Designing nanodiamonds for efficient siRNA vectorization: tuning of charge density and interface structure

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1. Introduction

Gene therapy comprises several technologies for modifying gene sequences to cure various diseases. In recent years, RNA interference mechanism (RNAi) based on specific repression of gene expression at the post-transcriptional level has become a major approach in various research applications. According to ClinicalTrials.gov, there is 46 studies to date, related to siRNA trials, but many of them have been terminated due to low efficacy or serious side effects as an unspecific immune response caused by recognition of naked siRNA by the innate immune system.[1,2]
In general, the incorporation of siRNA into RNAi machinery faces many physiological barriers. Furthermore, according to results from clinical trials, using of naked siRNA as RNAi therapeutic is strongly restricted to routes of administration and target organs. The common strategy on how to avoid siRNA recognition is based on the suitable delivery system, which is protecting fragile siRNA. Despite a broad range of materials serving as delivery agents (polymers, lipids, inorganic particles, viral vectors etc.) only three RNAi-based drugs (patisiran – 2018 – lipid nanoparticle formulation, givosiran – 2019 – GalNAc-siRNA conjugate, lumasiran – 2020 – GalNAc-siRNA conjugate) have been approved by FDA for clinical use. Apart from these, there are seven others siRNA drugs in late stages of Phase 3 clinical trials, which pave the way for other RNAi based nanotechnologies.

Lipid NPs/lipoplexes, cyclodextrin-based NPs, dynamic polyconjugates or GalNAc-siRNA conjugates are currently the most often using non-viral delivery systems in clinical trials due to their transfection efficacy and biodegradability. In contrast, nanodiamond (ND) as a hard/inorganic material is completely non-biodegradable and therefore it seems to be unsuitable delivery material for routine systemic administration. On the other hand, the major attractions for applying this material in gene therapy are based on extremely photo-stable intrinsic luminescence provided by diamond lattice and the large surface-to-volume ratio. On account of these properties, NDs can serve as a theranostic platform, enormously selective background-free in vivo imaging probe or as a long-term stable carrier providing antimicrobial activity. All these features make this material promising especially for the topic applications.

In comparison to the above-mentioned soft/organic carriers stabilized especially due to good solvation and interactions of electrical double layers, hard/inorganic nanoparticles need more demanding surface modification to control their colloidal properties. Raw NDs without the proper modification aggregate almost in all physiological solutions (see Figure S13). That makes the design of the surface coating (surface charge density, structure,
hydrophilicity, chemical composition etc.) the most crucial in the preparation of a well-working nanoparticle system. Figure 1 shows various engineering strategies for the production of a cationic coating, which are based on different types of colloidal stabilization and can be used for siRNA delivery.

**Figure 1.** Scheme of various engineering strategies for the production of cationic polymer-coated nanoparticles. Linear polymer chains represent only one example of many possible polymer structures (linear, branched, cross-linked etc.). Polymers can be also attached to ND surface via different approaches (electrostatic interaction, covalent grafting or hydrophobic interaction).

The first generation (Figure 1A) introduces electrostatic stabilization of NPs, which prevent aggregation by repulsive electrostatic forces. This approach can be typically achieved with a low molecular weight (LMW) cationic coating as 0.8 kDa polyethyleneimine (PEI branched, M<sub>W</sub>) or direct surface modification. The biggest disadvantage is a high sensitivity to the ionic strength of the dispersion media causing poor colloidal stability (aggregation). Biological media as 1xPBS, cell culture media etc., typically possess high ionic...
strength reducing the thickness of the electrical double layer, which is surrounding and protecting the particles against aggregation.

To improve the colloidal stability of the previous generation, the presence of high molecular weight (HMW) coating on the surface is required – 25 kDa branched PEI; ~30 kDa poly-L-lysine [32], 6.9 kDa polyamidoamine (PAMAM dendrimer) [33], poly(2-dimethylaminoethyl methacrylate) (PDMAEMA linear brush) [34,35]. This second-generation (Figure 1B) interface cover the particle surface in such a way that the charged polymer chains extend out into solution and provide additional sterical stabilization, which is based on microbrownian motion of the flexible ends of polymer chains. This electro-steric stabilization [36] usually resists in high ionic strength and provides sufficient stabilization in aqueous dispersants. In contrast to improved colloidal properties, it has been shown that cytotoxicity of free PEI [37] as well as PL [38], PAMAM [38] and PDMAEMA [39] increases with increasing molecular weight. The transfection efficacies above mentioned cationic polymers seem to be somewhat contradictory [40,37,41,42]. Despite ongoing efforts, the biological properties of polyplexes/cationic nanoparticles are not fully understood due to the complex dependence of biological activity on physicochemical properties such as polymer structure, molecular weight, buffer capacity, charge density, a degree of DNA/RNA complexation, colloidal stability, polydispersity etc. [40]

The development of third-generation (Figure 1C, 1D) closely relates to the application of functionalized nanoparticles in biological dispersants. Interaction of serum components with a cationic interface can strongly affect the colloidal stability and siRNA delivery itself [1]. In order to avoid nonspecific adsorption of serum components, polymer shells with shielding molecules were needed – PEI-PEG [43], poly-[2-(dimethylamino) ethyl methacrylate]-b-poly[N-(3-(methacryloylamino) propyl)-N,N-dimethyl-N-(3-sulfopropyl) ammonium hydroxide] (PDMAEMA-b-PMPDSAH) [44]. The shielding parts are exposed to the solution allowing effective protection (Figure 1C). On the other hand, serum proteins adsorbed on the surface
can be beneficial to improve cellular uptake as an endogenous targeting ligand, which interacts with the cellular receptors instead of a synthetic interface\textsuperscript{[45,46]}. However, it was also shown that opsonization effect results in uptake by reticuloendothelial system followed by phagocytes mediated clearance\textsuperscript{[9,47]}. In the present work, we demonstrate a systematic synthesis and biological testing of a cationic copolymer interface with a tuned charge density (third generation, \textbf{Figure 1D}). Statistical copolymer poly\{((2-dimethylaminoethyl methacrylate)-co-[N-(2-hydroxypropyl) methacrylamide]) (poly(DMAEMA\textsuperscript{+}-co-HPMA\textsuperscript{0})) grafted silica-coated NDs were prepared \textit{via} radical polymerization. PDMAEMA\textsuperscript{+} itself ensures good colloidal stability (second generation) but also decreases cell proliferation. To improve and optimize the efficacy/proliferation ratio by charge density tuning of the cationic shell, we have introduced HPMA\textsuperscript{0} monomers in the structure of the surface coating. PHPMA\textsuperscript{0} has been recently proven as a hydrophilic biocompatible polymer with no nonspecific protein interactions \textsuperscript{[25]} and successfully used as HPMA\textsuperscript{0} copolymer-drug conjugate \textsuperscript{[48]}. To show the colloidal robustness of this novel system, we have also chosen as a reference a very popular system from the first generation – ND-PEI (M\textsubscript{w} ~ 0.8 kDa, branched).

2. Results and Discussion

Covalently grafted NDs with a charge density tuned copolymer interface offer a novel approach for siRNA vectorization. We took advantage of our recently published methodology for silica coating of NDs \textsuperscript{[24]} to prepare a cationic linear-brush copolymer layer on the ND surface with a reduced positive charge density. Terminal methacrylate groups of silica coating allow a grow of dense copolymer layer, which is consisted of poly(DMAEMA\textsuperscript{+}-co-HPMA\textsuperscript{0}), directly from the surface \textit{via} radical polymerization (\textbf{Figure 2}).
Figure 2. Schematic structure of the (co)polymer coating on the nanodiamond surface.

This “grafting from” approach typically ensures denser and better-protecting coating than the attachment of presynthesized polymers (PEI$^{[27]}$, poly-L-lysine$^{[32]}$, PEG$^{[49]}$ etc.) to the surface as in case ND-PEI – “grafting to” approach $^{[23]}$. Cationic component DMAEMA$^+$ allows electrostatic immobilization of siRNA and HPMA$^0$ decreases overall charge density. Herein, we investigated the effect of the increasing amount of neutral HPMA$^0$ component in the cationic DMAEMA$^+$ polymer-brush to elucidate a relationship between physicochemical properties and biological activity of these complexes in a mouse 4T1 breast cancer cell line.

ND@(co)polymer characterization: We prepared (co)polymer nanoparticles of various compositions ND@silica@poly[DMAEMA(x%)$^+$-co-HPMA(y%)$^0$] abbreviated in following text into the form DMAEMA(x%)$^+$/HPMA(y%)$^0$: 100$^+$/0$^0$, 80$^+$/20$^0$, 45$^+$/55$^0$, 33$^+$/67$^0$, 0$^+$/100$^0$, or in general represented as ND@(co)polymer; number ratios describe the final percent mass fraction of each monomer in (co)polymer layer (100$^+$/0$^0$ – DMAEMA$^+$ only, 0$^+$/100$^0$ HPMA only) – Table S1. The resulting copolymer composition was characterized qualitatively by $^1$H NMR and quantitatively by thermogravimetric analysis (TGA) – Figure 3.
Figure 3. A) TGA curves of ND, ND-PEI and ND@(co)polymer samples. B) Copolymer composition according to $^1$H NMR data. C) $^1$H NMR spectra of ND@(co)polymer samples.

