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Nanoparticle Targeting to the Central Nervous System

Emily Baker

The University of Akron, emb113@zips.uakron.edu

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Emily Baker

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Abstract

Drug delivery to the central nervous system is complicated by the blood brain barrier, a vascular structure that prevents free diffusion of molecules into brain tissue. In this study, we examined the *in vitro* properties of a nanoparticle-based drug delivery system. We used several nanoparticle formulations, both LTP based and PLGA based, to test the cellular uptake and toxicity in microglial cells. Using immunofluorescence imaging, we show that LTP nanoparticles are taken up by microglia. We confirmed that our nanoparticle formulations are nontoxic by two cell viability assays. These results suggest that nanoparticle formulations may be a biocompatible method of delivering drugs to the brain.

Introduction

Drug delivery to the central nervous system (CNS) is a challenging goal that hinders the development of new therapeutics as well as imaging agents. A major difficulty for drug targeting to the brain is the presence of a specialized vascular structure termed the blood-brain barrier (BBB). The BBB is composed of endothelial cells that express tight junction proteins and inhibit the free diffusion of small molecules from the blood to the brain.² These cells also express ATP-dependent transporters that remove xenobiotics from the brain.¹ Due to the need to overcome this unique barrier, the physiochemical characteristics of drug formulations that successfully penetrate the brain endothelium and accumulate in the parenchyma have been extensively investigated. Compounds that are ligands for specific receptors expressed on the BBB as well as lipophilic compounds and some gases are able to readily penetrate into the brain parenchyma.²

A current area of active investigation for drug delivery is the packaging of compounds into nanoparticle formulations. Polymeric protein-based nanoparticles are being studied for

biomedical applications, such as MRI imaging and drug delivery.³ The main goal of this study was to test a nanoparticle delivery system for imaging agents and anti-inflammatory drugs that would increase CNS uptake. We focused on designing nanoparticle formulations that would not only penetrate the BBB, but also exhibit an affinity for microglia. Microglial cells are considered the major component of the immune system present within the CNS. These cells are multifunctional and can constantly scan the brain for damaged cells, debris and other indicators of trauma.⁴ Once the microglia sense injury or infection they will become activated (**Figure 1**). In an activated state, they promote wound healing via phagocytosis of debris and activate other immune cells via release of cytokines, such as transforming growth factor beta (TGF- β).² Activation of microglial cells occurs early after injury and this response is characteristic of a number of neurodegenerative and inflammatory conditions such as traumatic brain injury, Alzheimer's, and Parkinson's disease.⁵

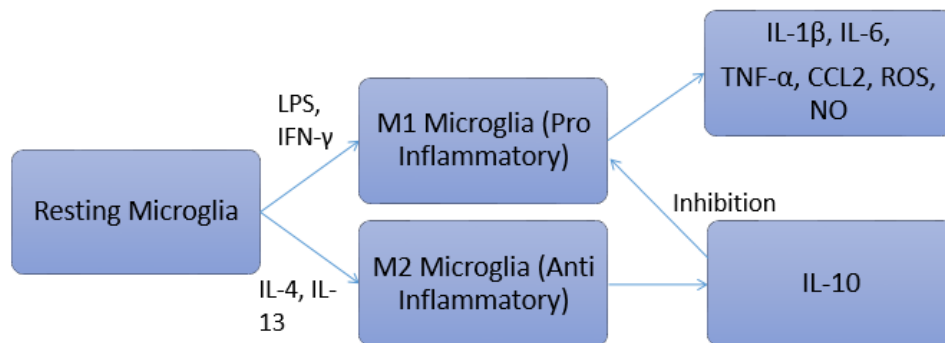


Figure 1: Microglial activation results from cytokine signaling and leads to release of pro-inflammatory (M1) or anti-inflammatory (M2) activity.

Recently, it has been shown that phagocytic cells such as macrophages can take up nanoparticles and deliver them through the BBB.⁶ We hoped to exploit the natural phagocytotic phenotype of the microglia in a similar strategy to induce uptake of our nanoparticles. A second

