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Improving the Enzymatic Stability and the Pharmacokinetics of Oligonucleotides via DNA-Backboned Bottlebrush Polymers

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Abstract

Herein, we design and synthesize site-specifically PEGylated oligonucleotide hairpins and demonstrate that their ability to undergo hybridization chain reaction is nearly unaffected by the PEGylation. The resulting DNA-backboned bottlebrush polymers with PEG side chains exhibit increased resistance against nucleolytic degradation, enhanced thermal stabilities, and elevated blood retention times in vivo, which collectively pave the way for more therapeutically focused DNA nanostructure designs.

Graphical Abstract

Keywords

Bottlebrush polymer; oligonucleotide; PEGylation; hybridization chain reaction; DNA nanotechnology

The field of DNA nanotechnology has made remarkable progress since its inception in the 1980s with the ability to now produce structures and morphologies with nearly arbitrary control.^{1–8} Nanostructured DNA bears an intrinsic advantage over its building block for medical use: the significantly increased molecular weight potentially allows for evasion of

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Supporting Information

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Materials, experimental procedures, instrumentation, and supplemental figures (PDF)

The authors declare no competing financial interest.

rapid renal clearance, which is the primary body elimination pathway for natural oligonucleotides under ~50 kDa in size.^{9,10} Still, the realization of DNA nanotechnology in medicine is occurring at a much slower pace compared to the development of new structures. Unmodified DNA exhibits poor nucleolytic stability in vivo.^{11,12} In addition, the compact DNA nanostructures often require high cation concentrations (Na^+ , Mg^{2+} , Ca^{2+} , and so forth) to minimize the repulsive interaction of the negatively charged phosphate on the DNA backbone.^{13,14} Several compatibilizing macromolecules, for example, cationic peptides, polymers, proteins, and nano-particles, have been developed to bind with preformed DNA nanostructures via electrostatic complexation to improve their solution and/or biological stabilities.^{15,16} Nevertheless, challenges exist regarding the control of the location and number of the compatibilizing agents on the DNA nanostructure, removal of excess unbound agent, and potential side effects associated with the agent.¹⁷

An alternative approach to improving the biopharmaceutical properties of nucleic acid-based materials is through covalent attachment of poly(ethylene glycol) (PEG), a biologically “stealth” polymer often adopted in pharmaceutical formulations.^{18,19} However, a single chain of linear or slightly branched PEG (40–100 kDa) is generally insufficient to shield oligonucleotides from interaction with proteins and thus cannot provide proper biopharmaceutical characteristics for systemic use.^{19,20} We have recently developed a novel form of brush polymer-oligonucleotide conjugate, termed pacDNA (polymer-assisted compaction of DNA), to address this challenge. The bottlebrush-architected PEG has a large number (typically >25) of shorter (5–10 kDa) PEG side chains emanating from a central backbone, which significantly increases local PEG density and thus steric protection of the conjugated oligonucleotide.^{21–24} Meanwhile, DNA hybridization kinetics and thermodynamics with a complementary sequence are not negatively affected.²⁰ The pacDNA exhibits high enzymatic stability (10–20× increase in half-life), can act as a single-entity antisense gene regulation agent without the need for a transfection agent, and minimizes side-effects associated with specific and nonspecific protein–DNA interactions, for example, unwanted activation of the innate immune system and coagulopathy.²²

While brush-architected PEG is highly effective for shielding conjugated oligonucleotides, it would not be feasible for a preassembled DNA nanostructure due to its large size (the level of shielding is inversely correlated to the distance from the brush backbone).²³ However, findings about the pacDNA imply that if one can precisely and evenly position many shorter PEG chains along the surface of a DNA nanostructure, achieving similar PEG densities to the pacDNA, similarly improved biopharmaceutical properties may be realized (see Scheme 1).^{20–24}