Result concentration of DMAEMA$^+$ in copolymer layer (relative to HPMA$^0$) as a function of added concentration of DMAEMA$^+$ in the reaction mixture was determined by a comparison of the area under peaks at 4.0 and 3.8 ppm in $^1$H NMR spectra, which are assigned to the hydrogens of the $–$O–CH$_2$– group in DMAEMA$^+$ and $–$CH–OH group in HPMA$^0$. Figure 3B shows a good agreement between added and final concentration in the (co)polymer. In addition, TGA indicates higher reactivity of the cationic component according to a relative weight loss of the (co)polymer.
**Figure 4.** NTA measurement of ND@(co)polymer samples in Milli-Q water. Displayed finite track-length adjusted (FTLA) histograms (A, B, C, D, E) represent data from five measurements ± sample standard deviation. Vertical red dashed lines coupled with the histogram represent 10th, 50th (D50) and 90th percentiles of the size distribution; PDI and SPAN values reflecting polydispersity were calculated from FTLA non-merged data (provided by software) according to equations (4) and (5) in Table S8 respecting error propagation approach for ± sample standard deviation values. All valid tracks from the five measurements (raw data – manual data processing is described in Supporting Information) are plotted in a scatter plot – each measurement is separated by a different color. Increased differentiation in y-axes of detected particles in the scatter plot is allowed due to measuring the peak intensity for each particle during the tracking analysis. (F) Box plots characterize a particle size distribution using 25th, 50th and 75th percentiles, and 90% and 10% whiskers; the horizontal red line in the box: median of the distribution; the cyan points: outliers defined as 3×interquartile range (IQR) > outliers > 1.5×IQR; the black points: extreme values > 3×IQR. A MATLAB routine was applied to unbin the FTLA data provided by software and construct the box plots; p – permutation-based p-value from one way PERMANOVA assessing the significance of the pseudo F-statistic (for more information see [50]); PERMANOVA tests the differences between ND@(co)polymer samples considering D50 and SPAN values as dependent variables at the same time. MATLAB implementation of the test [51] and a posteriori multiple-comparison test is provided in Supporting Information).
NTA and DLS results (Figure 4, Figure S2) also reveal a higher degree of polydispersity (considering sensitive PDI values) and a tendency to form bigger aggregates as the amount of DMAEMA⁺ in the copolymer layer grows. However the DLS intensity-based results indicate a significant difference between the (co)polymer variants (see Figure S2 and Table S8), the NTA results (Figure 4) clearly show that this difference is given by the extreme values in the size distributions (Figure 4F) – the mean value of the NTA median diameters over all (co)polymer-coated samples is 81.7 ± 7.3 nm. This value well corresponds with the mean value over all DLS number weighted median diameters 81.3 ± 11.6 nm (transformation was performed without inspecting change in optical properties due to (co)polymer coating) – see Table S11. Detailed discussion focusing on comparison of NTA, DLS and TEM results can be found in Supporting Information. The presence of these extreme values was probably caused due to the formation of oppositely charged nanoparticles during the polymerization process. Silica coated particles with terminal methacrylate groups before polymerization possess a negatively charged surface. For the thin silica layer [25], the resulting apparent zeta potential of the silica-coated ND is typically influenced by both the raw ND particle potential and coating potential [52], which likely contains uncondened silanolate groups. These negatively charged silica-coated particles can electrostatically interact with newly formed ND@(co)polymer particles covered by positively charged dimethylamino groups. Measured apparent ζ-potentials of all (co)polymer compositions including cationic DMAEMA⁺ possess positive values from +35 mV to +42 mV. Only the sample 0⁺/100⁰ exhibits slightly negative apparent ζ-potential -11 mV; all samples had an average conductivity of 3.54 μS/cm and were measured in RNase free water with pH 5.1.

The apparent ζ-potential reported in this study is a relative measure of the part of the double-layer charge characterizing interparticle interaction. It should be emphasized that apparent ζ-potential represents the electrostatic potential calculated from electrophoretic mobility following Smoluchowski approximation for spherical noncoated particles without inspecting the value of the ratio of particle size to Debye length. Such a description doesn't reflect
(co)polymer layer thickness and its permeability to solvent flow and therefore has no rigorous meaning for (co)polymer-coated particles. However, it is a valuable approach to reveal the colloidally unstable area of ND@(co)polymer:siRNA complexes (Figure 5). [52–54]

**ND-PEI characterization:** Despite easy preparation of abundantly used ND-PEI, the proper colloidal characterization of these particles (HPHT NDs) is still missing in the literature. We observed, that in comparison with ND@(co)polymer, this system suffers from i) reversible aggregation during preparation, ii) instability at high ionic strength and iii) colloidal aging resulting in aggregation. At the first glance, fast and easy preparation of ND-PEI complexes has predestinated this approach to be considered as an easy-to-use nano-tool for gene therapy [27–29]. Unfortunately, this system exhibits poor colloidal properties, which strongly limit further understanding of the relationship between physicochemical properties and the biological activity of nanocarriers. As reported recently [28], PEI coating is associated with an increase of ND hydrodynamic diameter from 50 nm to 130 nm (DLS Z-average) due to the formation of small aggregates during preparation. The mixing procedure of ND (HPHT) and PEI (0.8 kDa) typically results in aggregated milky dispersion that flocculates. Following centrifugation and water purification of the mixture serves as a deflocculation process for removing the excess electrolytes and free PEI molecules. Deflocculation recovers colloidal stability of ND-PEI complexes with the diameter 107 nm (DLS Z-average) and apparent ζ-potential +28 mV (RNase free water, sample conductivity 2.96 μS/cm). Unfortunately, ND-PEI without excess PEI at concentration 9 mg/mL, stored 3 days at 4 °C underwent colloidal aging, which causes further aggregation. Increased complex size around 200 nm (Z-average) was accompanied by apparent ζ-potential reduction to -11 mV (3.24 μS/cm). Further centrifugation and water purification of the stored sample led to previous diameter (108 nm, Z-average), but long-term testing (~20 min) at room temperature revealed slow aggregation with observable sedimentation.
Electrostatic complexation of siRNA onto cationic ND: Positively charged ND@(co)polymer nanoparticles have allowed a complexation with siRNA via electrostatic interaction providing ND@(co)polymer:siRNA complex, which possesses a positive or negative value of apparent ζ-potential. This interaction exhibited a typical mass ratio-dependent relationship \[26\] schematically shown on Figure 5.

![Figure 5](image)

**Figure 5.** Schematic relationship between cationic-coated ND:siRNA mass ratio, apparent ζ-potential, free fraction of siRNA and colloidal stability of the ND:siRNA sample. The schematic graph is divided into the three areas (marked as I, II and III) according to the colloidal state of the sample (stable, unstable). Depicted curves are illustrative and do not represent real experimental data.

In general, all ND@(co)polymer:siRNA complexes tend to be stable at mass ratio (ND:siRNA) 20:1 or higher in water possessing a positive value of (colloidal stability area III – Figure 5). The aggregation at lower mass ratios (< 20:1) was observed once the apparent ζ-potential of the complexes ND@(co)polymer:siRNA started to decrease from positive values to zero or became slightly negative (colloidal stability area II). This is caused due to effective compensation of positive surface charge by negatively charged siRNA. The aggregation process is then initiated due to the formation of oppositely charged nanoparticles during the complexation when the electrostatic interaction comes to dominate over steric stabilization. Further decreasing of the
mass ratio is leading to the restoration of colloidal stability accompanied by increasing fraction of free siRNA and apparent ζ-potential of complexes turns to negative (the colloidally stable area I). As expected, the colloidally unstable area is specific for each (co)polymer layer and depends on its composition. It is obvious, that as the number of positive charges within the (co)polymer shell grows, the lower mass ratio can be reached before the aggregation is observed (aggregation point (+)). In agreement with that, all ND@(co)polymer particles showed different aggregation points (+) (Figure 6A): 100*/0° (not shown), 80*/20° (~5:1), 45*/55° (~10:1), 33*/67° (~15:1), 0*/100° (intact). Aggregation point of 100*/0° (< 5:1) lies out of monitored range and 0*/100° do not electrostatically interact with siRNA and thus aggregation is not observed at all. In contrast to ND@(co)polymer:siRNA, the effective binding mass ratio of ND-PEI:siRNA is significantly higher and the apparent ζ-potential values for all monitored mass ratios are negative. The reason for such different behaviour is given by a relatively low amount of PEI on the ND surface (Figure 3A) compared to ND@(co)polymer. Furthermore, the repeating unit of PEI is ethylamine, resulting in a polymer structure of closely spaced amine groups. The interference of neighbouring groups causes that PEI protonation degree at pH 5 (~testing conditions) is only 45% [42,55]. Both factors contribute to enormously high optimal mass ratios (higher then 120:1 [28]).

