Characterization of a library of vitamin A- functionalized polymethacrylate-based nanoparticles for siRNA delivery

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ABSTRACT

A 60-membered library of vitamin A-functionalized P(MMA-*stat*-DMAEMA)-*b*-PPEGMA block copolymers was synthesized by RAFT polymerization. Subsequently, retinoic acid was coupled to hydroxyl groups present in the hydrophilic PPEGMA block. The polymers were investigated for their ability to encapsulate ribonucleic acids through nanoparticle (NP) formulation using the emulsion/solvent evaporation method. The localization of vitamin A in surface-near regions of the NPs was indicated by surface enhanced Raman spectroscopy, and the interaction of the NPs with a retinol binding protein was investigated by analytical ultracentrifugation. The systematic analysis of the NP library in terms of the encapsulation efficiency of the ribonucleic acids, the toxicity of the NPs, and the cellular uptake helped identifying suitable candidates for cellular internalization studies. The cell uptake was investigated by flow cytometry and fluorescence microscopy and reveals structure dependent uptake behavior of the examined particles.

TOC

INTRODUCTION

Liver fibrosis and liver cirrhosis caused more than two million deaths in 2010.¹ The most common causes of liver disease are infections with the human hepatitis B and C virus as well as alcohol abuse.²⁻⁴ Under physiologic conditions, hepatic stellate cells (HSCs) fulfil their function of storing vitamin A as retinyl esters into cytoplasmic lipid droplets and are the main source of hepatic collagen.⁵ During inflammation, HSCs are activated and produce an excess of collagen, also known as extracellular matrix (ECM), and proinflammatory and profibrogenic factors, e.g., cytokines, and lead to an impaired exchange of substances across the sinusoid and ultimately liver failure.⁶ One way to prevent the deposition of excessive ECM is to reduce the expression of ECM coding mRNAs or to induce chaperones critical for the correct folding and export of ECM.⁷⁻
¹⁰ However, a treatment of liver fibrosis in the living organisms with unprotected or unconjugated RNAs is not efficient because of its degradation by RNases and limited transfection efficacy.¹¹ Nanomedicines such as liposomes, polyplexes or polymeric nanoparticles (NPs) are common strategies to deliver ribonucleic acids. Targeting strategies may guide the nanomaterials to the

specific site of desired action. In liver fibrosis, activated HSC are known to overexpress certain receptors such as synaptophysin, the insulin growth factor 2 receptor, or the Retinol binding protein receptor 2 (RBPR).¹² Therefore, we aim to use retinol as targeting motif to develop NPs that deliver RNA to activated HSC. The functionalization of polymers with retinol is facile, efficient and well defined in comparison to, *e.g.*, surface modification of NPs with peptides or antibodies.¹³ However, there are only few studies that depict the targeting ability for HSC using retinol-modified NPs, *i.e.*, liposomal formulations, retinol-modified poly(ethylene imine), and a poly(methacrylate) based block copolymer.¹⁴⁻¹⁶ Promising transfection *in vitro* and fast body clearance *in vivo* have been shown for cationic nanogel particles¹⁷ without any targeting ligand.

In order to design polymeric NPs as carrier material for oligo-ribonucleotides such as small interfering RNA (siRNA), a cationic charge is required for efficient encapsulation, complexation and protection under desired physiological conditions. The cationic charge may further be useful for the intracellular release because of a solubility shift at lower pH values within the endosome. However, cationic charge also comes along with toxicity issues, thus it is important to find a suitable balance between encapsulation efficiency, endosomal release and cell damage.¹⁸ To prevent an immediate immune response triggered by the positively charged particle, the stealth polymer poly(ethylene glycol) (PEG)¹⁹ can be attached to the polymer backbone. The functionalization of PEG with retinoic acid (RA) is further envisioned to locate the targeting unit RA in the outer regions of the formulated NPs for better interaction with receptors or proteins. The optimization of the NPs with respect to *in vitro* and *in vivo* performance could be accomplished in a polymer library approach. For this, the reversible addition-fragmentation chain transfer (RAFT) polymerization represents a well-known tool to synthesize a larger number of well-defined polymers (#60) with tailored properties using different monomeric precursors, *e.g.* methacrylates,

in a straightforward way.^{20, 21} There are ample studies on polymeric NPs based on poly(methacrylate)s for delivery purposes of, *e.g.*, plasmid DNA, siRNA or the prime small molecule anticancer drug doxorubicin.^{17, 18, 22} For example, the commercially available Eudragit® E100, consisting of methyl methacrylate (MMA), *n*-butyl methacrylate (BMA) and *N,N*-dimethylaminoethyl methacrylate (DMAEMA), which is only slightly cytotoxic at appropriate concentrations, has also been used in gene delivery.^{22, 23}

Herein, we present a library approach based on block copolymers, *i.e.* poly(methacrylate)s, that vary systematically in their contents of the stealth constituent poly(ethylene glycol) (PEG) and the cationic functionality (based on DMAEMA) to identify the best candidates for an efficient RNA delivery to HSC. The polymers and NPs are characterized also in terms of their surface characteristics to proof the functionalization also after formulation and the accessibility of retinol moieties on the particle surfaces for a subsequent selective targeting of HSC.

EXPERIMENTAL PART

Materials

Methyl methacrylate (MMA, stabilized, Sigma Aldrich, 99%), (2-dimethylaminoethyl) methacrylate (DMAEMA, stabilized, Sigma Aldrich, 98%), poly(ethylene glycol) methacrylate $M_n = 360 \text{ g mol}^{-1}$ (PEGMA₃₆₀, stabilized, Sigma Aldrich) and poly(ethylene glycol) methacrylate $M_n = 500 \text{ g mol}^{-1}$ (PEGMA₅₀₀, stabilized, Sigma Aldrich) were used after removing the inhibiting monomethyl ether hydroquinone (MEHG) using the inhibitor remover for removing hydroquinone and monomethyl ether hydroquinone (Sigma Aldrich). 2,2'-Azobis(2-methylpropionitrile) (AIBN, Sigma Aldrich, 98%), 2-cyano-2-propyl benzodithioate (CPDB, Strem Chemicals, 97%), 1,3,5-trioxane (Sigma Aldrich, 99%), *all-trans*-retinoic acid (RA, TCI, 93%), *N,N'*-