To achieve such uniformly PEGylated DNA nanostructures, we have devised a “bottom-up” synthesis, wherein PEGylated oligonucleotide hairpins (HPs) are used to form nano-structured DNA. This process, in principle, will allow for accurate control over the number and position of the PEG chains over the entire surface of the nanostructure. Hybridization chain reaction (HCR) is selected as a model self-assembly reaction.^{25,26} HCR is a form of living polymerization with the monomers being a mixture of two stable HPs. The addition of an initiator strand opens up one of the HPs to expose a single-stranded region, which opens up the other HP and reveal another single-stranded region that is identical to the original

initiator. The resulting chain reaction leads to the consumption of the HPs and the formation of a nicked double helix (polymer). While HCR has been widely used in analytical chemistry owing to its ability for signal amplification, bottle brush-type materials based upon HCR backbones have not been reported, to the best of our knowledge. The choice of HCR is based on three factors. First, HCR uses only a pair of building blocks, simplifying the preparation of PEGylated versions. Second, HCR can readily generate high molecular weight structures with repetitive subunits, increasing the overall PEG density. Third, the repetitiveness of the structure reduces the complexity when analyzing thermal melting properties and enzymatic stability of the HCR brush polymer, as there are no non-PEGylated segments that may interfere with data interpretation.

The HCR motifs were designed using the software package NUPACK (Table S1).²⁷ In order to increase the PEG density postassembly, a notably short set of HPs were used (34 bases vs ~50 for typical HCR). Each HP consists of three domains: a 12 nucleotide (nt) stem, a 5 nt loop, and a 5 nt sticky end. HP1 was modified with a fluorescein tag at the 3' to enable fluorescent tracking. To synthesize PEGylated HPs, an aminemodified thymine (T) base in the stem domain was introduced during solid-phase oligonucleotide synthesis, which enables subsequent conjugation to N-hydroxysuccinimide ester-terminated PEG (5 kDa). A 17 nt initiator was used to trigger the HCR cascade, which generated an analogue of a linear alternating copolymer. In addition, a 4-arm initiator was also synthesized by coupling 3' dibenzocyclooctyne-terminated initiator strands to an azide-functionalized 4-arm 20 kDa PEG (each arm 5 kDa) via copper-free click chemistry.

The ability of the unmodified HPs to undergo HCR was first tested using both the linear and the 4-arm initiators across a series of HP/initiator ratios. The successful formation of high MW polymer was confirmed by agarose gel electrophoresis (AGE) (Figure 1a). The average degree of polymerization (DP) was a function of the HP/initiator ratio, and the highest DP was observed with 0.5 equiv of the initiator. Typical reactions converted ~80% of the monomers to the polymer, as determined by gel band densitometry analysis as well as peak integration using aqueous gel permeation chromatography (GPC, Figure 1b). The polymerization resulted in DPs in the range of 2–20 (Figure S4C).

We next investigated the ability of PEGylated HPs (HP_{PEG}) to participate in HCR. Monomer mixtures of both partial PEGylation (HP1_{PEG} + HP2) and full PEGylation (HP1_{PEG} + HP2_{PEG}) resulted in high MW polymer as a function of HP/initiator ratio (Figures 3c and S4). Aqueous GPC showed (Figure 1d) distinct polymer peaks but without baseline separation from the monomers, which are themselves polymers. By peak deconvolution, we calculated the yields of the polymerization to be ~75%, which is consistent with the yields calculated by gel band densitometry analysis (DP = 2 population: 75%–85%). These results suggest that the PEGylated monomers are able to incorporate into HCR, but their incorporation kinetics compared to non-PEGylated HPs remains unclear.

