Published in final edited form as:

Int J Pharm. 2018 September 05; 548(1): 698–706. doi:10.1016/j.ijpharm.2018.07.043.

Nanoparticle formulations that allow for sustained delivery and brain targeting of the neuropeptide oxytocin

Rokon Uz Zaman^a, Nihal S Mulla^b, Keegan Braz Gomes^a, Cherilyn D'Souza^a, Kevin Sean Murnane^a, and Martin J D'Souza^a

^aDepartment of Pharmaceutical Sciences, Mercer University College of Pharmacy, Mercer University Health Sciences Center, Atlanta, GA USA

^bPharmaceutical Sciences Department, Drake University, Des Moines, IA 50311 USA

Abstract

Oxytocin is a promising candidate for the treatment of social-deficit disorders such as Autism Spectrum Disorder, but oxytocin cannot readily pass the blood-brain barrier. Moreover, oxytocin requires frequent dosing as it is rapidly metabolized in blood. We fabricated four polymeric nanoparticle formulations using poly(lactic-co-glycolic acid) (PLGA) or bovine serum albumin (BSA) as the base material. In order to target them to the brain, we then conjugated the materials to either transferrin or rabies virus glycoprotein (RVG) as targeting ligands. The formulations were characterized *in vitro* for size, zeta potential, encapsulation efficiency, and release profiles. All formulations showed slightly negative charges and sizes ranging from 100 to 278 nm in diameter, with RVG-conjugated BSA nanoparticles exhibiting the smallest sizes. No formulation was found to be immunogenic or cytotoxic. The encapsulation efficiency was 75% for all nanoparticle formulations. Release studies demonstrated that BSA nanoparticle formulation exhibited a faster initial burst of release compared to PLGA particles, in addition to later sustained release. This initial burst release would be favorable for clinical dosing as therapeutic effects could be quickly established, especially in combination with additional sustained release to maintain the therapeutic effects. Our size and release profile data indicate that RVG-conjugated BSA nanoparticles are the most favorable formulation for brain delivery of oxytocin.

Keywords

Autism; Blood-brain barrier (BBB); Brain delivery; Oxytocin; Nanoparticle

Corresponding author: Martin D'Souza, Ph.D., Professor, Department of Pharmaceutical Sciences, Mercer University College of Pharmacy, Mercer University Health Sciences Center, 3001 Mercer University Dr., Atlanta, GA 30341, Phone: (678)547-6353, Fax: (678)547-6423, dsouza_mj@mercer.edu.

Disclosure

The authors report no conflicts of interest in this work. The authors alone are responsible for the content and writing of this article.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction:

Developing new treatments for central nervous system (CNS) diseases is a pressing public health concern [1–5]. To treat neurological diseases in the brain, one route of treatment is delivering beneficial therapies, such as neuropeptides, across the blood-brain barrier (BBB) and into the brain. Neuropeptides are small protein-like molecules that are commonly co-released with neurotransmitters to modulate neurotransmission. Neuropeptides vary in their size, the neuroanatomical distribution of their neurons of origin and their partnered neurotransmitters for co-release. They have shown particular promise as potential therapeutics for CNS disorders as observed in animal studies that demonstrate profound efficacy in treating a number of disease states. Although there are many neuropeptides with potential therapeutic benefits, one of particular interest is oxytocin (OT). OT is a nine-amino acid neuropeptide that is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. It is released into peripheral circulation by the posterior pituitary gland and also functions as a neurotransmitter throughout much of the mammalian brain [6]. Although traditionally recognized as being involved in uterine stimulation during parturition, OT's role as a brain neurotransmitter in the OT system has been linked to many social functions such as behavior, recognition, comfort, and altruism. [7–9]. This has led to considerable interest in the therapeutic use of OT for CNS disorders with social deficit components, including autism and Dravet syndrome.

Unfortunately, a fundamental obstacle for the development of OT as a CNS medication is that it 1) does not readily passively cross the BBB following exogenous administration and 2) its half-life in the blood is short, on the order of minutes. The BBB limits large or hydrophilic molecules from readily entering the brain. This limits the range of compounds and scaffolds available to medicinal chemistry programs in the development of new chemical entities for treating CNS disorders. One method to overcome the resistance of the BBB is surgical cannulation of the brain ventricles for widespread or region-specific brain delivery of large molecule compounds to treat CNS disorders in animal models. Some of these compounds include neurotrophins such as brain-derived neurotrophic factor (BDNF) [10, 11], secretase inhibitors for Alzheimer's disease [12], anti-inflammatory cytokines [13, 14], and therapeutic plasmid DNA for neuronal stimulation or myelin recovery and remodeling [15–17]. However, surgical cannulation is an invasive procedure to deliver these molecules. Alternatively, researchers have tried to utilize the mechanisms present in the BBB to deliver compounds to the brain without physical or surgical disruption of the barrier.

An attractive strategy for targeted delivery of drugs into the brain is endogenous active transport systems such as receptor-mediated transport (RMT) [18]. RMT is a class of transport system that transports macromolecules such as insulin and transferrin between the blood and the brain. Transferrin (Tf) has been widely studied and shown to be a promising molecular probe for targeted drug delivery to the brain through RMT as transferrin receptors are known to be expressed in the luminal membrane of capillary endothelium of the BBB [19–21]. Tf is a single chain 80kDa protein that facilitates the movement of iron between the blood and brain. Tf-bound iron from blood binds to the Tf receptor (TfR) expressed on the BBB. The TfR is expressed mostly on endothelial cells, intestinal cells, hepatocytes, and monocytes. Tf-conjugated drug delivery systems (nanoparticles, liposomes and micelles) can

improve drug transport across the BBB since Tf-receptors (TfR) are overexpressed in brain capillary endothelium [22–27]. Previous studies have extensively examined the Transferrin (Tf) system as a mean of targeting drug formulations to the brain [23, 24, 28, 29]. Tf can be adsorbed on or conjugated to different polymers including PLGA, polyethyleneglycol, lipopolyplexes, polymer-based cyclodextrin, and gold nanoparticles [30–33]. Building enthusiasm for RMT, recent studies have also described a new brain targeting ligand called Rabies Virus Glycoprotein (RVG), which putatively binds to nicotinic cholinergic receptors on the BBB to engage brain uptake of drug formulations [34]. Both Tf and RVG have been used previously to target therapeutic peptides to the brain, and are as such likely to be effective in the transport of neuropeptides into the brain. However, to the best of our knowledge, no previous studies have examined these ligands in the brain targeting of neuropeptides using nanoparticles.

