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Maximization of Loading and Stability of ssDNA:Iron Oxide Nanoparticle Complexes Formed through Electrostatic Interaction

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Abstract

The use of inorganic nanoparticles (NPs) as vectors for the delivery of oligonucleotides for *in vitro* and *in vivo* applications is rapidly gaining momentum. Some of the reasons making them especially good candidates for this purpose are their ease of synthesis in a range of sizes and surface coatings, their propensity to penetrate cell membranes, their stability and biocompatibility and their unique size-dependent physical properties that impart additional diagnostic and therapeutic tools. Notwithstanding these notable attributes, a major obstacle to their practical use is given by the typically low oligonucleotide loading levels attainable through conventional bioconjugation procedures. This shortcoming is especially worrisome as toxicity concerns have been associated with co-delivery of NPs. In this paper we are analytically analyzing the formation of electrostatic complexes between negatively charged ssDNA and positively charged iron oxide nanoparticles (SPIO-NP) with the purpose of identifying the optimal conditions leading to stable formulations at high oligo loading levels. The formation and loading levels of ssDNA:SPIO-NP complexes have been investigated at different oligo:NP ratios and under different ionic strengths through Dynamic Light Scattering, fluorescence quenching experiments and pull-down assays. Through these studies we have identified optimal conditions for attaining maximal oligo loading levels and we are proposing a simple model to explain an unusual behavior observed in the formation of the complexes. Finally we introduce an alternative loading method relying on the electrostatic co-loading of an oligo sequence in the presence of a negatively charged PEGylated block copolymer, yielding very stable and high loading PEGylated ssDNA:SPIO-NPs. The findings that we are reporting are of general validity and similar conditions could be easily translated to the electrostatic formation of ssDNA:NP complexes consisting of different NP materials and sizes.

Introduction

Oligonucleotide-based therapies are a promising approach for the treatment of many diseases, such as cancer, where the expression of a pathogenic protein can be downregulated by selectively silencing the encoding gene during transcription^{1, 2}. Ribozymes³⁻⁵, antigene⁶ and antisense^{4, 7} oligonucleotides, small interfering RNA (siRNA)^{8, 9} and, more recently, microRNA (mRNA)¹⁰ are among the most promising candidates for this purpose. Notwithstanding the enormous potential of this therapeutic approach, the translation into the clinical setting has been impaired by several shortcomings, mainly stemming from the

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Supporting information Available

Plots of weight of ssDNA absorbed vs. weight of nanoparticles, Table listing ssDNA surface densities and molecular ratios of immobilized strands, additional TEM Images. This material is available free of charge via the Internet at <http://pubs.acs.org>.

presence of multiple physiological barriers limiting the availability of free and intact oligonucleotides following *in vivo* administration¹¹. The mode of action for therapeutic oligonucleotides requires their internalization by the diseased cell and availability in the cytosol and presumes that they are not degraded or excreted before they reach the target site. Unfortunately, upon administration the oligos are rapidly degraded by nucleases, excreted by the kidneys and removed by the reticuloendothelial system. Further, diffusion through the blood capillary walls and through the extracellular matrix, cellular internalization and endosomal release are restricted by the polyelectrolytic and macromolecular nature of the oligos. In order to overcome these barriers cumbersome administration protocols, backbone modification and vectorization have been attempted but have so far resulted only in limited improvements¹². As a result, the exploration of new vectors for *in vitro* and *in vivo* delivery of therapeutic oligonucleotides is an area of active investigation¹²⁻¹⁴. Many approaches, including oligonucleotide-conjugates with cell penetrating peptides^{15, 16} or other transporter, such as cholesterol¹⁷, complexation with cationic polymers¹⁸, liposomes¹⁹ and viral vectors^{20, 21} have been explored, resulting to some extent in improved cell internalization and *in vivo* stability.