dicyclohexylcarbodiimide (DCC, Sigma Aldrich, 99%), 4-(dimethylamino)pyridine (DMAP, Sigma Aldrich, 98%), anhydrous dimethyl sulfoxide (DMSO, Sigma Aldrich, 99.9%), poly(vinyl alcohol) (Mowiol 4-88, Sigma Aldrich), tRNA (from brewer's yeast, Sigma Aldrich), siRNA and labeled siRNA (Eurofins Genomics) were used as received. Bio-Beads (S-X1, Bio Rad) were utilized according to the manufacturer's protocol using freshly distilled tetrahydrofuran (THF) as the eluent. The anhydrous solvents dichloromethane (CH_2Cl_2) and THF were dried in a solvent purification system prior to use (SPS, Pure solv. EN, InnovativeTechnology). All other solvents were purchased from standard suppliers and were used without any further purification. Agarose (HR-Plus), ethidium bromide solution (1%) and glycine were purchased from Carl Roth. DNA-Ladder (100 BP) and Gel Loading Buffer (6x Green) were purchased from Jena Bioscience. Heparin sodium salt was purchased from Alfa Aesar. RNase A (10 mg/mL) and RNase free water (UltraPure™) were purchased from Thermo Fisher Scientific and Bromophenol Blue from ACROS Organics™. Glycerol, TRIS, acetate-, citrate-, and phosphate buffer were purchased from Sigma-Aldrich. Coomassie Brilliant Blue R-250 solution was purchased from G Biosciences. Pre-casts PAGE gels (Mini-PROTEAN 4-15%) were purchased from Bio Rad. Hydrochloric acid (AnalaR® NORMAPUR®) was purchased from VWR. The retinol-binding protein (RBP) was purchased from MyBioSource, Inc. The bovine serum albumin (BSA, 98%) was purchased from Sigma Aldrich. The 0.01 M phosphate buffered saline (PBS) and the buffer solution (pH 7.4) were purchased from Sigma Aldrich.

Methods

Polymer synthesis

Synthesis of the hydrophobic core building block via RAFT polymerization (C1 to C13)

MMA was copolymerized with different amounts of DMAEMA using CPDB as chain transfer agent (CTA), AIBN as initiator and 1,3,5-trioxane as internal standard for the determination of conversion, see SI **Equation S1**, in dimethylformamide (DMF). The ratio of CTA to initiator ($[CTA]/[I]$) was 4. The polymerizations were performed at 70 °C for 8 h with a monomer concentration of 2 M. Different ratios of the monomers to the CTA ($[M]/[CTA]$) were selected, as indicated in the SI **Table S1**. The general procedure of the RAFT polymerization was as follows: Stock solutions of CPDB and AIBN in DMF were prepared. MMA and DMAEMA were added to a suitable reaction vessel. The corresponding volume of dissolved AIBN and CPDB were added from the respective stock solutions. The reaction solution was diluted with DMF to adjust the total monomer concentration to 2 M. 10 mg of trioxane were added to the reaction mixture and dissolved while stirring. The reaction vessel was closed and purged for 20 min with argon. An initial sample (t_o) of the reaction mixture was taken to enable determination of the monomer conversions, and the vial was heated for 8 h at 70 °C. The polymerization was stopped by opening the vial and cooling to room temperature. A second sample (t_{end}) was taken prior to purification. For purification of polymers with DMAEMA contents of $\leq 20\%$, the reaction mixture was precipitated twice in a large excess of cold diethyl ether (-80 °C) and collected by filtration. The resulting pink colored powder was dried under vacuum at 40 °C. For purification of polymers with DMAEMA contents of $\geq 30\%$, the solvent was removed by evaporation under reduced pressure. The residue was diluted with $CHCl_3$ and precipitated twice in a large excess of cold *n*-hexane (-80 °C) and collected by filtration. The resulting pink colored powder was dried under vacuum at 40 °C.

The polymers were characterized regarding their apparent average molar masses (M_n) and dispersity ($\mathcal{D}$) by SEC and 1H NMR spectroscopy (SI, **Table S4**). The overall monomer conversion

(%_{conv}) was calculated from the resulting signal integrals of the vinyl protons of the monomers in the ¹H NMR spectra of the t_0 and the t_{end} samples related to the integrals of trioxane as internal standard, see SI **Equation S1** to **S4**.

Block copolymer synthesis comprising a hydrophilic block via RAFT polymerization (P1 to P30)

Two PEGMAs with different molar masses (PEGMA₃₆₀ and PEGMA₅₀₀) were polymerized in various amounts using the synthesized copolymers **C1** to **C13** consisting of MMA and DMAEMA as macro chain transfer agents (macroCTA), AIBN as the initiator and 1,3,5-trioxane in DMF as internal standard. The [M]/[macroCTA] ratio was adjusted to 10 or 20, and the [macroCTA]/[I] ratio was adjusted to 4. Detailed amounts utilized are summarized in **Table S2** of the SI. The polymerizations were performed at 70 °C for 4 h at a monomer concentration of 0.15 M. The general RAFT polymerization procedure and the determination of the conversion were performed as described above. Polymers with a DMAEMA content ≤20% were purified by precipitation of the reaction mixture (twice) in a large excess of cold diethyl ether (-80 °C) and collected by filtration. The resulting pink colored powder was dried under vacuum at 40 °C. For the purification of polymers with DMAEMA contents of ≥30% and PEGMA₃₆₀, the solvent of the reaction mixture was removed by evaporation at reduced pressure by evaporation. The resulting residue was diluted with CHCl₃, precipitated twice in a large excess of cold *n*-hexane (-80 °C) and collected by filtration. The resulting pink colored powder was dried under high vacuum at 40 °C. Polymers with DMAEMA content ≥30% and PEGMA₅₀₀ were purified as follows: The solvent of the reaction mixture was removed by evaporation at reduced pressure. The residue was diluted with CHCl₃ and precipitated twice in a large excess of cold *n*-hexane and collected by filtration, dissolved in THF and purified *via* gel permeation chromatography using BioBeads S-X1 in THF to remove the unreacted monomer. The volatiles of the desired fractions were removed by

evaporation at reduced pressure. The resulting oil was diluted with CHCl_3 , precipitated once in a large excess of cold *n*-hexane ($-80\text{ }^\circ\text{C}$) and collected by filtration. The resulting pink colored powder was dried under vacuum at $40\text{ }^\circ\text{C}$.

The calculation of $\%_{\text{conv}}$, and molar masses, and the characterization of all polymers *via* SEC and $^1\text{H-NMR}$ spectroscopy was performed as for the macroCTA (*vide supra*), Equation S5. The characteristics of the polymers with the hydrophilic block are summarized in SI **Table S5** of the SI.

