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Tetanus toxin C fragment-conjugated nanoparticles for targeted drug delivery to neurons

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Abstract

The use of nanoparticles for targeted drug delivery is often facilitated by specific conjugation of functional targeting molecules to the nanoparticle surface. We compared different biotin-binding proteins (avidin, streptavidin, or neutravidin) as crosslinkers to conjugate proteins to biodegradable nanoparticles prepared from poly(lactic-*co*-glycolic acid) (PLGA)–polyethylene glycol (PEG)-biotin polymers. Avidin gave the highest levels of overall protein conjugation, whereas neutravidin minimized protein non-specific binding to the polymer. The tetanus toxin C fragment (TTC), which is efficiently retrogradely transported in neurons and binds to neurons with high specificity and affinity, retained the ability to bind to neuroblastoma cells following amine group modifications. TTC was conjugated to nanoparticles using neutravidin, and the resulting nanoparticles were shown to selectively target neuroblastoma cells *in vitro*. TTC-conjugated nanoparticles have the potential to serve as drug delivery vehicles targeted to the central nervous system.

Keywords: Nanoparticle; Drug delivery; Brain; Cell culture; Nerve; Protein

1. Introduction

Biodegradable polymers, including polylactic acid (PLA) and poly(lactic-*co*-glycolic acid) (PLGA), have been used to create nanoparticles and microparticles that encapsulate a variety of therapeutic compounds over time with favorable safety profiles [1]. Polyethylene glycol (PEG) reduces systemic clearance rates *in vivo* [2], and the functionalization of polymer end groups and subsequent conjugation of targeting moieties (proteins, aptamers, and peptides) permits local drug delivery and reduced systemic toxicity [3]. *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) are commonly used for protein conjugation and can generate a stable covalent bond. One problem when using this and other similar techniques is the presence of intermediaries with short half lives, which may lead to inefficient conjugations. In contrast, non-covalent interactions between biotin and its binding proteins (avidin, streptavidin, and neutravidin) are highly specific and do not involve unstable intermediaries. Biotin-binding proteins have previously been used to conjugate proteins to the surface of microparticles and nanoparticles [4–10].

A major challenge in treating neurodegenerative diseases is directly delivering therapies to neurons in the central nervous system (CNS). The CNS is difficult to penetrate

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because it is protected by the blood–brain barrier (BBB) [11]. Recently, nanoparticles synthesized from poly(butylcyanoacrylate) with polysorbate 80 [12], and in separate experiments liposomes conjugated to the antibody to the transferrin receptor, have been used to bypass the BBB [13].

Retrograde transport from distal axon terminals to the neuronal cell body is an essential process in neurons; it transports enzymes, vesicles, and mitochondria, and is exploited by viruses and bacterial pathogens as a route to intoxicate motor neurons [14]. It is apparent that retrograde axonal transport of substances from the periphery to motor neuron cell bodies can effectively penetrate the CNS and bypass the BBB [15]. Thus, it may be possible to target nanoparticles to CNS neurons by exploiting retrograde neuronal transport.

One important element in our early studies has been the use of a non-toxic fragment of tetanus toxin, known as tetanus toxin C fragment or TTC [16]. TTC is the neuronal-binding portion of the native tetanus toxin. TTC demonstrates extremely high affinity binding to the neuronal ganglioside $GT1_b$ that is the tetanus receptor, which is located selectively on the surfaces of neurons [17]. Moreover, once TTC binds to neurons, it is readily endocytosed and efficiently carried via retrograde transport from the distal axonal terminus to the neuronal cell body [17,18].

In this report, we compare the ability of different biotin-binding proteins (avidin, streptavidin, and neutravidin) to specifically conjugate a protein to the surface of PLGA-PEG-biotin nanoparticles. We describe the use of TTC-conjugated PLGA-PEG-biotin nanoparticles as a drug delivery system that selectively targets neuronal cells *in vitro*. This system may have applications for delivering therapeutics to neurons affected by neurodegenerative diseases and may allow retrograde transport delivery to the CNS.

2. Methods

2.1. Preparation of PLGA-PEG-COOH and PLGA-PEG-biotin

One gram of PLGA-COOH (20 kDa MW, Lactel Absorbable Polymers) was dissolved in 4 mL dichloromethane and stirred at RT in the presence of NHS (1:8 PLGA:NHS molar ratio) and EDC (1:8 PLGA:EDC molar ratio) to form an amine reactive ester. Unreacted NHS and EDC were removed using a solution containing 70% ethyl ether and 30% methanol. Trace solvents were removed under vacuum for 2 h. The polymer was re-dissolved in 5 mL chloroform and incubated under gentle stirring overnight with HCl.NH2-PEG-COOH (3400 MW, Nektar Therapeutics) or NH2-PEG-biotin (3400 MW, Laysan Bio) (1:1.3 PLGA:PEG molar ratio). *N*-ethyldiisopropylamine (DIEA) was also added to the HCl.NH2-PEG-COOH solution. The polymer was washed with methanol to remove unreacted PEG. The final PLGA-PEG-COOH/biotin product was recovered using ethyl ether, vacuum dried for 2 h, and stored at −20 °C until use.

