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Precision Tuning of DNA- and Poly(ethylene glycol)-Based Nanoparticles via Coassembly for Effective Antisense Gene Regulation

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Oligonucleotides (ONs) are considered a form of informational drug, where drug-like properties (pharmacophore) and target information (dianophore) are independent of each other.^{1–5} Therefore, ON therapeutics promises to dramatically reduce the cost of new drug development, as a change in disease target in principle requires only a change in the ON sequence.^{6–9} However, direct utilization of ONs as a drug is hampered by enzymatic degradation, poor cellular uptake, rapid liver clearance, unwanted activation of the immune system, and overall low biochemical efficacy.^{10,11} Various chemical modifications of the ON or vectors (of viral, polycationic, and liposomal formulations) have been utilized to improve the bioavailability of the nucleic acid.^{12–16} Despite exhaustive efforts, however, these strategies remain subject to several longstanding drawbacks, including toxicity, immunogenicity, ON instability, and off-target side effects.^{17–19}

Recently, we reported a novel form of brush polymer–DNA conjugate termed pacDNA (polymer-assisted compaction of DNA), which consists of ONs covalently attached to the backbone of a sterically congested brush polymer having polyethylene glycol (PEG) side chains.^{20–22} The compaction of DNA by the densely packed PEG side chains impart the DNA with selective accessibility, shielding DNA from proteins while maintaining the hybridization with a complementary DNA strand, both kinetically and thermodynamically.²¹ The steric selectivity of the pacDNA greatly lowers various side effects associated with DNA–protein interactions, including degradation, toll-like receptor 9 (TLR9) activation, coagulopathy (DNA binding to thrombin), and hepatic capture. Interestingly, the pacDNA

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.7b03520. Synthesis detail of DNA-*b*-PCL and PEG-*b*-PCL and their NMR, FTIR, reverse-phase HPLC, and agarose gel electrophoresis characterization data; preparation and characterization of polymer–DNA self-assembled and coassembled nanoparticles; calculation of PEG density; and in vitro cell experiments (PDF)

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can enter cells via endocytosis and effectively knock down target genes through an antisense mechanism.²² While this brush-architected PEG has been proven useful for the delivery of oligonucleotides, a deeper understanding of how various structural parameters affect hybridization kinetics, nuclease resistance, cellular uptake, and ultimately gene regulation efficacy is of great value for the further development of PEG-based ON vectors.^{23–25} It is, however, difficult to explore these parameters using brush-type architectures due to the nontrivial design and synthesis involved. Moreover, the backbone of the brush polymer is nondegradable. For biopharmaceutical use, it is desirable to adopt materials regarded as generally safe for drug formulations.^{26–29} In this context, a simple, efficient, and robust method to diversify the structure of PEG-based vectors is of significance in advancing the rational design of effective noncationic ON vectors.

Herein, we created a library of micelles with tunable PEG length and density to investigate the structure-dependent steric selectivity and ON bioactivity (Scheme 1). The micelles are coassembled from two amphiphilic diblock copolymers, DNA-*b*-poly(ϵ -caprolactone) (DNA-*b*-PCL) and PEG-*b*-PCL, as well as the PCL homopolymer ($M_n = 10.5$ kDa). A 21-base DNA sequence and three PEG lengths (M_n 2, 5, and 10 kDa) are used to create the diblock copolymers. The PCL homopolymer contributes to the micellar core volume but not the shell, thereby modulating the densities of micelle surface moieties.