*Procedure of the coupling of RA (**P1*** to **P30***)*

All block copolymers **P1** to **P30** were functionalized with RA using DCC and DMAP. Therefore, 1 eq. of PEGMA of the corresponding block copolymer, 1.5 eq. (or 0.75 eq.) of RA and 1.8 eq (or 0.9 eq.) of DMAP were added to a reaction vessel and purged for 10 min with argon. 470 eq. of anhydrous CH_2Cl_2 and 94 eq were added to dissolve all compounds. In **Table S3** the used amounts of the modifications are summarized. The reaction mixture was cooled to $0\text{ }^\circ\text{C}$ and 1.8 eq. (resp. 0.9 eq.) of DCC were added. After stirring for 10 min at $0\text{ }^\circ\text{C}$, the reaction mixture was allowed to reach room temperature and stirred for 18 h in the dark. The resulting yellow to orange colored suspension was filtered and the solid remainder was discarded. For the purification of polymers with a DMAEMA content $\leq 20\%$, the reaction mixture was precipitated twice in a large excess of cold diethyl ether and the product was collected by filtration. The resulting yellow colored powder was dried under vacuum at $40\text{ }^\circ\text{C}$. For the purification of polymers with DMAEMA contents of $\geq 30\%$, the solvent of the reaction mixture was removed by evaporation under reduced pressure. The resulting residue was diluted with CHCl_3 , precipitated twice in a large excess of cold *n*-hexane ($-80\text{ }^\circ\text{C}$), collected by filtration, dissolved in THF and purified *via* gel permeation chromatography

using BioBeads S-X1 in THF to remove the free RA. Volatiles from the desired fractions were removed by evaporation at reduced pressure. The resulting oil was diluted with CHCl_3 and precipitated once in a large excess of cold *n*-hexane ($-80\text{ }^\circ\text{C}$) and collected by filtration. The resulting yellow colored powder was dried under high vacuum at $40\text{ }^\circ\text{C}$.

Polymer characterization

Proton nuclear magnetic resonance (^1H NMR) spectra were measured in deuterated chloroform (CDCl_3) at room temperature on a 300 MHz Avance I spectrometer from Bruker. All chemical shifts are given in ppm in reference to the residual solvent signal of chloroform at 7.26. For the size exclusion chromatography (SEC) measurements, a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, a RID-10A refractive index detector, and a PSSSDV-linear S column ($5\text{ }\mu\text{m}$ particle size, Polymer Standards Service GmbH, Mainz, Germany) at $40\text{ }^\circ\text{C}$ was utilized. Chloroform (CHCl_3), triethylamine and 2-propanol at a ratio of 94:4:2 was used as the eluent at a flow rate of 1 mL min^{-1} . SEC-UV measurements were performed with an Agilent 1200 series system, including a PSS degasser, a G1310A pump, a Techlab oven ($40\text{ }^\circ\text{C}$), a wolfram Vis-lamp (Agilent), a deuterium UV-lamp (analytics shop) and a G1315D detector (Agilent). The UV-Vis intensity was measured from 300 to 500 nm and recorded at each elution volume. A solution of 0.21 wt% LiCl (Fisher Scientific) in dimethylacetamide (DMAc, VWR) was used as eluent at a flow rate of 1 mL min^{-1} . A PSS Gram 30 and a PSS Gram 1000 column (Polymer Standards Service GmbH, Mainz, Germany) placed in series served as the column combination. To determine the apparent molar masses and dispersity values $\bar{D}$, a calibration with PMMA standards (Individual Standards Kit, 410 to 88,000 g/mol, Polymer Standards Service GmbH, Mainz, Germany) was performed on both SEC systems. UV measurements for quantification of the RA content were done in 96 well plates made of polypropylene (VWR) using the Infinite

M200 Pro Platereader from Tecan Group Ltd. (Switzerland). The NP formulations were performed using the ultrasonic processor UP2000St (Hielscher, Teltow, Germany). The Zetasizer Nano ZS from Malvern Instruments (Herrenberg, Germany) was used for dynamic light scattering (DLS) in UV cuvettes consisting of polystyrene (Brand). The DLS was operating with a He-Ne laser at a wavelength of $\lambda = 633$ nm. The measurements were detected at an angle of 173° , and the size distributions of the NPs were calculated applying the nonlinear least-squares fitting mode. The hydrodynamic diameter was approximated as the effective (z-average) diameter and the width of the distribution as the polydispersity index of the NPs (PDI) obtained by the cumulants method assuming a spherical shape.

Determination of the degree of functionalization of RA (DF_{RA})

RA dissolved in DMSO was used as the calibration standard for the establishment of a calibration function *via* linear regression. Small amounts of RA functionalized and non-functionalized polymers were dissolved in DMSO. The samples were all measured at the absorption maximum of RA, *i.e.* at $\lambda = 360$ nm in triplicate. The DF_{RA} of each polymer was calculated using equation S6 shown in the SI.

NP formulation

The polymers (10 mg) were dissolved in 500 μ L of ethyl acetate and 50 μ L of water or cargo in water (tRNA, Cy3-siRNA) were added and emulsified using a sonicator tip (100 W, 10 sec). Subsequently, 1 mL of 3 wt% aqueous PVA solution was added, and the solutions were sonicated again for 10 sec. The resulting emulsion was poured into 4 mL deionized water (or RNase free water for ribonucleic acid entrapment) and stirred overnight to evaporate the ethyl acetate. The

volume of the resulting suspension was adjusted to 2.5 mL (final NP concentrations of $c_{NP} = 2 \text{ mg/mL}$). The samples were stored at $-20 \text{ }^{\circ}\text{C}$ in the freezer.

tRNA quantification

The QuantiFluor[®] RNA System (Promega) was used according to the manufacturer's protocol for low standard curves. The NP samples were added without any treatment or dilution. The QuantiFluor[®] dye is binding free RNA and gives a high fluorescence signal that can be quantified by a calibration curve (Ex.: 492 nm, det.: 540 nm). The measurements were done in triplicate.

Stability tests for NPs

The NPs' pH responsiveness was tested *via* DLS by dilution in different buffers. Therefore, the count rate was monitored over time at a fixed attenuator setting and scattering position. A decreasing overall count rate indicates NP degradation with decreasing scattering events caused by the originally present NPs. The results are shown in **Table S5** of the SI. Degradation experiments with RNase A are explained in detail in the SI (**Tables S10 - 12, Figure S12**).