Inorganic nanoparticles (NPs) are emerging as very attractive delivery vehicles for oligonucleotides thanks to their unique properties²². For instance, NPs are inherently prone to penetrate cell membranes²³, they are relatively easy to synthesize in a range of sizes and surface coatings and their surface properties and stability are usually better defined than those of micelles and liposomes. Furthermore, inorganic NPs often display unique size-dependent physical properties, such as quantum confinement²⁴, plasmon resonance²⁵ and superparamagnetism²⁶ that could be exploited as additional diagnostic or therapeutic tools. As for all drug delivery vehicles, a major caveat of using inorganic NPs as carriers of oligonucleotides lays in the necessity to maximize drug loading while maintaining a desirable pharmacokinetic profile. For inorganic NPs this aspect is especially important, as toxicity concerns have been associated with their co-delivery²⁷. Covalent immobilization of oligonucleotides on NPs is the most established loading method and has been extensively exploited^{28, 29}. Although synthetically effective, the loading levels for the oligos are unavoidably low as at best only a monolayer of oligonucleotide can be immobilized on the NP surface. More recently, the layer-by-layer (LbL) approach has also been utilized to form electrostatic NP-oligo complexes. In this case, the strong electrostatic field emanating from a positively charged nanoparticle extends well beyond the monolayer, permitting the electrostatic immobilization of multiple layers of oligonucleotides^{30, 31}. This factor, combined with the realization that oligonucleotides adopt a very compact conformation on positively charged surfaces³², allows in principle the electrostatic packing of oligonucleotides at very high density. However, both the formation and the colloidal stability of the resulting oligonucleotide-NP complex are dictated by a delicate balance of electrostatic forces³³ and it is often difficult to identify the ideal conditions (i.e. concentration, ionic strength, relative ratios of reagents) yielding stable formulations at high oligo loading levels. As a result, most ssDNA:NP complexes are obtained at fairly low oligo loading levels^{28, 30, 31}.

In this work we are investigating the formation and stability of electrostatic complexes formed between negatively charged ssDNA and positively charged superparamagnetic iron oxide nanoparticles (SPIO-NPs) under different ionic strength and ssDNA:SPIO-NP ratios. Using Dynamic Light Scattering (DLS), fluorescence quenching and a simple pull-down assay we have established a protocol that permits the identification of a set of optimal conditions yielding maximal oligo loading levels on positively charged 30 nm SPIO-NPs. We are also proposing a model of general validity for the formation of these complexes that might be applied to the identification of optimal conditions for the electrostatic immobilization of oligonucleotides on other types of nanoparticles. Finally, we will show

how the method that we have developed can be adapted to generate stable, high-loading ssDNA:SPIO-NP complexes through simultaneous co-loading of a poly(ethyleneglycol) block copolymer.

Materials and Methods

30 nm (50 nm hydrodynamic radius) poly(ethyleneimine)-coated Superparamagnetic Iron Oxide Nanoparticles (PEI-SPIO-NPs) were obtained from Ocean Nanotech (Springdale, AK). 5'-Cy5 labeled and unlabeled ssDNA sequences (5'-GAG CTG CAC GCT GCC GTC-3'), were custom synthesized at a 200 nmol scale by Eurofins EWG Operon (Huntsville, AL). 5 kDa poly(ethyleneglycol) block co-polymer with poly(glutamic acid)₁₀ (PEG_{5k}-b-PLE₁₀) was obtained from Alamanda Polymers (Huntsville, AL). Stock solutions of ssDNA (1 g/L), SPIO-NPs and PEG_{5k}-b-PLE₁₀ (1 µg/µL) at different ionic strengths were prepared with doubly distilled water (ddH₂O).

Dynamic Light Scattering (DLS) measurements were executed with a Zetatract particle size analyzer (Microtrac, Montgomeryville, PA). Fluorescence measurements on Cy5-labeled oligonucleotides were performed on a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) by measuring the emission at 660 nm ($\lambda_{ex} = 620$ nm). The amount of Cy5 oligo was kept constant at 2 ng/µL while increasing the amount of SPIO-NPs. Ultracentrifugation for the pull-down assay was carried on with a Beckmann Airfuge ultracentrifuge (Brea, CA) operating at a fixed speed of $199,000 \times g$.

Samples for the gel retardation assay were prepared by mixing 5 ng of Cy5-labeled ssDNA with increasing amounts of SPIO-NPs in 1 X PBS. Samples were loaded on a 2% agarose gel, loading 5 ng of Cy5-labeled ssDNA per well and running in 1 x TE at 10 V/cm for 20 min. The fluorescent bands, corresponding to unbound Cy5-labeled ssDNA were visualized with a Kodak 2000 mm scanner with the excitation filter set at 555 nm and the emission filter set at 700 nm.