The DNA-*b*-PCL amphiphile is synthesized in two steps. First, azide-terminated PCL is prepared via ring-opening polymerization (ROP) of ϵ -CL using O-(2-azidoethyl)-heptaethylene glycol as the initiator (for $^1\text{H}/^{13}\text{C}$ NMR and infrared spectra, see Figures S1–S3). Subsequently, an ON with a 5' dibenzocyclooctyl (DBCO) group and a 3' Cy3 reporter (Table S1) is coupled to the azide-capped PCL through copper-free click chemistry in dimethyl sulfoxide (DMSO):water mixture (9:1 v:v).³⁰ Unreacted PCL and DNA are removed by dialysis and reverse-phase HPLC, respectively, to yield pure conjugates (~85% conjugation yield) as determined by agarose gel electrophoresis (Figures 1, S4, and S5). For proof-of-concept, we choose an antisense DNA sequence (G3139 by Genta Inc.) that targets the antiapoptotic B-cell lymphoma 2 (Bcl-2) family proteins as the hydrophilic block. Bcl-2 is an important biomarker for many cancers, including several types of breast and ovarian cancers, for which only one small molecule inhibitor has obtained regulatory approval.^{31,32} The PEG-*b*-PCL amphiphiles are synthesized by using hydroxy terminated PEG to initiate the ROP of ϵ -CL. The degree of polymerization of the PCL is controlled via initiator:monomer stoichiometry to be 100, and successful synthesis was confirmed by ^1H NMR and dimethylformamide (DMF) gel permeation chromatography (GPC) (Figure S6 and Table S2).

Coassembly of the block copolymers and the PCL homopolymer is achieved via nanoprecipitation (gradual solvent exchange from DMSO to Nanopure water).^{34,35} To systematically probe the relationship between the structural parameters of DNA-PEG nanoparticles and their steric selectivity, two series of nanostructures were prepared: (1) micelles containing DNA-*b*-PCL and PEG-*b*-PCL copolymers having varying PEG lengths and (2) micelles containing DNA-*b*-PCL, PEG_{10k}-*b*-PCL, and varying amounts of PCL homopolymer. The first series emphasizes the effect of the relative lengths of the PEG and the DNA, while the second series enables the study of the surface PEG density.

To study the ability of the DNA component within the coassembled micelles to hybridize with a complementary (sense) sequence, we adopted a fluorescence quenching assay²⁰ in which a quencher (dabcyl)-modified sense strand is mixed with Cy3-labeled particles. Upon hybridization, the fluorophore–quencher pair is brought to proximity, leading to a reduction in the fluorescence signals (Figure 2A). The rate of fluorescence reduction is therefore an indicator of the hybridization kinetics. A scrambled strand is used as a control to rule out the possibility of nonspecific interactions. As shown in Figure 2B, both free DNA and micellar nanoparticles can hybridize with the sense strand, while the scrambled sequence causes no change in the fluorescent signals. The micelles, however, exhibit a two-population behavior: a population with rapid hybridization kinetics similar to that of free DNA, and a slow-hybridizing population, which increases with increasing PEG content (Figures 2B and C; see Supporting Information Section 3.6 for calculation of PEG density). Notably, micelles containing no PEG-*b*-PCL (spherical nucleic acid-like micelles, see Figure S7) comprise almost entirely the fast-hybridization population. We attribute the slow-hybridizing population to the submicellar microstructure,^{36,37} which causes excessive hindrance around the DNA and is affected by the density of the PEG. Indeed, by diluting the micelle core with PCL homopolymer (thus a decrease in surface PEG density), the fast-hybridizing population can be almost fully restored (Figures 2D and 2E).

We next probed the enzyme accessibility of the DNA component within the micelles using bovine pancreas DNase I as a model enzyme. Cy3-labeled micelles are prehybridized with quencher-labeled sense strands. Upon introduction of DNase I, the dsDNA is degraded, resulting in an increase in fluorescence (Figure 3A). As shown in Figure 3B, naked dsDNA is readily accessed and degraded in the presence DNase I, with a half-life ($t_{0.5}$) of 12.0 ± 0.5 min. In contrast, micelles consisting of pure DNA-*b*-PCL show slightly enhanced nuclease resistance ($t_{0.5}$: 36 ± 1 min) due to increased steric hindrance and local high salt concentrations, which is consistent with previous reports.^{38,39} Interestingly, adding PEG_{2k}-*b*-PCL to the micelle (10:1 m:m PEG:DNA amphiphile ratio) results in an increase in degradation rate ($t_{0.5}$: 22 ± 2 min). Amphiphiles of higher PEG molecular weight (5 and 10 kDa), on the other hand, enhance DNA stability under identical conditions. The highest stability is achieved with PEG_{10k}-*b*-PCL, which gives a $t_{0.5}$ of 142 ± 15 min at 10:1 PEG:DNA amphiphile ratio, and 240 ± 18 min at 20:1. One interpretation of these results is that longer PEG chains can more effectively shield the underlying DNA strands from enzymatic access (Figure 3C and Table S3). When the PEG block is too short, the diluting effect of the PCL block becomes dominant, which serves to increase the spacing of DNA strands, leading to more facile enzyme access and degradation.