Nanotechnology such as nanoparticles can be used to encapsulate our target neuropeptide and can be coated with Tf or RVG to utilize receptor-mediated transport, more specifically transcytosis to move the therapeutic across the BBB. Previous studies have used a variety of approaches for sustained delivery and brain targeting of drug formulations. Gan et al. developed a nanoparticulate system with transferrin conjugated on its surface as a molecular probe to deliver clinical Taxotere across the BBB; furthermore, they showed that transferrin-conjugated nanoparticles were more successful than unconjugated nanoparticles at reaching the brain. [19]. Molecules can be encapsulated within different polymers including PLGA, BSA, polyethyleneglycol, lipopolyplexes, polymer-based cyclodextrin, and gold nanoparticles [30–33]. Additionally, since size plays a role in brain penetrance, smaller nanoparticles (under 200 nm) can potentially increase the penetrance of our formulation through the BBB and into the brain. We focused our efforts on using PLGA and BSA for our nanoparticle polymers because they are biocompatible, biodegradable, and are approved by the US Food and Drug Administration (FDA) [35–40]. We also have experience and expertise working with these polymers. For example, we have previously used PLGA to develop nanoparticulate dosage forms to deliver the anti-cancer drug camptothecin to B16 cells *in vitro* [41]. Additionally, our previous studies in non-human primates have shown low levels of toxicity using BSA as the polymer for particulate drug delivery [40]. Furthermore, both PLGA and BSA nanoparticles have sustained release properties which can increase half-life of OT thereby decreasing the frequency of administration and increasing patient compliance. Our goal was to optimize a formulation that provides an initial burst release of the peptide to induce rapid symptom control followed by a sustained release for prolonged therapeutic effects.

In the present study, we designed two polymeric-based nanoparticle formulations in order to increase the brain penetrance and extend the duration of action of OT. After the optimization of the formulation, we determined the physical, physiochemical, and release properties of the formulations as well as their potential for undesirable immunogenic reactions and cytotoxicity. Also, we compared formulations composed of PLGA and BSA particles conjugated with Tf or RVG in order to select a formulation based on our criteria for a desired small particle size and optimal release profile. Of the four final formulations, we found that our OT-loaded BSA nanoparticles conjugated to RVG produced the smallest-sized particles and maintained our desired release profile of OT.

2. Materials and methods:

2.1. Materials

PLGA lactide:glycolide (50:50), polyvinyl alcohol, dichloromethane, indocyanine green, transferrin (Tf), and benzalkonium chloride were purchased from Sigma Aldrich (Milwaukee, WI, USA). Bovine serum albumin (Fraction V), glutaraldehyde (25% in water) and acetone were purchased from Fischer Scientific (Pittsburgh, PA, USA). Rabies Virus Glycoprotein (RVG) was purchased from GenScript (Piscataway, NJ, USA). All the chemicals were of analytical grade and used as is without further modification.

2.2. Experimental design

The size of the nanoparticles affects the ability of the nanoparticles to cross the BBB [42]. Given the importance of nanoparticle size in brain targeting, particle size of the nanoparticles were monitored to ensure uniform size distribution of the formulation [43]. Various formulation and process parameters including polymer concentration, ratio of antisolvent and solvent and pH of solvent can influence the size of the nanoparticles [44]. We performed a full factorial design to optimize the parameters for the BSA nanoparticle formulation such as pH of the aqueous solution, polymer concentration, and antisolvent (acetone): solvent (water) ratio. All three variables were evaluated at three different levels using a full factorial design of experiments. Results of the experiments were analyzed using JMP® (SAS) software as previously demonstrated by our group [45–48].

2.3. Preparation of nanoparticles

PLGA nanoparticles were prepared using a multiple emulsion solvent evaporation and lyophilization method. Briefly, 100 mg of PLGA and 20 µl of sorbitan monooleate were dissolved in 5 ml dichloromethane. 10 mg of OT was dissolved in 1 ml of deionized water. The water/oil emulsion was prepared by homogenization at 35,000 rpm for 1 minute and also followed by probe sonication for 5 minutes with a 30 second pause after each minute. This primary emulsion was added to 10 ml of a 1% w/v polyvinyl alcohol solution and homogenized using the same method as described for primary emulsion in order to obtain the final water/oil/water (w/o/w) emulsion. The emulsion was kept under stirring conditions for 6 hours to remove dichloromethane and the formulation was lyophilized and the dried particles were stored in glass vials at –20°C for future use.

BSA nanoparticles were prepared by a coacervation/ nanoprecipitation method followed by lyophilization. Briefly, 270 mg of BSA and 27 mg of OT were dissolved in 10 ml of 10mM NaCl at pH 9.3. Acetone was added drop wise at a speed of 1ml/minute to the aqueous solution at a ratio of 2:1 with stirring at 800 rpm. After desolvation, the nanoparticle suspension was crosslinked for 30 minutes with 25% (w/v) glutaraldehyde in water (200 µL of glutaraldehyde for 1000 mg of BSA). Unused glutaraldehyde was neutralized with sodium bisulphite. Trehalose (2% w/v) was added as a cryoprotectant and the nanoparticle suspension was lyophilized as above.

2.4. Tf and RVG conjugation to peptide-loaded nanoparticles

Previous studies have used Tf and RVG as brain targeting ligands [15, 19, 49] using other formulation technologies and to carry therapeutics other than neuropeptides into the brain. We attempted to adopt these approaches for nanoparticle formulations of neuropeptides. Tf and RVG were conjugated to PLGA and BSA nanoparticles in two steps. The first step required activation of the carboxylic acid terminal groups using 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) and NHS (98%) followed by conjugating Tf or RVG in the nanoparticles. Briefly, 1 ml (1 mg/ml) of EDC and NHS (1ml, 1 mg/ml) was added to 10ml of nanoparticle suspension containing 40 mg of OT-loaded PLGA or BSA nanoparticles. Carboxylic acid groups at the periphery were converted to amine-reactive esters by stirring the PLGA with EDC and sulfo- NHS reaction mixture at room temperature for 4 h. The activated NPs were then dispersed in 1 ml of PBS, and Tf (1ml, 1 mg/ml) or RVG (1ml, 1 mg/ml) was added drop-wise to the mixture. The mixture was stirred at room temperature for 2 h and incubated at 4°C overnight or 12 hrs. Finally, the samples were washed and lyophilized using a Labconco freeze dryer (Labconco corporation, Missouri, USA). The four lead formulations were prepared as listed in Table 1.

2.5. Particle size, zeta potential and surface morphology

To determine the size and zeta potential of our nanoparticle formulations, a 50 µL aliquot of the nanoparticle suspension was diluted with 1.5 mL distilled water. Malvern Zetasizer (Malvern Instruments Inc, Massachusetts, USA) was used to measure the particle size and zeta potential of the nanoparticles utilizing the principle of dynamic light scattering. The results reported are average values from triplicate runs of three independent experiments for each sample. To visualize surface morphology, microparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum coated with a thin layer (100–150Å) of gold, the nanoparticles were observed under 20kV using a scanning electron microscope (Phenom World Pure Scanning electron microscope, AZ, USA) to examine the morphology of the nanoparticles.

2.6. Peptide encapsulation efficiency

To determine the encapsulation efficiency, a known amount of each nanoparticle suspension was centrifuged at a speed of 35,000 rpm. The supernatant was obtained and the amount of peptide in the supernatant was determined by an OT-specific enzyme linked immunosorbent assay (ELISA) (Oxytocin ELISA kit, Catalogue no: ADI-901–153A-0001, Lot no: 12041404E, Enzo life Sciences, NY 11735). This amount was subtracted from the total amount of peptide used for the formulation to determine the peptide encapsulation efficiency inside the nanoparticles.

