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A novel approach for targeted delivery to motoneurons using cholera toxin-B modified protocells

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HIGHLIGHTS

- Cholera toxin-B (CTB) modified protocells provide a novel delivery method to target motoneurons.
- CTB-protocells display uptake by presynaptic axon terminals at neuromuscular junctions.
- CTB-protocells showed greater motoneuron uptake compared to unmodified protocells.
- CTB-protocells constitute a promising delivery vehicle for therapy in motoneuron diseases.

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ABSTRACT

Background: Trophic interactions between muscle fibers and motoneurons at the neuromuscular junction (NMJ) play a critical role in determining motor function throughout development, ageing, injury, or disease. Treatment of neuromuscular disorders is hindered by the inability to selectively target motoneurons with pharmacological and genetic interventions.
New method: We describe a novel delivery system to motoneurons using mesoporous silica nanoparticles encapsulated within a lipid bilayer (protocells) and modified with the atoxic subunit B of the cholera toxin (CTB) that binds to gangliosides present on neuronal membranes.
Results: CTB modified protocells showed significantly greater motoneuron uptake compared to unmodified protocells after 24 h of treatment (60% vs. 15%, respectively). CTB-protocells showed specific uptake by motoneurons compared to muscle cells and demonstrated cargo release of a surrogate drug. Protocells showed a lack of cytotoxicity and unimpaired cellular proliferation. In isolated diaphragm muscle-phrenic nerve preparations, preferential axon terminal uptake of CTB-modified protocells was observed compared to uptake in surrounding muscle tissue. A larger proportion of axon terminals displayed uptake following treatment with CTB-protocells compared to unmodified protocells (40% vs. 6%, respectively).
Comparison with existing method(s): Current motoneuron targeting strategies lack the functionality to load and deliver multiple cargos. CTB-protocells capitalizes on the advantages of liposomes and mesoporous silica nanoparticles allowing a large loading capacity and cargo release. The ability of CTB-protocells to target motoneurons at the NMJ confers a great advantage over existing methods.
Conclusions: CTB-protocells constitute a viable targeted motoneuron delivery system for drugs and genes facilitating various therapies for neuromuscular diseases.

1. Introduction

Targeted delivery systems to motoneurons are critical in developing effective and safe treatments for motoneuron diseases (*e.g.* amyotrophic lateral sclerosis), as well as in understanding causes of muscle denervation (*e.g.* spinal cord injury, spinal muscular atrophy

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or ageing muscle) (Boido and Vercelli, 2016; Comley et al., 2016; Dupuis and Loeffler, 2009; Hepple and Rice, 2015; Mantilla and Sieck, 2009). Despite efforts in the field, treatments remain hindered by lack of drug selectivity to neurons in the central nervous system (CNS), difficulty in targeted cellular delivery, poor penetration through biological membranes/barriers and insufficient stability (Misra et al., 2003). There is an advantage in targeting motoneurons over other CNS neurons in that they have peripherally located nerve terminals at neuromuscular junctions (NMJs). This characteristic makes motoneurons accessible to treatments that exploit retrograde neuronal transport. However, the transfer of promising molecules (*e.g.*, trophic factors) into the desired sites of action with high efficiency and uncompromised activity, while minimizing adverse reactions caused by their off-target effects, remains challenging (Weishaupt et al., 2012).

Nanoparticles are novel drug delivery systems with exceptional therapeutic potential (Simonato et al., 2013) that can encapsulate a variety of compounds and deliver them to target cells or tissues often with favorable safety profiles. In particular, mesoporous silica nanoparticles (MSNPs) have unique properties that make them a suitable treatment vehicle to target motoneurons, including: (1) the ability to independently modify pore size and the surface chemistry to enhance cargo loading when compared to other common drug delivery systems (*e.g.*, liposomes); and, (2) the possibility to engineer bio-functionality and bio-compatibility by modifying the MSNPs surface (Ashley et al., 2011; Tarn et al., 2013). MSNPs encapsulated within a supported lipid bilayer (so-called protocells) exhibit the combined beneficial features of MSNPs and liposomes with versatile cargo loading, controlled release and the possibility to introduce strategic targeting ligands in the supported lipid bilayer to enable cell specific delivery of molecular components (Ashley et al., 2011).

A number of natural toxins exist that target the nervous system and could be employed in a targeting strategy (Edupuganti et al., 2012b). Cholera toxin produced by the bacterium *Vibrio cholerae* has an atoxic subunit (CTB) formed by five identical B-subunit monomers each composed of 103 amino acids (Miller et al., 2004). CTB binds a cell-surface receptor, ganglioside GM1, present on neuronal membranes (Sheikh et al., 1999; Zhang et al., 1995), and is effectively transported retrogradely in neurons. Indeed, CTB has been extensively used as a reliable neuronal tracer (Dederen et al., 1994; Mantilla et al., 2009; Wan et al., 1982). We hypothesized that cargo loaded, CTB modified protocells (CTB-protocells) will target motoneurons and show axon terminal uptake at NMJs. In the present study, we demonstrate that CTB conjugated protocells using biotin-NeutrAvidin, predominantly target motoneurons *in vitro* compared to muscle cell controls. We also show that there is CTB-protocell uptake into nerve terminals at diaphragm muscle NMJs. In addition, we validate intracellular cargo delivery using a membrane impermeable molecule, demonstrating the efficacy of CTB-protocells as a vehicle for targeting and delivering cargo to motoneurons.