Surface enhanced Raman scattering (SERS) measurements

Ag NP decorated glass substrates were fabricated by microwave synthesis as previously described.²⁴ The fabricated SERS substrates were cleaned by sonication in deionized water and ethanol for 10 min each. The Ar dried substrates were stored in an Ar atmosphere. Directly before usage, the SERS substrates were further cleaned in an Ar plasma for 5 min. For each measurement freshly cleaned SERS substrates were used and 1 to 5 μL of the diluted (1:10) stock solutions were added. The reference sample was retinoic acid diluted in ethyl acetate. Due to fast evaporation, these reference measurements were carried out under ambient conditions. All other measurements

were conducted in the liquid state. To avoid fast evaporation, a humidity chamber was used for these measurements.

SERS spectra were acquired with a spectrograph (Acton Advanced SP 2750A, Princeton Instruments, USA) equipped with a Peltier-cooled CCD camera (Pixis 256, Princeton Instruments, USA). The laser excitation wavelength was 532 nm. The laser was focused, and the Raman signal was collected with the same NA 1.45 (60×) oil immersion objective (UPLAPO60XOHR, Olympus, Japan). The Raman signal is separated from the back-reflected excitation light by means of an edge filter (532 US LPF, Iridian, Canada). All SERS spectra were acquired with an excitation power of 97 μ W. The acquisition time per spectrum was 1 s and an average of 30 accumulated spectra were utilized, respectively. All presented SERS Raman spectra show the average of these accumulations, are baseline corrected with an asymmetric least square smoothing and normalized to the maximum intensity of the C=C stretching mode (1575 cm^{-1}).

AUC investigations

The RBP was dissolved in a 0.01 M PBS buffer solution. All sedimentation velocity experiments were performed using a ProteomeLab XL-I AUC (Beckman Coulter Instruments, Brea, CA) with an An-50 Ti eight-hole rotor at 20 °C. The optical solution path length of utilized ultracentrifuge cells equipped with Epon centerpieces was 12 mm. The reference sectors of the AUC cells were filled with ca. 440 μ L of a solution mixture of the exact same volume ratio of ultrapure water, freshly acquired from a Thermo Scientific Barnsted GenPure x CAD Plus water purification system (Thermo Electron LED GmbH, Langenselbold, Germany) and a 0.01 M PBS buffer solution (pH = 7.4) as present in the sample. All NP samples were diluted with 0.01 M PBS buffer solution. For AUC measurements, solutions of **P20** (0.5 mg mL^{-1}), **P20** (0.5 mg mL^{-1}) plus RBP

(0.28 mg mL⁻¹), **P20*** (0.55 mg mL⁻¹), **P20*** (0.12 mg mL⁻¹) plus RBP (0.21 mg mL⁻¹), **P24*** (0.54 mg mL⁻¹), **P24*** (0.5 mg mL⁻¹) plus RBP (0.29 mg mL⁻¹) were prepared. Ca. 420 µL of the diluted sample solution were filled in the sample sector. Rotor position eight was used as the counterbalance, also enabling the absorbance optical module calibration. Scans were acquired in 7.30 min time intervals and by the multi-detection, i.e. by universal refractive index (RI) detection and the absorbance optical detection system in terms of OD at wavelengths of $\lambda = 280$ nm and $\lambda = 350$ nm. The rotor velocity was 7,500 rpm in all cases. Sedimentation velocity scans were recorded for 24 h. **P24*** was analyzed in a further AUC experiment at the same settings. The obtained data were numerically evaluated with SEDFIT and the $ls - g^*(s)$ model by using least squares boundary modelling considering non-diffusing species.²⁵ In instances, rotor velocity was increased to 42,000 rpm.

AFM imaging

Atomic Force microscopy (AFM) was carried out with a Nanowizard 3 NanoOptics system (JPK Instruments AG, Germany). All AFM images were recorded in intermittent mode using the tip scanning option. Si cantilever probes with a resonance frequency of 190 kHz and a force constant of ~ 30 N m⁻¹ (TAP190, Budget sensors, Bulgaria) were used. Typically, an oscillation amplitude of ~ 30 nm was chosen, and the setpoint was set to 80% of the free oscillation. Scan velocities of 1 Hz were applied to image the NPs. All topography images were post-processed to correct for sample tilting by application of standard plane and line flattening procedures, disregarding the NP area.

TEM imaging

Cryo-TEM images were acquired on a FEI Tecnai G² 20 TEM. Samples were prepared on Quantifoil grids (Quantifoil R2/2), which were hydrophilized prior to use by a 2 min Ar plasma treatment. 8.5 μ L of the NP suspension were blotted onto the grid and plunged into liquid ethane, which served as the cryogen. The vitrification was performed with a Vitrobot Mark IV. After vitrification, the grids were transferred to the cryo-holder (Gatan 626) utilizing a Gatan cryo transfer stage and were measured at an acceleration voltage of 200 kV. Samples were maintained at a temperature below -175 °C for all steps after the vitrification process. Images were acquired with a CCD camera system (Olympus Soft Imaging Systems, Megaview G2, 1376 $\times$ 1024 pixels).

Cell culture

Mouse embryonic fibroblasts (MEFs) were cultured in cell culture flasks at 37 °C, 5% CO₂ (HeraCell CO₂ Incubator, Heraeus, Germany) and water vapor supplemented atmosphere. For the cultivation, Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Germany), 100 IU streptomycin, 100 IU penicillin (Gibco, Thermo Fisher Scientific, Germany) and 1 wt% stable glutamine (GlutaMax, Gibco, Thermo Fisher Scientific, Germany) were used. Cells were passaged when confluency reached 75% by pipetting. For further evaluation, the cells were plated in different well plates (Sarstedt, Germany) to perform the experiments.

Cytotoxicity assay

MEFs were incubated with different NPs (diluted to a concentration of 50 μ g mL⁻¹, 100 μ g mL⁻¹, 250 μ g mL⁻¹ and 500 μ g mL⁻¹) in growth media without fetal bovine serum (FBS) and antibiotics for 24 h. The supernatant was taken and lactate dehydrogenase (LDH) activity was assessed as a marker for membrane rupture and biocompatibility using the CytoTox96 Non-Radioactive

Cytotoxicity Assay (Promega, Germany). The absorbance of the formazan product from the conversion of the tetrazolium salt was quantified on a multi-plate reader (EnSpire Multimode Plate Reader, Perkin Elmer, USA) at 490 nm (bandwidth 5 nm). A 100% positive control cell lysis was used to determine the maximum amount of LDH present. The rate of lysis was calculated by subtracting the medium background from each value and relativizes the absorbance values of the treated cells to that of the positive control. Experiments were performed three times in triplicates.