2.7. Fourier Transform Infrared spectroscopy (FT-IR) to determine conjugation efficiency

The adsorption of Tf and RVG on the surface of PLGA and BSA nanoparticles was investigated using FT-IR spectroscopy in order to determine the conjugation efficiency. The FT-IR spectra were recorded using IRAffinity –1S Spectrometer (Shimadzu Corporation, Japan). FTIR spectra of transferrin and RVG coated PLGA and BSA nanoparticles were

obtained in the range of 4000 to 600 cm^{-1} . Spectral output was recorded by the transmittance as a function of wave number.

2.8. Immunogenicity

The potential of the formulations to induce an immunogenic reaction was assessed by measuring the amount of nitrite released by dendritic cells (DC) in presence of nanoparticulate formulations. Murine dendritic cells (DC 2.4, Millipore Sigma) were grown and maintained in DMEM with glucose, L-glutamine, and 10% FBS. DC cells were passaged at 70% confluence. For the assay, cells were plated in a 96 well-plate at 15,000 cells/well. The treatments consisted of untreated cells (negative control), the 4T1 immunogenic breast cancer vaccine (positive control), blank particles, and peptide particles. The cells were pulsed with 300 μg blank nanoparticles (1 mg/ml stock suspension) or peptide nanoparticles (1 mg/ml stock suspension) using incomplete DMEM in a 96-well plate for 16 hrs (n=3). After 16 hours, the supernatant was collected and analyzed for nitric oxide concentration using the Greiss chemical method [50]. The Greiss reagent was prepared by mixing equal volumes of 1% sulfanilamide and 0.1 % N-(1-naphthylethyldiamine) solutions. 100 μL of the supernatant was transferred to a 96 well plate, to which 100 μL of Greiss reagent was added. The plate was incubated for 10 minutes and read at 540 nm to assess the color change using a microplate reader (Synergy H1; BIO-TEK Instruments, Winooski, VT). The concentration of nitrite was calculated for each well of cells using the standard curve of NaNO_2 (1mM stock concentration in distilled water further diluted to the highest standard at 100 μM followed by serial dilutions to 1.56 μM).

2.9. Cytotoxicity

The cytotoxicity of blank and peptide loaded nanoparticles was assessed by the MTT (3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell suspension equivalent to 10,000 cells/well was plated in a 96-well cell culture plate, and complete DMEM medium was added to obtain a volume of 100 μL per well. Various concentrations of the nanoparticles ranging from 0.5 $\mu\text{g}/\text{mL}$ to 1000 $\mu\text{g}/\text{mL}$ were added to the plates in triplicates and incubated for specified time points (24 and 48 hours). Cells alone (untreated) and benzalkonium chloride were used as negative and positive controls, respectively. 10 μL of MTT Reagent was added to each well, including controls followed by incubation at 37°C for 2 to 4 hours. When the purple precipitate was clearly visible, 100 μL of detergent reagent was added to all wells, including positive and negative controls. Absorbance in each well was measured at 570 nm in a microtiter plate reader. The formation of a colored product quantified by measuring absorbance can be correlated to the percentage of living cells.

2.10. In vitro release

The release of OT from nanoparticles was studied in phosphate buffered saline (PBS, pH 7.4) and in 10% mice serum collected from Swiss-Webster mice by submandibular bleeding [51]. Briefly, 10 mg of nanoparticles were suspended in 1 mL of PBS or 10% mice serum and kept for continuous shaking (100rpm). Two different temperatures were used, 4°C and 37°C. At each time point, the tube was centrifuged at 35,000 rpm for 15 min, a sample of the supernatant was removed, after which the removed sample volume was replenished with

media. The supernatant ($n = 3$) was analyzed for the amount of peptide (OT) released using an OT ELISA as mentioned earlier.

3. Results:

3.1. Particle size, zeta potential and surface morphology

In general, the smaller the size of the nanoparticles, the more amenable they are for active transport across the BBB [42, 52–54]. The mean diameter of PLGA nanoparticles measured by Malvern zeta-sizer ranged between 197.7 and 278.3 nm. PLGA-based particles were negatively charged ranging from -11.9 to -19.6 mV. The mean diameter of the BSA nanoparticles ranged from 100.1 to 197 nm in diameter. All the BSA nanoparticles were negatively charged ranging from -15.4 to -22.8 mV. The lowest particle size (approx. 100nm) was obtained using a BSA formulation. Size distribution of RVG-conjugated OT-loaded BSA nanoparticles showed two peaks where intensity of the main peak was 99.24%. This may happen due to aggregation of some nanoparticles. As more than 99% particles were within a very narrow distribution range, it can be said that very few particles had aggregated. For Tf-conjugated OT-loaded BSA nanoparticles, the intensity of the main peak was 92.6%. For RVG- and TF-conjugated OT-loaded PLGA nanoparticles there was only one peak for each type of particles and the intensities of the peaks were 99.9% and 100% respectively.

Figure 1 shows representative data from the Zetasizer as well as the scanning electron microscope. As can be seen, the particle formulations exhibited an even spherical shape. Table 2 summarizes the sizes and zeta potentials of the various formulations that were assessed.

3.2. Optimization of formulation by full factorial design

Parameters that affects the particle size are rank ordered by analysis of the formulation design with JMP® (SAS) software. Significant parameters ($p < 0.05$) affecting particle size (in the order of importance) are interaction of BSA concentration and acetone: water ratio ($p < 0.0005$) pH of the aqueous solution ($p < 0.0006$), interaction of pH and acetone: water ratio ($p < 0.0013$), acetone: water ratio ($p < 0.0053$). The optimization is listed in Table 3.

3.3. Transferrin (Tf) and Rabies Virus Glycoprotein (RVG) conjugation to peptide loaded nanoparticles

Figures 2 (A and B) shows the FT-IR spectra of Tf- and RVG-unconjugated and conjugated PLGA nanoparticles. Figure 2 (A) shows the FT-IR spectrum of PLGA nanoparticles conjugated with Tf showing the amide bands assigned to the protein. The peak of the amide I band in the spectrum of PLGA-Tf nanoparticles occurred at 1648 cm^{-1} . The bands of the characteristic functional groups of PLGA nanoparticles are present (1750 cm^{-1} for C=O stretching and $1000\text{--}1300\text{ cm}^{-1}$ for the ester C-O stretching), with small shifts when compared to the spectrum of unconjugated nanoparticles. The presence of the amide bond confirms the presence of Tf on the Tf-coated PLGA nanoparticles. Figure 2(B) shows FT-IR spectrum of RVG conjugated to PLGA nanoparticles containing characteristic peaks of both PLGA and RVG.

Figure 2 (C and D) shows the FT-IR spectra of RVG and Tf conjugated BSA nanoparticles. Both Tf s and RVG's spectra show two main peaks that correlate with amide I and amide II vibrations at 1640 cm⁻¹ and 1535 cm⁻¹ respectively. The same two peaks are visible for BSA as well. Both RVG- and Tf-conjugated BSA nanoparticles shows intensified peaks for amide I and amide II vibrations indicating conjugation of RVG and Tf to BSA.

