $m$, thermal energy, $k_{B} T$, and the cutoff radius of the interaction potential, $r_c$. For convenience, the quantities are fixed as $m = k_{B} T = r_c = 1$, so that the characteristic timescale is defined as $\tau = r_c (m/k_{B} T)^{1/2}$ and also equals 1.\[3a\] By setting the standard value of number density $\rho = 3$, the interaction parameters $a_{ij}$ (in units of $k_{B} T/r_c$) can be mapped onto the Flory-Huggins parameters $\chi_{ij}$ by a linear relation: \[4\]

$$a_{ij} \approx a_{ij} + 3.27 \chi_{ij},$$  \hspace{1cm} (S6)

where $a_{ij} = 25$ for any two beads of the same type.\[2b\]
For lipid molecules and nanogels the bond force between the connected beads is applied. Such force is described by the harmonic potential:

\[ E^b = \frac{1}{2} k_s (r - r_0)^2, \]

with specified spring constant \( k_s \) and equilibrium bond length \( r_0 \).

In addition, the angle potential is applied for applied on the adjacent three beads to ensure the stiffness of lipid tails (see below).\(^{[3a]}\) The potential has the following form:

\[ E^\theta = k_a (1 - \cos \theta), \]

with specified angle constant \( k_a \). Additional information can be found elsewhere.\(^{[2]}\)

**Simulation models**

The Shillcock and Lipowsky model\(^{[5]}\) was used as a model of lipids forming the bilayer. The lipid molecule is represented by three hydrophilic head particles and two hydrophobic tails each having four particles (Figure S5A). A flat bilayer obtained by self-assembly of such lipids in water medium is shown in Figure S5B (the conditions of self-assembly are discussed below). In turn, the nanogels were designed using a model of ideal diamond-like network.\(^{[4, 6]}\) In brief, fully stretched subchains of the same length were arranged and connected through tetrafunctional cross-linkers to form a frame of modified diamond unit cells. Each subchain consists of identical beads equidistantly located from each other while the beads themselves represent either the crosslinkers or the monomers. The positions of the cross-links in the constructed frame repeat the pattern of a diamond cubic crystal structure. The number \( N \) of the beads in the subchain (between two neighbor cross-links) was chosen as \( N = 5 \) for the model of hard nanogel and \( N = 10 \) for the model of soft nanogel, which approximately corresponds to the networks with 10\%, and 5\% of cross-links, respectively. To obtain the spherical particles, a sphere of certain radius was inscribed into the frame, and all the beads, which are outside the sphere, were cropped out. The total number of beads was 50063 and 35091 for the respective hard and soft nanogels. These numbers were selected in order to obtain the colloidal networks of the same size in a good solvent and thus to correlate the simulation studies with the experiment. The images of nanogels equilibrated in solution are presented in Figures S5C, D.

**Interaction parameters, length and time scales**

The simulation systems contain four types of beads: water (W), lipid heads (H), lipid tails (T) and nanogel segments (N). As it was already mentioned, the interaction parameters for two beads of the same type
was set as $a_{ij} = 25$. Since the lipid heads are hydrophilic and the tails are hydrophobic, we chose the following values:\cite{3c, 3d, 5} $a_{HW} = 25$, $a_{HT} = a_{TW} = a_{TN} = 100$. To simulate the PEG-water interactions, the $a_{NW}$ parameter was set as 26.3.\cite{3c, 3d} To mimic the effective attraction between the lipid heads and nanogel segments, the value of $a_{HN}$ was set as 10. Here we assume that the attraction is a result of smeared electrostatic interactions between positively charged lipid heads and negative charges on the nanogel surface. The whole set of interaction parameters is done in Table S1.

Table S1. Interaction parameters between all types of DPD beads.

| Type         | W (water) | N (nanogel) | H (lipid head) | T (lipid tail) |
|--------------|-----------|-------------|----------------|----------------|
| W (water)    | 25        | 26.3        | 25             | 100            |
| N (nanogel)  | 25        | 10          | 100            | 100            |
| H (lipid head)|           | 25          | 100            | 100            |
| T (lipid tail)|           | 25          |                |                |

Finally, for bond and angle potentials, the following coefficients were chosen:\cite{3a} $k_s = 64$, $r_0 = 0.5r_c$, $k_A = 10$.

Along with the interaction parameters, the spatial and temporal mappings should be also done. In our simulations each water bead represents four water molecules, which gives $r_c \approx 0.71$ nm according to the conventional coarse-graining procedure in DPD.\cite{3d, 6} Simultaneously, the thickness of flat bilayer $d$ appeared to be $\sim 5r_c$ which gives $d \approx 3.55$ nm which is very close to the thickness of a membrane formed by dipalmitoylphosphatidylcholine (DPPC) phospholipids.\cite{5} By matching the in-plane diffusion coefficient of DPPC bilayer from experiments (around $5 \mu m^2/s$) and the diffusion coefficient of simulated membrane (around $0.015\ r_c^2/\tau$), we can yield the time unit as $\tau = 1.5$ ns.

