Synthesis and Assembly of Click-Nucleic-Acid-Containing PEG–PLGA Nanoparticles for DNA Delivery

Albert Harguindey, Dylan W. Domaille, Benjamin D. Fairbanks, Justine Wagner, Christopher N. Bowman, and Jennifer N. Cha*

Co-delivery of both chemotherapy drugs and siRNA from a single delivery vehicle can have a significant impact on cancer therapy due to the potential for overcoming issues such as drug resistance. However, the inherent chemical differences between charged nucleic acids and hydrophobic drugs have hindered entrapment of both components within a single carrier. While poly(ethylene glycol)-block-poly(lactic-co-glycolic acid) (PEG–PLGA) copolymers have been used successfully for targeted delivery of chemotherapy drugs, loading of DNA or RNA has been poor. It is demonstrated that significant amounts of DNA can be encapsulated within PLGA-containing nanoparticles through the use of a new synthetic DNA analog, click nucleic acids (CNAs). First, triblock copolymers of PEG-CNA-PLGA are synthesized and then formulated into polymer nanoparticles from oil-in-water emulsions. The CNA-containing particles show high encapsulation of DNA complementary to the CNA sequence, whereas PEG-PLGA alone shows minimal DNA loading, and non-complementary DNA strands do not get encapsulated within the PEG-CNA-PLGA nanoparticles. Furthermore, the dye pyrene can be successfully co-loaded with DNA and lastly, a complex, larger DNA sequence that contains an overhang complementary to the CNA can also be encapsulated, demonstrating the potential utility of the CNA-containing particles as carriers for chemotherapy agents and gene silencers.

While nucleic acid delivery has great potential for gene silencing and elimination of drug resistance in diseased cells such as cancer, significant challenges remain toward building effective delivery agents. Cationic polymers and lipids or DNA nanostructures have been widely studied for DNA delivery, but issues remain such as immunogenicity, difficulties in nucleic acid release, and cost, to name a few. Furthermore, a co-delivery system that can simultaneously release both DNA or RNA and drugs would ensure that each cancer cell receives both the drug and RNA without having to time sequential doses. However, due to the vast differences in chemical properties between nucleic acids and many drugs, such as charge and hydrophobicity, entrapment of both within a single carrier at high payloads has proven difficult. For example, common block copolymer polymer vehicles such as poly(ethylene glycol)-block-poly(lactic-co-glycolic acid) (PEG–PLGA) used for targeted drug delivery have shown poor loading capacity for nucleic acids. While in recent years, Mirkin and co-workers have pioneered the use of spherical nucleic acids for gene uptake and cells and regulation, many of these studies have primarily focused on building nucleic acid micellar or liposomal structures. To expand the materials set for co-encapsulating nucleic acids and hydrophobic drugs within a single carrier, we report here the design and implementation of synthetic DNA analogs—namely click nucleic acids (CNAs)—for synthesizing PLGA-based polymer nanoparticles that sequester high loadings of DNA.

CNAs are a recently reported synthetic analogs of DNA that are produced by photoinitiated thiolene chemistry. While synthetic polymers have been used to incorporate nucleobases for building assemblies and new materials, CNAs present a new class of nucleobase polymers that have the potential to be made with sequence control through thiol-click reactions. As compared to other synthetic DNA systems such as peptide nucleic acids, CNA monomer units can be synthesized at scale and both the monomer and oligomers have mutual solvent compatibility with other synthetic polymers such as PEG and PLGA. In this work, we describe methods to synthesize the triblock copolymer PEG–CNA(T10)–PLGA. These amphiphilic copolymers were used to produce ~200 nm polymer nanoparticles via an oil-in-water (O/W) emulsion technique. We also show the ability to encapsulate high amounts of nucleic acid strands within the PEG–CNA–PLGA nanoparticles whereas PEG–PLGA alone showed minimal loading of DNA and PEG–CNA–PLGA nanoparticles demonstrated no encapsulation of noncomplementary DNA, showcasing the specificity of the CNA–DNA interactions.

