

Article

Size-Based Targeting of Anti-Inflammatory Nanoparticles for Drug Delivery to Blast-Injured BBB for TBI Treatment

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide, with blast TBI (bTBI) particularly affecting military personnel and individuals exposed to explosive environments, yet there are no available curative treatments to date. While adrenergic receptor antagonists have shown promise in reducing neuroinflammation and improving TBI mortality rates, systemic administration of these drugs can have deleterious effects including bradycardia and hypotension. Here, we introduce a polymeric nanoparticle system for the delivery of adrenergic receptor antagonists, which allows for size-based targeting of the injured blood–brain barrier (BBB). These nanoparticles consist of chitosan-coated polylactic co-glycolic acid encapsulating the β -adrenergic receptor antagonist propranolol and/or the α -adrenergic receptor antagonist phenoxybenzamine. Particles designed with a 200 nm hydrodynamic diameter showed a 20–24% increase in permeability on an in vitro contact co-culture BBB model exposed to a 23 or 35 PSI acoustic blast when compared to uninjured controls, whereas 100 nm particles show no difference, suggesting blast injury induces BBB damage that enables the accumulation of larger particles. Treatment of blast-injured human brain microvascular cells with our nanoformulation reduced extracellular inflammatory cytokine levels and reduced the expression of pro-inflammatory markers in microglia. Moreover, these particles mitigated the upregulation of extracellular TNF α induced by free phenoxybenzamine in injured and uninjured microglia, suggesting nanoparticle drug encapsulation can reduce adverse drug reactions in the brain. Together, these findings provide proof-of-concept for size-based targeting and the potential anti-inflammatory effects of CS-PLGA nanoparticles containing adrenergic receptor antagonists for treatment of TBI and bTBI.

Keywords: traumatic brain injury; blast traumatic brain injury; neuroinflammation; polymeric nanoparticles; adrenergic receptor antagonists; targeted drug delivery



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1. Introduction

Traumatic brain injury (TBI) is a complex and multifaceted health problem that leads to nearly 65,000 deaths in the United States annually, a number that continues to grow [1,2]. Surviving patients are often faced with considerable cognitive, psychological, and psychosocial deficits, largely as a result of secondary injury defined by a series of cellular and biochemical changes that occur after the initial impact [3–5]. Additionally, military personnel are at an increased risk for TBI due to blast overpressure (BOP) from acoustic shock waves generated by explosives, known as blast TBI (bTBI) [6,7]. Despite the evident burden, there are currently no treatments available to attenuate TBI progression, highlighting an imperative need for further research.

Neuroinflammation is a key driver of both TBI and bTBI progression, thus serving as a potential target for effective treatment. In the hours or days following injury, microglia and astrocytes are activated [8–12] and release a variety of inflammatory cytokines, chemokines, and damage-associated molecular patterns (DAMPs), such as $\text{TNF}\alpha$ and IL8 [13–16]. While the early activation of these cells is critical for healing, long-term release of inflammatory biomolecules triggers oxidative stress, mitochondrial dysfunction, and apoptosis, heavily contributing to profound and prolonged neurological deficits and high mortality rates for TBI patients [11,13–15]. Furthermore, inflammatory cytokines disrupt essential tight-junction proteins (TJPs) [17–19], playing a key role in the blood–brain barrier (BBB) dysfunction that is characteristic of TBI [20–22]. This BBB dysfunction increases the patient’s risk of edema and stroke, while also increasing neurological impairment [21,22]. Importantly, microglia activation and BBB disruption are detectable for up to a year or more following initial injury [12,21,22].

The so called “sympathetic storm” or paroxysmal sympathetic hyperactivity (PSH) synergistically promotes TBI pathogenesis, forming a positive feedback loop with neuroinflammation. This PSH phenomenon describes a hyperadrenergic state in which increased catecholamine activity and subsequent activation of adrenergic receptors induces a series of psychological and physical symptoms, including increased agitation, blood pressure, and heart rate [23–25]. Moreover, PSH is associated with longer hospitalizations, worse clinical outcomes, and increased mortality for TBI patients [25–28]. While the specific mechanisms behind PSH are not fully understood, studies have shown a correlation between catecholamine activity and neuroinflammation [25,29–31]. TBI results in an increased release of norepinephrine and epinephrine [32,33], which triggers the activation of the pro-inflammatory $\alpha 1$ and $\beta 1$ adrenergic receptors. Individual activation of either receptor leads to an increased production of inflammatory cytokines, including $\text{TNF}\alpha$ [25,29,30]. Consequently, inhibition of α - and β -adrenergic receptors results in an anti-inflammatory and neuroprotective effect, positioning α - and β -adrenergic receptor antagonists as promising therapeutic agents for TBI [23,26,34–37]. In some instances, inhibition of just α - or β -adrenergic receptors results in overcompensation by the other due to an increased availability of their shared ligands and the additive effect of their downstream signaling, indicating a combination of α - and β -receptor antagonists may be of value [38]. However, systemic administration of such drugs can lead to unwanted side effects, including hypotension and bradycardia, causing their application to TBI to be limited [35,39].

Polymeric nanoparticles can passively target the injured brain [39–42], helping to overcome the limitations of α - and β -adrenergic receptor antagonists. In TBI, the breakdown of TJPs results in decreased BBB integrity and a subsequent increase in paracellular transport [21,22], analogous to the leaky vasculature seen in various tumors. Nanoparticles of a larger size that would not typically cross an intact BBB may take advantage of this dysfunction to passively accumulate at higher concentrations in the injured brain [39–42], in an effect similar to enhanced permeability and retention (EPR) [43,44]. We propose

that optimization of nanoparticle size can enhance these passive targeting effects in a size-based targeting approach, thus increasing local accumulation of the nanoparticles and their load while reducing off-target side effects. Furthermore, polymeric nanoparticles can co-encapsulate multiple drugs and/or imaging modalities, allowing for the simultaneous delivery of both α - and β -adrenergic receptor antagonists.

In this work, we discuss the design and investigation of a chitosan (CS)-coated poly(lactic-co-glycolic acid) (PLGA) nanoformulation (CS-PLGA) for the size-based targeting of the TBI-injured brain. The final nanoformulation (Figure 1) consists of the α -receptor antagonist phenoxybenzamine [38] and the β -receptor antagonist propranolol [36] co-encapsulated with Cy7 as an imaging agent. These drugs were selected as they are both FDA-approved and have demonstrated anti-inflammatory effects in pre-clinical models of TBI [37,45], with propranolol already being used off-label to reduce agitation in TBI patients [36]. While systemic administration of both drugs is associated with potential deleterious side effects including hypotension and bradycardia [46,47], we expect that the encapsulation within a biodegradable, biocompatible nanoparticle will enable selective accumulation of these drugs at the site of injury, minimizing these potential safety concerns. To investigate the targeting and therapeutic effects of our nanoformulation, we used a compressed-air-driven shock-tube model which mimics the BOP primary injury of bTBI [48–50] to administer injury to cultured cells. Our 200 nm particles exhibited selectivity towards an in vitro model of the blast-injured BBB, while 100 nm particles were indiscriminate, demonstrating a passive size-based targeting effect. Moreover, these particles reduced the expression of inflammatory markers in human brain microvascular cells (HBMVECs) as well as decreased pro-inflammatory activation in microglia, while mitigating unwanted cellular effects observed with free drugs. Together, these findings provide proof-of-concept for the use of polymeric nanoparticles as effective treatment systems for TBI and bTBI.

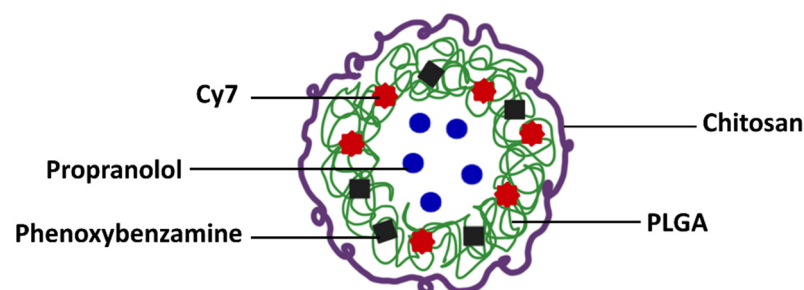


Figure 1. Nanoformulation—A schematic of our total nanoformulation consisting of a chitosan-coated PLGA nanoparticle co-encapsulating the α -receptor antagonist phenoxybenzamine, the β -receptor antagonist propranolol, and the NIR dye Cy7.

2. Materials and Methods

Materials: Poly(lactic-co-glycolic acid) (PLGA, 50:50 ester terminated, 7–17 kDa MW), poly(lactic-co-glycolic acid) (PLGA, 50:50 ester terminated, 24–38 kDa MW), chitosan oligosaccharide lactate (CS, 5 kDa MW), cyanine 7, pluronic-F127, phenoxybenzamine hydrochloride ($\geq 97\%$), and $\pm$ -propranolol ($\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylene chloride (MC, ACS reagent grade, $\geq 99.5\%$), and ethyl acetate (EA, ACS reagent grade, $\geq 99.5\%$) were purchased from Fisher Scientific (Waltham, MA, USA). All materials were used as received.