motivation for targeting microglial cells is that their early activation and differentiation into pro-inflammatory cells make them ideal targets for an anti-inflammatory compound.² The nanoparticle formulations that we test in this study consists of either a modified L-Tyrosine phosphate (LTP) monomer or poly(lactic-co-glycolic acid) (PLGA) (**Figure 2**). LTP was chosen due to a polyethylene glycol (PEG) coating that allows for greater systemic distribution.⁷ Both formulations have also been shown to be non-toxic.^{7,8} Compounds were subsequently loaded into the nanoparticle formulations to test delivery and toxicity. The imaging agent Gadolinium-Diethylenetriaminepentaacetic acid (Gd-DTPA), was incorporated into particles and examined for toxicity. Gd-DPTA (**Figure 2**) is an FDA-approved contrast agent for MRI. Gd-based contrast agents allow visualization of areas of blood brain breakdown and are used to detect inflammatory lesions in the CNS.⁹ In addition, the same polymer nanoparticles were tested uptake by confocal imaging. For these experiments, the particles contained BSA conjugated to the fluorophore FITC. The PLGA nanoparticle formulation was loaded with the anti-inflammatory drug Rolipram. Rolipram acts by inhibiting phosphodiesterase IV subtype, PDE IVB, increasing intracellular levels of cAMP that leads to suppression of CNS inflammatory responses.¹⁰

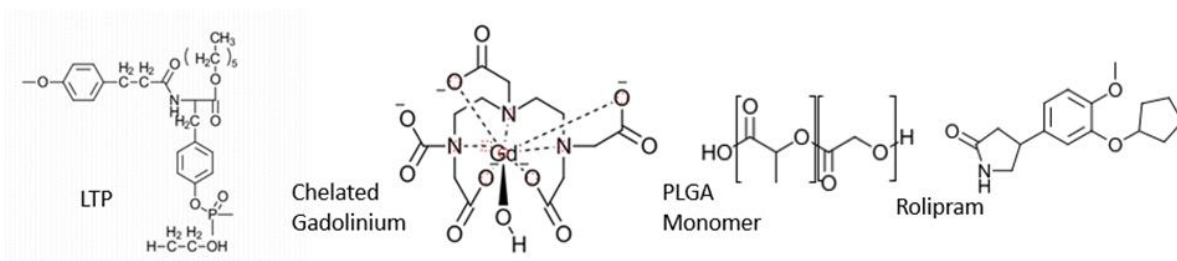


Figure 2: Structures of modified L-Tyrosine Phosphate (LTP), poly(lactic-co-glycolic acid) (PLGA) monomer, chelated gadolinium and rolipram. All structures were drawn using ChemDoodle software.

A microglia-like cell line, SIMA9, was used to test the biocompatibility of these particles. SIMA9 cells respond to inflammatory stimulation similar to primary microglial cells, providing a method of testing on primary microglia without the harvesting process.¹¹

We found that SIMA9 cells readily internalized nanoparticles. In addition, the formulations displayed limited toxicity even at high concentrations. Finally, we sought to measure whether nanoparticle uptake could trigger release of pro-inflammatory cytokines from cells, but we were unable to make a solid conclusion about cytokine release from our results.

Materials and Methods

Nanoparticle Synthesis and Characterization

The LPS and PLGA nanoparticles used in this study were synthesized by the Yun lab in Biomedical Engineering as previously described by using an oil and water emulsion via sonication and evaporation.¹² Physical characteristics of the nanoparticle, such as size and morphology, were then determined via scanning electron microscopy and dynamic laser light scattering.¹²

Cell Culture

All cells were cultured at 37°C and 5% CO₂ in DMEM media with 5% horse serum and 10% fetal bovine serum. Approximately every 4 days, a standard protocol for splitting adherent cells was used with minor additions for SIMA9 cells as they are semi-adherent. To account for the semi-adherence, all PBS wash steps were eliminated. Cell counting using a hemocytometer was also used to determine seed density for plates used in these experiments.

Immunofluorescence

In order to determine the uptake of nanoparticles by SIMA9 cells, confocal imaging was performed. Cells were plated at 10^5 cells/well on coverslips coated in poly-L-lysine, which was used to account for the semi-adherence of the cells as it acts as a synthetic attachment factor.¹³ Cells were subsequently treated with 20 $\mu\text{g}/\text{mL}$ or 200 $\mu\text{g}/\text{mL}$ of nanoparticle (LTP BSA-FITC) and left to incubate overnight (See Table 1 for plate set-up). If the cells were also being treated with LPS (100 ng/mL), this was done 24 hours prior to nanoparticle exposure. The cells were then fixed using 4% paraformaldehyde, blocked with 10 % FBS, and incubated with anti-F480 antibody conjugated to eFluro 570 (Texas Red) at a concentration of 5 $\mu\text{g}/\text{mL}$.