3.4. Encapsulation efficiency

The encapsulation efficiency was determined before the conjugation of the nanoparticles to Tf and RVG. The amount of peptide in the supernatant was subtracted from the total amount of peptide used for the formulation to determine the peptide encapsulation efficiency inside the nanoparticles. The oxytocin peptide content of the PLGA nanoparticles was $81 \pm 2.5\%$ (w/w) and for BSA nanoparticles it was $75 \pm 2.5\%$ (w/w).

3.5. Immunogenicity determination

Nitric oxide release is an important indicator of innate immune response [55, 56]. This can be used to determine the potential of nanoparticle formulations to induce immunogenic reactions. Figure 3 shows the effects of each formulation of oxytocin on nitric oxide release. Neither the blank nor the OT-loaded nanoparticles induced significant nitric oxide release demonstrating desirable non-immunogenicity. The level of nitric oxide release was compared to a positive control, 4T1 murine tumor antigen loaded microparticles which result in a large nitric oxide release and high immunogenicity.

3.6. Cell cytotoxicity

The cytotoxic effects of the four lead nanoparticle formulations (**as mentioned in Table 1**) at given concentrations are depicted in Figure 4. None of the formulations were cytotoxic within the tested concentration range (0.0625–1 mg/mL) after 48 hours. The percent cell viability is expressed relative to negative controls of cells treated with DMEM medium only. Benzalkonium chloride, used as the positive control was cytotoxic, as demonstrated by 21.44% and 20.089% cell viability after 24 and 48 hours, respectively.

3.7. In vitro release

The release of OT from PLGA nanoparticles in PBS was sustained over a period of 21 days (Figure 5). OT was released from PLGA nanoparticles in a sustained manner and 50% of peptide was released over a period of 10.8 days (Figure 5). The release of OT from BSA nanoparticles in PBS was sustained over a period of 18 days with 50% of OT released over a period of 9.8 days (Figure 5). In 10% serum at pH 7.4 OT is released from BSA nanoparticles in sustained fashion, with initial burst release of around 20% and 50% of OT released over a period of 3 days at both 4°C and 37°C. In order to get a clearer view of the burst release, we conducted a release study for 6 hours with sampling interval of 30 minutes. At the end of 6 hours 37.07% and 32.8% OT was released from BSA nanoparticles at 4°C and 37°C temperature, respectively (Figure 6). The release of OT from PLGA nanoparticles was very slow with only 5% release at the end of 6 hours at both 4°C and 37°C (Figure 6). These data show that BSA nanoparticles have a faster initial release profile than PLGA particles, and that temperature does not have a significant effect on the release profile over

the first 6 hours. Our goal was to find a release profile that provides an initial burst release of the peptide to induce rapid symptom control followed by a sustained release for prolonged therapeutic effects. Figure 7 demonstrates that the RVG-conjugated BSA nanoparticles have a mixed burst release / sustained release profile, with approximately 35% release within the first 6 hours, but 50% release not occurring until approximately 3 days in solution. In figures 5, 6 and 7, all points represent the mean $\pm$ SD, and any points without error bars indicate instances in which the SD is encompassed by the data point.

4. Discussion:

The purpose of this study was to extensively characterize several nanoparticle-based formulations of OT that can potentially cross the BBB. Our goal was to find a release profile that provides an initial burst release of the peptide to induce rapid symptom control followed by a sustained release for prolonged therapeutic effects. We focused our efforts on using PLGA and BSA for our nanoparticle polymers. This is because they are biocompatible, biodegradable and approved by the US Food and Drug Administration (FDA) [35]. Also, we have experience and expertise working with these polymers. We focused our efforts on conjugating our particles to Tf and RVG as these are documented brain targeting ligands, which provided us with four lead formulations: Tf-PLGA, Tf-BSA, RVG-PLGA, and RVG-BSA. We formulated and extensively characterized these four formulations. We used two criteria to decide upon an optimal formulation. First, we sought the smallest possible formulation, and used a minimum criteria of a size under 200 nm. Second, we sought a release profile that provides an initial burst release of the peptide followed by a sustained release, as we believe that this is the optimal profile for therapeutic effects, as well as the optimal profile for *in vivo* testing. On both accounts, our data shows that BSA particles conjugated to RVG offer optimal properties.

Receptor-mediated transport (RMT) is an appealing strategy for delivering drugs to the brain [18]. Transferrin and rabies virus glycoprotein (RVG) has been used in previous studies as brain targeting ligands. Transferrin receptors are known to be expressed in the luminal membrane of capillary endothelium of the BBB [19–21]. RVG is a short peptide that can bind to nicotinic cholinergic receptors on the BBB and it has been employed as a targeting ligand to deliver small interfering RNA (siRNA) to the brain [57, 58]. Another study used RVG tagged PEGylated cyclodextrin (CD)-based nanoparticle to deliver siRNA to the brain [59]. Although BBB represents one of the most difficult biological barriers to cross in order to deliver therapeutic agents/ drugs/ peptides for treatment of neurological diseases [29, 60, 61], these promising studies have created enthusiasm that feasible approaches are under development. If a reliable system for active transport into the brain could be developed, a broad range of compounds that have poor passive brain bioavailability would be allowed to readily penetrate the brain. This innovation would be a paradigm shift for CNS drug delivery. There are hundreds to thousands of compounds with potentially therapeutic use for CNS disorders that have been abandoned because of poor brain bioavailability. Some of these compounds include neurotrophins such as BDNF [10, 11], secretase inhibitors for Alzheimer's disease [12], antiinflammatory cytokines [13, 14], and therapeutic plasmid DNA for neuronal stimulation or myelin recovery and remodeling [15–17]. Neuropeptides

such as neuropeptide Y, BDNF, orexin, and others show profound therapeutic promise in defined disease states such as epilepsy, substance-use disorders, and obesity.

The size, shape, and charge of our particles are highly critical for delivering drugs across the BBB [62]. Nanoparticles smaller than 500nm are usually taken via the clathrin-mediated transport system whereas larger particles are usually transported via macropinocytosis [63]. In reference to charge, negatively-charged nanoparticles are endocytosed by interacting with the positively charged protein sites that flanks the membrane [63–65]. Previous studies report that negatively charged nanoparticles are highly endocytosed by cells due to their repulsive interactions with the negatively charged cell surface [63, 65], and positively charged particles show substantially reduced BBB penetrance. Our nanoparticle formulations exhibit a smooth, regular and spherical shape, which helps maximize uptake, as spherical particles are efficiently taken up via both clathrin and caveolae mediated transport [66, 67]. Particles below 200 nm are desirable to maximize the probability of efficient brain uptake. In initial studies, we have generated PLGA particles conjugated to Tf using homogenization. However, the particles size exceeded 200 nm with this approach. We modified the production method to sonication and were able to readily achieve particles sizes below 200 nm. Such sizes were also readily achievable with BSA nanoparticles. However, it is important to note the BSA particles conjugated to RVG clearly achieved the smallest particles sizes.