Nanoparticle Fabrication: Nanoparticles were fabricated using a water–oil–water emulsion–evaporation technique, with size being controlled by PLGA molecular weight and oil-phase composition.

100 nm Particles: The oil phase for 100 nm nanoparticles consisted of 30 mg PLGA (50:50 ester terminated, 7–17 kDa MW) and 2 mg phenoxybenzamine dissolved in 1 mL ethyl acetate and combined with 100 μ L Cy7 (0.5 mg/mL) in methylene chloride.

200 nm Particles: The oil phase for 200 nm nanoparticles consisted of 30 mg PLGA (50:50 ester terminated, 24–38 kDa MW) and 2 mg phenoxybenzamine dissolved in 650 μ L methylene chloride and 550 μ L ethyl acetate, then combined with 100 μ L Cy7 (0.5 mg/mL) in methylene chloride.

1 mL of the corresponding oil phase was then emulsified with 200 μ L of water containing 5 mg propranolol via probe sonication for 1.5 min at 30% power. 4 mL of aqueous 2.5% F127 was heated briefly, then mixed with the first emulsion using probe sonication for 3 min at 30% power. The final emulsion was stirred vigorously under air flow for 30 min until the organic solvents fully evaporated. After evaporation, 400 μ L of 3 mg/mL CS and 400 μ L of 0.1 M NaCH₃COO buffer at 4.4 pH were added to the suspension and stirred for 5 min. The nanoparticles were purified with a 30K MWCO Spin-X UF Concentrator (Corning Inc., Corning, NY, USA), then resuspended in phosphate-buffered saline (PBS) to the desired total volume/concentration. The drug(s) were omitted in the fabrication of empty nanoparticles or nanoparticles with only one encapsulated drug.

Nanoparticle Characterization: The nanoparticles' hydrodynamic diameter and surface charge were determined with dynamic light scattering (DLS) and zeta-potential analysis on a 90Plus zeta sizer (Brookhaven Instruments, Holtsville NY, USA). The nanoparticles' size and the morphology were characterized by transmission electron microscopy (TEM). Nanoparticles were prepared for TEM using a negative staining technique to account for the fact that they exhibit low electron density. The TEM samples were prepared by dropping 10 μ L of the nanoparticle suspension onto the surface of a 200-mesh formvar-coated copper grid (Ted Pella Inc., Redding, CA, USA). The suspension was allowed to sit for 2 min before being absorbed using the corner of a piece of 125 mm Whatman™ filter paper (GE Healthcare, Chicago, IL, USA) cut into triangles. Immediately following this, 10 μ L of a 2% phosphotungstic acid (Fluka Chemical, Ronkonkoma, NY, USA) solution (pH 7 using 0.1M NaOH) was dropped on to the grid and allowed to sit for 3 min. This was subsequently absorbed with an additional piece of filter paper. The grid was allowed to dry, covered, overnight before visualization using a JEM-2010 microscope (JEOL USA, Inc., Peabody, MA, USA) at an acceleration voltage of 200 kV.

Colloidal Stability in PBS: The colloidal stability of the 100 nm and 200 nm nanoparticle formulations was investigated in PBS at room temperature over a period of 10 days. Briefly, 200 μ L of each nanoparticle suspension was diluted with 800 μ L of PBS to obtain a final volume of 1 mL. The samples were stored at room temperature and analyzed on day 1, day 2, day 4, day 6, and day 10. At each time point, the hydrodynamic diameter and polydispersity index (PDI) were measured by dynamic light scattering (DLS) using a Malvern Zetasizer (Malvern Panalytical Ltd., Westborough, MA, USA). Prior to measurement, samples were mixed by gentle inversion to ensure homogeneous dispersion. Stability was determined by monitoring temporal changes in size and PDI, with minimal variation indicating good colloidal stability in PBS.

Drug Encapsulation Efficiency: After the nanoparticles were purified, both the supernatant and the nanoparticle suspension were analyzed using UV-Vis spectroscopy with an absorbance wavelength of 269 nm for phenoxybenzamine and 289 nm for propranolol. Separate calibration curves for propranolol (concentrations ranging from 0 to 0.25 mg/mL) and phenoxybenzamine (concentrations ranging from 0 to 0.4 mg/mL) were then used to calculate the corresponding concentration. Encapsulation efficiency was calculated using

Equation (1), where T_0 represents the concentration of TMZ added during synthesis and T_E represents the concentration of TMZ detected in the final nanoformulation.

$$\frac{T_E}{T_0} \times 100 = EE \quad (1)$$

Drug Release: Analysis of propranolol and phenoxybenzamine release was performed using the dialysis bag method. Molecular porous membrane tubing (Spectra/Por®, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) with a molecular weight cut off of 12–14 kDa and a 25 mm width was cut into 5 cm strips, then allowed to soak in diH₂O overnight. The following day, the tubing was filled with 500 µL of either 100 nm or 200 nm CS-PLGA particles co-encapsulating phenoxybenzamine and propranolol. The sealed tubing was incubated in 13 mL of pH 7.4 PBS or pH 5.5 0.1 M acetate buffer at 37 °C. Samples of 300 µL were collected at predetermined time points (0, 0.75, 1.5, 3, 6, 24, 48, 72 h) and stored at −20 °C until analysis. All samples were analyzed by a Thermo Fisher HPLC Linear Ion Trap (LTQ) mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with a reverse phase Kinetex 5 µm C18 column (4.6 × 150 mm, Phenomenex, Torrance, CA, USA). An isocratic method was used with a mobile phase consisting of 0.5% acetic acid and acetonitrile (50:50, HPLC grade, Fisher Scientific, Pittsburg, PA, USA) at a flow rate of 1.1 mL/min. Effluents were analyzed at 260 *m/z* and 286 *m/z*, corresponding to propranolol and phenoxybenzamine, respectively. All data was reported as percent release, normalized against the total encapsulated concentration.

Cells and Cell Culture: NHA (Cat # Lonza CC-2565 Product) Human astrocytes were obtained from Lonza RTP, Morrisville, NC, USA. HTHµ microglia cells were obtained from Jonathan Karn, CWRU, Cleveland, OH, USA. The cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ and maintained in a 50:50 DMEM: F-12K medium supplemented with non-essential amino acids, sodium pyruvate, antibiotic–antimycotic, 10% fetal bovine serum (FBS), and 1% Penicillin–Streptomycin. Human brain microvascular cells (HBMVECs Cat # ACBRI-376) were obtained from Cell Systems, Kirkland, WA, USA. The cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ and maintained in an RPMI medium supplemented with non-essential amino acids, sodium pyruvate, Penicillin–Streptomycin, 10% fetal bovine serum (FBS), and endothelial cell growth supplement (ECGS Cat # 1166 Cell Biologics, Inc., Chicago, IL, USA).

Cytotoxicity: Cytotoxicity was measured using a standard MTT assay. Briefly, HBMVECs or, separately, NHA cells were seeded at 3000 cells/well in 96-well plates. The next day, cells were incubated with fresh media containing the desired treatment concentration. Untreated wells served as the control. After 72 h, the medium was replaced with 0.5 mg/mL MTT for a 3 h incubation. Finally, formazan crystals were dissolved with DMSO. The cell plate was shaken for 15 min and read at 570 nm using an absorbance plate reader.

2D In Vitro BBB Model: The 2D in vitro BBB model consisted of a 24-well culture plate with upper and lower compartments in each well. The upper compartment was separated from the lower by a 3-µm PET insert (surface area = 0.3 cm²). On the upper side of the insert, human brain microvascular endothelial cells (HBMVECs) were grown to confluence. On the underside, a confluent layer of normal human astrocyte (NHA) cells was grown.

Blast Injury: A compressed-air-driven shock tube (Cauble Precision Inc., 75 cm compression tube, 76 cm expansion tube, 48 cm horn) was used to administer a shock wave which was generated with a Mylar®/Polyester Film (Piedmont Plastics, 0.002 in × 7 in × 9 in) (Figure S1, Top). Prior to blast injury, the cell medium was removed from each well and the cell culture plates were sealed with a sterile ThermalSeal® plate sealer (Excel Scientific, Inc., Victorville, CA, USA). The plates were then placed in a vertical

plate stand which was positioned 149 cm from the Mylar® film and 25 cm from the end of the shock tube's horn. A shock wave was rapidly administered to the cells at 23 PSI (~172 dB pSPL) and the impulse waveform used is represented in Figure S1 (obtained using a B&K Type 4938 ¼" microphone and Type 4941 ¼" microphone). Immediately following blast injury, the plate sealer was removed, and the cell medium was replaced. The maximum period that cells were without media was 5 min. All sham control cells also had their media removed for the duration of time it took to administer blast to the injured cells.