In order to compare their reactivity ratio, a “copolymerization” experiment was devised (Figure 2a), which involves using a mixture of two types of HP1: unmodified HP1 (Cy5-labeled, red) and PEGylated HP1 (fluorescein-labeled, green). The two monomers compete with each other for incorporation into the growing polymer, and their ratio in the polymer

versus the feed reflects their relative ability to participate in the HCR (Figure 2b). It was found that the content of HP1 in the polymer was marginally but consistently higher across a range of $\text{HP1}_{\text{PEG}}/\text{HP1}$ ratios, suggesting slightly greater reactivity. Analysis using the Mayo–Lewis equation and linear fitting via the Fineman–Ross method shows the ratio of incorporation kinetics ($k_{\text{HP1}}/k_{\text{HP1-PEG}}$) to be 1.32 (see Supporting Information for detailed analysis).²⁸ These results indicate that PEGylated HPs can effectively incorporate into HCR nanostructures with only a minor reduction in reactivity despite the steric congestion associated with the PEG chains both on the growing polymer and the monomer.

The DNA-backboned brush polymers (with 0.5 equiv initiator) were studied by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The number-average hydrodynamic diameter ($D_{\text{h(n)}}$) increased from ~15 nm (HP_{PEG}) to ~150 nm (linear polymers) or ~400 nm (4-arm assemblies) (Figure 2e). TEM of negatively stained samples (with 1% uranyl acetate) reveals worm-like morphologies for the assembled structures with or without PEGylation (Figure 2d). Interestingly, there is a transition in persistent length of the worms on a pyrolytic carbon substrate from $\sim 9 \pm 3$ nm for the non-PEGylated assembly ($\text{HP1}+\text{HP2}$) to 13 ± 1 nm for the partially PEGylated assembly ($\text{HP1}_{\text{PEG}}+\text{HP2}$) and to 37 ± 9 nm for the fully PEGylated structure ($\text{HP1}_{\text{PEG}}+\text{HP2}_{\text{PEG}}$) based upon particle end-to-end distance analyses via Easyworm.²⁹ The increased persistent lengths can also be seen in the star polymer initiated by the 4-arm initiator (Figure S7). We attribute the difference in persistent length to the steric congestion of the PEG side chains, which enhance the stiffness of the DNA backbone. Similar phenomena have been observed with other DNA-based materials as well as synthetic brush polymers and also through computation.^{15,16,30,31}

Next, we studied the effect of PEGylation on the thermal stability of HCR nanostructures. The melting curves of three assemblies (non-, partially, and fully PEGylated) were recorded by monitoring the absorbance at 260 nm as the temperature was slowly ramped up ($0.2\text{ }^{\circ}\text{C}/\text{min}$) from 25 to $95\text{ }^{\circ}\text{C}$ (Figure 3a,b). The first-order derivative of the melting transitions revealed that melting temperatures increased from $72.2\text{ }^{\circ}\text{C}$ (non-PEGylated) to $76.3\text{ }^{\circ}\text{C}$ (partially PEGylated) and to $80.2\text{ }^{\circ}\text{C}$ (fully PEGylated) (buffer: SPSC). The enhanced thermal stability upon PEGylation is likely due to the macromolecular excluded volume effect,^{20,32,33} which favors volume-reducing reactions such as DNA hybridization. The sharpness of the melting transition as determined by the full width at half-maximum also decreased from 36.5 to $22.6\text{ }^{\circ}\text{C}$, suggesting a degree of cooperativity.^{34,35}

A key aspect regarding the biomedical applications of DNA nanostructures is their in vitro and in vivo stability. To examine the enzymatic degradation of the HCR nanostructures, we selected the endonuclease, DNase I, as a model enzyme, which acts upon double-stranded DNA with little sequence selectivity (Figure 3d).³⁶ Because the assembled HCR structures were dispersed in SPSC buffer, which significantly inhibits DNase I activity, dialysis against a low-salt buffer (PBS with 2.5 mM MgCl_2 and 0.5 mM CaCl_2) was first performed. The DNA structures remain stable in the low-salt buffer for at least two months (Figure S8). Thereafter, DNase I (0.7 unit/mL) was incubated with the DNA nanostructures for up to 2 h. Analysis of the amount of remaining high MW fraction by AGE ($\text{MW} > 50\text{ kDa}$, important for avoiding renal clearance) as a function of time revealed that PEGylation can effectively protect the assembled structure with $4.3\times$ increase in enzymatic half-life. A similar trend

was found for the 4-armed assemblies (Figure S9). While these improvements over free DNA do not yet exceed that of the pacDNA, we anticipate that there is significant room for further optimization (e.g., number of PEG chains per HP, MW of PEG, etc).