NP uptake in mouse embryonic fibroblasts

MEFs cultivated in 24 well-plates as stated above were incubated with the NPs (diluted to a resulting siRNA concentration of 200 nM) in serum free medium at 37 °C in a humidified atmosphere. After 24 h, MEFs were detached in PBS (Lonza, Switzerland) containing 5 mmol L⁻¹ EDTA (Thermo Fisher Scientific, Germany). The uptake was then quantified by means of the Cy3 fluorescence intensity using flow cytometry (BD Accuri C6 Plus, BD Bioscience, Germany) (approximately 10,000 cells per sample). A correction factor was applied to the raw-data correcting for the differences in Cy3-loading. The experiments were performed independently three times in duplicates.

Imaging of NP uptake in mouse embryonic fibroblasts

MEFs were cultivated as stated above and incubated for 1 hour with the NPs (diluted to a resulting siRNA concentration of 200 nmol/L) in serum free medium at 37 °C in a humidified atmosphere. Fluorescence images were taken with a Laser scanning microscope (Leica SP8, Falcon, Mannheim, Germany), equipped with a 63x oil immersion objective and a confocal detector in epi direction, capable of single photon counting. The excitation laser (white light laser, Leica, Germany) was operated at a wavelength of 540 nm and the emission was recorded in a wavelength

range of 555 to 701 nm (**Figure 7**) or 550 to 650 nm in intervals of 5 nm (**Figure 8**). Precise acquisition parameters for every image are given in the SI. Z-resolved measurements were deconvoluted by Leica's Lightning algorithm using the 'adaptive' preset, in order to improve the spatial resolution.

Experimental data in the supporting information (SI)

Further tables, description and detailed data for polymer synthesis and polymer/NP characterization including NMR- and Raman spectroscopy, SEC, SERS, AUC, gel retardation assays, PAGE, fluorescence microscopy.

RESULTS

Polymer synthesis

A library of polymers based on methacrylates was synthesized in a two-step RAFT polymerization with subsequent post-polymerization modification to introduce retinoyl functionalities as the desired targeting moiety (**Scheme 1**). To that end, the three monomers MMA, DMAEMA and PEGMA were used to obtain well-defined block copolymers. The statistical copolymerization of MMA and DMAEMA was used to introduce cationic charges into the hydrophobic building block to form the core of the NPs that prospectively interact with the RNA for efficient loading. Chain extension with PEGMA was intended to form the hydrophilic shell of the NP while maintaining insolubility in water. In addition, the pendant hydroxyl groups in the PEGMA block provided the possibility to introduce RA as a targeting moiety to be present in the NP shell.

Scheme 1: Schematic representation of the synthetic route towards RA-functionalized block copolymers. I) Synthesis of P(MMA-*stat*-DMAEMA) (**C1 – C13**) via RAFT polymerization ($x = 50$ to 100%, $y = 0$ to 50%, $z = 92$ to 131). II) Chain extension with a hydrophilic block via RAFT polymerization of PEGMA (**P1 – P30**) ($t = 4$ to 18, $w = 6$ or 9). III) Esterification with RA (**P1* – P30***) ($u = 71$ to 96%, $v = 4$ to 29%).

To obtain hydrophobic blocks comprising different amounts of cationic charges, a series of P(MMA-*stat*-DMAEMA) with varied DMAEMA fractions of 0, 2, 6, 9, 20, 30, 40 and 50% were synthesized in DMF using AIBN as initiator and CPDB as the chain transfer agent. Relatively low molar masses below 14,000 g mol⁻¹ were targeted, because of the known toxicity of polycations at increased molar masses.²⁷ The reactions were quenched at moderate monomer conversions between 60% and 90% to ensure preservation of the dithiobenzoate ω -end groups (**Table S1**). It is known that the RAFT polymerization of the two monomers results in random copolymers.²⁸ The amount of incorporated DMAEMA monomers affects the pK_a value of the polymers and should, hence, influence also the desired interaction with the ribonucleic acids.²⁹ ¹H-NMR spectroscopy confirmed that the targeted comonomer ratios were reflected by the composition of the purified copolymers (**Figure 1**).²⁶ To that end, the fraction of MMA and DMAEMA was estimated by

integration of the methyl ester group originating from the MMA and the methylene proton signal adjacent to the ester moiety of the DMAEMA repeating units in the polymers.

SEC measurements showed a trend toward increased apparent molar masses for copolymers with higher DMAEMA content but a similar overall DP, which has to be critically evaluated due to the PMMA calibration of the SEC system (**Table S4**). However, M_n values from SEC were in reasonable agreement with the theoretically expected M_n values estimated from the monomer feed and conversion values. All polymers featured unimodal molar mass distributions with dispersities ($\bar{D}$) ranging from 1.19 to 1.36 from the SEC measurements (**Figure S1-S3**).

The different P(MMA-*stat*-DMAEMA) were hence used as macromolecular chain transfer agents (macroCTA) to polymerize PEGMA by using AIBN as the radical initiator. In order to determine the optimal stealth- and targeting effect through influencing the thickness and density of the to be created NP shell,^{30, 31} two PEGMAs with different molar masses, *i.e.* PEGMA₃₆₀ and PEGMA₅₀₀ were used at two [PEGMA]:[macroCTA]:[I] ratios, *i.e.* 20:1:0.25 and 10:1:0.25, respectively (**Table S2**). Aiming towards nanoparticle dispersions, a low DP of the PPEGMA blocks was targeted to circumvent micelle formation of the carrier material.

Figure 1: ^1H -NMR spectra (CDCl₃, 298 K, 300 MHz) of **C2**, **C9**, **P20** and **P20*** including assignments of the signals to the schematic representation of the polymer structures.

SEC analysis of the resulting P(MMA-*stat*-DMAEMA)-*b*-PPEGMA copolymers revealed clearly shifted molar mass distributions toward larger apparent molar masses, confirming the successful use of **C1-C13** as the macroCTA (**Figure S1-S3**). However, shoulders at high molar mass were observed, in particular for block copolymers obtained from PEGMA₃₆₀ when polymerized up to conversions above 80%. This is likely due to branching induced by the presence of traces of PEG dimethacrylate in the commercial PEGMA. Accordingly, the dispersity ranged from 1.17 to 1.65.

¹H-NMR spectroscopy also confirmed the presence of the PPEGMA block, indicating molar PEGMA fractions mostly below 10 mol%, as estimated from the respective integrals in the spectra (**Figure 1**).