Assessment of Size-Based Targeting: A set of three in vitro BBB models were prepared and subsequently exposed to either 23 PSI or 35 PSI blasts, with an uninjured or "no-blast" (NB) model serving as the control. Immediately following blast injury, the models were treated with either ~100 nm or ~200 nm chitosan-coated PLGA nanoparticles (CS-PLGAs) that encapsulate Cy7. Following injury and treatment, models were incubated at 37 °C with 5% CO₂. Samples of 100 µL were then collected from the lower chamber of the BBB model at predetermined time points (0.5, 1, 2, 4, 8, and 24 h), treated with 200 µL acetonitrile, and later analyzed via fluorescence spectroscopy using a FluoroLog-3.1.1 spectrofluorometer (Horiba Scientific, Jobin Yvon, Edison, NJ, USA) with a slit width spectral resolution of 5 nm, an excitation wavelength of 730 nm, and an emission wavelength of 805 nm. Values were normalized to a 100% crossing model, which was prepared by creating a nanoparticle suspension in cell media consistent with the concentration administered to the BBB model, collecting a 100 µL sample, and treating it the same as experimental samples. Prior to analysis, all samples were stored at −20 °C.

Cellular Uptake: HBMVECs and human microglial (HTHµ) cells were seeded in 35 mm glass-bottom dishes and allowed to reach ~70% confluency prior to treatment. Cells were then exposed to a 23 PSI blast injury, and the medium was immediately replaced with 2 mL of fresh culture medium containing 0.002 µg/mL Cy7-labeled nanoparticles encapsulating propranolol and phenoxybenzamine. Nanoparticle internalization was monitored at 0.5, 2.5, 4.5, and 6.5 h post-treatment by confocal microscopy. Confocal imaging was performed on a Leica STELLARIS 8 inverted confocal microscope (DMI8-CS, Leica Microsystems, Mannheim, Germany) using an HC PL APO 40×/0.95 dry objective (Leica Microsystems, Mannheim, Germany). Cy7 was excited at 752 nm, and emission was collected from 761.03 to 812 nm using the HyD R 5 detector. Transmission images were acquired simultaneously using the Trans PMT channel. Images were collected at a 512 × 512 resolution, 400 Hz scan speed, 3.1625 µs pixel dwell time, 0.97 zoom, and a pinhole of 89.4 µm (1 AU). Single-plane images were acquired with a pixel size of 0.59 µm and identical instrument settings across all experimental groups.

Investigation of Anti-Inflammatory Properties: HBMVECs or HTHµ cells were plated in 6-well plates and grown to 70% confluence. Immediately following blast injury, the models were treated with CS-PLGA particles containing phenoxybenzamine (−α), propranolol (−β), a combination of both (−αβ), or the corresponding free drug using a constant total drug concentration of 0.002 mg/mL. Following injury and treatment, models were incubated at 37 °C with 5% CO₂ and after 12 h the media were collected and stored at −20 °C until the TNFα could be quantified or our multiplexed cytokine panel could be run. Additionally, cells were lysed using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and the lysate was stored at −20 °C until extraction.

RNA Extraction and RT-qPCR: Total RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform method with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The RNA pellet was washed with 75% ethanol, briefly air-dried, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNA concentration and purity were determined by absorbance at 260 nm using a NanoDrop spectrophotometer, with acceptable

A260/A280 ratios of approximately 2.0. RNA samples were stored at -70°C until further analysis. Quantitative PCR (qPCR) was performed to assess the relative mRNA expression levels of hCCL20, hCD74, hCD80, hCD86, and β -actin. Complementary DNA (cDNA) synthesis was carried out using a reverse transcription kit (SuperScriptTM IV, Invitrogen). qPCR reactions were prepared using SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) with gene-specific primers (Table 1) and conducted on a real-time thermal cycler under optimized cycling conditions. β -Actin served as the housekeeping gene for normalization. The relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with treated samples normalized to control cells.

Table 1. List of primers as well as their associated accession numbers and sequences.

Primer Name	Gene Accession Number	Forward Sequence (5' to 3')
CD74	NG_029730.1	ACCCTGTACCTCATCCCAT
CD80	NM_005191.4	GGAAAGTGACGCCCTGTATAA
CD86	NG_029928.2	GATGAATGGAAGGAGGCTTAGG

Multiplex Cytokine Analysis by Flow Cytometry: The conditioned media (200 μL) were collected and analyzed using the RayPlex[®] Human Inflammation Flow Cytometry Bead Array Kit (FAH-INF, RayBiotech, Peachtree Corners, GA, USA) according to the manufacturer's instructions. Briefly, 25 μL of RayPlex Multiplex Bead Cocktail (Item 1) was added to each well, followed by 25 μL of conditioned medium, standard, or negative control. Samples were incubated for 2 h at room temperature with gentle agitation to allow cytokine capture. Following incubation, beads were washed and incubated with 25 μL of biotinylated detection antibody (Item 6) for 1 h at room temperature. After washing, 25 μL of streptavidin–phycoerythrin (PE) conjugate (Item 7) was added and incubated for 30 min at room temperature, protected from light. Beads were washed and resuspended in 150 μL of $1\times$ wash buffer prior to acquisition. Data were acquired on a BD Celesta flow cytometer (BD Biosciences, Paramus, NJ, USA) using forward and side scatter to identify bead populations and the PE channel to quantify cytokine-associated fluorescence. A minimum of 400 events per bead population was collected. Data analysis was performed using FlowJo (v11), where bead populations were gated based on size and singlet discrimination. The median fluorescence intensity (MFI) values were extracted for each analyte and converted to concentration using standard curves generated from serially diluted protein standards.

TNF α Quantification: The lytic effect of TNF upon the WEHI-13VAR fibroblast cell line was used to analyze supernatants from in vitro BBB models for the presence of biologically active TNF α [51,52]. Briefly, WEHI-13VAR fibroblast cells, a TNF α -sensitive cell line derived from a mouse fibrosarcoma (ATCC, Manassas, VA, USA), were grown in RPMI-1640 culture medium containing 2mM L-glutamine, 10% fetal bovine serum (Invitrogen, Chicago, IL, USA), and 3 $\mu\text{g}/\text{mL}$ gentamicin (Sigma-Aldrich Chemical, Burlington, MA, USA) in T75 flasks at 37°C , 95% relative humidity (RH), and 5% CO_2 . Cells used in the TNF α bioassay were cultured to approximately 90% confluency and were always below passage 25 to avoid loss of TNF α sensitivity. Cells were prepared for the assay by detaching with 0.25% trypsin and 0.02% EDTA (Sigma-Aldrich Chemical) and resuspension in culture medium supplemented with 1 $\mu\text{g}/\text{mL}$ actinomycin D (Calbiochem, La Jolla, CA, USA) to a concentration of 500,000 cells/ mL . One hundred microliters of cell suspension were added to each well of a flat-bottom 96-well tissue culture plate containing 100 μL of 2-fold serial dilutions of unknown samples, in duplicate, or known concentrations of human recombinant TNF α standards (R&D Systems, Minneapolis, MN, USA) in diluting medium, RPMI-1640, 2 mM L-glutamine, 1% fetal bovine serum, and 15 mM HEPES (Sigma-Aldrich Chemical). Following 20 h of incubation at 37°C , 95% RH, and

5% CO₂, 10 µL of Cell Proliferation Reagent WST-1 (a solution of the tetrazolium salt, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate) and the electron coupling reagent, mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate); Roche Diagnostics, Indianapolis, IN, USA) in diluting medium was added to each well. WST-1 counting solution was used as a cell viability indicator, which is quantified spectrophotometrically [53]. After incubating for 4 h at 37 °C, 95% RH, and 5% CO₂, the absorbance at 400 and 700 nm was measured using a SpectraMax 96 microplate reader with SoftMax Pro v.4.0 acquisition and analysis software (MDS Analytical Technologies, Sunnyvale, CA, USA). A standard curve (0.003 pg/mL–10,000 pg/mL, reverse sigmoid in shape) of the (OD440—OD700) vs. log [TNF] was plotted. The [TNF] of each sample was determined from the dilution closest to the inflection point of the standard curve. This assay has a detection limit of approximately 1 pg/mL [54]. The assay is based on the specific cytotoxicity of the WEHI-13VAR cells to TNF in the presence of actinomycin D. Increasing TNF concentration results in increased cell death and, therefore, a reduced absorbance at 440 nm. Results are expressed as [TNF] pg/mL.

Statistical Analysis: All experiments were performed in triplicate with an $n = 3$. All data were expressed as the mean $\pm$ SD. Data was plotted in GraphPad Prism (v.10.6.1) and statistical analysis was performed using a one-way or two-way ANOVA test, followed by either a Dunnett's or Šídák's multiple comparisons test. p values less than 0.05 were considered to be statistically significant. A detailed description and discussion of the statistical analyses can be found in the Supporting Material document.

3. Results

3.1. Characterization of CS-PLGA Nanoparticles Containing Adrenergic Receptor Antagonists

Nanoparticles ≤ 100 nm can typically transverse a healthy BBB while larger particles may only enter the brain parenchyma when BBB integrity is disrupted [55]. To leverage this selective permeability for targeted TBI treatment, we designed 100 nm and 200 nm CS-coated PLGA nanoparticles (CS-PLGA) encapsulating the α -receptor antagonist phenoxybenzamine and/or the β -receptor antagonist propranolol as well as Cy7 to offer image-guided drug delivery.

