Formulation/Preparation of Functionalized Nanoparticles for In Vivo Targeted Drug Delivery

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Formulation/Preparation of Functionalized Nanoparticles for In Vivo Targeted Drug Delivery

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Summary

Targeted cancer therapy allows the delivery of therapeutic agents to cancer cells without incurring undesirable side effects on the neighboring healthy tissues. Over the past decade, there has been an increasing interest in the development of advanced cancer therapeutics using targeted nanoparticles. Here we describe the preparation of drug-encapsulated nanoparticles formulated with biocompatible and biodegradable poly(D,L-lactic-co-glycolic acid)-*block*-poly(ethylene glycol) (PLGA-*b*-PEG) copolymer and surface functionalized with the A10 2-fluoropyrimidine ribonucleic acid aptamers that recognize the extracellular domain of prostate-specific membrane antigen (PSMA), a well-characterized antigen expressed on the surface of prostate cancer cells. We show that the self-assembled nanoparticles can selectively bind to PSMA-targeted prostate cancer cells in vitro and in vivo. This formulation method may contribute to the development of highly selective and effective cancer therapeutic and diagnostic devices.

Keywords

Aptamers; Nanoparticles; Chemotherapy; Targeted drug delivery; Bioconjugated chemistry

1. Introduction

Nanomaterials have unique physicochemical properties, such as large surface area-to-mass ratios and high surface reactivity, which are different from bulk materials of the same composition. These unique physical properties allow the materials to interact with the human body on the molecular scale with a high degree of specificity. The application of nanotechnology in medicine, also known as nanomedicine, involves the use of precisely engineered nanomaterials for medical diagnosis and therapeutic treatments (1). One of the most exciting research topics in nanomedicine is targeted drug delivery. By combining molecular targeting capabilities and controlled drug release properties, targeted drug delivery offers the possibility of achieving precision-guided drug delivery to individual diseased cells with minimal side effects on neighboring healthy cells (2, 3).

In this chapter, we describe the method for preparing prostate cancer-targeted nanoparticles (NPs) (4–7). We used an A10 2′-fluoropyrimidine ribonucleic acid aptamer (Apt) (8), which binds to prostate-specific membrane antigen (PSMA) on the surface of prostate cancer (PCa) cells, as a model hydrophilic targeting molecule; the poly(D,L-lactide-co-glycolide) (PLGA)

as a model controlled release polymer system; and polyethylene glycol (PEG) as a model hydrophilic polymer with antibiofouling properties, to develop a proof-of-concept NP-Apt that is potentially suitable for selectively targeting PSMA PCa cells in vitro and in vivo.

2. Materials

2.1. Polymer Conjugation

1. All chemical reagents used in this study were cell culture grade (purity >95%) and were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise noted.
2. PSMA A10 2′-fluoropyrimidine RNA Apt (sequence: 5′-NH_2-spacer GGG/AGG/ACG/AUG/CGG/AUC/AGC/CAU/GUU/UAC/GUC/ACU/CCU/UGU /CAA/UCC/UCA/UCG/GCiT-3′ with 2′-fluoro pyrimidines, a 5′-amino group attached by a hexaethyleneglycol spacer and a 3′-inverted T cap) was custom synthesized by RNA-TEC (Leuven, Belgium). The aptamers were stored as lyophilized powder at −80°C.
3. Heterobifunctional PEG (amine-PEG-carboxylate) (MW = 34,00 g/mol) (Nektar Therapeutics, San Carlos, CA, USA) was stored in the dark at −20°C.
4. Poly(D,L-lactide-co-glycolide) (PLGA) (Lactel Absorbable Polymers, Pelham, AL, USA) with terminal carboxylate groups (PLGA–carboxylate) was stored at −20°C.
5. Conjugationcross-linkers:1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was stored in the dark at −20°C, and *N*-hydroxysuccinimide (NHS) was stored at 4°C.
6. *N*,*N*-diisopropylethylamine (DIEA) was stored in the dark at room temperature.
7. Solvents: Dichloromethane (DCM), ethyl ether, acetonitrile, and methanol were molecular biology grade (>99% in purity or higher).
8. Amicon ultracentrifugation tubes with molecular weight cut-off of 100,000 Da (Millipore, Billerica, MA, USA).
9. Washing solution: Anhydrous ethyl ether and methanol (50/50, v/v).