For the determination of hydrodynamic radii, the SPIO concentration was kept constant at 1 µg/mL, while varying the concentration of ssDNA. The values and standard deviations reported for all graphs represent the average of three measurements.

Results and Discussion

An initial, qualitative evaluation of the maximum ssDNA loading level attainable through electrostatic complexation with positively charged PEI-SPIO-NPs was provided by a simple gel retardation assay. Cy5-labeled ssDNA was mixed at different weight-over-weight ratios (from now on defined as *R*) with PEI-SPIO-NPs in 1 x PBS and then run on a 2% agarose gel. As only the free Cy5-labeled ssDNA is fluorescent, and the nanoparticle complex does not migrate through the gel, the absence of a fluorescent band indicates quantitative complexation. The gel is shown in Fig. 1 and shows that for *R* < 25 all ssDNA is bound to the nanoparticles.

Although the gel retardation assay provides a qualitative assessment of the ratios corresponding to quantitative ssDNA loading, it does not provide any information on the nature or stability of the complex.

Effect of Ionic Strength on Size

It is well known that ionic strength plays a pivotal role in the formation of electrostatic complexes via the LbL approach^{33, 34}. In order to evaluate the stability of the ssDNA:SPIO-NPs complexes formed under different ionic strengths, we monitored the

changes in hydrodynamic radius under four representative ionic strength conditions, namely 10 mM, 100 mM and 1 M NaCl and 1 X PBS (corresponding to 137 mM NaCl), (Fig. 2). Hydrodynamic radii larger than 200 nm, roughly corresponding to a four-fold increase in size from the starting SPIO-NP, were arbitrarily considered a threshold value for aggregation.

At low ionic strength (10 mM NaCl, Fig. 2A) the size of the ssDNA:SPIO-NP complex did not increase significantly when compared to the initial value of 52.9 ± 8 nm up to $R = 5$ and began to show a gradual increase for $5 < R < 25$. For $R > 25$ a sharp increase in size was observed indicating a strong tendency to aggregation at this critical ratio. As ssDNA complexation also leads to charge neutralization and to destabilization of the colloidal suspension, aggregation was expected. However, for $R > 40$ the size of the complex decreased sharply. At intermediate ionic strength (100 mM NaCl, Fig. 2B) the complex was stable only up to $R = 2$, and started increasing in size steadily for $R > 2$. At $R \approx 10$, aggregation was already apparent but the hydrodynamic radius started decreasing gradually for $R > 25$. A similar trend was observed in 1 X PBS (Fig. 2D), although the hydrodynamic radius started to increase for $R > 1$, aggregation was already apparent for $R = 2$ and the decrease in size was obvious for $R > 10$. At high ionic strength (1 M NaCl, Fig. 2C), the hydrodynamic radius of the ssDNA:SPIO-NP complex did not change significantly up to $R = 30$. For $R > 30$ the complex showed a propensity to aggregation as indicated by the steady size increase while no decrease in hydrodynamic radius was observed at this ionic strength. From these data it is apparent that the ionic strength of the complexing medium plays a major role in the stability of the ssDNA:SPIO-NP complex. This is not surprising as it is well known that the screening length - the maximum distance at which two charged particles can still be influenced by their respective electrostatic field - hence the tendency of a colloidal suspension to aggregate, is dependent from the ionic strength. However, the apparent stability of the ssDNA:SPIO-NP complex at high ionic strength (1 M NaCl, Fig. 2C) seems to deviate from this pattern. The interpretation of these data must however keep into account two opposing effects that will be analyzed in more detail later on: on one hand electrostatic attraction between oppositely charged polyelectrolytes leads to formation of a complex; on the other hand the incoming polyelectrolyte (i.e. ssDNA) experiences repulsive forces, opposing the complexation, arising from ssDNA packing.