3.1.1. Physical Properties

The size, zeta potential, and encapsulation efficiency (EE) of the ~ 100 nm and ~ 200 nm particles were assessed for individual and combined encapsulation of our therapeutic agents, as summarized in Table 2, with all nanoparticles exhibiting a consistent spherical morphology (Figure 2A). The measured hydrodynamic diameters of the CS-PLGA particles containing propranolol (CS-PLGA- β) were ~ 68 nm and ~ 204 nm, with a zeta potential of $\sim +15$ mV and EE of $\sim 22\%$ for both particle sizes. For CS-PLGA particles containing phenoxybenzamine (CS-PLGA- α), the measured hydrodynamic diameters were ~ 74 nm and ~ 214 nm, consistent with CS-PLGA- β . Both 100 nm and 200 nm particles showed strong stability in PBS, demonstrating no measurable difference in nanoparticle size (Figure S2A,B), with only a slight differences in zeta potential (Figure S2C,D) and polydispersity (Figure S2E) over the course of 10 days. The EE of phenoxybenzamine was determined to be $\sim 94\%$ for the ~ 74 nm CS-PLGA- α particles and $\sim 44\%$ for the ~ 214 nm CS-PLGA- α particles, demonstrating that particle size impacts phenoxybenzamine encapsulation. Nanoparticle size also had a slight impact on CS-PLGA- α surface charge, with ~ 74 nm particles exhibiting a zeta potential of $\sim +9$ mV and ~ 214 nm particles exhibiting a zeta potential of $\sim +14$ mV. In CS-PLGA particles containing both propranolol and phenoxybenzamine (CS-PLGA- $\alpha\beta$), the measured hydrodynamic diameters were ~ 107 nm and ~ 189 nm with a surface charge of ~ 15 mV and ~ 11 mV, respectively. The EE of the ~ 107 nm CS-PLGA- $\alpha\beta$ particles was

~60% phenoxybenzamine and ~26% for propranolol, while ~189 nm CS-PLGA- $\alpha\beta$ particles showed an EE of ~16% and ~6% for phenoxybenzamine and propranolol, respectively. The cause of this decrease in encapsulation for ~189 nm CS-PLGA- $\alpha\beta$ is unknown, but could be due to intermolecular forces between the drugs and/or between the drugs and PLGA at different molecular weights. To account for these variations in encapsulation, nanoparticle concentration was normalized to drug concentration in all biological experiments. Additionally, it is important to note that from this point forward, our smaller nanoparticles will be referred to as ~100 nm particles and our larger particles will be referred to as ~200 nm particles for simplicity.

Table 2. Nanoparticle characterization—summary of nanoparticle encapsulation efficiency (EE), hydrodynamic diameter, polydispersity index (PDI), and zeta potential.

	EE (%)	Diameter (nm)	PDI	Zeta Potential (mV)
CS-PLGA- β (Propranolol)	22 $\pm$ 3	68 $\pm$ 5	0.17 $\pm$ 0.02	+15 $\pm$ 1
	21 $\pm$ 1	204 $\pm$ 7	0.13 $\pm$ 0.02	+15 $\pm$ 2
CS-PLGA- α (Phenoxybenzamine)	94 $\pm$ 4	74 $\pm$ 2	0.15 $\pm$ 0.03	+9 $\pm$ 1
	44.5 $\pm$ 3	214 $\pm$ 5	0.169 $\pm$ 0.003	+14 $\pm$ 1
CS-PLGA- $\alpha\beta$ (Phenoxy/Prop)	60 $\pm$ 8/26 $\pm$ 5	107 $\pm$ 7	0.13 $\pm$ 0.03	+15 $\pm$ 1
	16 $\pm$ 1/6.1 $\pm$ 0.2	189 $\pm$ 7	0.12 $\pm$ 0.02	+11 $\pm$ 1

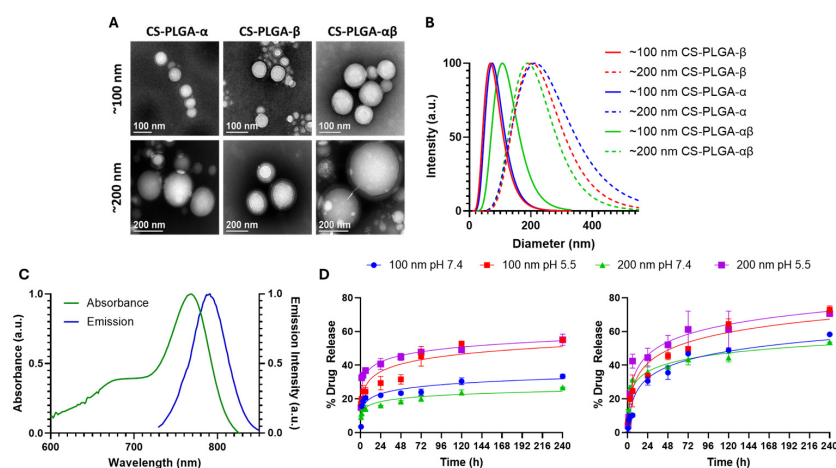


Figure 2. Nanoparticle characterization—(A) TEM images of ~100 nm (top) and ~200 nm (bottom) nanoparticles, containing either phenoxybenzamine (CS-PLGA- β), propranolol (CS-PLGA- α) or a combination of the two (CS-PLGA- $\alpha\beta$). (B) Graphical representation of nanoparticle PDI. (C) Absorption and emission spectra of 100 and 200 nm CS-PLGA- $\alpha\beta$. (D) Drug release profile of propranolol (left) and phenoxybenzamine (right) from 100 nm and 200 nm CS-PLGA- $\alpha\beta$ particles at pH 5.5 and 7.4.

3.1.2. Optical Properties

The optical properties of our nanoformulation were determined by measuring the absorption and emission spectra of the CS-PLGA- $\alpha\beta$ particles as shown in Figure 2C. Both ~100 nm and ~200 nm particles exhibited the same spectra, with a maximum absorbance wavelength of 770 nm and maximum emission wavelength of 790 nm. While this is a ~25 nm red shift from the accepted Cy7 absorbance and emission wavelengths ($\lambda_{\text{abs}} = 743$ nm, $\lambda_{\text{em}} = 767$ nm), the measured spectroscopic properties are still sufficient for biological imaging.

3.1.3. Drug Release Profile

PLGA-based nanoparticles are typically pH-responsive, allowing for a more rapid drug release under acidic conditions, such as that found in late-stage endosomes (pH 5.5) [56]. To investigate the pH-responsive drug release of our nanoformulations, we measured the propranolol (Figure 2D, left) and phenoxybenzamine (Figure 2D, right) release profiles from 100 nm and 200 nm CS-PLGA- $\alpha\beta$ particles at pH 5.5 and pH 7.4. Both 100 nm and 200 nm CS-PLGA- $\alpha\beta$ exhibited a more rapid release of propranolol and phenoxybenzamine at pH 5.5, suggesting that our particles are pH-responsive. In comparing drug release profiles between nanoparticle sizes, 100 nm particles exhibited a slightly faster release of propranolol than 200 nm particles at pH 7.4 and a slightly slower release at pH 5.5. However, no measurable difference was found in the phenoxybenzamine release from 100 nm and 200 nm particles at either pH. Thus, nanoparticle size has a minimal effect on drug release.

3.2. Biosafety of CS-PLGA Particles Containing Adrenergic Receptor Antagonists

It is critical that prospective nanoparticle-based drug delivery platforms do not cause adverse reactions in biological systems or, in other words, exhibit sufficient biosafety. Here we examine the preliminary biosafety of our nanoparticles by analyzing their cytotoxicity and biocompatibility, using cell viability and inflammatory responses in healthy cells as readouts.

3.2.1. Cytotoxicity

To ensure any observed biological effects are not due to cell death and to examine the biosafety of our nanoparticles, we analyzed their cytotoxicity on NHAs and HBMVECs, the main cell types that make up the BBB, using a standard cell viability assay. Twenty-four hours following treatment with CS-PLGA- α , CS-PLGA- β , or CS-PLGA- $\alpha\beta$ particles of 100 nm or 200 nm, NHAs (Figure 3A; 200 nm top left, 100 nm bottom left) and HBMVECs (Figure 3B; 200 nm top right, 200 nm bottom right) both exhibited a viability >90% over a range of treatment concentrations, indicating no detectable cytotoxicity.

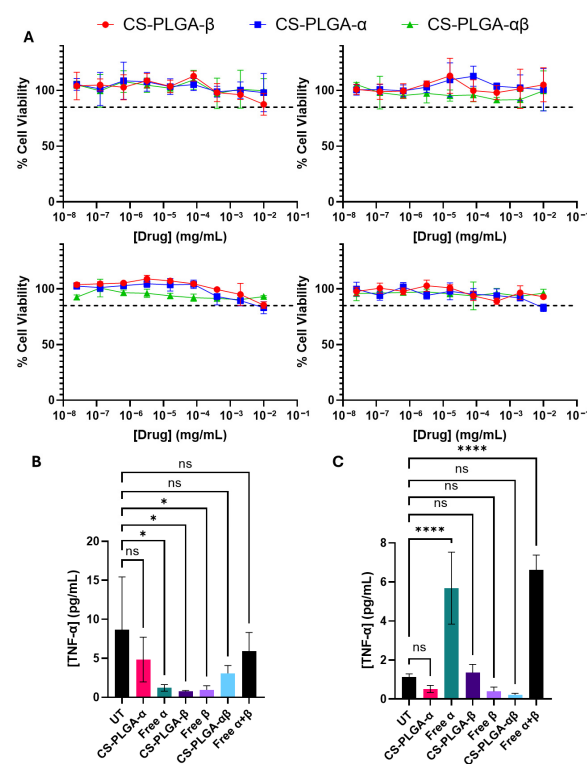


Figure 3. Biosafety—(A) Cell viability of NHAs treated with 200 nm particles (top left), HBMVECs treated with 200 nm particles (top right), NHAs treated with 100 nm particles (bottom left), and

HBMVECs treated with 100 nm particles (**bottom right**) 24 h after treatment (red circle = CS-PLGA- β , blue square = CS-PLGA- α , green triangle = CS-PLGA- $\alpha\beta$). Extracellular TNF α concentrations in uninjured (**B**) HBMVECs and (**C**) microglia (HTH μ) 12 h following treatment with CS-PLGA- α , CS-PLGA- β , CS-PLGA- $\alpha\beta$, phenoxybenzamine (free α), propranolol (free β), or a combination of phenoxybenzamine and propranolol (free $\alpha + \beta$) compared to untreated (UT) controls. (* = $p < 0.05$, *** = $p < 0.0001$, ns = non-significant).