In order to demonstrate the therapeutic viability of our nanoparticulate formulations, we have completed both immunogenicity and cytotoxicity studies in vitro. As confirmed in our past research, our nanoparticle formulations have shown to be both safe and non-toxic in non-human primates. In primates, all parameters related to liver toxicity (such as SGOT, SGPT) and kidney toxicity (such as serum creatinine, serum bilirubin) were normal after repeated nanoparticulate administrations when compared to the control group. Also, no antibody titers in response to our polymeric particle matrix were detected demonstrating the absence of an immune response to the polymer matrix formulation [40]. For confirmation of a potential innate inflammatory response, nitric oxide assays are an important tool. We have demonstrated that there is no significant difference in the amount of nitric oxide released in the supernatant of cells exposed to peptideloaded nanoparticles compared to blank nanoparticles or the cells alone. This indicates minimal if not negligible immunogenicity of our formulation. For reference, nitric oxide release in response to our OT-loaded particles was substantially lower compared to cells that were exposed to a well-known highly-immunogenic breast cancer vaccine formulation. Likewise, cytotoxicity of blank nanoparticles was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay in a macrophage (RAW 246.7) cell line. We found no significant cell death following 48 hours of exposure to our formulations indicating that our formulation was not significantly toxic to the cells.

A major advantage of a nanoparticle formulation for peptide delivery is that the encapsulation protects the peptide from degradation, and sustains the delivery of the therapeutic agent over an extended period of time [68]. This sustained delivery allows for fewer administrations and easier compliance, an issue of particular importance in the treatment of chronic substance abuse disorders or in patients that have difficulty with

compliance. Resultantly, the OT can provide long-lasting therapeutic effects in the brain, improving the health of those suffering with various cognitive ailments. We have demonstrated that our formulations maintain a sustained release profile of OT *in vitro* in both PBS and plasma, which was confirmed using ELISA (Figure 5 and 7). During our release studies, we took samples in 30-minute interval initially, then hourly, and then daily to establish a release profile covering several weeks. In order to select the most viable formulation to test *in vivo*, we explored the two factors discussed prior 1) size and 2) suitable sustained release criteria. We found that the RVG-conjugated BSA nanoparticles were smaller than our PLGA particles or Tf-conjugated BSA particles. The rank order of size of our nanoparticulate formulations was as follows: RVG-conjugated BSA (100.1 nm), Tf-conjugated BSA (196.3 nm), Tf-conjugated PLGA (191.7 nm), RVG-conjugated PLGA (201.2 nm). For the purpose of our treatment with OT, we desired an initial burst release followed by a sustained for several days. The OT release in 6 hours at 37°C serum was as follows: BSA nanoparticles (32.8% release), and PLGA nanoparticles (5% release). Hence, we determined that our PLGA nanoparticle formulations would not be viable candidates since they released OT very slowly compared to BSA particles thereby reducing the therapeutic window for OT in the brain. Therefore, when selecting the final formulation to test *in vivo*, we determined that our BSA nanoparticles conjugated to RVG formulation was the most suitable candidate. Although the two BSA-based formulations demonstrated desirable release profiles, the RVG-conjugated BSA nanoparticles were almost half the size of Tf-conjugated BSA nanoparticles. Hence when taking into account size and the OT-release profile, we concluded that RVG-conjugated BSA nanoparticles would be the most appropriate formulation to move forward with in our *in vivo* murine models.

5. Conclusion:

In this study, biodegradable Tf or RVG-conjugated PLGA and BSA nanoparticles loaded with oxytocin were successfully prepared by solvent evaporation and nanoprecipitation/coacervation methods respectively. Our formulations were also characterized using various methods such as zeta sizing and scanning electron microscopy. Significant parameters affecting particle size were ranked and optimized to produce the final desirable formulations for future proposed *in vivo* testing. We found that our nanoparticles were uniformly distributed in nm range, spherically shaped, and individually dispersed. We also found that the nanoparticles formulations were non- immunogenic and non-toxic. All four formulations showed sustained the delivery of the therapeutic agent allowing for fewer administrations and easier compliance. Therefore, our nanoparticulate formulation could serve as a potential candidate for delivering drugs and therapeutic peptides across the BBB non-invasively. Among the four formulations, the OT-loaded BSA nanoparticles conjugated to RVG formulation produced particles that were the smallest in size and demonstrated a desirable release profile, advocating it as a very strong candidate for drug delivery to the brain. We further propose testing our selected nanoparticle formulation *in vivo* in well-characterized murine disease models.

Acknowledgements

The authors acknowledge the use of facilities of Vaccine Nanotechnology Laboratory, Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Mercer University, Atlanta, GA. The results of the study will be a part of Rokan Uz Zaman's dissertation.

Funding

These studies were supported by the National Institutes of Health [NS100512 (KSM and MJD)] and by funding from the Mercer University College of Pharmacy.

References:

- [1]. Misra A , Ganesh S , Shahiwala A , Shah SP . Drug delivery to the central nervous system: a review. *Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques*. 2003;6:252–73.
- [2]. Broderick DF , Schweitzer KJ , Wszolek ZK . Vascular risk factors and dementia: how to move forward? *Neurology*. 2009;73:1934–5. [PubMed: 19949045]
- [3]. Herrmann N , Chau SA , Kircanski I , Lanctot KL . Current and emerging drug treatment options for Alzheimer's disease: a systematic review. *Drugs*. 2011;71:2031–65. [PubMed: 21985169]
- [4]. Strecker K , Schwarz J . Parkinson's disease: emerging pharmacotherapy. *Expert opinion on emerging drugs*. 2008;13:573–91. [PubMed: 19046128]
- [5]. Cai Q , Wang L , Deng G , Liu J , Chen Q , Chen Z . Systemic delivery to central nervous system by engineered PLGA nanoparticles. *American journal of translational research*. 2016;8:749–64. [PubMed: 27158367]
- [6]. Carson DS , Guastella AJ , Taylor ER , McGregor IS . A brief history of oxytocin and its role in modulating psychostimulant effects. *Journal of psychopharmacology (Oxford, England)*. 2013;27:231–47.
- [7]. Donaldson ZR , Young LJ . Oxytocin, vasopressin, and the neurogenetics of sociality. *Science (New York, NY)*. 2008;322:900–4.
- [8]. Engelmann M , Wotjak CT , Neumann I , Ludwig M , Landgraf R . Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. *Neurosci Biobehav Rev*. 1996;20:341–58. [PubMed: 8880728]
- [9]. Modi ME , Young LJ . The oxytocin system in drug discovery for autism: animal models and novel therapeutic strategies. *Horm Behav*. 2012;61:340–50. [PubMed: 22206823]
- [10]. Nagahara AH , Merrill DA , Coppola G , Tsukada S , Schroeder BE , Shaked GM , et al. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nature medicine*. 2009;15:331–7.
- [11]. Dawbarn D , Allen SJ . Neurotrophins and neurodegeneration. *Neuropathology and applied neurobiology*. 2003;29:211–30. [PubMed: 12787319]
- [12]. O'Brien RJ , Wong PC . Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci*. 2011;34:185–204. [PubMed: 21456963]
- [13]. Opal SM , DePalo VA . Anti-inflammatory cytokines. *Chest*. 2000;117:1162–72. [PubMed: 10767254]
- [14]. Walker D , Lue LF . Anti-inflammatory and immune therapy for Alzheimer's disease: current status and future directions. *Current neuropharmacology*. 2007;5:232–43. [PubMed: 19305740]
- [15]. Blesch A , Tuszynski MH . Transient growth factor delivery sustains regenerated axons after spinal cord injury. *J Neurosci*. 2007;27:10535–45. [PubMed: 17898225]
- [16]. Sloane E , Ledebor A , Seibert W , Coats B , van Strien M , Maier SF , et al. Anti-inflammatory cytokine gene therapy decreases sensory and motor dysfunction in experimental Multiple Sclerosis: MOG-EAE behavioral and anatomical symptom treatment with cytokine gene therapy. *Brain, behavior, and immunity*. 2009;23:92–100.
- [17]. Soderquist RG , Mahoney MJ . Central nervous system delivery of large molecules: challenges and new frontiers for intrathecally administered therapeutics. *Expert opinion on drug delivery*. 2010;7:285–93. [PubMed: 20201735]