2.2. Cell Culture

1. Prostate cancer cell lines LNCaP and PC3 were both purchased from ATCC (Manassas, VA, USA).
2. LNCaP cells were cultured in RPMI-1640 (ATCC) supplemented with 100 U/mL aqueous penicillin G, 100 g/mL streptomycin, and 10% fetal bovine serum (FBS).
3. PC3 cells were cultured in F-12K (ATCC) supplemented with 100 U/mL aqueous penicillin G, 100 g/mL streptomycin, and 10% FBS.
4. Phenol-red-reduced OptiMEM media (Invitrogen, Carlsbad, CA, USA).

2.3. Immunohisto-chemistry for Tracking Nanoparticle Endocytosis

1. PLGA-*b*-PEG (50 mg/mL in acetonitrile [ACN]).

2. Fixative: 4% formaldehyde in phosphate-buffered saline (PBS) (freshly prepared).
3. Blocking solution: 1% bovine serum albumin (BSA) in PBS.
4. Blocking and permeabilization solution: 0.1% Triton-X100 in blocking solution.
5. Alexa phalloidin (5 U/mL) (Invitrogen).
6. 4′,6-diamidino-2-phenylindole (DAPI): 0.1 mg/mL.
7. Vectashield mounting media kit (Vector Laboratories, Burlin-game, CA, USA).

2.4. Tumor Preparation In Vivo

1. LNCaP cells were cultured in T-175 flasks (BD Biosciences, Franklin Lakes, NJ, USA).
2. Matrigel (BD Biosciences) was stored at −20°C.
3. 8-week-old balb/c nude mice (Charles River Laboratories, Wilmington, MA, USA) (*see* Note 1).

3. Methods

3.1. Polymer Conjugation Chemistry

1. 5 g of PLGA–carboxylate (0.28 mmol) was dissolved in 10–20 mL DCM (*see* Note 2).
2. NHS (135 mg, 1.1 mmol) and EDC (230 mg, 1.2 mmol) were dissolved in 2 mL DCM (*see* Note 3).
3. PLGA–carboxylate was converted to PLGA–NHS by adding the EDC/NHS solution prepared in **step 2** to the PLGA–carboxylate solution.
4. PLGA–NHS was precipitated with 10 mL ethyl ether/methanol washing solvent to remove residual NHS and EDC.
5. The precipitated PLGA–NHS was collected by centrifugation at 4,000 × *g* for 20 min.
6. Washing and centrifugation (**step 4** and **5**) were repeated two times.
7. The PLGA–NHS pellet was dried under vacuum for 30 min to remove the residual ether and methanol.
8. After drying under vacuum, PLGA–NHS (1 g, 0.059 mmol) was dissolved in DCM (4 mL) followed by addition of amine–PEG–carboxylate (250 mg, 0.074 mmol) and DIEA (28 mg, 0.22 mmol).

[1]All animal studies were carried out under the supervision of the Massachusetts Institute of Technology's Division of Comparative Medicine and in compliance with the National Institutes of Health's Principles of Laboratory Animal Care.
[2]The PLGA viscosity can influence the rate of PLGA-*b*-PEG conjugation. For high-viscosity PLGA, dilute PLGA in DCM to 0.1–0.25 g/mL before adding EDC/NHS.
[3]For maximum conjugation efficiency, dissolve EDC/NHS in DCM immediately before adding to PLGA–carboxylate.

9. The resulting PLGA-*b*-PEG block copolymer was precipitated with ether/methanol washing solvent and washed with the same solvent to remove unreacted PEG.

10. The resulting purified PLGA-*b*-PEG block copolymer was dried under vacuum and used for NP preparation without further treatment (*see* Note 4).