2.2. NMR analysis

Polymer was dissolved in deuterated chloroform (5–10 mg/mL) and placed in a glass NMR tube. Polymer was analyzed on a Bruker Avance 400 Mhz NMR spectrometer using standard proton NMR to verify PEG conjugation to PLGA. Samples were analyzed for the presence of any intermediary products and to quantify the extent of conjugation.

2.3. Preparation of nanoparticles (nanoprecipitation)

Ten milligrams of PLGA-PEG-COOH or PLGA-PEG-biotin was dissolved in 1.5 mL acetone, and fluorescent nanoparticles were made by also adding 200 μL of coumarin-6 (1 mg/mL in acetone, Sigma Aldrich) [19]. Nanoparticles made of different mixtures of –COOH and –biotin polymers were prepared in the same way, similarly to methods previously described [20]. Five aliquots of 0.3 mL of the polymer solution were continuously injected with a glass syringe to each of five stirring vials of 0.9 mL deionized water to form nanoparticles. The tip of the syringe was submerged during particle formation. The vials were pooled, the acetone solvent was evaporated at RT for 1 h, and nanoparticles were briefly centrifuged (2000 rcf, 10 s) to remove any visible aggregates. Nanoparticles were concentrated and washed to remove any remaining acetone in an Amicon Ultra-4, 100 kDa centrifugal filter (Millipore). Particles were redissolved in a minimal volume of water and stored at 4 °C until use.

2.4. Protein attachment to nanoparticles

Five hundred microliters of nanoparticle solution (~20 mg polymer/mL) was incubated with 2 mL avidin (Invitrogen) solution (2 mg/mL) and gently stirred for 30 min at RT to allow avidin conjugation to the nanoparticles. Neutravidin (Pierce Biotechnology) or streptavidin (Invitrogen) were used analogously for experiments using these as the crosslinker. Nanoparticles were washed and free biotin-binding protein was removed by three centrifugal washes (4000 rcf, 25C, ~10 min) in an Amicon filter. Nanoparticles were resuspended in 500 μL of water, and biotinylated bovine serum albumin (BSA) or TTC (2 mg/mL in PBS) were incubated with the nanoparticles at RT under gentle stirring. Product was washed three times with PBS by centrifugation using an Amicon filter (4000 rcf, 25C, ~20 min) to remove unbound protein. Nanoparticles were resuspended in a minimal volume of PBS and stored at 4 °C until analysis. For all free biotin conjugation experiments, free biotin was mixed with biotinylated TTC in different concentrations, and conjugated analogously. All fluorescent measurements were made on a 1420 VICTOR3 plate reader (Perkin-Elmer) and read in a 96-well plate in triplicate.

2.5. Preparation of BSA–FITC and TTC–FITC

Ten milligrams of BSA was dissolved in PBS (10 mg/mL) or 1 mg of TTC was dissolved in PBS (2 mg/mL), and incubated with EZ-Link NHS–FITC (Pierce Biotechnology) (1–24:1 FITC:protein molar ratio) under gentle stirring for 2 h at RT. Reacted product was collected using a Zeba Desalt Spin Column (Pierce Biotechnology), according to manufacturer's directions.

2.6. Biotinylation of BSA and TTC

Ten milligrams of BSA was dissolved in PBS (10 mg/mL) or 1 mg of TTC was dissolved in PBS (2 mg/mL), and incubated with EZ-Link NHS–PEG_4–biotin (Pierce Biotechnology) under gentle stirring for 2 h at RT. Reacted product was collected using a Zeba Desalt Spin Column (Pierce Biotechnology), according to manufacturer's directions. Biotinylated BSA was incubated at a ratio of 20:1 NHS–PEG_4–biotin:BSA and biotinylated TTC was incubated at a ratio of 10:1 NHS–PEG_4–biotin:TTC in all experiments unless otherwise noted.

2.7. Biotinylation quantification

The extent of BSA and TTC biotinylation was determined using a HABA–biotin quantitation assay (Pierce Biotechnology), according to manufacturer's directions. Briefly, the absorbance of the HABA–avidin