- [18]. Pulicherla KK , Verma MK . Targeting therapeutics across the blood brain barrier (BBB), prerequisite towards thrombolytic therapy for cerebrovascular disorders-an overview and advancements. *AAPS PharmSciTech*. 2015;16:223–33. [PubMed: 25613561]
- [19]. Gan CW , Feng SS . Transferrin-conjugated nanoparticles of poly(lactide)-D-alpha-tocopheryl polyethylene glycol succinate diblock copolymer for targeted drug delivery across the blood-brain barrier. *Biomaterials*. 2010;31:7748–57. [PubMed: 20673685]
- [20]. Pardridge WM . Blood-brain barrier delivery. *Drug discovery today*. 2007;12:54–61. [PubMed: 17198973]
- [21]. Gatter KC , Brown G , Trowbridge IS , Woolston RE , Mason DY . Transferrin receptors in human tissues: their distribution and possible clinical relevance. *Journal of clinical pathology*. 1983;36:539–45. [PubMed: 6302135]
- [22]. Pang Z , Gao H , Yu Y , Guo L , Chen J , Pan S , et al. Enhanced intracellular delivery and chemotherapy for glioma rats by transferrin-conjugated biodegradable polymersomes loaded with doxorubicin. *Bioconjugate chemistry*. 2011;22:1171–80. [PubMed: 21528923]
- [23]. Qian ZM , Li H , Sun H , Ho K . Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacological reviews*. 2002;54:561–87. [PubMed: 12429868]
- [24]. Ulbrich K , Hekmatara T , Herbert E , Kreuter J . Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood-brain barrier (BBB). *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV*. 2009;71:251–6.
- [25]. Wiley DT , Webster P , Gale A , Davis ME . Transcytosis and brain uptake of transferrin-containing nanoparticles by tuning avidity to transferrin receptor. *Proc Natl Acad Sci U S A*. 2013;110:8662–7. [PubMed: 23650374]
- [26]. Yan F , Wang Y , He S , Ku S , Gu W , Ye L . Transferrin-conjugated, fluorescein-loaded magnetic nanoparticles for targeted delivery across the blood-brain barrier. *Journal of materials science Materials in medicine*. 2013;24:2371–9. [PubMed: 23793566]
- [27]. Ying X , Wen H , Lu WL , Du J , Guo J , Tian W , et al. Dual-targeting daunorubicin liposomes improve the therapeutic efficacy of brain glioma in animals. *Journal of controlled release : official journal of the Controlled Release Society*. 2010;141:183–92. [PubMed: 19799948]
- [28]. Broadwell RD , Baker-Cairns BJ , Friden PM , Oliver C , Villegas JC . Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Experimental neurology*. 1996;142:47–65. [PubMed: 8912898]
- [29]. Pardridge WM . Blood-brain barrier drug delivery of IgG fusion proteins with a transferrin receptor monoclonal antibody. *Expert opinion on drug delivery*. 2014:1–16.
- [30]. Li Y , Ogris M , Wagner E , Pelisek J , Ruffer M . Nanoparticles bearing polyethyleneglycol-coupled transferrin as gene carriers: preparation and in vitro evaluation. *International journal of pharmaceutics*. 2003;259:93–101. [PubMed: 12787639]
- [31]. Pan X , Guan J , Yoo JW , Epstein AJ , Lee LJ , Lee RJ . Cationic lipid-coated magnetic nanoparticles associated with transferrin for gene delivery. *International journal of pharmaceutics*. 2008;358:263–70. [PubMed: 18384982]
- [32]. Sahoo SK , Labhasetwar V . Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. *Molecular pharmaceutics*. 2005;2:373–83. [PubMed: 16196490]
- [33]. Zhang X , Koh CG , Yu B , Liu S , Piao L , Marcucci G , et al. Transferrin receptor targeted lipopolyplexes for delivery of antisense oligonucleotide g3139 in a murine k562 xenograft model. *Pharmaceutical research*. 2009;26:1516–24. [PubMed: 19291371]
- [34]. Liu Y , An S , Li J , Kuang Y , He X , Guo Y , et al. Brain-targeted co-delivery of therapeutic gene and peptide by multifunctional nanoparticles in Alzheimer's disease mice. *Biomaterials*. 2016;80:33–45. [PubMed: 26706474]
- [35]. Makadia HK , Siegel SJ . Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers*. 2011;3:1377–97. [PubMed: 22577513]