Stability testing of ND-siRNA in biological conditions: The long-term stability testing of all ND-siRNA complexes was performed in the full cell culture media (9% FBS, 37°C) a full FCS (90% FCS, 37 °C) to mimic in vitro and in vivo conditions. DLS results in the cell culture media show the same stability range of ND@(co)polymer particles as observed in water, however, the aggregation points (+) were slightly shifted due to the presence of serum components. The positive surface charge of nanoparticles and high abundance of the serum albumins (isoelectric point < 5.5) most probably leads to nonspecific protein adsorption, which provides further stabilization of ND@(co)polymer:siRNA complexes. This stabilization effect was observed in
typical cell culture media as DMEM and RPMI (see Figure S5). On the other hand, tested samples in full FCS reveals slow sedimentation except 0\%/100\% which stay intact. Interestingly, sample 45\%/55\%:siR in the full FCS exhibits particularly poor stability for all monitored ratios in contrast to copolymer analogue 80\%/20\%:siR with a higher content of cationic component, which showed almost the same stability trend as 100\%/0\%:siR. On the contrary, the colloidal behaviour of 33\%/67\%:siR with a dominant fraction of neutral component seems to be similar to 0\%/100\%:siR if one compares the stable regions. Surprisingly, the simple combination of DMAEMA\textsuperscript{+} and HPMA\textsuperscript{0} (45\%/55\%:siR) does not lead to the optimal connection of their inherent properties and rather negatively affects colloidal stability in harsh biological conditions. To provide more realistic insight into the colloidal behaviour, the sample 80\%/20\%:siR was also tested after incubation with HeLa cells at different time points and in various solvents (see Figure S6). Stabilization effect was observed without any aggregation in full cell culture media after 40 min. However, serum-free media caused sample destabilization after the same time.

Enhanced stabilization in full FCS compared to full cell culture media was also observed for the complex ND-PEI:siRNA possessing the negative apparent ζ-potential in the stable area due to electrostatic repulsion with negatively charged serum components.
Figure 6. Colloidal properties of ND-PEI:siRNA and ND@(co)polymer:siRNA complexes at different mass ratios. DLS intensity weighted mean diameters (NNLS) of tested complexes A) in RNase free water at 25 °C – data points represent the mean value of intensity weighted mean diameters ± sample standard deviation over three measurements; B) in full cell culture media for 4T1 cell line (9% FCS) at 37 ° – data points represent the first measurement of intensity mean diameter and the top/bottom part of the superimposed dashed line the 10th one (~ after 20 min) ; D) in full fetal calf serum (90% FCS) at 37 °C – meaning of data points is the same as in B). C) ELS apparent ζ-potential in RNase free water (pH 5.1) at 25 °C. Detailed discussion of these results is provided in Supporting information.

Biological testing of ND-siRNA: To evaluate the transfection capability of the synthesized complexes, we have utilized the mouse 4T1 breast tumour cell line as a testing model (Figure 7). All experiments were carried out with a constant amount of siRNA.
Figure 7. A) Amount of isolated total RNA (48 h after stimulation) from 4T1 cells before qPCR analysis – displayed ratios above the bars represent ND@polymer:siRNA optimal mass ratios. B) In vitro inhibition of mouse GAPDH mRNA expression in 4T1 cells using 560nM siRNA, measured by qPCR 48 h after stimulation. C) Inhibition of mGAPDH mRNA and amount of isolated total RNA during qPCR experiment displayed as a function of ND@polymer:siRNA complex concentration in the plate well; r – Pearson's linear correlation coefficient; p tests the null hypothesis of no correlation at 0.05 significance level. All data (A), (B), (C)) are presented as mean ± sample standard deviation over biological triplicate. D) Cluster dendrogram of the group means (standardized z-scores) based on the Euclidean metric containing PERMANOVA results (p-values); c – cophenetic correlation coefficient reflects an agreement between original data distances and cluster tree cophenet distances; p – permutation-based p-value assessing the significance of the pseudo F-statistic (for more information see [50]; PERMANOVA tests the differences between tested samples considering isolated total RNA and mGAPDH mRNA level values as dependent variables at the same time. Visual observation of the cells in light microscope after stimulation, a posteriori multiple-comparison test and MATLAB implementation of the test [51] are provided in Supporting Information).
Consequently the optimal mass ratios of ND@(co)polymer:siRNA complexes vary due to different aggregation points (+) (Figure 5). Optimal mass ratio combines (i) maximal siRNA binding efficacy of ND@(co)polymer sample and (ii) ND@(co)polymer:siRNA mass ratio as low as possible, but still keeping the complex in colloidally stable area III (for more detailed discussion about estimation of the optimal mass ratio see Supporting information – Colloidal stability assessment and choosing of the optimal mass ratio). Sample optimized this way exhibits positive potential. To show the importance of this optimization, we tested two variants of 100\(^+\)/0\(^0\):siR complex – 100\(^+\)/0\(^0\):siR(+) and 100\(^+\)/0\(^0\):siR(-) possessing opposite polarity of apparent \(\zeta\)-potential. Additionally, both samples 100\(^+\)/0\(^0\):siR(+) and 80\(^+\)/20\(^0\):siR(+) reveal a similar stability trend in 90% FCS at 37 °C (see Figure 6D), which makes them potentially feasible for in vivo testing. Samples with a low content of cationic units were not considered as perspective due to the relatively high optimal mass ratios. More detailed characterization of ND@(co)polymer:siRNA complexes for qPCR testing is available in Table S2.

To provide a more complex comparison between biologically tested samples, the positively correlated amount of isolated total RNA and mGAPDH mRNA levels from the qPCR experiment (Figure 7C) were used to assess the overall transfection capability of the tested samples. Hierarchical clustering of both data sets using Euclidean dissimilarity measure between each pair of group standardized z-scores (each group represents one tested sample) was applied to create a graphical representation in the form of the dendrogram (see Figure 7D). The separation between groups (\(p\)-values above links) was evaluated using one-way permutational multivariate analysis of variance (PERMANOVA) for each cluster – for MATLAB implementation see Supporting Information. This non-parametric approach allows using the above-mentioned dissimilarity measure and has no assumption of multivariate normality\(^{[50]}\). In this study, we consider that separation between groups is significant if less than 5\% of the permuted pseudo F- statistics have values smaller than observed pseudo F- statistic \((p < 0.05)\). If the branches of the dendrogram are cut at dissimilarity level slightly above 1.8,
two clusters are obtained: 1. cluster [control 10%, 100*/0*:siR(-)] and 2. cluster [HP:siR, 100*/0*:siR(+), 80*/20*:siR(+), 45*/55*:siR(+)]. Interestingly, sample 100*/0*:siR(-) possessing negative apparent ζ-potential is significantly separated from the rest of the tested samples with no observable inhibition of mGAPDH mRNA level and is comparable with a not treated control. On the other hand, the same sample with the opposite value of apparent ζ-potential (100*/0*:siR(+)) shows the same dissimilarity level as commercial transfection reactant (HP:siR). These results justify the established concept of the optimal mass ratio (Figure 5) for cationic (co)polymer-coated nanoparticles bearing negatively charged siRNA. Nevertheless, we want to emphasize that polarity of the apparent ζ-potential cannot serve as a general parameter for assessing the transfection capability – e. g. spherical nucleic acids represent a new effective transfection system with inherently high negative potential [56, 57]. On the contrary, (3-aminopropyl) trimethoxysilane-terminated-NDs:pDNA complexes possessing positive potential do not reveal sufficient transfection response in comparison with ND-PEI (0.8 kDa) [27]. Visual observation of the 4T1 cells 48 h after stimulation also well corresponds with the dendrogram structure (Figure S3). The highest dissimilarity level in the 2. cluster belongs to sample 45*/55*:siR(+) correlating with an increased tendency of the cells to form clumps and also losing their adherence. As indicate in Figure 7C and Table S4, these adverse effects seem to be strongly related to a concentration of the complexes rather than with the number of cationic units per well. Thus, overall transfection capability (considering HP:siR as a standard) can be written in following order: [HP:siR, 100*/0*-siR(+)] > 80*/20*:siR(+) > 45*/55*:siR(+) > 100*/0*-siR(-).
3. Conclusions

We have demonstrated a systematic synthesis and robust colloidal testing of the new designed cationic (co)polymer poly(DMAEMA\(^{+}\)-co-HPMA\(^{0}\)) growth from silica-coated nanodiamond bearing methacrylate groups. This novel platform was utilized for efficient siRNA delivery into mouse 4T1 breast cancer cell line. We have shown a stabilization effect of the serum proteins on the tested ND:siR complexes in the presence of living cells. That makes this system feasible for the cell culture (in vivo) experiments where the serum proteins are essential. Additionally, we are offering our practical experiences with the DLS colloidal stability assessment and providing practical examples of typical and anomalous DLS results in the complex biological media (see Supporting information). Assessing the overall transfection capability reveals that charge diluting of the surface coating leads to an increasing of the optimal ND:siR mass ratio, which is related to adverse effects on tested cells.

4. Experimental Section

Materials: Polyethyleneimine (PEI, MW = 800, branched), polyvinylpyrrolidone (PVP, MW = 10,000), tetraethyl orthosilicate (TEOS, redistilled and stored under an argon atmosphere), 3-(trimethoxysilyl)propylmethacrylate (TMSPMA), 2-dimethylaminoethyl methacrylate (DMAEMA), were purchased from Sigma-Aldrich. Aqueous ammonia (25% w/w), ethanol (super-pure for UV, 96% v/v) purchased from Penta Chemicals. N-(2-hydroxypropyl)methacrylamide (HPMA) synthesized according to published procedures\(^{[58]}\) and freshly recrystallized prior to use (2g HPMA was dissolved in 20mL of ethyl acetate, gently heated to dissolve it in the solvent, filtered using 0.2 μm polytetrafluorethylene microfilter (PTFE) filter, hexane was dropwise added until HPMA precipitation initiated and then cooled in freezer (-20 °C) at least 2 hours). 2,2’-Azobis(2-methylpropionitrile) (AIBN) purchased from Sigma-Aldrich freshly recrystallized prior to use (1.3g AIBN was dissolved in 65mL of ethanol
a recrystallized by thickening an ethanol solution on a rotary evaporator at a maximum temperature of 30 °C until crystallization initiated, then cooled down in freezer (-20 °C) at least 2 hours). Duplexed mouse GAPDH siRNA (MW = 13968) with the following sequence was purchased from Sigma-Aldrich in the form of desalted pellet:

sense strand: 5'-r(GAAGGUGCUGUGAAGCGAU)d(TT)-3'
antisense strand: 5'-r(AUCCGUUCACACCGACCUCU)d(TT)-3'.