Effect of Ionic Strength on ssDNA Packing Density

In order to gather more information on the nature of the ssDNA shell, its formation was followed via fluorescence quenching using Cy5-labeled ssDNA. The emission of fluorophores is known to be very sensitive to the surroundings and compression of a fluorophore in a crowded environment can result in quenching due to non-radiative decay pathways. Quenching can therefore be employed as a direct measure of the extent of DNA condensation³⁵. Through these experiments, an interesting behaviour was observed. With the exception of the measurements obtained at 1 M NaCl, for which no or very little quenching was observed for all values of R , the emission of Cy5 decreased gradually with increasing R up to a critical value where the fluorescence was $> 90\%$ quenched. When the experiment was conducted in 1 X PBS decay in the emission intensity of Cy5 was observed already for $R > 0.5$. Increasing R past this point resulted only in a small increase in fluorescence intensity. This behaviour, and especially the decrease in emission intensity observed for low values of R , is counterintuitive. As a matter of fact the expected trend should be the opposite, as the emission intensity should be quenched at low R (corresponding to high ssDNA complexation due to excess of SPIO-NPs) then gradually increase as an excess of unbound ssDNA builds up. This predicted behaviour corresponds however to a model where the packing of DNA on the SPIO follows a linear relationship and is independent from factors other than electrostatic attraction. This model obviously

does not fit the experimental observations and a different interpretation must be adopted. The data in Fig. 3 indicate that at low values of R the packing density of ssDNA on the nanoparticle is low. However, the packing density increases by increasing the amount of ssDNA (so does the R value) and by increasing the ionic strength. This observation seems to indicate that minimization of electrostatic repulsion through ionic screening is a predominant factor in the formation of the complex. The differing trend observed for 1 M NaCl seem to indicate that ssDNA does not pack at all on the nanoparticles under these conditions, probably because electrostatic attraction is completely screened under these conditions.

Effect of Ionic Strength on ssDNA Loading Levels

In order to determine quantitatively the loading levels of ssDNA attainable under different conditions, we developed a simple pull-down assay, separating the ssDNA:SPIO-NP complexes obtained at different ratios from the supernatant through ultracentrifugation. The concentration of ssDNA was kept constant, while varying the amount of SPIO-NPs added. Unbound ssDNA in the supernatant after centrifugation was determined by measuring the absorbance at 260 nm and the amount of ssDNA effectively bound to the SPIO-NPs determined by subtraction. Once again we observed that the ssDNA:NP ratios (R) corresponding to the quantitative formation of the ssDNA:SPIO-NP complex were strongly dependent on the ionic strength of the complexing medium (Fig. 4). The ssDNA loading curves, plotted as percentage of ssDNA absorbed vs. R , showed that the amount of ssDNA absorbed increased with increasing R , highlighting the need of an excess of ssDNA in solution to achieve maximum loading. Another important observation was that the complexation efficiency is poor at low R although loading levels for the same low R -values improved at intermediate ionic strength.

Plotting the weight of ssDNA absorbed vs. weight of SPIO yields a linear relationship, where the slope of the linear regression can be interpreted as the propensity of ssDNA to form complexes with the SPIO-NPs under a given set of conditions, where a steeper slope corresponds to a higher tendency to absorb DNA (see Supporting Information – Fig. S1). Approximate values for the number of ssDNA molecules absorbed per nanoparticle can be estimated by calculating the molar ratio between ssDNA complexed and nanoparticles (Supporting Information – Table S1).

A Model for the Formation of ssDNA:NP complex

On the basis of these observations we are proposing a model for the formation of ssDNA:SPIO-NP conjugates through electrostatic complexation. This model is most likely valid for other nanoparticles as well, as it is derived from basic considerations on electrostatic interactions.

As it was mentioned before, the formation of complexes between polyelectrolytes and charged surfaces, or spheres, is driven by two opposing forces³⁶: electrostatic attraction and repulsion. Electrostatic attraction between opposite charges is responsible for the initial interaction leading to the formation of the complexes. However, as the complex forms, polyelectrolytes of similar charge are forced in close proximity and this gives rise to strong repulsive forces opposing the electrostatic attraction between the surface of the nanoparticle and the incoming polyelectrolyte. Often, additional constructive interactions within the forming polyelectrolyte layer facilitate the formation of the complex and ultimately result in charge reversal, where the final electrostatic complex has a net charge opposite to that of the starting surface³⁷. At low ionic strength charge screening effects are small and electrostatic attraction is favored while electrostatic repulsion between incoming polyelectrolyte chains is not shielded very effectively. Incoming negatively charged ssDNA is attracted effectively by