To formulate CNA-containing polymer nanoparticles for DNA encapsulation and delivery, the triblock copolymer

A. Harguindey, Prof. D. W. Domaille, Prof. B. D. Fairbanks, J. Wagner, Prof. C. N. Bowman, Prof. J. N. Cha
Department of Chemical and Biological Engineering
University of Colorado
3415 Colorado Ave, Boulder, CO 80303, USA
E-mail: jennifer.cha@colorado.edu
Prof. C. N. Bowman, Prof. J. N. Cha
Materials Science and Engineering Program
University of Colorado
3415 Colorado Ave, Boulder, CO 80303, USA
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PEG–CNA(T10)–PLGA was first synthesized ([Figure 1](#)). The thymine CNA monomer 1 was synthesized as previously described,[35] and a photoinitiated thiol-ene polymerization between 1 and PEG-thiol (10 kDa) in the presence of 2,2-dimethoxy-2-phenylacetophenone produced PEG–CNA, 2, as a white solid. Small oligomers and uncoupled CNA polymers were separated by dialysis against acetone.

Characterization by proton nuclear magnetic resonance spectroscopy (1H NMR) ([Figure S1](#), Supporting Information) confirmed the structure, and gel permeation chromatography (GPC) revealed an average molecular weight of ≈12 kDa ([Figure S2](#), Supporting Information) against a poly(methylmethacrylate) standard, which corresponds to an average degree of polymerization of 10 CNA bases.

Next, prior to coupling the PEG–CNA with PLGA, a heterobifunctional amine–vinyl sulfone linker (3) was synthesized from 2-Boc-amino(ethanethiol) and divinyl sulfone ([Figure S3](#), Supporting Information). Then the Boc group was removed with trifluoroacetic acid (TFA), followed by amide coupling between 3 and the N-hydroxysuccinimide ester of PLGA (PLGA–NHS) afforded PLGA vinyl sulfone (4) ([Figure S4 and S5](#), Supporting Information). Finally, the terminal thiol of PEG–CNA 2 was attached to vinyl sulfone 4 by thiol-Michael addition to yield the final triblock polymer, PEG–CNA–PLGA (5). The final product was precipitated into ether and dialyzed with a 15 kDa dialysis membrane to remove small molecular weight impurities as well as mono- and diblock impurities. As shown in [Figure S5–S8](#) in the Supporting Information, GPC, NMR, and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) confirmed the successful synthesis of the PEGₙ–CNA(T10)–PLGAₘ triblock copolymer with an average molecular weight of 23 kDa.

Next, the PEG–CNA(T10)–PLGA polymers were formulated into nanoparticles using an O/W emulsion approach. In this approach, the polymer is first dissolved in an organic solvent and then introduced to an aqueous solution with stirring to form an emulsion dispersion. Here, the polymer was first dissolved in methylene chloride at 10 mg mL⁻¹. Next, a polymer solution (0.2 mL) was added dropwise to a stirring solution of 0.5 mL 0.1 M phosphate buffered saline (PBS). The temperature was raised to 37 °C and maintained for 2 h, followed by increasing the temperature to 45 °C and maintaining for 1 h to remove any remaining organic solvent. After cooling to room temperature, the particles were collected by centrifuging and characterized using nanoparticle tracking analysis and transmission electron microscopy (TEM). As shown in [Figure 2](#), using an O/W approach yielded the successful formation of polymer nanoparticles ≈200 ± 90 nm in diameter in its hydrated state. As a comparison, PEG–PLGA nanoparticles formulated using the same O/W procedures produced particles ≈154 nm ± 100 nm in diameter ([Figure S9](#), Supporting Information).

Next, the entrapment of DNA oligonucleotides within the preformed PEG–CNA–PLGA nanoparticles was studied. The reason for choosing this method was that if successful, it would allow encapsulating a chemotherapy drug such as Paclitaxel into the PLGA domain first, followed by incorporation of a therapeutic oligonucleotide, such as a gene silencer. As most drugs are mainly soluble in organic solvents, whereas DNA prefers a highly aqueous environment, this process would enable loading each component almost independently into the same particle.
via a compatible solvent. PEG–CNA(T10)–PLGA polymer nanoparticles were formulated as above and to the washed pellets, 5 µL of $1 \times 10^{-7}$ M Cy3-labeled A10 DNA was added to 45 µL of a mix of dimethyl sulfoxide (DMSO) and buffer ranging from 5% to 30% (v/v). This was done because we hypothesized that the inherent insolubility of CNA in aqueous environments would prevent CNA–DNA association if pure water or buffer was used alone, thereby hindering loading. Thus, DMSO was added to promote CNA–DNA interactions and help entrap DNA within the PEG–CNA(T10)–PLGA polymer nanoparticles.