3.2.2. Effect on Inflammation in Healthy Cells

Tight regulation of inflammatory responses and low levels of neuroinflammation are essential for normal brain function [57]. Therefore, we determined the effects of our nanoparticles and their corresponding unencapsulated drugs on neuroinflammation in uninjured brain cells using extracellular concentrations of the inflammatory cytokine, TNF α [13,16], as our readout. In HBMVECs, 200 nm CS-PLGA- α particles had no significant effect on extracellular TNF α , while free phenoxybenzamine reduced TNF α by 86% (free α , mean diff. = 7.48 ± 2.4 , $p = 0.0350$) 24 h after treatment (Figure 3B). CS-PLGA- β (mean diff. = 7.95 ± 2.4 , $p = 0.0243$) and free propranolol (free β , mean diff. = 7.75 ± 2.4 , $p = 0.0284$) also reduced extracellular TNF α in HBMVECs by 85–90%, while CS-PLGA- $\alpha\beta$ and a combination of free phenoxybenzamine and propranolol (free $\alpha + \beta$) induced no significant difference (Figure 3B). In microglia, the brain's resident immune cells, free phenoxybenzamine (mean diff. = -4.56 ± 0.64 , $p < 0.0001$) as well as a combination of free propranolol and phenoxybenzamine (mean diff. = -5.48 ± 0.64 , $p < 0.0001$) caused a 4.5–5-fold increase in extracellular TNF α 24 h after treatment that was mitigated by the corresponding CS-PLGA- α or - $\alpha\beta$ particles, as they showed no significant effect in TNF α in microglia (Figure 3C). CS-PLGA- β and free propranolol also showed no significant effect on extracellular TNF α in uninjured microglia (Figure 3C). Together, these results suggest that free phenoxybenzamine and propranolol, alone or in combination, as well as CS-PLGA- β , may lead to dysregulated inflammatory pathways in the healthy brain. CS-PLGA- α and CS-PLGA- $\alpha\beta$, on the other hand, appear to have no significant effect on basal levels of inflammation, indicating superior biosafety.

3.3. CS-PLGA Nanoparticles Exhibit Size-Based Targeting and Rapid Cellular Uptake

3.3.1. BBB Permeability

To analyze size-based targeting of the injured brain with our nanoparticles, we utilized a well validated in vitro 2-D BBB model [58–60], which was subsequently injured by an acoustic blast from a compressed-air-driven shock tube to mimic the primary injury of bTBI [48–50]. This bTBI model has previously been shown to alter the gene expression of TJPs and the viability of HBMVECs, demonstrating its ability to simulate a TBI-injured BBB [20]. To emulate two degrees of injury, a 23 PSI or 35 PSI blast was used. The 100 nm nanoparticles exhibited no significant difference in permeability between uninjured (NB) and injured conditions at any time point (Figure 4A, left). The 200 nm particles, however, demonstrated a ~1.6- (mean diff. = -18.71 $p < 0.0001$) and 1.8-fold (mean diff. = -23.45 , $p < 0.0001$) increase in permeability 24 h following injury with 23 PSI and 35 PSI blasts, respectively (Figure 4A, right). This suggests that the ~200 nm particles selectively permeate an injured BBB. Furthermore, in the uninjured (NB) BBB model, the ~200 nm particles exhibited BBB permeability ~1.9-fold (mean diff. = -25.06 , $p < 0.0001$) lower than that of ~100 nm particles 24 h after treatment, indicating that the ~200 nm particles are less likely to permeate a healthy BBB. Ultimately, these findings support size-based targeting of a BBB affected by bTBI.

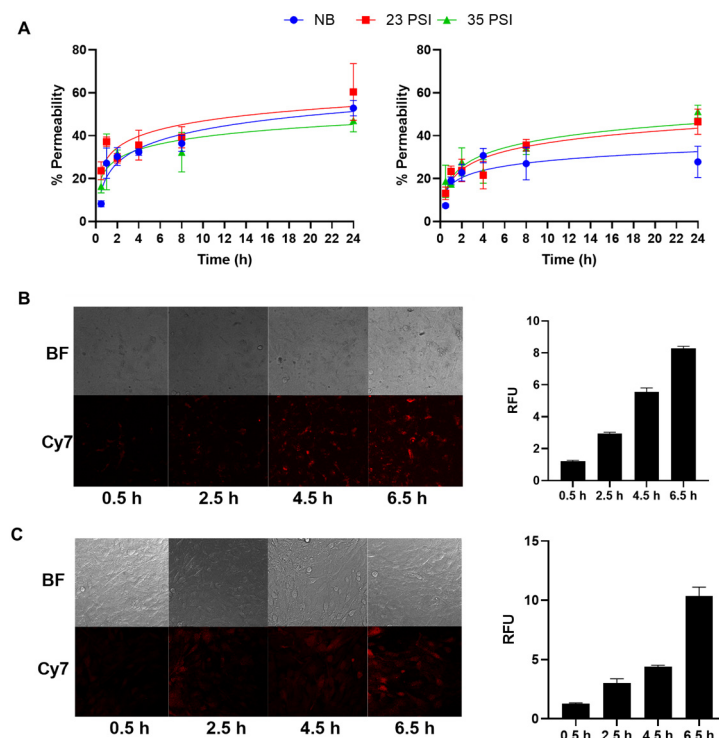


Figure 4. Nanoparticle targeting and cellular uptake—(A) BBB permeability of ~100 nm nanoparticles (left) and ~200 nm nanoparticles (right) in an in vitro 2-D BBB model either uninjured (NB) or injured with a 23 PSI or 35 PSI acoustic blast. The cellular uptake of 200 nm CS-PLGA nanoparticles in (B) HBMVECs and (C) microglia cells 0.5, 2.5, 4.5, and 6.5 h following 23 PSI blast injury.

For a detailed statistical discussion of each time point and condition, please refer to the Supporting Document.

3.3.2. Nanoparticles Exhibit Rapid Cellular Uptake

We next investigated the ability of our 200 nm nanoparticles to be taken up by HBMVECs and microglia with and without blast injury. After just 30 min, nanoparticle uptake was detectable in both HBMVECs (Figure 4B) and microglia (Figure 4C) and continued to increase over the course of 6.5 h.

3.4. Anti-Inflammatory Effects of CS-PLGA Nanoparticles Containing Adrenergic Receptor Antagonists Following Blast-Induced Injury

3.4.1. Anti-Inflammatory Effect in HBMVECs

Given that neuroinflammation is a major contributor to prolonged BBB dysfunction following TBI [12,15], we examined the anti-inflammatory effects of our nanoparticles on blast-injured HBMVECs, the main cell type comprising the BBB. Blast injury (23 PSI) resulted in a significant increase in the extracellular concentration of several inflammatory cytokines, namely $\text{INF}\gamma$ (Figure 5A, mean diff. = -33.25 , $p = 0.0001$) and $\text{IL1}\beta$ (Figure 5B, mean diff. = -33.43 , $p < 0.0001$). After 12 h of treatment immediately following injury, CS-PLGA- α reduced extracellular $\text{INF}\gamma$ by 50% (Figure 5A, mean diff. = 37.41 , $p < 0.0001$), $\text{IL1}\beta$ by 50% (Figure 5B, mean diff. = -6.257 , $p = 0.0177$), and $\text{TNF}\alpha$ by 77% (Figure 5C, mean diff. = 3.21 ± 0.61 , $p = 0.0007$). This impact on extracellular $\text{TNF}\alpha$ is a 17% improvement from the 60% reduction exhibited by free phenoxybenzamine (Figure 5D, mean diff. = 2.47 ± 0.61 , $p = 0.0072$). CS-PLGA- β also reduced extracellular $\text{INF}\gamma$ by 35% (Figure 5A, mean diff. = -10.16 , $p = 0.0021$), $\text{IL1}\beta$ by 45% (Figure 5B, mean diff. = -10.16 , $p = 0.0021$), and $\text{TNF}\alpha$ by 66% (Figure 5C, mean diff. = 2.78 ± 0.61 , $p = 0.0029$), with nanoparticle encapsulation showing an observable but statistically insignificant improvement in efficacy

compared to the 51% (Figure 5C, mean diff. = 2.11 ± 0.61 , $p = 0.0228$) reduction in TNF α exhibited by free propranolol. The effects of CS-PLGA- β on extracellular IL4 was similarly observable, albeit insignificant. In most cases, CS-PLGA- $\alpha\beta$ had comparable effects to CS-PLGA- α , resulting in a 50% reduction in extracellular INF γ (Figure 5A, mean diff. = 35.42, $p < 0.0001$) and a 50% reduction in IL1 β (Figure 5B, mean diff. = 24.92, $p < 0.0001$). Surprisingly, however, CS-PLGA- $\alpha\beta$ and combined treatment with free phenoxybenzamine and propranolol had no significant impact on extracellular TNF α in blast-injured HBMVECs.