The hydroxyl side chain end groups of the PPEGMA blocks of **P1** to **P30** were functionalized with RA *via* esterification using DCC as the activation agent and DMAP as the catalyst. 1.5 eq. and 0.75 eq. of RA with respect to each hydroxyl group were chosen to investigate the influence of the amount of coupled RA on the polymer properties. ¹H-NMR spectroscopy revealed the presence of signals between 5.5 and 7.2 ppm, which are assigned to the retinoyl moieties (**Figure 1**). The covalent attachment of RA was confirmed by SEC with UV-DAD (SI, **Figure S4**). The corresponding 3D plots in the SI in **Figure S5** depict the UV-Vis absorption spectra at each elution time in a wavelength range from 360 to 500 nm. The successful esterification with RA was evident from the high absorption at $\lambda = 360$ nm, corresponding to the maximum absorption wavelength of the retinoyl moiety (**Figure S6**). A quantification of the RA coupled to the polymer was hence accomplished by means of UV-Vis spectroscopy. Degrees of functionalization (DF) between 5 and 29% were obtained. However, the combination of high DPs of PEGMA and high DF of RA often resulted in insoluble materials unsuitable for further studies (**P2***, **P6***, **P10***, **P14***, **P18***, **P22***, **P26***, **P30***). Raman spectroscopy of selected macroinitiators, unmodified- and RA-modified polymers, further proofed the modification of the polymers with RA (**Figure S7**).

Figure 2: Overview of the polymer library showing selected polymer and particle characteristics (for detailed information see SI, **Tables S4** and **S5**). MMA: methyl methacrylate; DMAEMA: dimethylaminoethyl methacrylate (content in magenta); PEGMA_{360/500}: polyethylene glycol methacrylate #: low (light pink) and high (dark red) degree of polymerization of PEGMA; #*: functionalization with retinoic acid; Tox: cell toxicity: ✓ non-toxic ($\geq 70\%$ viability), ☠ toxic ($< 70\%$ viability); EE (encapsulation efficiency of tRNA measured by gel electrophoresis and intercalating dye): ✓ encapsulation, × no encapsulation.

Nanoparticle formulation and characterization

Figure 2 (turquoise, red and green boxes) summarizes the characteristics of the polymer library in terms of polymer composition excluding the insoluble batches **P2***, **P6***, **P10***, **P14***, **P18***, **P22***, **P26*** and **P30***. All other polymers were water insoluble but could be dissolved in organic solvents such as DMSO or ethylacetate. It needs to be mentioned that due to the water insolubility no common polyplexes can be formed. Therefore, we have chosen a double emulsion/solvent evaporation formulation where the aqueous phase (bearing the RNA) is encapsulated in a solid polymer matrix while the RNA is hindered from leakage due to the positive charges of the polymer. The nitrogen to phosphor (N/P) ratio is consequently not the main parameter to be adjusted. In a first set of experiments, double emulsion/solvent evaporation with either water (as control) or tRNA in water (as the model ribonucleic acid) as first aqueous phase was performed. The NP suspensions were used without further purification steps while assuming that free (non-encapsulated) RNA will immediately be degraded by RNAses in biological systems. The organic solvent is completely removed during the procedure, and the poly(vinyl alcohol) (PVA) as stabilizer is known to be biocompatible.³² DLS investigations of the NP suspensions show mean hydrodynamic diameters between 60 and 210 nm and varying polydispersity indices (PDI) between 0.12 and 0.38. AFM and cryo-TEM imaging confirmed the spherical shape of the NPs (exemplified for **P20***) and supported the results obtained by DLS (**Figure 3**). The zeta potential for all formulations was within +/-10 mV and appeared not being dependent on the degree of positive charges (DMAEMA) within the polymers used for NP formulation. The functionalization of the polymers with RA did not result in pronouncedly different NP size distributions after formulation.

Figure 3: A) AFM topography image (z-scale 0 to 75 nm) and B) cryo-TEM image of NPs prepared from **P20***.

The encapsulation ability for ribonucleic acids was tested for all polymers using a model tRNA originating from brewer's yeast. Gel electrophoresis experiments provided first evidence for the encapsulation efficiency (EE%) of the tRNA (**Figures 4A and B**). The tRNA that was encapsulated in or adsorbed at the NP remained in the pocket of the gel whereas free tRNA was separated on the gel. The resulting gels showed that NPs with less than 30% DMAEMA content did not contain tRNA within the resolution limit. NPs comprising 30% or more DMAEMA resulted in fluorescence in the pocket and none on the gel (**Figures 4A and B**). For quantification of the tRNA, an assay with a fluorescent RNA-binding dye (QuantiFluor®) was performed. Consequently, the amount of free tRNA could be determined as an indirect method to apparently quantify the amount of encapsulated and adsorbed tRNA. The suspensions were used without purification for the assay because the scattering properties of the NPs as well as of PVA or blank particles itself did not influence the resulting fluorescence intensity. As expected, the EE% directly correlated with the

percentage of DMAEMA (**Figure 4C**). The tRNA EE% increased to almost 100% at 30% DMAEMA contents and higher.

Figure 4: Encapsulation efficiency was assessed by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide and a fluorescent RNA-binding assay. A) With ethidium bromide stained agarose gel electrophoresis, the encapsulation efficiency was calculated by the quotient of the run length of free tRNA and the NPs (data are depicted as median box plot. Data are depicted as median $\pm$ SD, individual values are depicted as dots, $n=2-6$). B) Representative picture of an agarose gel showing the retardation of the encapsulated tRNA. C) Encapsulation efficiency of all NP suspensions **P1-P30** determined by fluorescence of the free tRNA that intercalates with a dye. Data are depicted as median $\pm$ SD, individual values are depicted as dots, $n=2-6$.

The protection of the encapsulated tRNA against degradation was tested when incubating the NPs with RNase A. Heparin is able to release ribonucleic acids after complexation/encapsulation but also protects it against RNase A. The addition of heparin after 60 min proofed that the tRNA remained stable during RNase treatment and can still be released in an intact form after treating with heparin (**Figure S12**). The stability of the NPs was also tested at different pH values. NPs containing no DMAEMA remained stable over weeks at relevant pH values between 4.5 and 7.4 whereas NPs with 30% and 50% DMAEMA dissolved rapidly at pH values between 4.5 and 6. At

pH 7.4, NPs with 0% and 30% DMAEMA were stable while 50% DMAEMA particles started to dissolve within a week (SI, **Table S9**).