4T1 cell line obtained from the ATCC (CRL-2539) was incubated in full cell culture media containing RPMI 1640 (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco), 44 mg/mL gentamicin (Sandoz), 44 mg/L gentamicin (Sandoz), 4.5 g/L glucose (Sigma Aldrich), 1.1 % pyruvate (Sigma Aldrich). Control transfection of GAPDH siRNA was carried out using X-tremeGENE HP DNA Transfection Reagent (Roche). Total RNA was isolated using RNeasy® Mini Kit (Qiagen) and reverse transcribed using random primers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR quantification of cDNA was carried out with gene-specific primers Mm99999915_g1 GAPDH (FAM, Life Technologies), Mm00437762_m1 B2M (FAM, Life Technologies) and a TaqMan Universal PCR Master Mix II, no UNG (Life Technologies). Unless stated otherwise, all reagents used in this work were directly used without further purification.

**ND Pretreatment:** HPHT NDs were supplied by Microdiamant Switzerland (MSY 0-0.05). The NDs were oxidized by air in a furnace (Thermolyne 21100 tube) at 510 °C for 5 h. Subsequently, the NDs were treated with a mixture of H2SO4 and HNO3 (9:1) at 90 °C for 3 days and washed with Milli-Q water, 1 M NaOH and 1 M HCl. They were washed an additional 5 times with Milli-Q water and then freeze-dried. Prior to use, the particles were dissolved in Milli-Q water (2 mg/mL) and sonicated with a probe (Cole-Parmer CPX 750, 20 kHz – tapered microtip 1/8" (3 mm)) – selected parameters: 30 min (ON cycle), amplitude 40%, duty cycle 02:02 s. The
resulting transparent colloid (ND) was incubated 30 min at room temperature and filtered using a 0.2 μm polyvinylidene fluoride (PVDF) filter.

*Coating of NDs with Methacrylate-terminated Thin Silica Layer (part I)*: To avoid precipitation of ND during silication caused by increased ionic strength (ammonia), PVP was used as a non-ionic surfactant[23]. PVP (187 mg, 0.0187 mmol) was dissolved in Milli-Q water (165 mL) and sonicated for 10 min in an ultrasonic bath (Ultrasonic Cleaner Elmasonic P60H, power 100%, 37 kHz, pulse mode). All samples sonicated in ultrasonic bath were placed in a cavitation hotspot (see Figure S1). Unless stated otherwise, all coated ND samples after centrifugation were sonicated in cup horn until dissolved (Cole-Parmer CPX 750, 20 kHz – cup horn; samples were placed on the bottom of the cup) – selected parameters: amplitude 60%, duty cycle 100%. ND colloid (33 mL, 2 mg/ml) was added, and the mixture was stirred for 24 h. The colloid was then concentrated by two-step centrifugation. In the first step (45,000 g, 1 hour), the mixture was centrifuged in 25 mL centrifuge tubes. The volume was reduced to approximately 22.4 mL. The second centrifugation step (30,000 g, 30 min) was performed in microvials (16 x 1.4 mL), and the solvent volume was reduced to approximately 5 mL. Resuspended NDs were mixed with ethanol (66 mL, super-pure for UV) in a round bottom flask during stirring. TEOS (495 μL, 2.22 mmol at 20 °C) and TMSPMA (165 μL, 0.694 mmol at 20 °C) were added to the round bottom flask during stirring and sonicated in an ultrasonic bath for 2-4 min. Aqueous ammonia (25% w/w, 2.75 mL, 37 mmol at 20 °C) was added and gently shaken. The reaction mixture was stirred for 14 h and then centrifuged in 25 mL tubes. First centrifugation was done in three steps - firstly 15,000 g, 15 min, secondly the supernatant was removed and centrifuged again at 20,000 g, 20 min, the resulting supernatant from the second step was centrifuged at 50,000 g, 20 min. The final supernatant was discarded and pellets were pooled. Following centrifugations were performed in at least two steps – 25,000 g, 25 min and 50,000 g, 25 min. The sample was centrifuged 5x and purified by methanol (MeOH, HPLC quality). The volume
of the purified sample was reduced to approximately 2 mL by two-step centrifugation in 2 mL microvials (25,000 g, 25 min and 50,000 g, 25 min) and stored in the fridge (4 °C) overnight. Methacrylate-terminated ND nanoparticles in MeOH were transferred into 414 μL of dimethyl sulfoxide (DMSO) using rotary evaporator.

Coating of NDs with a Copolymer Layer (part II): According to Table S1, different weight ratios of DMAEMA⁺ (100, 75, 50, 25, 0 % w/w) and HPMA⁰ (0, 25, 50, 75, 100 % w/w) were dissolved in 563 μL of DMSO – sample prepared as 75% DMAEMA⁺ / 25% HPMA (80°/20° in text) was used in⁴⁵⁹; AIBN (56.4 mg, 0.343 mmol) was added to the mixture. The mixture was filtered using a 0.2 μm PTFE filter. Methacrylate-terminated NDs (part I) in DMSO (57 μL, 9 mg of ND – considering 100% yield after silication) were added to each mixture. The rest of the methacrylate-terminated NDs (129 μL, 20 mg) were purified by MeOH (preventing silica layer against hydrolysis) and stored at -20 °C. Vacuum degassing of the reaction mixture under argon filling cycles of the stirred mixture was performed (three cycles vacuum – argon, 1 min – 1 min using disposable needles 0.80x0.50 mm) – this step allows regulation of the (co)polymer thickness. Degassed mixture proceeded for 3 days under argon at 55 °C. The reaction was stopped by MeOH addition. The ND@copolymer samples were centrifuged 5x (1x = 20,000 g, 20 min and 40,000 g 20 min) and purified by RNase free water (dilution factor ~100x).

Coating of NDs with a PEI: PEI (100 μL, 0.9 mg/mL, 0.1 μmol) was dissolved in Milli-Q water and filtrated using 0.2 μm PVDF filter; ND colloid (100 μL, 2 mg/mL) was added. The completely aggregated mixture (Figure S1B) was vortexed for at least 30 min and sonicated in an ultrasonic bath (power 100%, 37 kHz, pulse mode) which was filled with the mixture of the ice and water (water-ice level in the bath was approximately 2.5 cm). Two-step centrifugation of the aggregated mixture was carried out; first centrifugation 9,000 g, 15 min and 30,000 g,
15min. Collected supernatants containing an excess PEI were discarded and replaced with the same volume of Milli-Q water. Following centrifugation (4x: 15,000 g, 15min and 30,000 g, 15min) and purification with RNase free water serves as a defloculation process resulting in transparent colloid ND-PEI (similar to Figure S1A); final volume was 100 μL. Importantly, a pellet of ND-PEI after each centrifugation must be dissolved in the ultrasonic bath (hotspot – Figure S1C), not in the cup horn.

*Complexation of siRNA for DLS, ELS analysis and Qubit assay* – Various amount of ND@(co)polymer (9 mg/mL) was diluted by RNase free water resulting in a final volume of 22.2 μL and added into 2.8 μL (100 μM, 0.28 nmol, 3.91 μg) GAPDH siRNA (to reach given ND@(co)polymer:siRNA mass ratio) – siRNA stock solution was dissolved in RNase free water, pH 5.1. The final mixture (25 μL or scalable volume) was gently sonicated 5-10 s in the ultrasonic bath, incubated at room temperature for 20 min and gently sonicated again. To obtain the desired concentration of ND@(co)polymer:siRNA complexes for further testing, the sample was centrifuged 20,000 g, 20 min and the necessary amount of supernatant was removed. The supernatant was analyzed using Qubit miRNA assay kit to quantify the amount of free siRNA relative to control (particle-free). Importantly, the complexation of siRNA with cationic ND@(co)polymer seems to be provider dependent likely due to a different way of siRNA purification. Except for siRNA from Sigma Aldrich utilized in this study, other siRNAs/DNAs provided from different sources typically led to the aggregation of the mixture. A significant raising of ND@(co)polymer:siRNA mass ratio was required to fix this issue, e.g. from optimal mass ratio 25:1 (Sigma Aldrich) to 65:1 (Dharmacon) – this problem was also reported in [59]. To reflect this situation, the complexation protocol was developed to normalize the preparation method regardless of the source of siRNA (see Supporting Information – *Complexation protocol*). *Short-term DLS/ELS testing (Figure 6A, C)* – dissolved ND@(co)polymer:siRNA pellet was added directly (not dropwise) into the RNase free water (pH 5.1) with the final
volume 0.6 mL. Long-term DLS testing (Figure 6B, D) – freshly prepared complexes ND@(co)polymer:siRNA (final mixture, 25 μL) were mixed directly with the 4T1 cell culture media (10% FCS) or with the 100% FCS (media containing FCS were centrifuged 5,000 g, 10min prior to use and supernatant was used for measurement); dilution of the media by the testing sample was always 8% (rounded to 10% in the text); particle concentration differs from 0.07 mg/mL (5:1 coated-ND:siRNA mass ratio) to 0.34 mg/mL (40:1). Samples were preheated at 37 °C, 2 min and mixed with a pipette before analysis.