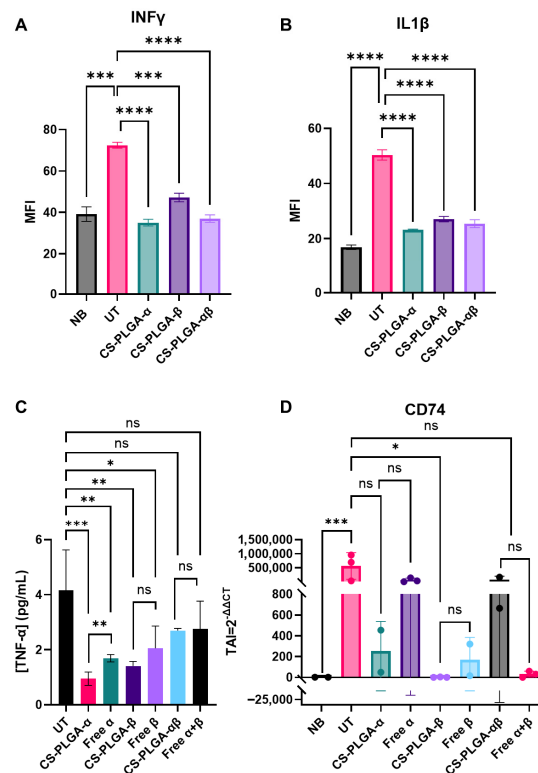


Figure 5. Therapeutic potential—Extracellular (A) INF γ , (B) IL1 β , and (C) TNF α levels as well as (D) gene expression of CD74 in HBMVECs injured with 23 PSI acoustic blast. Treatment conditions include CS-PLGA- α , CS-PLGA- β , CS-PLGA $\alpha\beta$, phenoxybenzamine (free α), propranolol (free β), or a combination of phenoxybenzamine and propranolol (free $\alpha + \beta$). (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = non-significant).

We also analyzed the impact of our nanoformulation on CD74 gene expression (Figure 5C), a critical modulator of microvascular inflammatory and immune response [61,62]. A blast injury of 23 PSI triggered a drastic 5.6×10^5 -fold increase (mean diff. = -5.45 ± 1.56 , $p = 0.0170$) in CD74 expression in HBMVECs 12 h following injury. Treatment with CS-PLGA- α (mean diff. = 3.30 ± 1.56 , $p = 0.488$), CS-PLGA- β (mean diff. = 5.859 ± 1.39 , $p = 0.0257$), as well as free phenoxybenzamine and propranolol either individually (phenoxybenzamine mean diff. = 0.994 ± 1.40 , $p = 0.0490$; propranolol mean diff. = 3.86 ± 1.40 , $p = 0.0487$) or in combination (mean diff. = 4.47 ± 1.40 , $p = 0.0257$) resulted in a 90–99% reduction in CD74 expression when compared to untreated controls. CS-PLGA- $\alpha\beta$, on the other hand, showed an observable 80% reduction in CD74 expression that was determined to be statistically insignificant due to large variations between samples, suggesting inconsistent effects. Thus, CS-PLGA- α and CS-PLGA- β show consistent anti-inflammatory effects on blast-injured HBMVECs.

3.4.2. Anti-Inflammatory Effect in Microglia

Microglia are the brain's resident immune cells that can remain activated in a pro-inflammatory state for over a year following TBI, largely driving long-term neurological deficits [12,57]. We therefore examined the effect of our nanoformulations on microglia inflammation and found that 23 PSI blast injury increased the extracellular release of the inflammatory cytokines $\text{INF}\gamma$ (Figure 6A, mean diff. = -57.09 , $p = 0.0095$), IL2 (Figure 6B, mean diff. = -86.43 , $p < 0.0001$), IL6 (Figure 6C, mean diff. = -17.25 , $p < 0.0001$), and IL1 β (Figure 6D, mean diff. = -47.81 , $p = 0.0002$). Treatment with CS-PLGA- α (mean diff. = 42.13 , $p = 0.0338$) or CS-PLGA- $\alpha\beta$ (mean diff. = 50.10 , $p = 0.0167$) for 12 h following injury reduced the extracellular $\text{INF}\gamma$ levels by 50%, while CS-PLGA- β (mean diff. = 62.33 , $p = 0.0065$) lead to a 70% reduction (Figure 6A). In the case of IL2, CS-PLGA- α had a marginal but insignificant inhibitory effect, whereas CS-PLGA- β (mean diff. = 83.09 , $p < 0.0001$) and CS-PLGA- $\alpha\beta$ (mean diff. = 69.97 , $p = 0.0002$) reduced extracellular levels of this cytokine by 55% and 45%, respectively (Figure 6B). Extracellular IL6 decreased 80% following treatment with CS-PLGA- α (mean diff. = 16.18 , $p < 0.0001$), 90% with CS-PLGA- β (mean diff. = 14.43 , $p < 0.0001$), and 70% with CS-PLGA- $\alpha\beta$ (mean diff. = 12.97 , $p < 0.0001$) (Figure 6C). All three particles also had an inhibitory effect on IL1 β , with CS-PLGA- α reducing the extracellular levels of this cytokine by 70% (mean diff. = 47.06 , $p = 0.0002$), CS-PLGA- β by 30% (mean diff. = 21.51 , $p = 0.0094$), and CS-PLGA- $\alpha\beta$ by 60% (mean diff. = 39.88 , $p = 0.0005$) (Figure 6D). However, CS-PLGA- α , CS-PLGA- β , and CS-PLGA- $\alpha\beta$ all exhibit no significant effect on extracellular $\text{TNF}\alpha$ whereas free phenoxybenzamine results in an observable, but statistically insignificant 7.75-fold increase (Figure 6E). Free propranolol (mean diff. = -54.75 ± 10.08 , $p = 0.0005$) as well as a combination of free propranolol and phenoxybenzamine (mean diff. = -74.08 ± 10.08 , $p < 0.0001$) similarly resulted in a 52–62-fold increase in extracellular $\text{TNF}\alpha$ (Figure 6E), indicating that unencapsulated adrenergic receptor antagonists may activate inflammatory pathways in microglia.

To investigate the impact of our nanoformulation on microglia polarization towards a pro-inflammatory state, we also examined their impact on the gene expression of the pro-inflammatory markers CD80 and CD86 [63–65]. A blast injury of 23 PSI induced a 2.0×10^6 -fold (mean diff. = -6.26 ± 0.365 , $p < 0.0001$) and 5.0×10^3 -fold (mean diff. = -3.64 ± 0.61 , $p = 0.0004$) increase in CD80 (Figure 6F) and CD86 (Figure 6G) gene expression, respectively, 12 h after injury, indicating microglia enter a pro-inflammatory state in our bTBI model. CS-PLGA- α particles resulted in a nearly 100% reduction in CD80 (Figure 6F, mean diff. = 5.95 ± 0.37 , $p < 0.0001$) and CD86 (Figure 6G, mean diff. = 2.39 ± 0.67 , $p = 0.0188$) expression in contrast to free phenoxybenzamine, which induced a 23-fold increase in CD86 expression compared to injured untreated controls Figure 6G. CS-PLGA- β particles also reduced CD80 (Figure 6F, mean diff. = 5.91 ± 0.40 , $p < 0.0001$) and CD86 expression (Figure 6G) by nearly 100% compared to injured untreated controls and, while free propranolol drastically reduced CD80 expression levels (mean diff. = 2.20 ± 0.40 , $p = 0.0009$), CS-PLGA- β treatment was 4.1×10^3 -fold more effective (Figure 6F). Following a similar trend, CS-PLGA- $\alpha\beta$ particles reduced CD80 (Figure 6F, mean diff. = 5.08 ± 0.36 , $p < 0.0001$) and CD86 (Figure 6G, mean diff. = 4.35 ± 0.61 , $p < 0.0004$) expression by about 100% in injured microglia and was 4.6-fold more effective than a combination of unencapsulated phenoxybenzamine and propranolol with respect to CD80. Together, these findings suggest that in our system, CS-PLGA- α , CS-PLGA- β , and CS-PLGA- $\alpha\beta$ prevent the pro-inflammatory polarization of microglia following blast injury.