- [36]. Jalil R , Nixon JR . Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. *Journal of microencapsulation*. 1990;7:297–325. [PubMed: 2200861]
- [37]. Jain RA . The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials*. 2000;21:2475–90. [PubMed: 11055295]
- [38]. Ma XY , Sun XC , Hargrove D , Chen J , Song DH , Dong QC , et al. A Biocompatible and Biodegradable Protein Hydrogel with Green and Red Autofluorescence: Preparation, Characterization and In Vivo Biodegradation Tracking and Modeling. *Sci Rep-Uk*. 2016;6.
- [39]. Wang ZM , Dong L , Han L , Wang KF , Lu X , Fang LM , et al. Self-assembled Biodegradable Nanoparticles and Polysaccharides as Biomimetic ECM Nanostructures for the Synergistic effect of RGD and BMP-2 on Bone Formation. *Sci Rep-Uk*. 2016;6.
- [40]. Oettinger CW , D'Souza M J , Akhavein N , Peer GT , Taylor FB , Kinasewitz GT . Pro-inflammatory cytokine inhibition in the primate using microencapsulated antisense oligomers to NF-kappaB. *J Microencapsul*. 2007;24:337–48. [PubMed: 17497387]
- [41]. Tong W , Wang L , D'Souza MJ . Evaluation of PLGA microspheres as delivery system for antitumor agent-camptothecin. *Drug development and industrial pharmacy*. 2003;29:745–56. [PubMed: 12906332]
- [42]. Saraiva C , Praca C , Ferreira R , Santos T , Ferreira L , Bernardino L . Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases. *Journal of controlled release : official journal of the Controlled Release Society*. 2016;235:34–47. [PubMed: 27208862]
- [43]. Hornig S , Bunjes H , Heinze T . Preparation and characterization of nanoparticles based on dextran- drug conjugates. *Journal of colloid and interface science*. 2009;338:56–62. [PubMed: 19635622]
- [44]. Chorny M , Fishbein I , Danenberg HD , Golomb G . Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. *Journal of controlled release : official journal of the Controlled Release Society*. 2002;83:389–400. [PubMed: 12387947]
- [45]. Kolluru LP , Rizvi SA , D'Souza M , D'Souza MJ . Formulation development of albumin based theragnostic nanoparticles as a potential delivery system for tumor targeting. *Journal of drug targeting*. 2013;21:77–86. [PubMed: 23036042]
- [46]. Chablani L , Tawde SA , D'Souza MJ . Spray-dried microparticles: a potential vehicle for oral delivery of vaccines. *Journal of microencapsulation*. 2012;29:388–97. [PubMed: 22283700]
- [47]. Shastri PN , Ubale RV , D'Souza MJ . Implementation of mixture design for formulation of albumin containing enteric-coated spray-dried microparticles. *Drug development and industrial pharmacy*. 2013;39:164–75. [PubMed: 22591196]
- [48]. Kolluru LP , Gala RP . Design of Experiments: A Valuable “Quality by Design” Tool in Formulation Development. In: D'Souza M J, editor. *Nanoparticulate Vaccine Delivery Systems*. New York Pan Stanford; 2015.
- [49]. Kim JY , Choi Wl , Kim YH , Tae G . Brain-targeted delivery of protein using chitosan- and RVG peptide- conjugated, pluronic-based nano-carrier. *Biomaterials*. 2013;34:1170–8. [PubMed: 23122677]
- [50]. Gala RP , D'Souza M , Zughaier SM . Evaluation of various adjuvant nanoparticulate formulations for meningococcal capsular polysaccharide-based vaccine. *Vaccine*. 2016;34:3260–7. [PubMed: 27177946]
- [51]. Golde WT , Gollobin P , Rodriguez LL . A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab animal*. 2005;34:39–43.
- [52]. Etame AB , Smith CA , Chan WC , Rutka JT . Design and potential application of PEGylated gold nanoparticles with size-dependent permeation through brain microvasculature. *Nanomedicine : nanotechnology, biology, and medicine*. 2011;7:992–1000.
- [53]. Hanada S , Fujioka K , Inoue Y , Kanaya F , Manome Y , Yamamoto K . Cell-based in vitro blood-brain barrier model can rapidly evaluate nanoparticles' brain permeability in association with particle size and surface modification. *International journal of molecular sciences*. 2014;15:1812–25. [PubMed: 24469316]

- [54]. Sonavane G , Tomoda K , Makino K . Biodistribution of colloidal gold nanoparticles after intravenous administration: effect of particle size. *Colloids and surfaces B, Biointerfaces*. 2008;66:274–80. [PubMed: 18722754]
- [55]. Zughaier SM , Shafer WM , Stephens DS . Antimicrobial peptides and endotoxin inhibit cytokine and nitric oxide release but amplify respiratory burst response in human and murine macrophages. *Cellular microbiology*. 2005;7:1251–62. [PubMed: 16098213]
- [56]. Bogdan C , Rollinghoff M , Diefenbach A . The role of nitric oxide in innate immunity. *Immunological reviews*. 2000;173:17–26. [PubMed: 10719664]
- [57]. Kumar P , Wu H , McBride JL , Jung KE , Kim MH , Davidson BL , et al. Transvascular delivery of small interfering RNA to the central nervous system. *Nature*. 2007;448:39–43. [PubMed: 17572664]
- [58]. Son S , Hwang DW , Singha K , Jeong JH , Park TG , Lee DS , et al. RVG peptide tethered bioreducible polyethylenimine for gene delivery to brain. *Journal of controlled release : official journal of the Controlled Release Society*. 2011;155:18–25. [PubMed: 20800628]
- [59]. Gooding M , Malhotra M , McCarthy DJ , Godinho BM , Cryan JF , Darcy R , et al. Synthesis and characterization of rabies virus glycoprotein-tagged amphiphilic cyclodextrins for siRNA delivery in human glioblastoma cells: in vitro analysis. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*. 2015;71:80–92. [PubMed: 25703259]
- [60]. Abbott NJ , Patabendige AA , Dolman DE , Yusof SR , Begley DJ . Structure and function of the blood- brain barrier. *Neurobiology of disease*. 2010;37:13–25. [PubMed: 19664713]
- [61]. Ballabh P , Braun A , Nedergaard M . The blood-brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiology of disease*. 2004;16:1–13. [PubMed: 15207256]
- [62]. Kulkarni SA , Feng SS . Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. *Pharmaceutical research*. 2013;30:2512–22. [PubMed: 23314933]
- [63]. Kou L , Sun J , Zhai Y , He Z . The endocytosis and intracellular fate of nanomedicines: Implication for rational design. . *Asian Journal of Pharmaceutical Sciences*. 2013;8:1–10.
- [64]. Lerch-Bader M , Lundin C , Kim H , Nilsson I , von Heijne G . Contribution of positively charged flanking residues to the insertion of transmembrane helices into the endoplasmic reticulum. *Proceedings of the National Academy of Sciences*. 2008;105:4127–32.
- [65]. Yeung T , Gilbert GE , Shi J , Silvius J , Kapus A , Grinstein S . Membrane phosphatidylserine regulates surface charge and protein localization. *Science*. 2008;319:210–3. [PubMed: 18187657]
- [66]. Tan J , Shah S , Thomas A , Ou-Yang HD , Liu Y . The influence of size, shape and vessel geometry on nanoparticle distribution. *Microfluidics and nanofluidics*. 2013;14:77–87. [PubMed: 23554583]
- [67]. Truong NP , Whittaker MR , Mak CW , Davis TP . The importance of nanoparticle shape in cancer drug delivery. *Expert opinion on drug delivery*. 2015;12:129–42. [PubMed: 25138827]
- [68]. Mudshinge SR , Deore AB , Patil S , Bhalgat CM . Nanoparticles: Emerging carriers for drug delivery. *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*. 2011;19:129–41. [PubMed: 23960751]

Figure 1.

Representative particle sizes and morphology of our nanoparticle formulations. (Left) Size distribution of PLGA nanoparticles conjugated to Tf or RVG, (Middle) size distribution of BSA nanoparticles conjugated to Tf or RVG, and (Right) scanning electron microscopy images of the BSA nanoparticles conjugated to Tf at a 3μm resolution. The BSA nanoparticles conjugated to RVG were too small to image at a 3μm resolution.

Tf = Transferrin; RVG = Rabies Virus Glycoprotein; PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin; OT = Oxytocin

Figure 2.