Label-free SERS investigations can be used to proof that the retinol-substituted PEGMA units assembled in a way that retinol is present at the NPs' surface and, thus, enables the retinol accessibility toward the retinol binding protein for targeting the HSC. Therefore, SERS investigations of RA containing NPs were conducted in order to specify whether the retinol is located in surface-near regions of the NP making use of the high surface sensitivity of this approach.³³ The SERS substrates were composed of a single layer of isolated Ag NPs, with an average diameter of approximately 40 nm, which proved to be stable in aqueous solutions. Therefore, the SERS investigation of all NP samples was performed in an aqueous suspension, to avoid morphology changes of the NPs due to drying artifacts. **Figure 5A** shows a typical SERS spectrum of retinoic acid. The retinoic acid specific Raman peaks can be assigned to the C=C stretching vibrations, the C-C stretching modes, the C-CH₃ in plane rocking modes and the hydrogen out-of-plane wagging modes (peak assignment **Table S8**).³⁴ For RA containing NPs **P20***, the corresponding SERS spectra revealed also the main characteristic modes, while for the reference sample of NPs of the same polymer without any retinoyl moieties (**P20**), none of the retinoic acid specific marker bands were detected. A comparison of the Raman spectra of the single RA and the NP bound RA revealed some changes. In particular, new Raman bands appeared which might be assigned to Raman modes of the PEGMA units. However, these bands could not be detected for the polymer NPs without retinoyl moieties using the same excitation power. Therefore, it is unlikely that these modes exclusively originate from the PEGMA. Besides these new Raman modes, a modification of the relative intensity of the C-C stretching mode at 1191 cm⁻¹ was observed. While the relative intensity ratio I_{1569} / I_{1191} increased from 6 in **Figure 5A** top to 11

in **Figure 5A** middle, the relative intensity ratio I_{1569} / I_{1151} remained constant (SI, **Table S8**). The different behaviors of the intensity of these modes allowed for assigning specific vibrations to the polyene chain and the contained ring structure by comparing with the mode assignment for all-trans-retinal structures (see SI for peak assignment Table S8).³⁵ In conclusion, the SERS investigations demonstrated the presence of RA in the RA positive samples. Furthermore, the comparison with pure RA demonstrated that RA is covalently bound to the block copolymer. The chosen excitation wavelength of 532 nm used in these experiments leads to Pre-Resonance Raman (PRR) scattering,³⁶ which results in a depth information of 2 to 8 nm for the SERS Raman measurements.³⁷ Consequently, the signatures of the RA detected in the SERS experiments originate from retinoyl functionalities that are located in the outer PPEGMA shell of the block copolymer NPs. This confirms the hypothesis that the RA is incorporated at or close to the surface of the NPs.

Figure 5: A) Averaged SERS spectra of RA acquired after evaporation of the ethyl acetate, RA-functionalized NPs of **P20*** and **P20** NPs without RA measured in aqueous solution. B)

Normalized differential distributions of sedimentation coefficients, $ls-g^*(s)$, from sedimentation velocity data recorded at a wavelength of $\lambda = 280$ nm in terms of optical density (OD) of **P20**, **P20***, and **P24*** by itself and additionally with RBP.

Interaction with RBP

The presence of RA at the surface of the NP is essential for its interaction with the retinol-binding protein (RBP) to form a complex that is recognized by the RBPR receptors in the cellular membrane. We first executed native PAGE and discovered that RBP in combination with RA-functionalized polymers migrated slower than the non-functionalized polymers (SI, **Figure S13**), which gave an indication for specific binding of RBP to the RA-functionalized NPs. In addition, experiments by analytical ultracentrifugation (AUC) were performed to indicate the interaction of the NPs with RBP utilizing the absorbance detection system in terms of OD. **Figure 5B** displays that NPs prepared from **P20**, not having any retinoyl moiety, were not affected by the presence of the RBP in solution. The differential distributions of sedimentation coefficients of **P20** itself and **P20** with RBP revealed a very similar shape, covering similar sedimentation coefficient values. At higher sedimentation coefficients, aggregates were apparent for both samples. Despite, for **P20***, possessing a 29% RA-functionalization, a distinct shift in average sedimentation coefficients was observed in the presence of RBP in the sample. The differential distribution of sedimentation coefficients of **P20*** has a maximum located at about 105 S. By adding the RBP to **P20***, the maximum of the differential distribution of sedimentation coefficients shifted to about 230 S. This change in the differential distribution of sedimentation coefficients of **P20*** by itself compared to the sample **P20*** including RBP occurs due to the increase of apparent size by the aggregation of **P20***, possibly originating from the interaction with the protein. Considering the relation $d_h \sim \sqrt{s}$, the increase between the maxima of the distributions would roughly lead to a size increase of about

50%. Thus, the RA-functionalized **P20*** interacted with RBP while **P20** did not reveal such indication, apparently due to the absence of the retinoyl moiety. The large size change might be due to multiple complexation of the RBP with RA moieties of several NPs, possibly also promoting aggregation. However, the RBP has only one binding site for RA moieties, and future studies will focus on this aggregation phenomenon.³⁸ Furthermore, NPs with a lower concentration of RA were investigated in comparison to **P20*** and **P20**. The differential distribution of sedimentation coefficients of **P24*** comprising 5% retinoyl moieties by itself showed a narrow distribution of sedimentation coefficients with a maximum at about 15 S. Compared to that, the sample containing **P24*** and additionally RBP showed a differential distribution of sedimentation coefficients with a maximum shifted roughly two-fold to 32 S. Thus, the RA present in **P24*** may cause some interaction with the RBP. Notwithstanding, due to the smaller amount of RA incorporated in **P24***, resulting in reduced possibility of RA-RBP interaction, the shift in the distributions was less pronounced compared to **P20***. The reduced RA amount of **P24*** caused less absorbance and, therefore, could hinder the observation of eventually present differences between the samples. The size change after binding of RBP on the NP surface may also be difficult to grasp due to small size changes in the absence of aggregation. In addition, aggregates were not observable, indicated by the absence of larger sedimentation coefficients. The specific interaction could also be indicated by a study of the same NPs with BSA, where interestingly the RA-functionalized NP do not change their apparent hydrodynamic properties (SI, **Figure S10A,B**) but the non-modified NPs **P24** interact with BSA resulting in agglomeration (SI, **Figure S10C**). At higher rotational speed, as expected the sedimentation of the remaining proteins becomes assessable (SI, **Figure S11**).

***In vitro* performance of the NPs**

The cytotoxicity of the non-modified and RA-modified NPs was tested in MEF cells after 24 h (Figure 6). At a concentration of $100 \mu\text{g mL}^{-1}$, the NPs containing none to 40% DMAEMA showed no toxic effects, whereas at 50% DMAEMA, the NPs revealed severe toxicity. NP concentrations up to $500 \mu\text{g mL}^{-1}$ were tested and provide the same results (data not shown). The modification of the polymers with retinoic acid had no influence on the toxicity of the particles. The results point out that the NPs containing 30% or 40% DMAEMA exhibit preferable properties with high encapsulation efficiencies of ribonucleic acids while being non-toxic to the examined cells. Consequently, further *in vitro*- and *in vivo* experiments will focus on these polymers as materials for NP formulation.