Cell stimulation (transfection efficiency) – The complexation procedure was without further optimization upscaled to 50 μL (final volume) containing 5.6 μL (100 μM) GAPDH siRNA. All ND samples for transfection were freshly prepared prior to use and incubated at room temperature for approximately 60 min. Control transfection of GAPDH siRNA without NDs was carried out using 2 μL of X-tremeGENE HP DNA Transfection Reagent diluted by 42.4 μL of RNase free water and mixed with 5.6 μL (100 μM) GAPDH siRNA; the sample was incubated 20-30 min at room temperature prior to use.

ND Colloidal Stability Studies (DLS, ELS, NTA): Particle size distributions and average values of apparent ζ-potential were obtained on a Zetasizer Nano ZSP (Malvern Instruments). Intensity weighted mean/median diameters were calculated from the second-order time intensity autocorrelation function $G^{(2)}(\tau)-1$ (where $G^{(2)}(\tau) = \langle I(t)I(t+\tau) \rangle \langle \cdot \rangle$ denoting time average) using General purpose algorithm (NNLS) in Zetasizer Software 7.11. The intensity mean diameters were calculated from the dominant (first) PSD peak. Z-average diameters were calculated from the fit by the first cumulant of a 3rd-order cumulant analysis. Data transformation into number-weighted size distribution was also performed in Zetasizer Software using Mie theory without inspecting change in optical properties due to (co)polymer coating; real ($n$) and imaginary ($k$) part of the complex refractive index was set as $n = 2.41$ and
$k = 0.00$ for nanodiamond samples; viscosity and refractive index values for different dispersants can be found in Table S11; Data were collected at a backscatter angle of 173° (NIBS system) using quartz cuvette ZEN2112. In short-term testing (Figure 6A, C), each sample was measured three times with an automatic duration; reported size represents an average value of these measurements. In long-term testing (Figure 6B, D), each sample was measured ten times (approximately 20 min); viscosity and refractive index of full FCS (90% FCS) and full cell culture media (9% FCS) for 4T1 cell line at 37 °C was set at 0.861 [60] and 0.740 cP [61] resp. – referred values represent viscosity for 100% FCS and cell culture media without diluting; dilution of media by the testing sample was always 10%. Electrophoretic light scattering at a forward angle 13° with a phase analysis of scattered light (PALS) was used for the determination of an apparent ζ-potential, which represents the electrostatic potential calculated from electrophoretic mobility following Smoluchowski approximation for spherical noncoated particles without inspecting the value of the ratio of particle size to Debye length. All samples were measured in RNase free water (pH 5.1) using a disposable cuvette with a dip cell; monomodal analysis with two measurements and twenty subruns was chosen; an average sample conductivity was 41.3 μS/cm for all samples tested with siRNA. For both type of measurements particle concentration was lower than 0.1 mg/mL for RNase free solvent or 0.4 mg/mL for FCS or full cell culture media for 4T1 cell line (see Figure S12). NTA measurements were performed with Nanosight NS300 equipped with a low-volume cell connected to a linear pump and a 532 nm green laser (60 mW). Stock solutions of ND@(co)polymer nanoparticles (9 mg/mL) were diluted in Milli-Q water with dilution factor $10^5$ resulting in measurements with 21-40 particles per frame and number of valid tracks > 930. Camera level was set to level 16 (shutter/gain: 1300/512), detected threshold to level 5 and syringe pump flow rate to level 12; blur, maximum jump distance, and minimum expected particle size (MEPS) were set to “auto”. Each sample was measured 5 x 60 s at ~25 °C. Captured data in “AllTracks.csv” files were manually analyzed; Measurements signed as FALSE
(column: “Included in distribution?”) were excluded; Unique values in column: “Particle ID” were found, and all repetitions were excluded – final number of particles per measurement was equal to number of valid tracks provided by software; Obtained raw data (without applying finite track-length adjustment (FTLA)) were plotted as a scatter plot (Figure 4) using column data “Ln(Adjusted intensity)/AU”. NTA size distributions (Figure 4) shows normalized FTLA plots provided by software. Prior to use, all ND@(co)polymer samples were sonicated in the cup horn.

Electron microscopy (TEM): Transmission electron microscopy was performed with JEOL JEM-1200EX electron microscope operated at 80 kV. The sample was prepared on carbon-coated copper grids according to [62]. Homemade MATLAB code implementing automatic/semiautomatic analysis of TEM images will be available from the corresponding author.

Cell Culture and ND-siRNA Stimulation: The 4T1 cell line was stored in the full cell culture media comprises 10% (v/v) DMSO at -80°C. First day (after thawing), the cells were incubated with a fresh full media under a humid atmosphere containing 5% CO₂ (incubation conditions) overnight. Second day, the full media was changed and the cells were incubated two days without media changing. Fifth day, the cells (~250, 000 cells/mL/well – Bürker counting chamber) were plated onto 12-well plates and incubated with fresh full media overnight.

Transfection Efficiency – RNA Isolation, Reverse Transcription and Real-Time Quantitative RT-PCR (qPCR): Sixth day, stimulation of the cells (confluence 70-80%) was performed; 50uL of the sample was added dropwise and uniformly across the wells. The final volume per well was 1 mL (950 μL of full cell culture media and 50 μL of testing sample dissolved in RNase free water); an amount of mouse GAPDH siRNA was constant for all samples (7.84 μg/well).
More detailed information can be found in Table S2 and Table S3. Stimulated cells were incubated for 48 hours. All samples were analyzed in biological triplicate. The isolated total RNA fraction (5 μg – Nanodrop quantification) were reverse transcribed in cDNA form. PCR quantification was carried out with 2 μL of prepared cDNA. B2M was used as an internal control for quantitation of GAPDH expression. During qPCR, all samples were analyzed in technical triplicate using an Bio-Rad CFX Real Time PCR Detection System. The obtained data were analyzed using Bio-Rad CFX Manager 3.1. The expression of the GAPDH was normalized to the expression of internal control, fold change was calculated by the software (based on 2−ddct method) and graph data is depicted relative to zero.

Other Analytical Methods: 1H NMR spectra were recorded on a Bruker Avance III 500 spectrometer (499.88 MHz for 1H and 125.71 MHz for 13C) equipped with a 5 mm PFG cryoprobe; 5 mg of sample was centrifugated 3x and transferred in heavy water resulting in final volume approximately 50 μL. The signals were assigned by using a combination of 1D and 2D (H,HCOSY and H,C-HSQC) techniques. TGA was measured with TG 750 Stanton Redcroft instrument. Approximately 1 mg powder sample was heated under air with a heating rate of 20 °C/min.

Acknowledgements
The authors are grateful to Dr. Martin Dracinsky (Institute of Organic Chemistry and Bichemistry of the CAS) for the measurement and help with analysis of 1H NMR spectra, to Dr. Helena Raabova (Institute of Organic Chemistry and Bichemistry of the CAS) for her support with TEM measurements, and to Dr. Jaroslav Hanus (University of Chemistry and Technology Prague) for providing us the access to the NanoSight NS300. This work was supported by the Czech Science Foundation Project No. 18-17071 S (to P.C.), MSM Project No. 8C18004 (NanoSpin) (to P.C.), European Regional Development Fund; OP RDE; Projects: Chem-BioDrug (No. CZ.02.1.01/0.0/0.0/16_019/0000729) (to P.C., M.K.) and CARAT (No. CZ.02.1.01/0.0/0.0/16_026/0008382) (to P.C., M.G.).
References


Supporting Information

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Nanodiamonds with charge density tuned copolymer interface for efficient siRNA vectorization

Marek Kindermann, Jitka Neburkova, Eva Neuhoferova, Jan Majer, Miroslava Guricova, Veronika Benson and Petr Cigler*

Figure S1. Pictures of A) colloidally stable B) aggregated nanodiamond dispersions.[59] For more pictures showing colloidal state before/after coating, siRNA adsorption etc. see [59]. C) Aluminum foil test revealing cavitation hotspots (e.g. intersection of red lines) inside of the sonication bath for the optimal sample sonication.
Table S1. Amounts of DMAEMA⁺/ HPMA⁰ in the reaction mixture

<table>
<thead>
<tr>
<th>ND@poly[(DMAEMA)⁻“co-HPMA”)</th>
<th>DMAEMA⁺ (mg / mmol)</th>
<th>HPMA⁰ (mg / mmol)</th>
<th>Mass fraction DMAEMA⁺/HPMA⁰ (%)</th>
<th>Mole fraction DMAEMA⁺/HPMA⁰ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0°</td>
<td>196.8 / 1.252</td>
<td>0.0 / 0.0</td>
<td>100 / 0</td>
<td>100 / 0</td>
</tr>
<tr>
<td>80/20°</td>
<td>147.6 / 0.9339</td>
<td>49.2 / 0.344</td>
<td>75 / 25</td>
<td>73 / 27</td>
</tr>
<tr>
<td>45/55°</td>
<td>98.4 / 0.626</td>
<td>98.4 / 0.687</td>
<td>50 / 50</td>
<td>48 / 52</td>
</tr>
<tr>
<td>33/67°</td>
<td>49.2 / 0.313</td>
<td>147.6 / 1.031</td>
<td>25 / 75</td>
<td>23 / 77</td>
</tr>
<tr>
<td>0°/100°</td>
<td>0.0 / 0.0</td>
<td>196.8 / 1.374</td>
<td>0 / 100</td>
<td>0 / 100</td>
</tr>
</tbody>
</table>

