ARTICLE

Upholding hyaluronic acid's multi-functionality for nucleic acid drug delivery to target EMT in breast cancer

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Synthetic nanoparticles can stably encapsulate nucleic acids as active pharmaceutical payloads. Recently, mRNA- and siRNA-based medicines have been successfully approved for preventing or treating infectious or orphan diseases. RNA interference is particularly relevant for cancer therapy, as tumors often involve up- or dys-regulated proteins that drive malignancy. This study aimed to develop a nanoparticulate delivery vehicle that targets EMT-phenotypic breast cancer cells, which lack effective treatment options. These "undruggable" tumors may be addressed by nanoparticles that target EMT-specific cell surface receptors. CD44, a transmembrane protein linked to cancer malignancy and EMT, was identified as a promising candidate. This work investigated the use of hyaluronic acid (HA) in HA-modified polyelectrolyte complexes (polyplexes, Px) for its dual roles as a targeting ligand and a stabilizing stealth-molecule. Various strategies for non-covalently immobilizing HA on the particle surfaces were tested. HAPx nanoparticles demonstrated HA:PEI-ratio dependent stability against competing anionic biomolecules, improved colloidal stability in protein-rich environments mimicking *in vivo* conditions, and enhanced selectivity and efficacy in targeting E/M-hybrids and EMT-positive cells via CD44-HA mediated endocytosis. Finally, our results indicate different internalization kinetics and efficiencies between CD44v and CD44s isotypes, highlighting the need to consider CD44 heterogeneities in the clinical development of HA-based drug delivery systems.

Supplementary Data

Figure S1: Course of hydrodynamic diameter as function of [%] size increase over 6-weeks of storage at room temperature of differently HA-coated Px. a) 2-step coating; b) Inverse 2-step coating; c) 1-step coating..

Figure S2: (**a**) Zeta potential of nanoparticles after incubation in 2% FBS compared to Px in 10 mM HEPES (pH 7.4) as a function of HA:PEI-ratio and coating procedure (**b**) Change of hydrodynamic diameter [± %] of nanoparticles after incubation in 2% FBS compared to sizes in 10 mM HEPES (pH 7.4) as a function of HA:PEI-ratio and coating procedure. Colors of bars indicate distinct (HA)Px-groups: Px (grey), HAPx$^{+\zeta}$ (green), HAPx$^{\pm\zeta}$ (orange), HAPx$^{-\zeta}$ (red). (**b**) Self-quenching of AF647 dye based on the labeled/unlabeled siRNA-ratio (%) during polyplex assembly: Px (squares) with 50 (blue), 100 (green) and 200 pmol (orange) siRNA at ratios of 25/75, 50/50, 75/25 and 100/0 were compared to respective amounts of free siRNA (triangles). Error bars represent SD (n = 3)

Figure S3 Additional independent replicates of Hyaluronic Acid (HA) coated nanoparticles (HAPx) and uncoated Px in 90% FBS.

(A, C, E) ACFs of naked Px particles at different time points of incubation in 90% FBS obtained from three independent experiments as indicated in the parentheses. The ACF of siRNA-ATTO643 in 90% FBS is shown as a reference. It should be noted that, for the replicate 1 in panel A, the particles were prepared with 15% non-coding siRNA labeled with ATTO643, as usual, but the remaining unlabeled 85% was constituted by a 25/27mer siRNA against eGFP. For replicates 2 and 3, the particles were prepared with 15% non-coding siRNA labeled with ATTO643 and 85% unlabeled non-coding siRNA.

(B, D, F) ACFs of HA coated particles at different time points of incubation in 90% FBS obtained from three independent experiments as indicated in the parentheses. The ACF of siRNA-ATTO643 in 90% FBS is shown as a reference. HAPx particles are formulated with a HA: PEI ratio of 2.5: 1. It should be noted that, for the replicate 1 in panel B, the particles were prepared with 15% non-coding siRNA labeled with ATTO643, as usual, but the remaining unlabeled 85% was constituted by a 25/27mer siRNA against eGFP. For replicates 2 and 3, the particles were prepared with 15% non-coding siRNA labeled with ATTO643 and 85% unlabeled non-coding siRNA.