FT-IR spectrum data collected to confirm the conjugation of the brain targeting ligands to the nanoparticles. (A): FT-IR spectrum of Tf conjugated to PLGA nanoparticles. In the spectra of Tf shows two main peaks at 1648 cm^{-1} and 1550 cm^{-1} that can be assigned to the amide I and amide II vibrations, respectively. FT-IR spectrum of blank and unconjugated PLGA nanoparticles shows characteristic bands of carbonyl $\text{C}=\text{O}$ stretching (1750 cm^{-1}) and the ester $\text{C}-\text{O}$ stretching ($1000\text{--}1300\text{ cm}^{-1}$). FT-IR spectrum of PLGA nanoparticles conjugated to Tf shows the amide bands assigned to the protein. The peak of the amide I

band in the spectrum of PLGA- Tf nanoparticles occurs at 1648 cm^{-1} . The bands of the characteristic functional groups of PLGA nanoparticles are present (1750 cm^{-1} and $1000\text{--}1300\text{ cm}^{-1}$) with small shifts when compared to the spectrum of the unmodified nanoparticles. The presence of amide I band in the PLGA-Tf spectrum demonstrates that Tf has been conjugated to the PLGA nanoparticles. (B): FT-IR spectrum of RVG conjugated to PLGA nanoparticles. (C): FT-IR spectrum of Tf conjugated to BSA nanoparticles. (D): FT-IR spectrum of RVG conjugated to BSA nanoparticles. Both BSA spectra show two main peaks near 1650 cm^{-1} and 1540 cm^{-1} , which are the two peaks of the amide I and amide II vibrations of Tf and RVG, indicating conjugation of RVG and Tf to BSA. Tf = Transferrin; RVG = Rabies Virus Glycoprotein; PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin; OT = Oxytocin; FT-IR = Fourier Transform Infrared spectroscopy

Figure 3. Induction of nitric oxide release by our lead oxytocin formulations. The oxytocin formulation was generated by double emulsion solvent evaporation by sonication. All points represent the mean $\pm$ standard error. *Abcissae*: Various formulations compared to the negative control of untreated cells and the positive control of the 4T1 breast cancer vaccine that induces a robust immune response. *Ordinates*: Measured concentration of nitric oxide expressed on an absolute scale in units of micromolar. All experiments were conducted in triplicate.

Figure 4.

Cytotoxicity profile of the nanoparticle formulations. After 48 hours of exposure, none of the four formulations were cytotoxic across the tested concentration range (0.0625–1 mg/mL). As there were no differences between the concentrations tested, only the highest concentration (1 mg/mL) is shown. *Abscissae*: Various formulations compared to the negative control of untreated cells and the positive control of benzalkonium chloride (a cationic surfactant that has biocidal activity). *Ordinates*: Cell viability expressed as a percentage of the negative control at 6 hours. All experiments were conducted in triplicate.

Figure 5.

Release of OT from PLGA and BSA nanoparticles in PBS, pH 7.4. The release of OT from PLGA nanoparticles was sustained over a period of 21 days. OT was released from PLGA nanoparticles in sustained fashion with 50% of the OT released over a period of 10.8 days. The release of OT from BSA nanoparticles was sustained over a period of 18 days and 50% of OT was released over a period of 9.8 days. *Abscissae*: Day following addition of the formulation to solution. *Ordinates*: Measured concentration of free oxytocin in solution expressed as a percentage of the maximally measured release. All points represent the mean $\pm$ SD, and any points without error bars indicate instances in which the SD is encompassed by the data point. PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin; OT = Oxytocin

Figure 6.

Release OT from PLGA (prepared by sonication) and BSA nanoparticles in 10% serum, pH 7.4 over 6 hours at 4°C and 37°C. At the end of 6 hours, 37.07% and 32.8% of the OT has been released from the BSA nanoparticles at 4°C and 37°C temperature, respectively. In contrast, only 5% of the OT had been released from the PLGA nanoparticles at either temperature. *Abcissae*: Hour following addition of the formulation to solution. *Ordinates*: Measured concentration of free oxytocin in solution expressed as a percentage of the maximally measured release. All points represent the mean $\pm$ SD, and any points without error bars indicate instances in which the SD is encompassed by the data point. PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin; OT = Oxytocin;

Figure 7.

Release OT from BSA nanoparticles in 10% serum, pH 7.4 at 4°C. 50% of the OT was released over a period of 3 days. *Abcissae*: Day following addition of the formulation to solution. *Ordinates*: Measured concentration of free oxytocin in solution expressed as a percentage of the maximally measured release. All points represent the mean $\pm$ SD, and any points without error bars indicate instances in which the SD is encompassed by the data point. BSA = Bovine serum albumin; OT = Oxytocin;

Table 1:

PLGA and BSA nanoparticle formulations

Number	Method of preparation	Formulation
1	Sonication/Solvent evaporation	TF conjugated to PLGA nanoparticles
2	Sonication/Solvent evaporation	RVG conjugated to PLGA nanoparticles
3	Coacervation/nanoprecipitation	TF conjugated to BSA nanoparticles
4	Coacervation/nanoprecipitation	RVG conjugated to BSA nanoparticles

This table shows the four lead formulations developed in the present study. The abbreviations include Tf = Transferrin; RVG = Rabies Virus Glycoprotein; PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin

Table 2:

Size distribution and Zeta potentials of different PLGA and BSA nanoparticle formulations of oxytocin.

Formulation				Characteristics	
Base material	Loading material	Targeting ligand	Preparation method	Size (nm)	Zeta Potential (mV)
PLGA	Blank	Tf	Homogenization	240.00±0.72	-20.10±0.51
PLGA	OT	Tf	Homogenization	278.30±2.62	-16.40±0.26
PLGA	Blank	Tf	Sonication	205.20±0.42	-16.30±0.62
PLGA	OT	Tf	Sonication	191.70±0.71	-19.60±0.25
PLGA	Blank	RVG	Sonication	203.44±0.94	-16.50±0.71
PLGA	OT	RVG	Sonication	201.20±3.35	-17.40±0.70
BSA	Blank	Tf	Coacervation/Nanoprecipitation	240.00±0.72	-19.60±0.50
BSA	OT	Tf	Coacervation/Nanoprecipitation	240.00±0.72	-21.10±0.38
BSA	Blank	RVG	Coacervation/Nanoprecipitation	240.00±0.72	-20.50±0.59
BSA	OT	RVG	Coacervation/Nanoprecipitation	240.00±0.72	-16.00±0.30

Abbreviations include, Tf = Transferrin; RVG = Rabies Virus Glycoprotein; PLGA = poly(lactic-co-glycolic acid); BSA = Bovine serum albumin; OT = Oxytocin

Table 3:

Parameters affecting particle size

Parameters affecting particle size	Significance
Interaction of BSA concentration and acetone: water ratio	$p < 0.001$
pH of the aqueous solution	$p < 0.001$
Interaction of pH and acetone: water ratio	$p < 0.001$
Acetone: water ratio	$p < 0.005$

Parameters that significantly affect the particle size as revealed by full factorial design of formulation variables for BSA nanoparticles.