the positive particle at low ionic strength and undertakes surface-induced condensation, leading potentially to high packing density but also to significant electrostatic repulsion, counterbalanced only partially by positive hydrophobic interaction between nucleobases. Under low ionic strength conditions, and at low R the net balance of electrostatic attraction and repulsion yields a loosely bound ssDNA layer, suggesting that repulsive effects are predominating the process and hamper the formation of a complex. However, when a critical R is reached, a critical mass of ssDNA induces the collapse of the forming ssDNA shell resulting in increased compaction and efficient loading, albeit at the expense of colloidal stability. Our observations at low ionic strength support this model (Fig. 2A, 3A and 4A). The amount of fluorescence quenching, proportional to compaction, decreases gradually until a minimum is reached, indicating low packing density at low ssDNA:SPIO-NP ratio (Fig. 2A). R for maximal quenching also corresponds to maximum aggregation (Fig. 3A) and maximum loading efficiency (Fig. 4A). Past this point a rapid collapse in size was observed and we attributed this to a mass-driven compaction of the ssDNA shell, where the loosely bound ssDNA reaches a critical mass able to overcome the electrostatic repulsion arising from close proximity of negatively charged ssDNA strands.

At intermediate ionic strength (100 mM NaCl and 1 x PBS), electrostatic repulsion between incoming ssDNA strands begins to be more effectively shielded than at low ionic strength while still maintaining a constructive electrostatic attraction between SPIO-NP and ssDNA. As a result the ssDNA layer at lower R is more compact than the corresponding layer obtained at low ionic strength as can be inferred from the rapid drop in fluorescence emission (Fig. 2-3B) that persists until the ssDNA layer is saturated. Correspondingly, ssDNA is quantitatively absorbed at multiple ratios although the increase in complexation efficiency comes at the expense of stability, as the complexes obtained at intermediate ionic strength have a tendency to aggregate already at low R . From our graph it is apparent that colloidal stability for this system can be maintained only for $R < 1$. Even at intermediate ionic strength the effect of reaching a critical mass of ssDNA can be observed albeit less obviously. For instance, looking at Fig. S1 and Table S1 it can be noticed that the loading of ssDNA per unit weight of nanoparticle (or the surface density of ssDNA), keeps increasing for increasing R although the size of the complex does not increase appreciably. In our proposed model this corresponds once again to a collapse of the ssDNA shell due to “mass effects” (i.e. the compression of the complex due to the presence of a large excess of ssDNA in the electrostatic sphere of attraction).

At high ionic strength (1 M NaCl), electrostatic repulsion between ssDNA is very effectively shielded, but so is electrostatic attraction. The fluorescence quenching plot (Fig. 3C) indicates that the ssDNA layer does not compact very effectively for any value of R , likely because of the lack of effective absorption on the NP surface. This is especially obvious when looking at the loading levels obtained through the pull-down assay (Fig. 4C), the amount of DNA absorbed is independent from R values. DLS indicate that the particle size does not increase significantly, and all the observations taken together suggest that formation of the complex is not efficient at high ionic strength.

At all ionic strengths kinetic effects were evaluated by monitoring the hydrodynamic radiuses over the course of several days. While a small size increase (10-20 %) was observed for all values of R , the observed trends did not change, similarly to what is shown in Fig. 5. We attributed this behaviour to a restructuring of the ssDNA shell over time to reach equilibrium, following the kinetically-driven initial formation of the complex.

Collectively, these observations indicate that the formation of the electrostatic complex is facilitated at low to intermediate ionic strengths. Low ionic strength (10 mM NaCl) however does not permit the loading of ssDNA below a critical R value and therefore require an

excess of ssDNA to achieve good loading levels. Intermediate ionic strengths are more efficient at capturing DNA at multiple ratios, however the stability of the resulting complexes, defined as their tendency to aggregate, is very low, limiting the practical use to low loading levels of ssDNA.