Although higher molecular weight PEG-*b*-PCL amphiphiles can better protect the DNA strands from degradation, they also increase the proportion of the slow-hybridizing population. For biopharmaceutical applications, it is desirable to maximally retain the hybridization capability while minimizing protein access.^{44,45} To this end, we systematically investigated how a homopolymer PCL in the nanoparticle formulation can be used to tune the spacing of particle surface moieties and maximize DNA binding selectivity. Micelles consisting of PEG_{10k}-*b*-PCL and DNA-*b*-PCL (20:1 m:m) are used as a base composition, to which different molar ratios of PCL (10–200, relative to DNA-*b*-PCL) are added. It is found that PCL significantly increases particle core size from 62 ± 3 nm (base composition,

estimated from TEM images) to 155 ± 5 nm (with 200 equiv PCL), which correlates with a drop of surface PEG density from 6.3×10^{13} to 1.5×10^{13} PEG/cm² (see Table S4). The reduction in PEG density results in acceleration in both hybridization and enzymatic access, with the change in hybridization being faster. With 20 equiv of PCL (density: 5.0×10^{13} PEG/cm²), hybridization can be mostly restored (>85% hybridized in 5 min), while protein shielding remains nearly unaffected ($t_{0.5}$ of 238 min vs. 240 min for base composition, Figures 3D and E). In contrast, excessive of PCL (200 equiv) results in incremental improvements in hybridization but substantial loss in enzyme stability, showing a $t_{0.5}$ of ~40 min. These results are consistent with our hypothesis that PCL homopolymer can be used to tune the PEG density of the micelles, thereby opening a window in which DNA binding selectivity can be maximized.

In addition to nuclease stability, efficient cellular uptake is another important aspect in high antisense efficacy. To examine the extent of endocytosis, SKOV3 cells are incubated with a library of Cy3-labeled micelles and free DNA having the same DNA concentration (1 μ M) for 4 h. Flow cytometry shows that, compared with free DNA, coassembled micelles have enhanced cell uptake (20–90 $\times$ relative to that of free DNA, Figure 4), with shorter PEG and lower PEG densities leading to more uptake (Figure 4A). Micelles with no surface PEG (DNA-*b*-PCL only) show the highest uptake at ~150 $\times$ that of free DNA. These PEG-free micelles are structurally analogous to spherical nucleic acids (SNAs), which have been shown to exhibit high, nonspecific cell uptake due to recognition by class A scavenger receptors and endocytosis via a lipid-raft-dependent, caveolae-mediated pathway.^{40–44} With the addition of moderate amounts of PCL homopolymer in the micellar core, cellular uptake is increased, but only to a small extent (Figure 4B). However, when 200 equiv of PCL is added, cell uptake is augmented to SNA-like levels. The unusually high cellular uptake is likely associated with the exposed hydrophobic regions of the micelle surface caused by excessive loading of PCL, which interacts with cells in a different mechanism than PEG- and DNA-dominated surfaces.⁴⁵ These results are corroborated by confocal laser scanning microscopy. While cells treated with free DNA show no or very weak fluorescence, DNA micelles give much stronger signals under the identical imaging settings, with increasing PEG contents reducing the intensities of fluorescent signals (Figures 4C and S10). The same experiments were also performed with HeLa cells, which show a similar general trend (Figure S11). Collectively, the results here indicate that the uptake of DNA-PEG-PCL micelles can be tuned by adjusting the surface composition exposed to the cell from being PEG-like to SNA-like and hydrophobic.