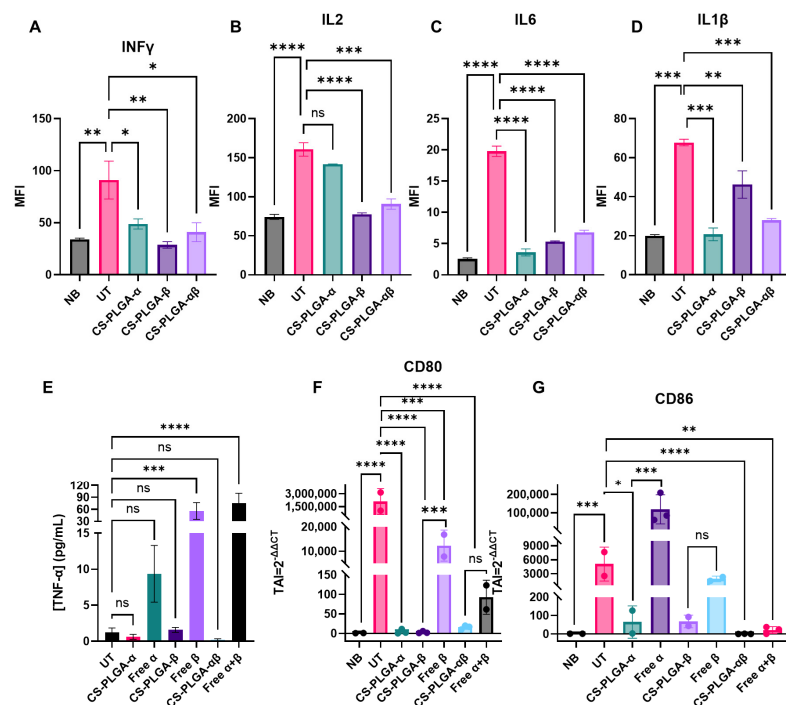


Figure 6. Therapeutic potential in microglia—Extracellular (A) INF γ , (B) IL2, (C) IL6, (D) IL1 β , and (E) TNF α levels as well as gene expression of (F) CD80 and (G) CD86 in microglia (HTH μ) injured with 23 PSI acoustic blast. Treatment conditions include CS-PLGA- α , CS-PLGA- β , CS-PLGA $\alpha\beta$, phenoxybenzamine (free α), propranolol (free β), or a combination of phenoxybenzamine and propranolol (free $\alpha + \beta$). (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = non-significant).

4. Discussion

Currently, there are no clinically available treatments to attenuate the progression of TBI. Adrenergic receptor antagonists have shown initial anti-inflammatory and neuroprotective effects in pre-clinical models [25,34–36]; however, their application is limited by deleterious side effects in systemic administration [23,36]. In this work, we hypothesized that the use of a size-controlled nanoparticle would allow for passive targeting of the injured brain by taking advantage of the BBB breakdown characteristic of TBI, thus mitigating said side effects. Therefore, we aimed to develop a nanoformulation that would offer size-based targeting of the TBI-injured brain for the image-guided delivery of the β -adrenergic antagonist propranolol and/or the α -adrenergic antagonist phenoxybenzamine. Further, we proposed that these nanoparticles would offer anti-inflammatory effects to attenuate TBI progression.

Our resulting series of nanoformulations, deemed CS-PLGA- α , CS-PLGA- β , and CS-PLGA- $\alpha\beta$, consist of the corresponding drug(s) co-encapsulated with Cy7 in a CS-PLGA particle. By modulating polymer molecular weight and the composition of the organic phase during nanoparticle fabrication, we were able to achieve control over nanoparticle size to produce nanoparticles ≤ 100 nm and ~ 200 nm (Table 2, Figure 2A,B). As expected for a water-soluble drug, propranolol reached $\sim 20\%$ EE in CS-PLGA- β and in the 100 nm CS-PLGA- $\alpha\beta$ particles (Table 2), consistent with its tendency to diffuse into the aqueous phase during fabrication [66,67]. In contrast, the markedly lower 6% EE observed in the 200 nm CS-PLGA- $\alpha\beta$ (Table 2) particles likely reflects drug saturation effects, as polymer-to-drug ratios dominantly dictate encapsulation efficiencies [68,69]. A similar mechanism likely underlies the reduction in phenoxybenzamine EE from $>90\%$ in 100 nm to 44% in 200 nm CS-PLGA- α (Table 2), with changes in organic-phase composition potentially increasing drug diffusion during particle hardening and exacerbating the decreased drug

loading [70,71]. Drug saturation may also contribute to the decrease in phenoxybenzamine EE upon co-encapsulation in both the 100 nm and 200 nm CS-PLGA- $\alpha\beta$ particles; however, intermolecular forces between drugs may be a contributing factor. Optimization of the polymer-to-drug and drug-to-drug ratios as well as solvent composition will improve EE, while use of a more intricate fabrication system like a continuous flow microfluidics platform will ensure scalability and batch-to-batch reproducibility.

With the physicochemical properties defined, we examined how these formulations perform under biologically relevant conditions. CS-PLGA- $\alpha\beta$ particles exhibit pH-responsive drug release (Figure 2D), suggesting that the encapsulated load would be more readily released in the acidic pH of late-stage endosomes. This could reduce the release of the encapsulated drug(s) during blood circulation and allow for controlled and sustained release following cellular uptake, helping to reduce systemic side effects. Moreover, CS-PLGA- α and CS-PLGA- $\alpha\beta$ particles exhibited excellent biosafety under our measured conditions, demonstrating no observable cytotoxicity in HBMVEC and NHA cells (Figure 3A) and having no significant effect on extracellular TNF α in uninjured HBMVEC (Figure 3B) and microglia (Figure 3C). These particles even mitigated an increase in TNF α induced in microglia by free phenoxybenzamine either alone or in combination with propranolol (Figure 3C). Given that TNF α is a key indicator of neuroinflammation, this implies that nanoparticle drug encapsulation can reduce adverse drug reactions in the brain. CS-PLGA- β particles, however, were an exception to this trend, as free propranolol and nanoparticle treatment reduced extracellular TNF α in uninjured HBMVECs (Figure 3A), representing a capacity to dysregulate the homeostatic level of TNF α necessary for normal brain function. Finally, the spectroscopic properties of Cy7 are largely maintained upon encapsulation (Figure 2C), with excitation and emission wavelengths in the first optically transparent biological window (650–950 nm), thus offering the potential for biomedical optical imaging. While in vivo studies will be necessary to better understand the biosafety and drug delivery capabilities of our nanoformulations, the observed properties of our CS-PLGA- α and CS-PLGA- $\alpha\beta$ suggest they are suitable for image-guided drug delivery.

Specifically leveraging the use of our nanoparticles in the treatment of TBI is the successful size-based targeting of the injured in vitro BBB, enabling selective drug accumulation at the injury site. BBB permeability is highly dependent on the size of the permeating substance, which is typically limited to ≤ 100 nm [55]. In TBI, however, TJP breakdown, mechanical sheering, cell death, and alterations in transport mechanisms cause a loss of BBB integrity [21,22,72]. Our particles take advantage of this loss, resulting in 200 nm particles selectively permeating our injured BBB model (Figure 4A). Although transcytosis is the accepted route for BBB permeation of nanoparticles, this effect is likely due to an increase in paracellular transport following injury. Transcytosis can only occur with particles ≤ 100 nm, as the intracellular transport vesicles used in this route are mostly ~ 30 – 150 nm [73,74]. The BBB dysregulation and mechanical damage in TBI, however, can result in larger gaps between cells and allow for greater paracellular transport of larger substances. This theory is supported by the limited effect of injury on the permeability of our 100 nm particles (Figure 4A), as they would already permeate the healthy BBB through transcytosis, diminishing the impact of any alternative injury-driven transport routes on total particle accumulation. The 200 nm particles, on the other hand, would be too large to enter the intracellular vesicles and would instead have to rely on other routes across the BBB. An increase in intercellular space upon injury could provide this alternative route, allowing for significantly greater permeability of the larger particles in the TBI-affected BBB.

While increased intracellular space following injury is plausible, further investigation into the mechanisms involved in nanoparticle transport across the injured BBB in vitro and

in vivo are necessary to better understand the process of size-based targeting. Notably, there have recently been contradictory reports regarding passive accumulation of nanoparticles following brain injury, with some suggesting 100–200 nm particles are ideal and others suggesting particles larger than 100 nm are rapidly cleared from the injury site [39–42]. These reports span multiple types of polymeric nanoparticles with various surface modifications and employ injury systems ranging from standard controlled cortical impact (CCI) to alternative models of TBI, demonstrating that multiple factors influence the efficacy of nanoparticle-based drug delivery. Given that the BOP primary injury of bTBI significantly differs from the blunt-force primary injury of traditional TBI, the degree of damage to the BBB due to mechanical force may also vary. Therefore, the size-based targeting effect observed in our studies may be specific to bTBI. Additionally, our studies utilize a 2D trans-well contact co-culture model of the BBB, which accurately models the tight junctions and major cell composition of the BBB in a high-throughput format but does not mimic the curvature of vasculature nor the flow of blood in the human brain [75,76]. The lack of curvature in 2D BBB models likely influences the tension and forces between cells at tight junctions, possibly impacting the effect of blast injury on BBB breakdown, and the static nature of treatment incubation may overestimate the percent permeation. Therefore, while our BBB model is ideal for initial screening of nanoparticle series, alternative models that account for the architecture of vasculature and blood flow, such as 3D microfluidic-based models or organoids [75–78], may more closely represent BBB permeability in the body. Future studies should include multiple in vitro and in vivo injury models and intensities to account for the nuances of BBB permeability in the injured brain and identify the scope of application to TBI treatment. Nevertheless, our current data supports the size-based targeting of the bTBI-injured brain.