Stabilization of High-loading complexes via PEGylation

The data collected are helpful in clarifying the interdependence between loading levels and stability of the resulting ssDNA:SPIO-NP complexes and the role of ionic strength in their formation. It is however worth reminding that the premise of this work was to maximize the loading levels of DNA on NPs for future *in vitro* and *in vivo* applications and this implies that the resulting complex must be stable. The loading levels attainable for 1 x PBS are actually quite satisfactory, as a ssDNA:SPIO-NP weight-over-weight ratio of up to 40 can be reached, however the colloidal stability profile has to be improved. To this end, we attempted to increase the colloidal stability of a ssDNA:SPIO-NP complex (for $R = 10$) by co-loading a block copolymer consisting of a 10-residue poly(glutamic acid) oligomer linked to a 5,000 Da poly(ethyleneglycol) (PEG_{5k-b-E10}). Adding a PEG coating to nanoparticles is a well-established procedure for the stabilization of colloidal suspensions through steric exclusion³⁸. PEGylation also increases the circulatory half-life by minimizing the interaction with serum proteins³⁹. Electrostatic complexes of SPIO-ssDNA-PEG were prepared at constant $R = 10$ by increasing the relative amount of PEG_{5k-b-E10}. The tendency to aggregate of the complexes for the different PEGylation ratios and at different time points was followed by DLS and the results are shown in Fig. 5. It is evident that increasing the amount of PEG_{5k-b-E10} results in a decrease in the size of the complex, indicating higher colloidal stability and diminished propensity to aggregation (Fig. 5A). Although the negatively charged poly(glutamic acid) chain competes with ssDNA for the positively charged NP surface, and could result in strand displacement the pull-down assay indicated that relatively high loading levels ($\approx 80\%$) of ssDNA were attainable for PEGylation ratios between 5 and 10.

The stability of a representative PEGylated ssDNA:SPIO-NP complex in 10% fetal bovine serum (FBS) was assessed by forming a ssDNA:PEG:SPIO complex at a 10:50:1 ratio in 1 X PBS and following the hydrodynamic radius over time for 24h. The initial size of the complex was 98.3 ± 39.4 nm, and became 216.0 ± 81.2 nm when exposed to 10 % FBS. After 24 h the size of the complex in 10% FBS was 293.42 ± 95.0 nm. The observed increase in size is to be expected, as absorption of serum proteins cannot be completely prevented^{40, 41}. The size of the complex in 10% FBS however did not indicate uncontrolled aggregation and this was regarded as evidence of the stabilization imparted by PEGylation. A similar experiment was repeated exposing the complex to 90% FBS obtaining similar results (final size of the complex in 90% FBS was 266.2 ± 76.9 nm).

Transmission Electron Microscopy (TEM) for the ssDNA-SPIO complexes revealed the formation of a stable corona of ssDNA or ssDNA/PEG (Fig. 6). Very few aggregated nanoparticles were spotted in the field of view, and some complexes showed a disaggregated shell for the PEGylated sample (see Supporting Info), probably resulting from sample preparation or caused by the high-vacuum inside the TEM chamber. The corona in the PEGylated complex (Fig. 6C) appeared fainter than for the non-PEGylated complex (Fig. 6B) and we attributed this to the lower DNA density in the PEGylated shell, leading to less staining and therefore to lower contrast.

Conclusions

In this work we have highlighted the importance of ionic strength and reagent ratio for the formation of electrostatic complexes between ssDNA and positively-coated SPIO-NP at