After incubating the Cy3 A10 with the PEG–CNA(T10)–PLGA nanoparticles in the various co-solvent mixtures at 4 °C for 1 h, the particles were collected by bulk centrifugation. Excess or non-encapsulated DNA was removed by centrifuging washing the polymer nanoparticles three times with PBS. Control reactions were also performed by reacting the PEG–CNA(T10)–PLGA polymer nanoparticles with noncomplementary Cy3-T10. As shown in Figure 3 and Figure S10 in the Supporting Information, the PEG–CNA(T10)–PLGA polymer nanoparticles demonstrated observable encapsulation of Cy3-A10, in which a distinct red color was seen in the polymer pellet. Color was completely absent from polymer nanoparticles reacted with the noncomplementary Cy3-T10 DNA (Figure 3 and Figure S10, Supporting Information). In addition, PEG–PLGA nanoparticles were incubated with Cy3-labeled A10 or T10 to measure DNA loading in the absence of a CNA domain. These also resulted in no visible Cy3-T10 or Cy3-A10 encapsulation (Figure 3).

To quantify DNA loading, the Cy3-A10 or Cy3-T10 reacted polymer nanoparticles were next dissolved in 1 M phosphate-hydroxide pH 12 buffer to isolate DNA from each set of polymer nanoparticles upon PLGA degradation. After incubating for 3 h at 37 °C, the now optically transparent solutions were centrifuged at high speed to remove any remaining particulates. The supernatants were then collected and UV–vis spectra were obtained to determine the amount of Cy3-labeled DNA in solution. To control for any effects of pH on photophysical properties, the UV–vis calibration curve of the Cy3 conjugated A10 and T10 was determined at pH 12 (Figure S11, Supporting Information). As shown in Figure 3, Cy3-A10 was only detected in the supernatants of the PEG–CNA(T10)–PLGA nanoparticles, where ≈57.2 ± 9.5 pmol of DNA were determined to be entrapped per mg of polymer nanoparticles. In direct contrast, little Cy3-T10 was detected in the supernatants from the PEG–CNA(T10)–PLGA nanoparticles, demonstrating the specificity of the CNA(T10) for A10 DNA. Lastly, as controls, the diblock PEG–PLGA showed almost no entrapment of either dye conjugated DNA, highlighting the role of CNA for loading nucleic acids into the polymer nanoparticles.

Figure 2. Nanoparticle tracking analysis of PEG–CNA(T10)–PLGA nanoparticles produced by an oil/water emulsion method. (Inset) Representative TEM image of the PEG–CNA(T10)–PLGA nanoparticles. Uranyl acetate staining was used to image the particles.