Aside from size-based targeting, our nanoformulation demonstrated promise in attenuating the inflammatory response that drives both TBI and bTBI progression. TBI-induced catecholamine activity leads to the activation of pro-inflammatory α 1- and β 1-adrenergic receptors. Antagonism of these pathways using drugs such as propranolol and phenoxybenzamine has, correspondingly, been shown to reduce neuroinflammation and improved patient prognosis in pre-clinical TBI studies [25,36–38]. Given that individual blockade of α - or β -adrenergic receptors is thought to result in overcompensation by the uninhibited pathway, we hypothesized that a nanoparticle containing both phenoxybenzamine and propranolol would provide the most robust anti-inflammatory response. In injured HB-MECs, however, CS-PLGA- α and CS-PLGA- β performed comparably to CS-PLGA- $\alpha\beta$ in reducing extracellular $\text{INF}\gamma$ (Figure 5A) and $\text{IL1}\beta$ (Figure 5C) following injury. Moreover, both reduced extracellular $\text{TNF}\alpha$, with CS-PLGA- α outperforming free phenoxybenzamine, while CS-PLGA- $\alpha\beta$ and a combined treatment with unencapsulated propranolol and phenoxybenzamine failed to do so (Figure 5D). Similarly, CS-PLGA- α and CS-PLGA- β significantly decreased the injury-induced HBMVEC gene expression of CD74, a surface protein that facilitates macrophage recruitment and activates inflammatory pathways in vasculature [61,62], but CS-PLGA- $\alpha\beta$ had variable effects (Figure 6E). In microglia, CS-PLGA- α and/or CS-PLGA- β once again performed comparably to CS-PLGA- $\alpha\beta$ in reducing extracellular levels of $\text{INF}\gamma$ (Figure 6A), IL2 (Figure 6B), IL6 (Figure 6C), and $\text{IL1}\beta$ (Figure 6D). All three nanoformulations also attenuated an increase in extracellular $\text{TNF}\alpha$ concentrations induced by free drugs in microglia (Figure 6E) as well as inhibited the injury-induced expression of CD80 (Figure 6F) and CD86 (Figure 6G), markers of pro-inflammatory microglia activation [63–65]. Ultimately, CS-PLGA- α , CS-PLGA- β , and CS-PLGA- $\alpha\beta$ all demonstrated robust anti-inflammatory effects in HBMVECs and microglia following blast injury.

To better understand why the co-encapsulated CS-PLGA- $\alpha\beta$ nanoformulation did not consistently outperform the single-drug formulations as predicted, we considered the underlying pharmacological mechanisms of adrenergic receptor signaling. Adrenergic receptor subtypes, particularly $\alpha 1$ versus $\alpha 2$ and $\beta 1$ versus $\beta 2$, exhibit complex and often opposing roles in neuroinflammation. $\alpha 1$ - and $\beta 1$ -adrenergic receptors are generally associated with pro-inflammatory effects [79–82], whereas $\alpha 2$ - and $\beta 2$ -adrenergic receptors are more commonly linked to anti-inflammatory responses [81,83–86]. Because phenoxybenzamine and propranolol are non-selective α - and β -adrenergic receptor antagonists, respectively, they inhibit both receptor subtypes [46,47]. As a result, combined treatment may produce competing effects, where simultaneous blockade of $\alpha 2$ and $\beta 2$ signaling partially offsets the benefits of $\alpha 1$ and $\beta 1$ blockade, while still allowing an overall anti-inflammatory effect to predominate. These findings suggest that more selective treatment strategies, such as $\alpha 1$ - or $\beta 1$ -selective antagonists or the incorporation of $\alpha 2$ - and $\beta 2$ -agonists, may yield more synergistic anti-inflammatory outcomes.

Notably, our nanoformulations often outperformed the free drug and, in some cases, even induced an anti-inflammatory effect where the free drug induced a pro-inflammatory effect. These differences between the encapsulated and free drug are likely due, in part, to nanoparticle encapsulation protecting the drug(s) from degradation and enabling a prolonged receptor blockade through sustained drug release, thereby improving drug efficacy [86]. However, the opposing nature of the $\alpha 1$ - versus $\alpha 2$ - and $\beta 1$ - versus $\beta 2$ -adrenergic receptor subtypes likely also plays a role. While phenoxybenzamine is considered a non-selective α -antagonist, it exhibits a slightly higher affinity for $\alpha 1$ -adrenergic receptors [87]. This higher affinity suggests that the overall effects of phenoxybenzamine may be dose-dependent, with lower doses preferentially blocking $\alpha 1$ signaling and reducing neuroinflammation. Treatment with a free drug delivers a single, higher dose immediately after administration, leading to a mixed or pro-inflammatory effect due to the simultaneous blockade of $\alpha 2$. The extended drug release profile of our nanoformulation, on the other hand, enables a more sustained, lower exposure to phenoxybenzamine that favors a consistent anti-inflammatory effect.

A similar dose-dependent mechanism may apply to propranolol, as norepinephrine is released in high amounts during TBI [32,33,88] and preferentially activates $\beta 1$ -adrenergic receptors [89]. $\beta 2$ -adrenergic receptors have a much lower affinity for norepinephrine and are more responsive to epinephrine, which also stimulates $\beta 1$ -receptors and is typically present at lower levels in this context [88,89]. As a result, $\beta 1$ -mediated pro-inflammatory signaling may predominate following injury. Thus, at lower, sustained drug exposures, inhibition of the more dominantly active $\beta 1$ -receptor may drive an anti-inflammatory effect. Whereas, under higher drug exposures, concurrent suppression of $\beta 2$ -mediated anti-inflammatory signaling may become more consequential, resulting in a mixed or even pro-inflammatory outcome despite continued $\beta 1$ inhibition. Further mechanistic study into the potential dose-dependent effects of phenoxybenzamine and propranolol will help delineate their impact on neuroinflammation. Importantly, despite the complexity and opposing roles of adrenergic receptor subtypes, all nanoformulations consistently exhibited anti-inflammatory effects across both endothelial and microglial models. Given that $\text{INF}\gamma$, IL2, IL6, IL1 β , and $\text{TNF}\alpha$ are major cytokines involved in the progression of TBI [13,16,90,91] and that chronic pro-inflammatory polarization of microglia is a key driver of prolonged neurological dysfunction for TBI patients [12,57], these findings suggest that each of our nanoformulations could reduce neuroinflammation and TBI progression.

In conclusion, we designed CS-PLGA nanoparticles containing Cy7, propranolol, and/or phenoxybenzamine, which exhibit pH-controlled drug release and optical imaging properties. We achieved size-based targeting of a TBI-injured *in vitro* BBB model by tuning

particle size, with 200 nm particles exhibiting selective permeability following injury. While each of the nanoparticles in our series demonstrated anti-inflammatory effects, CS-PLGA- α particles showed a slight advantage in attenuating TBI-induced neuroinflammation while also mitigating the unwanted effects of a free drug in vitro. We expect that these particles will mitigate the unwanted side effects associated with phenoxybenzamine, given that the observed size-based targeting should allow for increased drug accumulation at the site of injury. This targeting effect may be further enhanced using intranasal administration, as CS-PLGA nanoparticles have been shown to have rapid systemic clearance with predominant brain accumulation using this delivery route [92,93]. Future studies must assess the in vivo biodistribution and anti-inflammatory properties of these particles as well as examine their broader impacts on TBI progression. Nevertheless, these findings provide proof-of-concept for the use of CS-PLGA- α nanoparticles in TBI and bTBI treatment.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/immuno6020029/s1>, Figure S1: Blast TBI Model; Figure S2: Colloidal Stability; Statistical Discussion.

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Abbreviations

The following abbreviations are used in this manuscript:

TBI	Traumatic Brain Injury
bTBI	Blast Traumatic Brain Injury
BOP	Blast Overpressure
DAMPs	Damage-Associated Molecular Patterns
TJPs	Tight-Junction Proteins
BBB	Blood–Brain Barrier
PSH	Paroxysmal Sympathetic Hyperactivity
EPR	Enhanced Permeability and Retention
CS	Chitosan
PLGA	Poly(lactic-co-glycolic) Acid
CS-PLGA	Chitosan-Coated PLGA Nanoparticle
CS-PLGA- α	Nanoparticle Containing Phenoxybenzamine
CS-PLGA- β	Nanoparticle Containing Propranolol
CS-PLGA- $\alpha\beta$	Nanoparticle Containing a Combination of Phenoxybenzamine and Propranolol

NHAs	Normal Human Astrocytes
HBMVECs	Human Brain Microvascular Endothelial Cells
EA	Ethyl Acetate
MC	Methylene Chloride
PBS	Phosphate-Buffered Saline
DLS	Dynamic Light Scattering
TEM	Transmission Electron Microscopy
NB	No Blast
RH	Relative Humidity
qPCR	Quantitative Polymerase Chain Reaction
CCI	Controlled Cortical Impact

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