Next, we examined how different micelle structures affect antisense gene silencing efficacy. Because cellular endosomes are associated with digestive environments that can degrade the antisense sequence,^{14,46} higher stability may contribute to greater overall efficacy. SKOV3 cells were treated with the two series of micelles at an equal dose of DNA (1 μ M) for 24 h, followed by culturing for 48 h in fresh media. Lipofectamine-complexed DNA and free DNA were used as positive and negative controls, respectively. The levels of Bcl-2 were analyzed by Western blotting. As shown in Figures 5A and S12A, the Bcl-2 levels are significantly reduced by micelles having 10 kDa PEG amphiphiles. The best of these is PEG_{10k}-*b*-PCL:DNA-*b*-PCL:PCL (20:1:20), showing 92% reduction in expression (band densitometry analysis, normalized to β -actin). These micelles coincide with those showing

the highest nuclease stability but moderate cellular uptake and hybridization readiness, suggesting that protein inhibition is a key factor in achieving high efficacy. Indeed, low-stability micelles having 5 kDa PEG show considerably lower efficacy (22% knockdown for PEG_{5k}-*b*-PCL:DNA-*b*-PCL, 10:1) even though they exhibit better cell uptake and hybridization. Interestingly, although pure DNA-*b*-PCL micelle (SNA-like) only shows a small level of nuclease resistance, its antisense activity is significant (75% knockdown). This phenomenon is attributed to the unusually high cellular uptake associated with the SNAs.^{42–44} We also performed a dose-dependent study for the two-component system, PEG_{10k}-*b*-PCL:DNA-*b*-PCL (20:1) and the corresponding three-component system with 20 equiv of PCL. It is found that both systems are effective at high concentrations (>500 nM), but at a lower concentration (100 nM), the three-component system shows higher gene knockdown efficacy (84 vs 18%, Figures 5B and S12B), signifying that increased hybridization availability (Figure 2D) of the antisense ON is important because these two systems show roughly the same nuclease stability. Because the micelles consist primarily of DNA, PEG, and PCL, components regarded by the United States Food and Drug Administration as generally safe for pharmaceuticals, we anticipate that these coassembled nanoparticles are noncytotoxic. Indeed, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay of SKOV3 cells treated with the micelles show that viability after 48 h of incubation remains at nearly 100%. In contrast, DNA complexed with Lipofectamine exhibits significant cytotoxicity (>50% cell death at 100 nM of DNA), as expected from typical polycationic carrier systems (Figure S13).

In summary, we demonstrated a simple yet novel form of nucleic acid-based block copolymer micelles that can be used for effective antisense gene regulation. By screening a library of micelle compositions, we revealed a series of structure–property relationships. It was found that DNA hybridization availability and enzyme shielding are affected by the molecular weight of the PEG block and the surface PEG density. These parameters also affect cellular uptake with PEG-dominated surfaces showing moderate uptake and DNA-dominated surfaces showing high uptake. Correlations were also established between antisense activity and the degree of protein shielding, hybridization availability, and cellular uptake. Among these, protein shielding appears to have the strongest influence on antisense activity. Consisting of biodegradable and biocompatible components, these DNA block copolymer micelles represent an important departure from cationic systems which have been exhaustively investigated as nucleic acid delivery vectors. The general strategy employed here can also be expanded to include other types of biomolecules such as siRNA, microRNA, and peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Agarose gel (1%) electrophoresis of free DNA and various micelle compositions. Certain samples with high 10 kDa PEG contents migrate in the opposite direction of free DNA due to the transient interactions between PEG and the passing cations.³³

Figure 2.

(A) Schematics of the fluorescence assay used for quantifying DNA hybridization. (B and D) Hybridization kinetics for free DNA vs two- and three-component nanoparticles. (C and E) Relationship between PEG density and the percentage of the fast-hybridizing DNA population.

Figure 3.

(A) Schematics of the fluorescence assay used for monitoring the kinetics of DNA degradation by DNase I. (B and D) DNA degradation kinetics for free DNA vs two- and three-component nanoparticles. (C and E) Relationship between PEG density and the DNA nuclease half-life.

Figure 4.
(A and B) Flow cytometry measurements of SKOV3 cells treated with Cy3-labeled free DNA and micellar nanoparticles (conc: 1 μ M of DNA). (C) Corresponding confocal fluorescence images. Scale bar: 20 μ m.

Figure 5.

(A) Efficacy for antisense gene knockdown using coassembled micelles and controls (1 μ M DNA). (B) Dose response of two coassembled micelles with high efficacy.

Scheme 1.

Schematics of the Surface Composition for the Two- and Three-Component Micelles with Arrows Showing Observed Trends