11. The composition of PLGA-*b*-PEG was characterized using a 400 MHz ^{1}H nuclear magnetic resonance (Bruker, Bill-erica, MA, USA). The nuclear magnetic resonance (NMR) characterization sample was prepared by dissolving 5 mg of the PLGA-*b*-PEG diblock copolymer in 1 mL of deuterated chloroform ($CDCl_3$). An example of a PLGA-*b*-PEG NMR spectrum is shown in Fig. 1.

3.2. Nanoparticle Preparation Methods

3.2.1. Nanoprecipitation for Encapsulating of Hydrophobic Compounds

1. PLGA-*b*-PEG (10 mg/mL) and docetaxel (0.1 mg/mL) were dissolved in acetonitrile.

2. The PLGA-*b*-PEG and docetaxel mixture was added drop wise to three to five volumes of stirring water (*see* Note 5), giving a final polymer concentration of 3.3 mg/mL.

3. The NPs were stirred for 2 h, and the remaining organic solvent was removed in a rotary evaporator at reduced pressure.

4. The NPs were concentrated using Amicon ultracentrifugation at 4,000 × *g* for 15 min and washed with deionized water and reconstituted in PBS.

5. The particle size and size distribution can be measured by dynamic light scattering (Brookhaven Instruments Corporation 90 plus particle sizer, 676-nm laser) at 25°C and at a scattering angle of 90° at a concentration of approximately 1 mg NP/mL water (*see* Note 6).

3.2.2. Double Emulsion (w/o/w) for Encapsulating Hydrophilic Compounds

1. An aqueous solution of rhodamine-labeled dextran (2.5 mg/mL, 0.4 mL) was emulsified in 2 mL PLGA-*b*-PEG dissolved in DCM (50 mg/mL) using a probe sonicator (Fisher Scientific, Pittsburgh, PA, USA) at 20 W for 45 s.

2. The emulsion was then transferred to an aqueous solution of PVA (0.1%, w/v, 50 mL), and sonicated at 20 W for 1 min.

3. The w/o/w emulsion formed was gently stirred at room temperature for 2 h or until the evaporation of the organic phase was complete.

4. The nanoparticles were then recovered using Amicon ultracentrifugation as described in **step 4** of **Subheading 3.2.1** (*see* Note 6).

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[4]To achieve more efficient polymer conjugation, use a high-power vacuum pump to rapidly evaporate residual solvents in the polymer formulation.
[5]To avoid nanoparticle aggregation, the acetonitrile:water volume should be greater than 2:1.
[6]To maintain NP colloidal stability, always formulate NPs in pure water, then reconstitute NPs in PBS or other desired media.

5. The particle size and size distribution can be measured as described in **step 5** of **Subheading 3.2.1**.

3.2.3. NP–Apt Conjugation

1. PLGA-*b*-PEG NPs (10 mg/mL) were suspended in DNase- and RNase-free water, and were mixed with EDC (400 mM) and NHS (200 mM) for 20 min.
2. NPs were then washed three times in DNase- and RNase-free water using Amicon ultracentrifugation tubes.
3. The resulting NHS-activated NPs were reacted with PSMA A10-Apt (1 mg/mL) for 2 h.
4. The NP–Apt bioconjugates were washed 3 times as described in **Subheading 3.2.3**, **step 2**.
5. NP–Apt bioconjugates were denatured at 90°C and allowed to assume binding conformation during snap-cooling on ice.
6. The NP suspensions were kept at 4°C until use.
7. NP–Apt conjugation was confirmed using 10% TBE–urea poly-acrylamide gel electrophoresis (PAGE). Samples containing nanoparticles surface functionalized with aptamers (NP+Apt), native aptamers (Apt), and nanoparticles without surface modification (NP) were loaded in PAGE. A sample gel image is shown in Fig. 2.