Figure 3. (Top left) Optical image of pellets obtained after reacting preformed PEG–CNA(T10)–PLGA and (bottom Left) PEG–PLGA nanoparticles with solutions of Cy3-A10 or Cy3-T10. (Right) The amount of Cy3 DNA released into solution after completely dissolving the PEG–CNA(T10)–PLGA nanoparticles in 1 M phosphate-hydroxide, pH 12 buffer for 3 h. The average amount entrapped and standard deviation was determined by reacting the preformed PEG–CNA(T10)–PLGA and PEG–PLGA nanoparticles with either Cy3-T10 or Cy3-A10 in three separate runs.
As one of the advantages of using a PEG–PLGA system for therapy delivery is the ability to load chemotherapy drugs in the PLGA core, we next studied the use of the PEG–CNA–PLGA nanoparticles for co-entrapping drugs and DNA within the same delivery vehicle. To do this, pyrene was used as a model compound for a hydrophobic drug and PEG–CNA(T10)–PLGA was first dissolved with pyrene in dichloromethane. Next, the polymer and dye solution (10%, w/w) were added to PBS to produce dye-loaded PEG–CNA(T10)–PLGA nanoparticles. After thorough washing with buffer, half of the particles were lyophilized and then dissolved in DMSO to determine the amount of pyrene encapsulated by photoluminescence measurements. By building calibration curves of pyrene in DMSO, we were able to measure 69% of the initial pyrene could be entrapped inside the PEG–CNA(T10)–PLGA nanoparticles (Figure 4 and Figure S12, Supporting Information). To the other half of nanoparticles that had pyrene entrapped, Cy3-A10 or Cy3-T10 were added in 30% DMSO/buffer at 4 °C for 1 h. As before, after washing excess DNA, the particles were treated with 1 m phosphate-hydroxide, pH 12 buffer to accelerate degradation and release the DNA entrapped. As shown in Figure 4, in addition to pyrene being entrapped within the PLGA cores, Cy3-A10 could also be successfully encapsulated within the PEG–CNA(T10)–PLGA nanoparticles while Cy3-T10 could not. Lastly, in order to test the use of the PEG–CNA–PLGA nanoparticles for loading complex DNA or RNA sequences typically used for nanomedicine applications such as gene silencing, we next measured the ability to encapsulate the double stranded DNA (dsDNA) sequence Cy3-A10-CTTACGCTAGTACTTC-GATT hybridized with AAT CGA AGT ACT AGC GTA AG into the particles. We chose this nucleic sequence as its RNA form has been used for silencing luciferase expression in cells.[43] As many siRNA sequences can be tagged at their end with additional deoxynucleotides such as dT or dA, these PEG–CNA(T10)–PLGA (or PEG–CNA(A10)–PLGA) nanoparticles can be used as a universal carrier for a wide variety of gene silencers without the need to synthesize a unique CNA strand each time.[44,45] As described earlier, PEG–CNA(T10)–PLGA nanoparticles were first formulated using the O/W emulsion method followed by incubating the particles with the dye-labeled dsDNA for 1 h at 4 °C in 30% DMSO/buffer. After centrifuging the particles and removing unbound DNA, the particles were degraded in basic pH and as shown in Figure 5, UV–vis analysis showed =10 pmol of the complex dsDNA sequence could be entrapped per mg of polymer. The lower amount of DNA loading with the larger DNA sequence is most likely due to steric hindrance or charge repulsion between the longer nucleic acid strands. In conclusion, we have demonstrated the successful synthesis of PEG–CNA(T10)–PLGA polymers and its formulation into polymer nanoparticles for encapsulating large amounts of complementary DNA. First, the scalability of the CNA monomer coupled with its solvent compatibility with polymers such as PEG and PLGA enabled production of triblock copolymers containing PEG, CNA, and PLGA. The polymer nanoparticles were formulated using an oil-in-water approach, followed by loading nucleic acid strands complementary to the CNA sequence. With noncomplementary DNA or PEG–PLGA, little to no DNA entrapment was observed. These methods will enable future work in co-loading hydrophobic drugs and therapeutic nucleic acids such as siRNA within the interior of a single delivery agent. Since DNA loading within preformed polymer nanoparticles was shown to be successful, it was facile to load hydrophobic groups such as pyrene within the PLGA core first followed by encapsulating nucleic acids. Lastly, to demonstrate that the PEG–CNA(T10)–PLGA nanoparticles could be used to load complex nucleic acid sequences such as silencing genes, the polymer nanoparticles were reacted with Cy3-A10-CTTACGCTAGTACTTC-GATT hybridized with AAT CGA AGT ACT AGC GTA AG and showed successful entrapment of larger dsDNA. These results show the use of the PEG–CNA–PLGA polymers for building nanoparticles that can co-encapsulate hydrophobic drugs and hydrophilic nucleic acid strands within the same polymer nanoparticle carrier. Future studies will investigate this as well as

![Figure 4. (Left) Photoluminescence calibration and measurements to determine the amount of pyrene entrapped within the PEG–CNA(T10)–PLGA nanoparticles. (Right) Amount of Cy3-A10 or Cy3-T10 encapsulated within the pyrene loaded PEG–CNA(T10)–PLGA nanoparticles. The average amount entrapped and standard deviation was determined by reacting the preformed PEG–CNA(T10)–PLGA nanoparticles containing the pyrene with either Cy3-T10 or Cy3-A10 in three separate runs and measuring UV–vis of the solutions after completely dissolving the nanoparticles in 1 m phosphate-hydroxide, pH 12 buffer for 3 h.](https://www.advmat.de/newsletter/2017/29/1700743/1700743_4_of_6)
completely dissolving the nanoparticles in 1 m phosphate-hydroxide, pH 12 buffer for 3 h. The average amount entrapped and standard deviation was determined by reacting the preformed PEG–CNA(T10)–PLGA nanoparticles with the nucleic acid strands in three separate runs and measuring UV–vis of the solutions after completely dissolving the nanoparticles in 1 m phosphate-hydroxide, pH 12 buffer for 3 h.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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CNA, DNA, delivery vehicles, PLGA, polymer nanoparticles

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Figure 5. (Left) Optical image showing the final polymer nanoparticle pellets obtained after reacting PEG–CNA(T10)–PLGA nanoparticles with Cy3-A10, Cy3-A10-CTTACGCTAGTACTTCGATT hybridized with AAT CGA AGT ACT AGC GTA AG (labeled as Cy3-dsDNA) and Cy3-T10. (Right) Amounts of DNA encapsulated within the PEG–CNA(T10)–PLGA nanoparticles as a function of pH.

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