high ssDNA loading ratios. Intermediate ionic strength and higher ssDNA:SPIO-NP ratios give easier access to high-loading complexes. Ratios that are too low result in poor loading levels and ratios that are too high result in excess unbound ssDNA in solution. The tendency to aggregate of the resulting complexes is directly related to the loading levels, where high loading levels also correspond to maximal aggregation. By introducing a PEGylation layer we have shown that the stability of the complex can be increased without sacrificing the loading efficiency. Although the procedure here reported has been optimized for 50 nm PEI-coated SPIO-NPs, the process is most likely adaptable to other NP sizes and materials. Further studies in this direction are being currently carried on in our laboratory to determine the *in vitro* efficacy of these nanocarriers as transfection agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Devi GR. Cancer Gene Ther. 2006; 13:819–829. [PubMed: 16424918]
2. Gleave ME, Monia BP. Nat. Rev. Cancer. 2005; 5:468–479. [PubMed: 15905854]
3. Citti L, Rainaldi G. Curr. Gene Ther. 2005; 5:11–24. [PubMed: 15638708]
4. Scanlon KJ. Curr. Pharm. Biotechnol. 2004; 5:415–420.
5. Thompson JD, Macejak D, Couture L, Stinchcomb DT. Nat. Med. 1995; 1:277–278. [PubMed: 7585047]
6. Frank-Kamenetskii MD, Mirkin SM. Annu. Rev. Biochem. 1995; 64:65–95. [PubMed: 7574496]
7. Dias N, Stein CA. Mol. Cancer Ther. 2002; 1:347–355. [PubMed: 12489851]
8. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Nature. 1998; 391:806–811. [PubMed: 9486653]
9. Hannon GJ. RNA interference. Nature. 2002; 404:293–296. [PubMed: 10749213]
10. Nergini M, Ferracin M, Sabbioni S, Croce CM. J. Cell. Sci. 2007; 120:1833–1840. [PubMed: 17515481]
11. White PJ, Anastasopoulos F, Pouton CW, Boyd BJ. Expert Rev. Mol. Med. 2009; 11:e10. [PubMed: 19302730]
12. Juliano R, Alam MR, Dixit V, Kang H. Nucleic Acids Res. 2008; 36:4158–4171. [PubMed: 18558618]
13. El-Aneed A. J. Control. Rel. 2004; 94:1–14.
14. Zhao XB, Pan F, Holt CM, Lewis AL, Lu JR. Expert. Opin. Drug. Del. 2009; 6:673–686.
15. Hassane FS, Saleh AF, Abes R, Gait MJ, Lebleu B. Cell. Mol. Life Sci. 2010; 67:715–726. [PubMed: 19898741]
16. Zatselin TS, Turner JJ, Oretskaya TS, Gait MJ. Curr. Pharm. Design. 2005; 11:3639–3654.
17. Holasova S, Mojzisek M, Buncek M, Vokurkova D, Radilova H, Safarova M, Cervinka M, Haluza R. Mol. Cell. Biochem. 2005; 276:61–69. [PubMed: 16132686]
18. Merdan T, Kopecek J, Kissel T. Adv. Drug Deliver. Rev. 2002; 54:715–758.
19. Fattal E, Couvreur P, Dubernet C. Adv. Drug Deliver. Rev. 2004; 56:931–946.
20. Cooper A, Shaul Y. Biochem. Biophys. Res. Comm. 2005; 327:1094–1099.
21. Buchschacher GL, Wong-Staal F. Blood. 2000; 95:2499–2504. [PubMed: 10753827]
22. Sokolova V, Epple M. Angew. Chem. Int. Edit. Engl. 2007; 47:1382–1395.
23. Verma A, Stellacci F. Small. 2010; 6:12–21. [PubMed: 19844908]

24. Tolbert SH, Herhold AB, Johnson CS, Alivisatos AP. *Phys. Rev. Lett.* 1994; 73:3266–3269. [PubMed: 10057333]
25. Eustis S, El-Sayed MA. *Chem. Soc. Rev.* 2006; 35:209–217. [PubMed: 16505915]
26. Mikhaylova M, Kim DK, Bobrysheva N, Osmolowsky M, Semenov V, Tsakalakos T, Muhammed M. *Langmuir.* 2004; 20:2472–2477. [PubMed: 15835712]
27. Jeng HA, Swanson J. J. *Environ. Sci. Heal. A.* 2006; 41:2699–2711.
28. Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AKR, Han MS, Mirkin CA. *Science.* 2006; 312:1027–1030. [PubMed: 16709779]
29. Cutler JI, Zheng D, Xu XY, Giljohann DA, Mirkin DA. *Nano Lett.* 2010; 10:1477–1480. [PubMed: 20307079]
30. Song WJ, Du JZ, Sun TM, Zhang PZ, Wang J. *Small.* 2010; 6:239–246. [PubMed: 19924738]
31. Elbakry A, Zaky A, Liebk R, Rachel R, Goepferich A, Breunig M. *Nano Lett.* 2009; 9:2059–2064. [PubMed: 19331425]
32. Fang Y, Hoh JH. *Nucleic Acids Res.* 1998; 26:588–593. [PubMed: 9421520]
33. Schneider G, Decher G. *Langmuir.* 2008; 24:1778–1789. [PubMed: 18225923]
34. Kovacevic D, van der Burgh S, de Keizer A, Stuart MAC. *J. Phys. Chem. B.* 2003; 107:7998–8002.
35. Kombrabail MH, Krishnamoorthy G. J. *Fluoresc.* 2005; 15:741–747. [PubMed: 16341792]
36. Messina R, Holm C, Kremer K. *Langmuir.* 2003; 19:4473–4482.
37. Decher G. *Science.* 1997; 29:1232–1237.
38. Lourenco C, Teixeira M, Simoes S, Gaspar R. *Int. J. Pharm.* 1996; 138:1–12.
39. Schipper ML, Iyer G, Koh AL, Cheng Z, Ebenstein Y, Aharoni A, Keren S, Bentolila LA, Li J, Rao J, Chen X, Banin U, Wu AM, Sinclair R, Weiss S, Gambhir SS. *Small.* 2009; 5:126–134. [PubMed: 19051182]
40. Casals E, Pfaller T, Duschl A, Oostingh GJ, Puentes V. *ACS Nano.* 2010; 4:3623–3632. [PubMed: 20553005]
41. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Mueller RH. *Colloid. Surface B.* 2000; 18:301–313.