3.3. Targeted Nanoparticle Uptake In Vitro

1. PCa LNCaP andPC3 cells were grown in 8-well chamber slides in RPMI 1640 and Ham's F12K medium, respectively, supplemented with 100 U/mL aqueous penicillin G, 100 μg/mL streptomycin, and 10% fetal bovine serum at concentrations to allow 70% confluence (i.e., LNCaP: 40,000 cells/cm^2).
2. LNCaP and PC3 cells were washed with prewarmed PBS and incubated with phenol red-reduced OptiMEM media for 30 min at 37°C.
3. Cells were incubated with 50 μg of NP–Apt prepared as described in **Subheading 3.2.2** and **Subheading 3.2.3** for 15 min to 1 h at 37°C.
4. Cells were washed with prewarmed PBS three times.
5. Cells were fixed with 4% paraformaldehyde for 20 min, followed by washing with PBS.
6. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and Alexa-Fluor phalloidin.
7. The cell culture chamber slides were then mounted and visualized by fluorescent microscopy (*see* Note 7).

[7]To preserve the imaging quality, we recommend imaging the mounted slides right away, or at most within 5 days after mounting, in which case slides should be stored in the dark at −20°C until imaging.

8. Where indicated, the number of nanoparticle aptamer biocon-jugates or control nanoparticles attached to individual LNCaP or PC3 cells was quantified by fluorescent microscopy under oil immersion at 100× magnification (*see* Note 8). A sample figure of targeted NP uptake by LNCaP and PC cells is shown in Fig. 3.

3.4. Efficacy of Tumor Reduction In Vivo

1. The NPs were traced by encapsulating docetaxel using the nanoprecipitation method explained in **steps 1–5** of **Subheading 3.2.1**.
2. The NP formulations were suspended in 200 μL PBS before administration.
3. LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin.
4. Mice were anesthetized by intraperitoneal injection of Avertin (200 mg/kg body weight), and dosed with 3 million LNCaP cells suspended in 600 μL of 1:1 (v/v) media and Matrigel (*see* Note 9).
5. LNCaP tumors were induced in 8-week-old balb/c nude mice (Charles River Laboratories).
6. Mice were injected subcutaneously in the right flank with 3×10^6 LNCaP cells suspended in a 1:1 mixture of media and Matrigel (BD Biosciences).
7. Tumor-targeting studies were carried out after the mice developed ~100 mg tumors (*see* Note 10).
8. Mice were randomly divided into different groups, minimizing tumor size variations between groups.
9. Mice were anesthetized by intraperitoneal injection of Avertin (200 mg/kg body weight), and dosed with NP formulations via intratumoral injection.
10. After dosing, the mice were monitored for weight and implanted tumor size change daily for 2 weeks and every 3 days thereafter.
11. If body weight loss (BWL) persisted beyond 20% of predosing weight, the animals were euthanized.
12. The length and width of the tumors were measured by digital calipers, calculating tumor volume by the following formula: $(\text{width}^2 \times \text{length})/2$.
13. Mice were monitored for a maximum of 109 days, until the tumor was completely regressed or until the tumor volume exceeded 800 mm^3, for which the mice were euthanized for excessive tumor load.
14. For animals that were euthanized because of tumor load or BWL, the tumor size at the time of euthanasia was used for the purpose of mean tumor size calculation.

[8]The fluorescent dyes encapsulated in NPs are released in a time-dependent manner. Always prepare a fresh batch of NPs for the in vitro release study to maximize the amount of dyes encapsulated in the NPs.
[9]To obtain fast growing tumors, always reconstitute LNCaP cells in full growth media containing serum before mixing with Matrigel.
[10]For maximum LNCaP growth, ensure all media are phenol free.

Initial volume of the tumors averaged 328 mm^3. Tumor efficacy study results are shown in Fig. 4.