9 mg (57 uL) of methacrylate-terminated NDs in DMSO were added to each mixture

Table S2. Mass yields of NDs after (co)polymer coating

<table>
<thead>
<tr>
<th>ND@poly[(DMAEMA)⁻“co-HPMA”)</th>
<th>Apparent mass yield (%m1)</th>
<th>Mass of (co)polymer (%m2)</th>
<th>Real mass yield of ND (%m3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0°</td>
<td>65</td>
<td>28.66</td>
<td>46</td>
</tr>
<tr>
<td>80/20°</td>
<td>52</td>
<td>25.46</td>
<td>39</td>
</tr>
<tr>
<td>45/55°</td>
<td>54</td>
<td>22.81</td>
<td>42</td>
</tr>
<tr>
<td>33/67°</td>
<td>55</td>
<td>21.22</td>
<td>43</td>
</tr>
<tr>
<td>0°/100°</td>
<td>73</td>
<td>22.86</td>
<td>56</td>
</tr>
</tbody>
</table>

Calculation

\[
\%m1 = \frac{\text{final mass (ND@(co)polymer)}}{\text{initial mass (ND)}} \times 100 \\
\%m3 = \frac{\%m1}{100} \times \left(100 - \%m2\right) \times 100
\]

a) Values of the (co)polymer mass were obtained from TGA thermal curve (Figure 3A) at 600 °C. To collect percentual weight loss values at 600 °C, the linear regression was used in the range from 590 to 610 °C.

NTA, DLS and TEM results comparison

Size measurement interpretation of (co)polymer samples is facilitated due to repeated centrifugal purification of the samples. This treatment leads to the mean weight loss of raw NDs around 55% (Table S2) – specifically, loss of the small particle fraction from PSD results in decreasing of sample polydispersity and optimal particle size for discussed methods. NTA number weighted PSDs well correspond with the DLS transformed number weighted PSDs (Figure S2B, C, D, E, F) assuming sufficiently small particles, where the actual refractive index and particle shape are not critical for the transformation (Mie theory). On the other hand, the NTA results show low sensitivity for small particles in the case of the highly polydisperse raw NDs (Figure S2A, NDs) and mismatch with DLS PSD. However, even for the ND@co)polymer samples, the DLS and NTA PSD do not match with TEM PSD. Presented size distributions (Figure S2) and the mean/median diameters in the text measured in Milli-Q/RNase free water suffer from apparent increase in size (free diffusion is restricted) due to low ionic strength of water causing extension of the electrical double layers (EDLs) of charged particles resulting in decrease of diffusion coefficients. Moreover, this size increase is supported by swelling effect of charged linear chains. To remove this effect, measurement at high ionic strength (10xPBS) were performed. Collapsing of the EDL but preserving of the steric stabilization allows more correct measurements, which are comparable with TEM distributions. Measurement in pure water without adjustment of the proper ionic strength can be acceptable when the particles are stabilized only electrostatically and the colloidal stability is threatened. Other reason can be presence of unknown aggregation processes and monitoring of colloidal stability trend rather than precise size measurement itself.
Figure S2. Particle size distributions (PSD) of ND and ND@(co)polymer samples provided by different techniques (DLS (NNLS), NTA (FTLA), TEM (MATLAB image analysis script)). DLS intensity weighted PSD was converted, using Mie theory (see Experimental Section), to a number weighted DLS distribution. DLS – reported data represent a mean value of three measurements ± standard deviation; NTA – mean value of five measurements ± standard deviation.

Table S3. Characterization of ND@(co)polymer:siRNA complexes (in RNase free water at 25 °C) for biological testing

<table>
<thead>
<tr>
<th>Sample</th>
<th>N:P</th>
<th>Optimal mass ratios ND@(co)polymer:siR</th>
<th>DLS(i) intensity weighted diameter (mV)</th>
<th>ELS apparent ζ-potential (mV)</th>
<th>Qubit assay fraction of free siRNA (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(median) ± σ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mean ± σ&lt;sub&gt;b&lt;/sub&gt;</td>
<td>mean ± RSD&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>100°/0°:siR(-)</td>
<td>1:1</td>
<td>1:1</td>
<td>115.0 ± 4.2</td>
<td>-31.6 ± 0.6</td>
<td>61.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100°/0°:siR(+)</td>
<td>3:1</td>
<td>5:1</td>
<td>130.0 ± 2.8</td>
<td>34.1 ± 1.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>80°/20°:siR(+)</td>
<td>5:1</td>
<td>12:1</td>
<td>113.0 ± 2.1</td>
<td>34.5 ± 0.4</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>45°/55°:siR(+)</td>
<td>4:1</td>
<td>20:1</td>
<td>108.0 ± 7.9</td>
<td>37.7 ± 1.6</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean value of intensity weighted median diameters ± sample standard deviation over two measurements; <sup>b</sup> mean ± sample standard deviation over two measurements; <sup>c</sup> fraction of free siRNA calculated relative to control (sample without nanoparticles), RSD value represents relative standard deviation collected from three measurements of the same sample; <sup>d</sup> free fraction of siRNA was centrifugally removed and replaced with bounded one to keep a constant amount of bounded siRNA per well (7.84 µg) – see Table S4.
Table S4. Amount of transfection material per well for qPCR testing

<table>
<thead>
<tr>
<th>Sample</th>
<th>μg(ND)/well</th>
<th>μg(siRNA)/well</th>
<th>μg((co)polymer)/well</th>
<th>μg(DMAEMA+)/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%/0%:siR(-)</td>
<td>14.34</td>
<td>7.84</td>
<td>5.48</td>
<td>5.76</td>
</tr>
<tr>
<td></td>
<td>0.0161 ± 0.0006</td>
<td>560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%/0%:siR(+)</td>
<td>28.0</td>
<td>7.84</td>
<td>11.3</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>0.0805 ± 0.0032</td>
<td>560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%/20%:siR(+)</td>
<td>71.5</td>
<td>7.84</td>
<td>24.6</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>0.168 ± 0.007</td>
<td>560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45%/55%:siR(+)</td>
<td>124</td>
<td>7.84</td>
<td>36.5</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>0.314 ± 0.013</td>
<td>560</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Final volume per well is 1 mL; calculated according to a)NTA; b)TGA; c)NMR.

Figure S3. A) Cluster dendrogram of the group means with one-way PERMANOVA results (analysis of the amount of isolated total RNA and mGAPDH mRNA levels from the qPCR experiment for all samples). B) Light microscope images of 4T1 cells treated by ND(co)polymer:siRNA complexes for qPCR quantification; 48 h after stimulation. Optimal mass ratios for ND(co)polymer:siRNA complexes are presented in Table S3.
Table S5. One-way PERMANOVA outputs used in Figure S2. The number of iterations for permutation test was 1,000 for all analyzed clusters

<table>
<thead>
<tr>
<th>Pseudo F-ratio</th>
<th>Permutation-based p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.3975</td>
<td>0.0010</td>
</tr>
<tr>
<td>7.7493</td>
<td>0.0030</td>
</tr>
<tr>
<td>3.2565</td>
<td>0.0360</td>
</tr>
<tr>
<td>2.4322</td>
<td>0.1750</td>
</tr>
</tbody>
</table>

Implementation of hierarchical clustering and PERMANOVA in MATLAB (MathWorks, Inc.). This implementation contains `f_permanova()` and `f_permanovaPW()` functions, which are part of the ‘FATHOM TOOLBOX FOR MATLAB’ developed by David L. Jones [51]. This function performs one-way PERMANOVA using the modified F-statistic described in Anderson et al. (2017) [50]

```
% Tested qPCR groups
%--------------------------------------------------
% group index = sample name
%--------------------------------------------------
% group 1 = control 10%;
% group 2 = HP:siR;
% group 3 = 100/0:siR(-);
% group 4 = 100/0:siR(+);
% group 5 = 80/20:siR(+);
% group 6 = 45/55:siR(+);
%--------------------------------------------------

% Hierarchical clustering
%--------------------------------------------------
% averages_all is a group mean matrix (6,2)
% rows - group means; columns - mRNA levels, total RNA concentrations
% X is z-score matrix of group means over biological triplicates
% X = zscore(averages_all);
% the Euclidean distance between pairs of observations
% Y = pdist(X', 'euclidean');
% data clustering
% Z = linkage(Y, 'average');
% cophenic correlation coefficient
% c = cophenet(Z, Y);
% dendrogram(Z);
% box on;
%--------------------------------------------------

% one-way PERMANOVA
%--------------------------------------------------
% X_raw is z-score matrix (18,3) of indexed raw data (biological triplicates)
% rows - z-scores; columns - group indexes, mRNA levels(z-score), total RNA(z-score)
% X_raw = zscore(raw_indexed(:, 2:end));
% group = raw_indexed(:, 1);
% Y = pdist(X_raw, 'euclidean');
% square symmetric dissimilarity matrix
% yDis = squareform(Y);
% f_permanova function is part of the 'FATHOM TOOLBOX FOR MATLAB';
% f_permanova(yDis, group, 1000, 1);
% the smallest possible "p-value" for 1,000 permutations is ~ 0.001
```
function is part of the 'FATHOM TOOLBOX FOR MATLAB';
perform a posteriori multiple-comparison tests
\[
\text{f\_permanovaPW(yDis,group,1000,1)};
\]