Figure 1. Gel retardation assay for various ssDNA-Cy5:SPIO-NP ratios. The control is free Cy5-labeled ssDNA. No ssDNA band was visualized for $R < 25$ indicating that ssDNA can be quantitatively immobilized on SPIO-NPs up to this ratio.

Figure 2.

Effect of ionic strength over the size of ssDNA:SPIO-NP complexes formed at different weight-over-weight ratios (R). The horizontal scale is plotted in logarithmic format for clarity. The apparent instability of the complexes, as shown by size increase due to aggregation, is greatest at intermediate ionic strength (100 mM and 1 X PBS) and lowest at high ionic strength (1 M NaCl). Larger ssDNA:SPIO-NP ratios also have a destabilizing effect over the complex. The ratio at which the complexes aggregate (arbitrarily set for sizes > 200 nm) is dependent on ionic strength and was determined to be 32.5 (10 mM NaCl), 10 (100 mM NaCl), 45 (1 M NaCl) and 2 (1 X PBS).

Figure 3.

Fluorescence quenching analysis of the Cy5-ssDNA shell electrostatically bound on positively charged SPIO-NPs cores at different ionic strengths: **A.** 10 mM NaCl; **B.** 100 mM NaCl; **C.** 1 M NaCl; **D.** 1 x PBS. Higher quenching (lower emission intensity) was attributed to higher packing density, or condensation, of ssDNA. The quenching profile observed at 10 mM and 100 mM NaCl is not substantially different, and indicates maximal quenching for $25 < R < 50$, corresponding to tightly packed ssDNA at those values. At 1 M NaCl almost no quenching was observed indicating a very loosely bound ssDNA shell. In 1 X PBS buffer at pH 7.4 the quenching is very rapid, and Cy5 is 90% quenched for $2 < R < 25$.

Figure 4.

ssDNA loading curves obtained at different ionic strength with a pull-down assay, isolating the ssDNA/SPIO complex from unbound ssDNA: **a)** 10 mM NaCl; **b)** 100 mM NaCl; **c)** 1 M NaCl; **d)** 1 X PBS. The dashed line in the plots indicates the threshold ssDNA/SPIO ratio (R_t) after which ssDNA is quantitatively complexed to the SPIO-NP. Increasing the ionic strength from 10 mM (**a**) to 100 mM (**b**) has an effect on the ssDNA loading levels, as R_t shifts from 25 to 10. Increasing the ionic strength to 1 M NaCl (**c**) has a negative effect on the ssDNA loading levels never going past 50% and seemingly independent from R . In 1 X PBS (**d**) quantitative complexation of ssDNA was already observed for $R_t = 5$. Under all conditions, $R > 40$ results in lower complexation.

Figure 5. PEGylation by co-loading of PEG_{5k}-b-E₁₀ increases steric stabilization. **A.** Effect of increasing the PEG:ssDNA ratio. At a ratio of 5 the extent of stabilization is maximized, although increasing the ratio results in increase stability over time; **B.** The loading level of ssDNA decreases with increasing PEGylation, as a result of competitive strand displacement. PEG:ssDNA ratios between 5 and 10 seem ideal as the stability is fair while still maintaining high loading levels.

Figure 6. Transmission electron micrographs obtained after staining with phosphotungstic acid (PTA) of the commercial PEI-coated SPIO-NPs (**A**), 10:1 ssDNA-SPIO complex (**B**) and 10:50:1 ssDNA-PEG-SPIO complex (**C**)