The greater availability of the high MW fraction under digestive conditions may lead to reduced renal clearance rate and longer the blood retention times in vivo. To test this hypothesis, we performed a pharmacokinetic study in immunocompetent (C57BL/6) mice by administering Cy5-labeled HCR nanostructures and controls via the tail vein, and subsequent monitoring of the Cy5 concentration in the blood (Figure 3f). The HCR HP precursors (including PEGylated) and the non-PEGylated assembly (HP1 + HP2) were all rapidly cleared from blood circulation with less than 15% remaining after 0.5 h. In contrast, brush-type assemblies of the PEGylated HPs resulted in increased blood availability. The highest area-under-the-curve (AUC_{∞}) was achieved with the 4-armed PEGylated structure, which is 2.6 $\times$ that of the free DNA (Figure 3g). These data suggest that a favorable plasma pharmacokinetics is positively correlated with the fraction of high-MW DNA nanostructures in the blood. Both the assembly and the use of PEGylated monomers are important; DNA self-assembly alone does not prolong blood circulation likely due to rapid digestion.

In summary, we demonstrate a novel method to impart better biopharmaceutical properties to self-assembled DNA-backboned bottlebrush polymers by incorporating PEGylated building blocks. The strategy in principle allows for precise control over the number and position of the PEG chains on the surface of the DNA nanostructure. Using HCR as a model system, we show that the PEGylated subunits self-assemble nearly as effectively as the non-PEGylated versions. The multiple PEG side chains improve the thermal stability of the DNA nanostructure while protecting the DNA from enzymatic degradation. The increased nuclease resistance allows for a higher fraction of nondigested DNA nanostructure to remain in the blood, which leads to a more favorable pharmacokinetics in mice. Collectively, our strategy reported herein points to an attractive possibility of utilizing complex DNA-based materials in nanomedicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
(a,c) AGE (4.5%) analysis of the HCR assemblies as a function of initiator:HP ratio and composition. (b,d) Aqueous GPC analyses of the HCR assemblies and their precursors.

Figure 2.

(a) Schematic illustration of the competitive incorporation of PEGylated and non-PEGylated HP1 into the growing polymer with an opened HP2_{PEG} chain-end. (b) Multiplexed AGE (4.5%) analysis of the incorporation rate for HP1 (red) and HP1_{PEG} (green) into the growing HCR polymer. (c) Relationship between the feed ratio of monomers (HP1_{PEG} and HP1) and fraction of them in the polymer. (d) TEM images of HCR nanostructures (negatively stained with 1% uranyl acetate) of varying PEG content initiated with the linear initiator. (e) DLS number-average size distribution of the precursor (HP1_{PEG}) and the assemblies (linear and 4-arm), showing the increased hydrodynamic diameter after HCR.

Figure 3.

(a) Schematic representation of DNA thermal melting. (b) UV-vis melting profile of PEGylated and unmodified HCR systems. (c) Boltzmann fitting of the first-order derivative of the for each HCR combination. (d) Stability against DNase I as characterized by AGE (4.5%). (e) Percentage of assembled HCR structures maintaining high MW (>50 kDa) in the presence of DNase I as a function of time, determined by gel band densitometry analysis. (f) Plasma half-life of HCR monomers and assembled nanostructures in mice and (g) blood availability as expressed in AUC_{∞} (** $p < 0.01$).

Scheme 1.
Hybridization Chain Reaction of PEGylated Hairpins and the Structure of DNA-Backboned Bottlebrush Polymer