2. Materials and methods

2.1. Preparation of mesoporous silica-supported lipid bilayer nanoparticles (protocells)

2.1.1. MSNP synthesis

Fluorescently labeled MSNPs with hexagonal prismatic shape composed of close packed 2.8 nm diameter cylindrical pores were synthesized via a solution-based surfactant-directed self-assembly method, as reported by Lin et al. (2005). Briefly, MSNPs were fluorescently modified by dissolving 1 mg of rhodamine B isothiocyanate (Sigma-Aldrich, St. Louis, MO) in 1 mL of *N,N*-dimethyl formamide (DMF; Sigma-Aldrich) followed by addition of 1 μL 3-aminopropyltriethoxysilane (APTES; Sigma-Aldrich). Next, 290 mg of *n*-cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich) was dissolved in 150 mL of 0.50 M ammonium hydroxide (Sigma-Aldrich) solution in a 250 mL beaker, sealed, and heated at 50 °C while stirring for 1 h. Next, 3 mL of dilute ethanolic tetraethyl orthosilicate (TEOS; Sigma-Aldrich) solution and 1 mL of RITC-APTES solution were added simultaneously to the surfactant/ammonium hydroxide solution. After 1 h of continuous stirring, the particle solution was kept at 50 °C for ~18 h under static conditions. Next, the as-synthesized MSNPs were stored in a sealed container at 70 °C for 24 h. CTAB was removed by acidic solvent extraction as described in literature (Townson et al., 2013). Briefly, MSNPs were washed and refluxed in ammonium nitrate solution, followed by wash and reflux in ethanolic HCl solution, then washed and transferred to ethanol solution for storage.

2.1.2. Supported lipid bilayer formation and CTB conjugation

The procedure used to fuse liposomes to MSNPs (protocell assembly) was based on a modified method described by Liu et al. (Ashley et al., 2011; Liu et al., 2009). Briefly, 1,2-distearoyl-*sn*-*glycero*-3-phosphocholine (DSPC; Avanti Polar Lipids, Birmingham, AL) phospholipids, cholesterol (Chol; Avanti Polar Lipids, Birmingham, AL) and 1,2-distearoyl-*sn*-*glycero*-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (DSPE-PEG2000-NH2) phospholipids were pre-dissolved in chloroform, dried under vacuum for 24 h to remove residual solvent, rehydrated in Phosphate-Buffered Saline (PBS) (Life Technologies, Grand Island, NY), bath sonicated, and passed through a 100 nm filter (minimum of 21 passes). MSNP cores (25 mg/ml) were mixed with an excess of liposomes (1:4 mass ratio of MSNP:liposomes) for 30–90 min at room temperature. To remove excess liposomes, protocells were washed twice by centrifugation (15,000×*g* for 10 min) and re-suspended in PBS. CTB was conjugated to the protocells using a NeutrAvidin/biotin conjugation strategy (Nobs et al., 2004a,c). Briefly, CTB-biotin (Life Technologies, Grand Island, NY) (50 μg in PBS) was added to NeutrAvidin modified protocells (1 mg in PBS) and incubated at room temperature for 1 h. The non-conjugated CTB-biotin was removed by centrifugation (15,000×*g* for 10 min) and protocells were re-suspended in PBS. In order to estimate the number of CTB molecules on the surface of an individual protocell, we adapted equations and values previously derived (Durfee et al., 2016). Briefly, the surface area of MSNP supported lipid bilayers was calculated as a hexagonal prism with dimensions determined from EM images and was used to determine the molar ratio of DSPE-PEG-Amine (10%) per protocell as this is the moiety that is functionalized by binding to neutravidin. Complete saturation of DSPE-PEG-Amine with neutravidin was assumed on the protocell surface, with all four neutravidin binding sites conjugated by CTB-biotin based on the absence of steric hindrance.

2.1.3. YO-PRO-1 cargo loading

To study loading and release of a small molecule drug model, YO-PRO-1 (Thermo Scientific, Waltham, MA) was chosen since it can be detected by fluorescence and is membrane impermeable. YO-PRO-1 loading was achieved by soaking MSNP cores (1 mg/ml in water) in 1% volume YO-PRO-1 (1 mM in DMSO) for 12 h at 4 °C. Protocells were assembled following the supported lipid bilayer formation and CTB conjugation method described above. YO-PRO-1 loading was quantified by bath sonicating the protocells in DMSO, followed by centrifugation (repeated twice). Supernatants were saved and pooled; the concentration was determined using a microplate reader fluorescence measurement at 480/510 nm.