\%

**Table S6.** PERMANOVA a posteriori multiple-comparison test for the Figure 4F considering amount of isolated total RNA and mGAPDH mRNA level as dependent variables

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample</th>
<th>Permutation-based p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 10%</td>
<td>vs</td>
<td>HP:siR</td>
</tr>
<tr>
<td>control 10%</td>
<td>vs</td>
<td>100(^{\circ}):siR(-)</td>
</tr>
<tr>
<td>control 10%</td>
<td>vs</td>
<td>100(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>control 10%</td>
<td>vs</td>
<td>80(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>control 10%</td>
<td>vs</td>
<td>45(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>HP:siR</td>
<td>vs</td>
<td>100(^{\circ}):siR(-)</td>
</tr>
<tr>
<td>HP:siR</td>
<td>vs</td>
<td>100(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>HP:siR</td>
<td>vs</td>
<td>80(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>HP:siR</td>
<td>vs</td>
<td>45(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>100(^{\circ}):siR(-)</td>
<td>vs</td>
<td>100(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>100(^{\circ}):siR(-)</td>
<td>vs</td>
<td>80(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>100(^{\circ}):siR(-)</td>
<td>vs</td>
<td>45(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>100(^{\circ}):siR(+)</td>
<td>vs</td>
<td>80(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>100(^{\circ}):siR(+)</td>
<td>vs</td>
<td>45(^{\circ}):siR(+)</td>
</tr>
<tr>
<td>80(^{\circ}):siR(+)</td>
<td>vs</td>
<td>45(^{\circ}):siR(+)</td>
</tr>
</tbody>
</table>

**Table S7.** PERMANOVA a posteriori multiple-comparison test for the Figure 7D considering D50 and SPAN values as dependent variables

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample</th>
<th>Permutation-based p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>100(^{\circ}):0(^{\circ})</td>
<td>vs</td>
<td>80(^{\circ}):20(^{\circ})</td>
</tr>
<tr>
<td>100(^{\circ}):0(^{\circ})</td>
<td>vs</td>
<td>45(^{\circ}):55(^{\circ})</td>
</tr>
<tr>
<td>100(^{\circ}):0(^{\circ})</td>
<td>vs</td>
<td>33(^{\circ}):67(^{\circ})</td>
</tr>
<tr>
<td>100(^{\circ}):0(^{\circ})</td>
<td>vs</td>
<td>0(^{\circ}):100(^{\circ})</td>
</tr>
<tr>
<td>80(^{\circ}):20(^{\circ})</td>
<td>vs</td>
<td>45(^{\circ}):55(^{\circ})</td>
</tr>
<tr>
<td>80(^{\circ}):20(^{\circ})</td>
<td>vs</td>
<td>33(^{\circ}):67(^{\circ})</td>
</tr>
<tr>
<td>80(^{\circ}):20(^{\circ})</td>
<td>vs</td>
<td>0(^{\circ}):100(^{\circ})</td>
</tr>
<tr>
<td>45(^{\circ}):55(^{\circ})</td>
<td>vs</td>
<td>33(^{\circ}):67(^{\circ})</td>
</tr>
<tr>
<td>45(^{\circ}):55(^{\circ})</td>
<td>vs</td>
<td>0(^{\circ}):100(^{\circ})</td>
</tr>
<tr>
<td>33(^{\circ}):67(^{\circ})</td>
<td>vs</td>
<td>0(^{\circ}):100(^{\circ})</td>
</tr>
</tbody>
</table>
Figure S4. TEM images of ND samples. Size distributions are displayed in Figure S1 and the results of image analysis are summarized in Table S8. The scale bar represents 50 nm.
Table S8. Comparison of different techniques to estimate the equivalent diameter of particles

<table>
<thead>
<tr>
<th>Sample</th>
<th>DLS(i) intensity weighted diameter (nm)</th>
<th>DLS(n) number weighted diameter (nm)</th>
<th>NTA(n) number weighted diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>⟨D50⟩ ± σₚ / ⟨PDI⟩ ± σₚ a)</td>
<td>⟨D50⟩ ± σₚ / ⟨PDI⟩ ± σₚ a)</td>
<td>⟨D50⟩ ± σₚ / ⟨PDI⟩ ± σₚ b)</td>
</tr>
<tr>
<td>ND</td>
<td>56.9 ± 1.6 / 0.16 ± 0.04</td>
<td>29.6 ± 2.9 / 0.09 ± 0.00</td>
<td>66.5 ± 1.2</td>
</tr>
<tr>
<td>0°/100°</td>
<td>114.0 ± 1.0 / 0.11 ± 0.01</td>
<td>69.2 ± 4.2 / 0.10 ± 0.01</td>
<td>70.8 ± 1.9</td>
</tr>
<tr>
<td>33°/67°</td>
<td>132.3 ± 3.2 / 0.16 ± 0.02</td>
<td>71.1 ± 3.9 / 0.14 ± 0.01</td>
<td>80.3 ± 1.2</td>
</tr>
<tr>
<td>45°/55°</td>
<td>146.7 ± 3.2 / 0.13 ± 0.02</td>
<td>83.7 ± 6.7 / 0.15 ± 0.01</td>
<td>83.7 ± 2.0</td>
</tr>
<tr>
<td>80°/20°</td>
<td>170.0 ± 4.0 / 0.13 ± 0.02</td>
<td>97.6 ± 2.3 / 0.17 ± 0.01</td>
<td>82.8 ± 1.9</td>
</tr>
<tr>
<td>100°/0°</td>
<td>163.0 ± 0.0 / 0.19 ± 0.03</td>
<td>84.8 ± 3.3 / 0.17 ± 0.00</td>
<td>91.1 ± 2.6</td>
</tr>
</tbody>
</table>

Average number of analyzed particles per ND@(co)polymer sample:

- Merged data (5 measurements)

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Calculation

(1) ⟨D⟩ = \frac{\sum A_i D_i}{\sum A_i},

(2) ⟨D^2⟩ = \frac{\sum A_i D_i^2}{\sum A_i},

(3) \sigma_p = \sqrt{⟨D^2⟩ - ⟨D⟩^2},

(4) PDI = \left(\frac{\sigma_p}{⟨D⟩}\right)^2,

(5) SPAN = \frac{D_{90} - D_{10}}{D_{50}}

Mark a) Mean value of intensity weighted median diameters ± sample standard deviation / mean polydispersity index ± sample standard deviation over three measurements; b) number weighted median diameter / polydispersity index over five merged measurements (Experimental Section describes more detailsly data treatment ); c) number weighted median diameter / polydispersity index from merged data collecting all sample images. PDI values for all techniques (without ) were obtained directly from the size histograms according to calculations in the last row: ⟨D⟩ – particle weighted mean diameter; D_i – diameter of i-th particle; A_i – normalized height of i-th bar in histogram; \sigma_p – standard deviation of PSD; D10, D50, D90 – 10-th, 50-th and 90-th percentiles of the size distribution.
Table S9. Electrophoretic light scattering analysis of tested particles

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELS (apparent) ζ-potential (mV) mean ± σζ</th>
<th>Electrophoretic mobility (µm·cm/V·s) mean ± σζ</th>
<th>Conductivity (µS/cm) mean ± σζ</th>
<th>Applied voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>-40.0 ± 2.6</td>
<td>-3.14 ± 0.21</td>
<td>3.18 ± 0.06</td>
<td>9.98</td>
</tr>
<tr>
<td>0°/100°</td>
<td>-9.1 ± 3.0</td>
<td>-0.71 ± 0.24</td>
<td>4.27 ± 0.04</td>
<td>9.98</td>
</tr>
<tr>
<td>33°/67°</td>
<td>36.9 ± 3.0</td>
<td>2.89 ± 0.23</td>
<td>2.99 ± 0.05</td>
<td>9.98</td>
</tr>
<tr>
<td>45°/55°</td>
<td>38.4 ± 2.5</td>
<td>3.01 ± 0.20</td>
<td>3.21 ± 0.05</td>
<td>9.98</td>
</tr>
<tr>
<td>80°/20°</td>
<td>40.5 ± 3.1</td>
<td>3.17 ± 0.24</td>
<td>3.54 ± 0.06</td>
<td>9.98</td>
</tr>
<tr>
<td>100°/0°</td>
<td>35.3 ± 0.6</td>
<td>2.77 ± 0.04</td>
<td>3.19 ± 0.09</td>
<td>9.98</td>
</tr>
</tbody>
</table>

All data represent the mean value ± sample standard deviation over four measurements.