Acknowledgments

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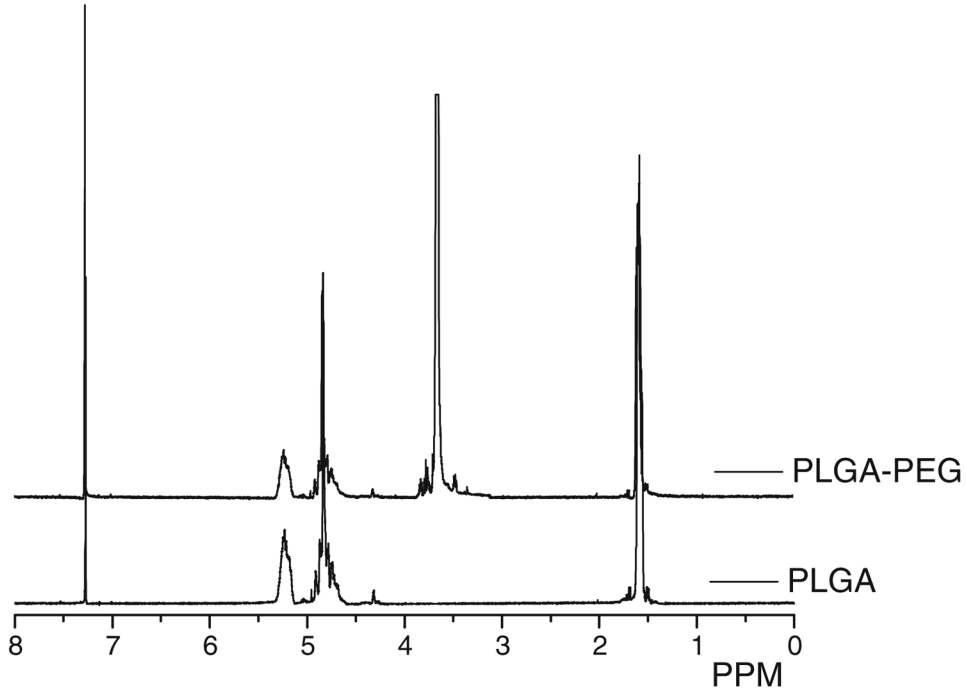

Fig. 1.

PLGA-*b*-PEG characterization using nuclear magnetic resonance (NMR). The presence of PLGA and PEG were visualized in using a 400 MHz ^{1}H NMR ppm 5.2 (m, ((OCH(CH3)C(O)OCH2C(O))n–(CH2CH2O)m), 4.8 (m, ((OCH(CH3)C(O)OCH2C(O))n–(CH2CH2O)m), 3.7 (s, ((OCH(CH3)C(O)OCH2C(O))n–(CH2CH2O)m), 1.6 (d, ((OCH(CH3)C(O)OCH2C(O)) n–(CH2CH2O)m) (reproduced from **ref**.(7) with permission from PNAS, Copyright (2008) National Academy of Sciences, U.S.A.).

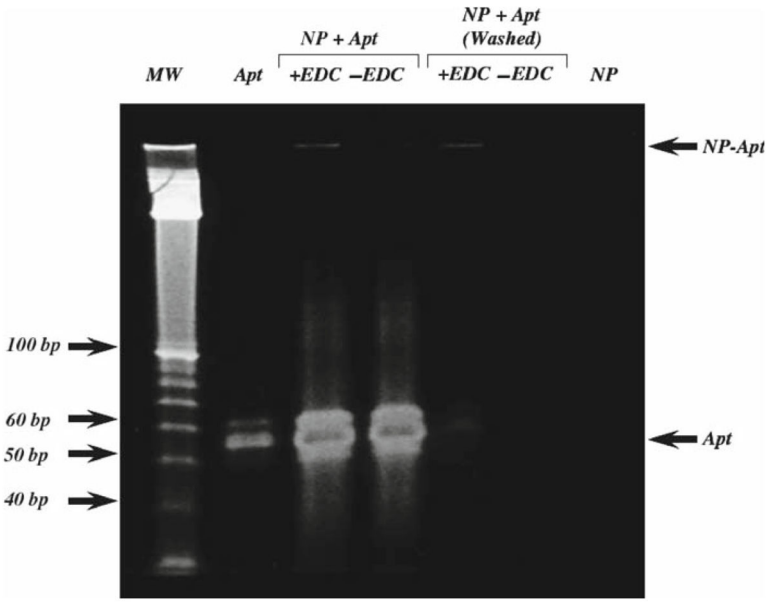

Fig. 2.