Figure 6: A marker for membrane rupture was assessed in MEFs by the quantification of lactate dehydrogenase activity from the supernatant 24 h after addition of various NPs. This assay indicates toxicity by means of membrane damage. Data shows the toxicity only for a concentration of $100 \mu\text{g mL}^{-1}$ of the NPs. The experiment was performed independently at least three times in

triplicates, data are depicted as mean bar plot (positive standard deviation), one-way ANOVA between groups, * $p < 0.05$.

Figure 7: A) Uptake of NPs containing 30% (**P22**) and 40% (**P26**) DMAEMA in MEF cells after 24 h incubation. Uptake was quantified by flow cytometry. The experiment was performed independently at least three times in duplicates each, data are depicted as median box plot (IQR = standard deviation, median line), unpaired t-test, * $p < 0.05$. B-D) Fluorescence intensity (in photons) of fixed MEF cells after incubation for 1 h with B) 30% DMAEMA (**P22**), C) 40% DMAEMA (**P26**), D) medium (control). The insert in B) has a dimension of 3 μm x 3 μm . Cells

incubated with NPs show drastically increased fluorescence intensities. Photon numbers above 255/pixel are displayed as white. Ex.: 540 nm, det.: 555 nm – 701 nm. The NPs were loaded with Cy3-labeled siRNA.

The uptake of the NPs containing 30% or 40% DMAEMA was further examined, because of their high encapsulation efficiencies of ribonucleic acids and no cytotoxic effects on MEF cells after 24 h. For NPs containing 30% DMAEMA (**P22**) as well as 40% DMAEMA (**P26**), a cellular uptake in MEF cells after 24 h was observed (**Figure 7A**) via FACS analysis. **P26** containing 40% DMAEMA delivered its cargo more efficient into the cell. To show whether the NPs were internalized by the cells and not only attached to the cell membrane, fluorescence imaging was performed. **Figure 7** depicts representative example images of fixed MEF cells after incubation for 1 h with 30% DMAEMA (**P22**) (**Figure 7B**) and 40% DMAEMA (**P26**) NP that were loaded with Cy3-labeled siRNA (**Figure 7C**), alongside a control sample (**Figure 7D**). Further images are part of the SI (**Figures S14 to S21**). Control samples in which cells were incubated with Cy3-labeled siRNA only were also measured and showed brightnesses between those of the untreated control samples and samples treated with 30% DMAEMA NP. The respective images are provided in the SI, **Figures S17 to S21**. Cells incubated with NPs show drastically increased fluorescence intensities, indicating an uptake. In accordance with the flow cytometry experiments (**Figure 7A**), 40% DMAEMA particles lead to stronger fluorescence. Individual NPs can be seen as bright dots at high magnification (**Figure 7B** inset). To confirm these dots' nature, spectrally resolved measurements were performed (**Figure 8A, B**). The spectra of individual dots could be obtained and assigned to Cy3 (**Figure 8D**). To assess their location precisely, z-resolved measurements were performed (**Figure 8C**), which show particles in the cytoplasm, as well as attached to the nuclear membrane. Few individual particles have also penetrated into the nuclei (**Figure 8C**).

Figure 8: Average intensity projection of a stack of spectrally resolved fluorescence images of fixed MEF cells after incubation for 1 h with 30% DMAEMA (**P22**) NPs loaded with Cy3-labeled siRNA. Ex.: 540 nm, det.: 550 to 650 nm in 20 intervals of 5 nm. Contrast and brightness were altered linearly to enhance visibility. B) Enlarged view of the area indicated by the white square in A). Individual NPs can be distinguished. Five particularly large dots are marked by red squares (901 x 901 nm²). The insert (top right) shows them in the above specified spectral windows. C) Deconvoluted (*Lightning* algorithm, Leica, Germany) z-stack (3.43 μm, sampled in 28 steps), taken in the same area as **Figure 8B**. The orthogonal views show clearly the nuclear (xz, yz) and cellular (yz) membranes. Individual NPs can be found in the cytoplasm and the nuclear membrane.

Some particles also penetrated into the nucleus. Excitation: 540 nm, detection: 555 to 701 nm. D) Normalized fluorescence spectra of five individual dots as indicated in **Figure 8B** (grey curves), the combined area of all five dots (red) and the entire image (**Figure 8A**, blue). The spectrum of Cy3 is shown for reference (orange). The combined dot spectrum shows the spectral characteristic of Cy3. The combined emission spectrum of the entire image is dominated by backscattered excitation laser light in the first spectral window (550 to 555 nm).

CONCLUSION

In this study, a library of functional polymethacrylate copolymers was synthesized by RAFT polymerization. The first hydrophobic block contained different ratios of MMA (50 to 100%) and DMAEMA (0 to 50%) and was used as macroCTA for a hydrophilic block consisting of PEGMA (**Pi**), which was functionalized with RA (**Pi***), a physiological metabolite of vitamin A, in different degrees of functionalization (DF_{RA}). It was found that a high DF_{RA} leads to crosslinking of the polymer and partial insolubility of the products. All polymers were formulated with tRNA to NPs with a diameter between 60 and 210 nm using a double emulsion approach. Our studies on encapsulation efficiency and toxicity found an optimum for the polymer compositions with a DMAEMA content of 30% and 40% (**P19** to **P28**). The derived NPs were able to quantitatively encapsulate and, thus, protect tRNA and did not have toxic effects in MEF cells (overview of polymer library see **Figure 2**). SERS measurements confirmed that RA on the particles is located at or close to the surface (max. 8 nm depth) of the NPs. The interaction of RA at the NPs with a retinol binding protein was confirmed by AUC. NPs with low RA modification reveal an interaction with the retinol binding protein whereas no or high modification results in no interaction or accumulation of the NPs, respectively. Consequently, a low DF_{RA} is again preferred, also when considering the solubility issues of high DF_{RA} . Initial *in vitro* experiments proved an enhanced uptake of 40% DMAEMA NPs compared to 30% DMAEMA NPs, however both NP

modifications show internalization in intracellular compartments. Such an initial screening of important parameters allows the identification of specific polymers that have the potential to target HSC and deliver a ribonucleic acid (*e.g.* anti-Col1 α 1-siRNA) to reduce the collagen production of HSC for treatment of liver injury.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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