**Simulation systems**

All simulations were performed using the LAMMPS software\cite{7} in a huge box with dimensions $L_x \times L_y \times L_z = 88.04$ nm $\times 88.04$ nm $\times 56.8$ nm with periodic boundary conditions in all directions. The simulation time step was set as $\Delta t = 0.02\tau = 30$ ps. Initially, a bilayer was self-assembled during $60\ \mu s$ in water. The total number of lipids was chosen as 23040 so that the area per lipid equals to $A_{PL} = 1.3$. With such amount of molecules an almost tensionless membrane was obtained.\cite{3a, 5} Then, a single nanogel was placed in a close contact with a bilayer (from 1 to 10 beads). The hydrodynamic radii $R_H$ of both hard and soft particles in the solution was estimated through the radial density profiles\cite{8} as $R_H \sim 15$ nm. In order to keep the membrane surface under zero tension, the N-varied DPD method was applied.\cite{3c, 9} In brief, the
lipids were inserted or deleted into a boundary region of a bilayer surrounding its central region. The $A_{PL}$ in the boundary region is kept in a certain range from $A_{PL}^{\text{min}} = 1.21$ to $A_{PL}^{\text{max}} = 1.41$. When $A_{PL} > A_{PL}^{\text{max}}$ the new lipid molecules are inserted into the region. Oppositely, if the area per lipid is lower than $A_{PL}^{\text{min}}$, a few lipid molecules are deleted randomly from the boundary region. Simultaneously, a corresponding number of water beads is randomly added or deleted to keep the whole density of the beads in the simulation box constant. The value of $A_{PL}$ is checked every 2000 time steps allowing to propagate the membrane tension to the whole membrane. The total simulation time was $340 \mu s$.

**Calculation of wrapping ratio**

The wrapping ratio is a general characteristic in computer simulations, which allows quantitative determination of the different interaction regimes between the lipid bilayers and various nanoparticles. In our simulations this parameter is defined as the NGs’ surface area in contact with the membrane to the total NGs’ surface area. In addition, we calculated the NGs' asphericity $\delta$ which is defined through the eigen values of the gyration tensor. Here the proximity to zero indicates the spherical shape, whereas the proximity to unity corresponds to the case of a 1D rod. In turn, the proximity to the value of 0.25 corresponds to the case of a 2D disk. The values of $\delta$ as functions of time are shown in Figure S6B. As can be seen, for soft NG $\delta$ is already equals to 0.2 at $t = 3 \mu s$, and this value increases slowly to 0.24 afterwards. Meanwhile, for hard NG $\delta$ does not exceed the value of 0.12 at $t = 3 \mu s$ and then continuously decreases during endocytosis.

**Figure S1:** Characterization of soft non-reducible NGs. A) Storage modulus of the hydrogel cross-linked via Michael addition using PEG-diacrylate. B) Confirmation of soft non-reducible NG formation by raman spectroscopy depicting the thioester band at 672 cm$^{-1}$. 

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*A Note*: The image of the figure is not included in the text. The combined text is detailed enough to understand the context and content without requiring visual reference.
**Figure S2:** Deformability of the NGs in wet state. A) Average length and height profile plotted for hard reducible NG. B) Average length and height profile plotted for soft reducible NG.

**Figure S3:** Stimulation of THP-1 cells using PMA for in vitro studies. A) Flow cytometry histogram depicting an increased side scatter upon THP-1 stimulation. B) MFI of THP-1 and stimulated THP-1 cells.
Figure S4: Nanogel uptake by stimulated THP-1 cells investigated by TEM. A) Internalization of hard reducible NG (i) Magnified image of the internalized nanogels and B) Soft reducible NG (i) Magnified image of the internalized nanogels. Scale bar: 3 µm and for magnified images: 0.5 µm. The white arrow indicates internalized NG. C) Quantification of number of NGs internalized obtained from ImageJ by counting an average of 10 images. D) Length (Diameter) of the nanogels from all the images using ImageJ software (The length was measured in 3 directions each: Vertical, horizontal, and diagonal). From all the obtained values, 15 values from each were randomly chosen and plotted.
**Figure S5**: A, B) The model of single lipid molecule A) and the snapshot of simulated flat bilayer B); C, D) equilibrium structures of simulated hard C) and soft D) nanogels.

**Figure S6**: A) The contacts fraction (the ratio between number of contacting NGs’ segments to the total number of NGs’ segments) and asphericities $\delta$ B) as functions of simulation time.
Figure S7: Top-view and bottom-view snapshots of hard nanogel on the lipid membrane at different simulation times.
Figure S8: Top-view snapshots of soft nanogel on the lipid membrane at different simulation times.

Figure S9: Side-view snapshots of hard nanogel after the endocytosis: (a) with all NG segments; (b) cross section through the NG’s center of mass.
Figure S10: A) Radio-TLC of different NGs. Radiolabeled NGs remain at the origin and unbound $^{68}$Ga was detected at the front. Depending upon the length of the silica gel strip used, unbound $^{68}$Ga is detected between 70 mm-95 mm. B) Schematic depicting the process of radiometal labeling. A bifunctional chelator (NOTA, DOTA) is covalently coupled to the thiolated pre-polymer. A solution of $^{68}$GaCl$_3$ is added to the construct to achieve radiolabeled NGs.

Figure S11: Characterization of radiolabeled hard reducible NG. A) Concentration-dependent increase in radiolabeling yield of hard reducible NG. B) Stability of hard reducible NG in serum and PBS for 120 minutes.
Figure S12: Average activity (KBq/cc) of Liver obtained by drawing regions of interest (ROIs) on the whole body images. One-way ANOVA was used for statistical analysis.

Figure S13: A) SDS-PAGE/Phosphor imager analysis of soft reducible NGs before and after ultracentrifugation using 50 kDa filtrate. B) SDS-PAGE of urine analysis at the time of finalization.

Ethical Statement

All animal experiments were approved by a German competent authority (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) for compliance with the Animal Protection Act, in conjunction with the regulation for the protection of animals used for experimental and other scientific purposes. (File Number 84-02.04.2016.A211)
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