Figure S4: (a) Surface CD44 expression in four breast cancer cell lines measured via flow cytometry. Bars show mean fluorescence intensity (MFI). Error bars indicate SD (n = 2). **(b)** RHAMM surface expression in the four breast cancer cell lines quantified by flow cytometry. Error bars indicate SD (n = 2). **(c)** Comparison of protein levels of epithelial marker CDH1, CD44s/v isotypes and GAPDH measured via Western blot.

Figure S5: CD44 (isotype, expression level) and HA:PEI-ratio dependent cellular uptake of HAPx particles produced via (**a**) Inverse 2-step coating and (**b**) 1-step coating measured by flow cytometry. Mean relative uptake normalized to Px (black, right y-axis) is shown as dotted line with grey (CD44^{null} MCF7), purple (CD44s^{high} MB-231), light blue (CD44v^{low} HCC1954) and dark blue (CD44v^{high} MB-468) color indicating the cell line/CD44-isotype expression level. Solid lines represent mean physico-chemical properties of respective HAPx species with hydrodynamic size (green) and zeta potential (orange) shown on the left and right y-axis, respectively. Black dotted line ($y_{right,\ black}$) indicates uptake levels of uncoated Px particles (y = 1). Orange dotted line ($y_{right,\ orange}$) depicts zeta potential at 0 mV.

Figure S6: **(a)** Surface pan-CD44 expression in various breast cancer cell lines after growth factor-mediated EMT induction measured via flow cytometry. Error bars indicate SD (n = 2) **(b)** Protein levels of CDH1, CD44s/v isotypes and GAPDH depending on EGF concentration in MDA-MB-468 cells.

Table S1. Control FCS measurement: diffusion of ATTO643 dye and ATTO643-labelled siRNA in HEPES, 10% FBS or 90 % FBS. The values obtained from the FCS measurements of ATTO643 and ATTO643-siRNA in the three solutions are reported as an average ± standard deviation of three independent replicates. The autocorrelation function was fit using a 2-component diffusion model. For the dye sample, A_1 and A_2 are the (size-weighted) relative amplitudes of ATTO643 and, when present, of the fluorescent components of FBS. D_1 and D_2 refer to the respective diffusion coefficients of A_1 and A_2. For the siRNA sample, A_1 and A_2 are the (size-weighted) relative amplitudes of siRNA-ATTO643 and free ATTO643. In this case, D_2 was fixed according to the mean D value of free ATTO643.

Table S2. FCS measurement: diffusion of labelled Px and HAPx in HEPES, or 10% FBS. The values obtained from the FCS measurements of Px and HAPx particles (HA:PEI ratio of 2.5:1 with 2-step coating) in HEPES and 10% FBS right after dilution and 2 hours later are reported as an average ± standard deviation of four and two independent replicates (for HEPES and 10% FBS respectively). The autocorrelation function was fit using a 1- or 2-component diffusion model where A_1 and A_2 are the (size-weighted) relative amplitudes of the particles and the free siRNA-ATTO643. D_1 and D_2 refer to the respective diffusion coefficients of A_1 and A_2, respectively. Note that D_2 was fixed according to the mean values previously determined for siRNA-ATTO643 in 10% FBS (table S1).

Table S3. FCS measurement: diffusion of labelled Px and HAPx in 90% FBS at different time points. The values obtained from the FCS measurements of Px and HAPx particles (HA:PEI ratio of 2.5:1 with 2-step coating) in 90% FBS shown in Figure 4 are reported. The autocorrelation function was fit using a 3-component diffusion model where A_1, A_2 and A_3 are the (size-weighted) relative amplitudes of the particles, free siRNA-ATTO643 and free dye. It should be noted that for Px particles, D_1 from 30 min of incubation onwards is too high to correspond to compact particles: this indicates complete release of siRNA.

*D_2 and D_3 were fixed according to the mean values previously determined for siRNA-ATTO643 and free ATTO643 in 90% FBS, respectively (table S1).