Figure S5. Raw DLS intensity autocorrelation functions of the sample 80°/20°:siRNA(+) in A) RPMI at 37 °C; B) RPMI+FCS (10%) without cell culture supplements at 37 °C; C) DMEM at 37 °C; D) DMEM+FCS (10%) without cell culture supplements at 37 °C. The samples were measured ten times (~ 20 min) except C) due to obvious aggregation of the sample.
Colloidal stability assessment and choosing of the optimal mass ratio

Here we are providing our experimental experiences with the colloidal stability assessment in the complex biological media as full cell culture media or full FCS. This task has rather a complex character and requires monitoring of a number of parameters e.g. polydispersity index (cumulant based PDI), an indicator of the quality of the baseline, and the intercept of the $G^2(\tau) - 1$ (In Range value; In Range = 100(1-$|X|^{0.5}$), where $X = 0.5$(Background Ratio/Signal to Noise Ratio) – all these parameters are available under these names in the software), detection of the statistically significant trend in $Z$-average values (Mean size trending – see Expert Advice in Zetasizer software), a change of the count rate value (Derived count rate), etc. – all these characteristics are available in the Zetasizer software. Moreover, one has to also assess the sample visually, which requires a certain experience. To quantify our empirical experiences, we have decided to heuristically choose data revealing "sufficient" colloidal stability (suitable for further experiments) and those that are aggregated (as shown in Figure S7, Figure S8). These two groups were described using the above-mentioned parameters and data were summarized in the form of box plots (Figure S10, Figure S11). Such an approach quantifies our decisions about colloidal stability in complex solvents and allows other researchers a more precise comparison of their stability measurements to those published in this work.

Figure S7 shows examples of typical raw DLS data for different colloidal states in the full cell culture media (9% FCS) at 37 °C. However, due to media complexity, anomalous data can occur as presented in Figure S8. Example #1: Figure S8B – at the first glance ($\Delta$PDI = 0.032; $\Delta$(In Range) = -0.5; Mean size trending not detected - see Figure S10), the $G^2(\tau) - 1$ is well shaped with a little bit lower value of the intercept, however, the sample looked cloudy and the estimated mean size is far from the original size before complexation with siRNA. Based on these observations, the sample belongs to the aggregation area. Example #2: S8. Figure S8C – in this case, the scattering background of the FCS must be taken into account (see Figure S12A), which is one of the factors causing extremely high PDI value. This issue can be overcome by increasing the particle concentration or utilizing more advanced techniques as depolarized DLS to remove the scattering background [63]. Example #3: S8. Figure S8D – when considering the complete data set, one could expect a rather stable sample at such a high mass ratio (also compare with 100+0/0). We observed that repeating complexation of the same sample at the same mass ratio can result in some cases in different DLS results as shown in Figure S9. That can be likely given by the complexity of the tested solvent and the potential presence of aggregation centres. All these effects (and some others) make the determination of the optimal mass ratio between coated-ND and siRNA for biological applications rather complicated and often remains on the authors to heuristically choose one in order to fulfil
conditions defined by the ideal optimal mass ratio. In our experience, to estimate the optimal mass ratio, the repeated screening over several estimated mass ratios (higher than for aggregation point (+) – see Figure 5) is required and strongly recommended.

Figure S7. Examples of typical raw DLS data representing different colloidal states (A): blue boxes: * – aggregated, ** – aggregation process, *** – stable) and the key parameters for assessing of the degree of aggregation. Control sample 40:1 0*/1000:siRNA in full cell culture media for 4T1 cells (9% FCS) serves as an example of a colloidal stable sample. Autocorrelation functions for each sample represent 1st and 10th measurement (~20min). The key parameters for assessing of the colloidal stability: cumulant based polydispersity index (PDI); In Range value – describing the quality of the intercept and baseline of the autocorrelation function; The size trending value is calculated over all ten measurements – Z-average size testing to reveal a statistically significant trend. A) Heuristically chosen data surrounded by the green line (see Figure 6B) represent acceptable data in the view of colloidal stability (data selection was also based on the visual observation of the colloidal state). Box plots (Figure S10) summarize the key parameters for these acceptable points (testing in full cell culture media) merged with acceptable data from testing in full FCS (see Figure S8). Analogous, Figure S11 describes the key parameters of the merged data for the samples in the aggregation area (grey area in Figure S7A and Figure S8A).
Figure S8. Anomalous raw DLS data (A): red boxes: *, **, ***). These measurements deviate from typical measurements presented in Figure S7. Control sample 40:1 0^7/100^6:siRNA in full cell culture media for 4T1 cells (9% FCS) serves as an example of a colloidally stable sample. Autocorrelation functions for each sample represent 1^st and 10^th measurement (~ 20 min). The key parameters for assessing of colloidal stability: cumulant based polydispersity index (PDI); In Range value – describing the quality of the intercept and baseline of the autocorrelation function; The size trending is calculated over all ten measurements – Z-average size testing to reveal a statistically significant trend. A) Heuristically chosen data in green circles represent acceptable data in the view of colloidal stability (data selection was also based on the visual observation of the colloidal state). Box plots (Figure S10) summarize the key parameters for these acceptable points (testing in full FCS) merged with the acceptable data from Figure S7 (testing in full cell culture media). Analogous, Figure S11 describes the key parameters of the merged data for the samples in the aggregation area (grey area in Figure S7 and S8A).
Figure S9. Raw DLS intensity autocorrelation functions of the sample 12:1 80+/20:siR(+) in 4T1 full cell culture media. Each graph represents freshly prepared sample.

Figure S10. Summarized DLS parameters for the heuristically chosen data set (see Figure S7A and S8A), which should contain “sufficiently” stable samples in the cell culture media (9% FCS) and full FCS (90%) at 37 °C. Values in A), B), D) represent differences between the 10th and 1st measurement of the sample (~ 20 min). (e.g. the key parameters (green text) in Figure S7D shows the following differences: $\Delta PDI = 0.047$; $\Delta (In\ Range) = -0.3$; Mean size trending = 5.7%, and therefore the sample is considered as a stable).
Figure S11. Summarized DLS parameters for the heuristically chosen data set (see Figure S7A and S8A), which should contain aggregated samples in the cell culture media (9% FCS) and full FCS (90%) at 37 °C. Values in A), B), D) represent differences between the 1st and 10th measurement of the sample. (e.g. the key parameters (red text) in Figure S7C shows the following differences: \( \Delta PDI = 0.445 \); \( \Delta (\text{In Range}) = -7.9 \); Mean size trending = 42.5%, and therefore the sample can not be considered as a stable).

Figure S12. DLS intensity size distributions from long-term testing (Figure 6D). Each sample is represented by the 1st and 10th measurement. Measured particle concentrations were \( \sim 0.1 \) mg/mL and \( \sim 0.3 \) mg/mL for 5:1 33*/67°:siRNA and 30:1 100*/0°:siRNA.
Figure S13. Raw DLS intensity autocorrelation functions of all ND@(co)polymer samples and ND-PEI at 25°C in 10x PBS. NDs without any coating reveal similar behaviour as ND-PEI at given conditions. Each sample was measured three times – $G^{(2)}(\tau)$ curves represent single measurements.

Complexation protocol

Sample 80+/200 dissolved in RNase-free H₂O was tested with mGAPDH siRNA (Sigma Aldrich, 22 bp, duplex), Luc2 siRNA (Dharmacon, 21 bp, duplex), and ssDNA (IDT, 40 nt, single strand). All samples were delivered as lyophilizates and dissolved in RNase-free H₂O with a stock concentration of 100 µM (according to Nanodrop). The complexation at different ratios was performed according to section: *Complexation of siRNA for DLS, ELS analysis and Qubit assay*. The most stable complexes were observed when using mGAPDH siRNA. Samples with Luc2 siRNA and ssDNA were significantly compromised with aggregation. The following procedure was utilized to get rid of aggregation during complex preparation. The ssDNA samples were lyophilized and redissolved in 25 mM MgCl₂ with the final concentration of ssDNA 100 µM. To obtain mass ratio 25:1:3.9 µL (21.9 mg/mL in RNase free water) 80+/200 was diluted with 18.3µL (25 mM MgCl₂). Directly into this mixture (not dropwise) was added 2.8 µL ssDNA (100 µM, 1.2 mg/mL in 25 mM MgCl₂) resulting in a stable transparent colloid (the procedure is scalable and also well working for Luc2 siRNA). Further addition of MgCl₂ can also help to stabilize the dispersion. After quantitative centrifugation, the supernatant of 80+/200:siRNA/ssDNA was quantified relative to the control sample (nanoparticle free) not revealing a significant amount of free fraction of siRNA/DNA.
**Table S10.** Overview of colloidal stabilities before/after application of the complexation protocol

<table>
<thead>
<tr>
<th>Sample</th>
<th>ND@copolymer:siRNA/DNA mass ratio</th>
<th>Colloidal stability without MgCl₂</th>
<th>Colloidal stability with MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>80°/20°:mGAPDH siRNA</td>
<td>25:1</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>80°/20°:Luc2 siRNA</td>
<td>25:1</td>
<td>Aggregated</td>
<td>Stable</td>
</tr>
<tr>
<td>80°/20°:ssDNA</td>
<td>25:1</td>
<td>Aggregated</td>
<td>Stable</td>
</tr>
</tbody>
</table>

**Table S11.** Solvent properties for DLS measurements

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temperature (°C)</th>
<th>Viscosity (cP)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>25</td>
<td>0.8872&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.330&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1xPBS</td>
<td>25</td>
<td>0.9082&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.332&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10xPBS</td>
<td>25</td>
<td>1.0982&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.347&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4T1 cell culture media</td>
<td>37</td>
<td>0.8610&lt;sup&gt;[61]&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>37</td>
<td>0.7400&lt;sup&gt;[61]&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Default values from Zetasizer software; <sup>b</sup> Complex solvent calculated in Zetasizer software;