Confirmation of NP–Apt conjugation. The A10 PSMA aptamer (Apt) was incubated with PLGA–*b*–PEG NP in the absence (−) or presence (+) of EDC and the reactions were resolved on a 10% TBE–urea PAGE directly, or after washing to remove any unconjugated Apt. The bands corresponding to the A10 PSMA Apt and NP–Apt are indicated by *arrows*. The molecular weight (MW) DNA marker and free aptamer served as standards for a 57-base pair band on the gel and are shown on the *left* (reproduced from **ref.**(4) with permission from Elsevier).

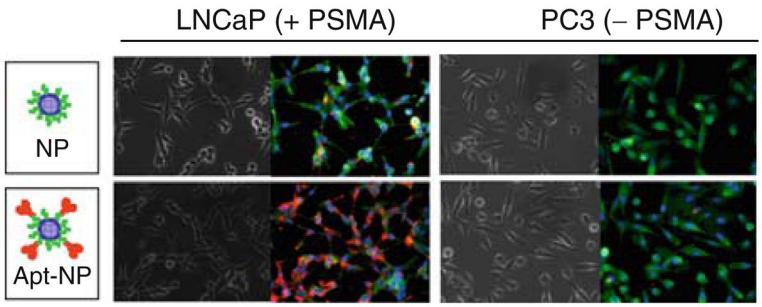

Fig. 3.

Binding of NP–Apt bioconjugates to prostate epithelial cells. LNCaP cells and PC3 cells were grown on chamber slides and incubated in culture medium with rhodamine-labeled dextran-encapsulated pegylated NP (*red*), or rhodamine-labeled dextran-encapsulated pegylated NP–Apt bioconjugates (*red*). Cells were washed in PBS three times, fixed, and permeabilized, stained with 4′,6-diamidino-2-phenylindole (nuclei, *blue*) and Alexa-Fluor phalloidin (cytoskeleton, *green*), washed, and analyzed by light transmission or fluorescent microscopy (reproduced from **ref.**(6) with permission from American Association for Cancer Research).

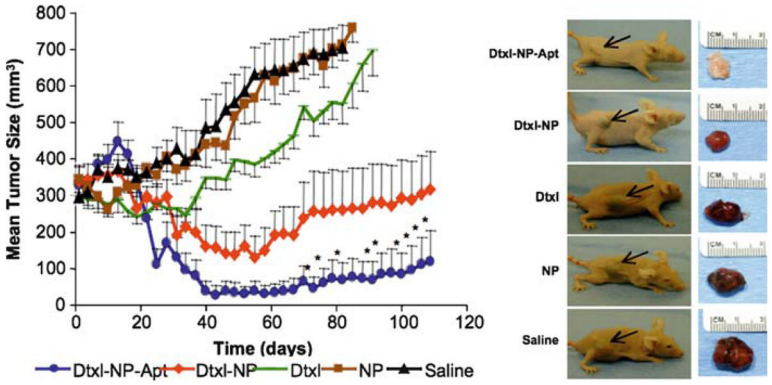

Fig. 4.

Comparative efficacy study in LNCaP subcutaneous xenograft nude mouse model of PCa. (**a**) The comparative efficacy study of single intratumoral injection (day 0) of (i) saline (*black*); (ii) pegylated PLGA NP without drug (NP, *brown*); (iii) emulsified Dtxl (Dtxl, *green*), 40 mg/kg; (iv) Dtxl–encapsulated NPs (Dtxl–NP, *red*), 40 mg/kg; or (v) Dtxl–encapsulated NP–Apt bioconjugates (Dtxl–NP–Apt, *blue*), 40 mg/kg was evaluated over 109 days and demonstrated that targeted NPs are significantly more efficacious in tumor reduction as compared with other groups. Data represent mean ± SEM of seven mice per group. Data points labeled with "*" for the Dtxl–NP–Apt group were statistically significant compared with all other groups by analysis of variance (ANOVA) at a 95% confidence interval. (**b**) A representative mouse at the end point for each group is shown (*left*) alongside images of excised tumors (*right*). For the Dtxl–NP–Apt group, which achieved complete tumor regression, the scar tissue and underlying skin at the site of injection are shown. *Black arrows* point to the position of the implanted tumor on each mouse (reproduced from **ref.**(5) with permission from PNAS, Copyright (2008) National Academy of Sciences, U.S.A.).