Determination of Cell Viability

To test the potential cytotoxicity of the nanoparticle two cell viability assays were used, the Thiazolyl Blue Tetrazolium Bromide (MTT) Assay (ThermoFisher Scientific) and a resazurin assay were performed. Spectrophotometry is used along with this assay to quantify MTT color change.¹⁴ Details on the nanoparticle treatments are given in **Table 1**. The absorbance of the plate was read at 590 nm. For each of the treatments, percent viability was calculated using the treatment mean divided by the control mean times 100.

Table 1: Detailed method for the preparation of nanoparticle solutions for MTT and resazurin assay treatment.

well #	1	2	3	4	5	6	7	8	9	10	11	12
Stock (ug/mL)	5000					2500	1000	500	100	50	50	10
vol stock (uL)	100	50	20	15	10	10	10	10	10	10	5	10
final conc (ug/mL)	5000	2500	1000	750	500	250	100	50	10	5	2.5	1
final volume (uL)	100	100	100	100	100	100	100	100	100	100	100	100
vol PBS (uL)		50	80	85	90	90	90	90	90	90	95	90
treatment (uL)	10	10	10	10	10	10	10	10	10	10	10	10

A resazurin assay was subsequently used because the MTT assay involved removing the media before testing and likely disrupted the cells, giving inconsistent results due to the semi-adherent nature of SIMA9 cultures. This resazurin assay involves using resazurin dye, which is reduced by metabolically active cells. This reduction visibly changes the dye from blue to red, which can be measured by spectrophotometric methods.¹⁵ This assay does not involve removing any media and does not have any wash steps, which was ideal for the semi-adherent cell line.

The protocol for the In Vitro Toxicology Assay Kit; Resazurin Based, from Sigma Aldrich, was used.¹⁵ The SIMA9 cells were plated on a 96-well plate and left to incubate overnight (see Appendix B for plate set up). Cells were treated with a range of concentrations of PLGA and PLGA-Rolipram nanoparticles (**Table 2**). After a second 24-hour incubation, each well was treated with 10 μ L of resazurin dye solution and incubated for 4 hours. The plate was then read for fluorescence at 590 nm. The fluorescence was corrected for by using the fluorescent reading of PBS subtracted from the average fluorescence reading for each treatment

of the two nanoparticles. This value was then divided by the fluorescence of PBS and multiplied by 100 to obtain % viability.

Table 2: Detailed method for the preparation of nanoparticle solutions for the Resazurin-based cell viability assay.

Well #		1	2	3	4	5	67	8	9	10	11	12
Stock (ug/mL)	5000				2500	1000	500	500	100	50	10	10
vol stock (uL)	100	50	20	10	10	10	10	5	10	10	10	5
final conc (ug/mL)	5000	2500	1000	500	250	100	50	25	10	5	1	0.5
final volume (uL)	100	100	100	100	100	100	100	100	100	100	100	100
vol PBS (uL)		50	80	90	90	90	90	95	90	90	90	95

Quantification of Cytokine Release after Nanoparticle Treatment

To measure cytokine release by SIMA9 cells after nanoparticle treatment, an enzyme-linked immunosorbent assay, or ELISA, was used. ELISA uses an enzymatic assay to determine the relative abundance of a particular protein in a sample.¹⁶ An ELISA was done on two different samples of SIMA9 cells that were treated with either a PLGA formulation or a PLGA-Rolipram formulation (each at 20 µg/mL) in the presence or absence of LPS (100 µg/mL). The media from these treated cells was collected and used in a 96-well plate set up (Appendix B). For the ELISA, the protocol from Affymetrix eBioscience ELISA kit was followed.¹⁷

Results and Discussion

We first examined the uptake and internalization of our nanoparticle formulations in SimA9 cells. Cultures were treated with nanoparticles containing BSA-FITC in the presence and absence of the immune stimulator LPS, a bacterial product that induces a pro-inflammatory M1

phenotype in these cells. Resting cells showed evidence of nanoparticle uptake and this was not accompanied by changes in activation state such as alterations in membrane morphology (**Figure 3A-B**). The treatment with nanoparticle and LPS also displayed nanoparticle uptake; however, there was evidence of microglial activation due to LPS stimulation. Prominent membrane protrusions are observed on the microglia and this indicates the cells are activated and internalizing the nanoparticles (**Figure 3C**). While activation altered the shape of the nanoparticles, there did not appear to be an increase in the amount of nanoparticles internalized, although quantification would be necessary to definitively determine this.

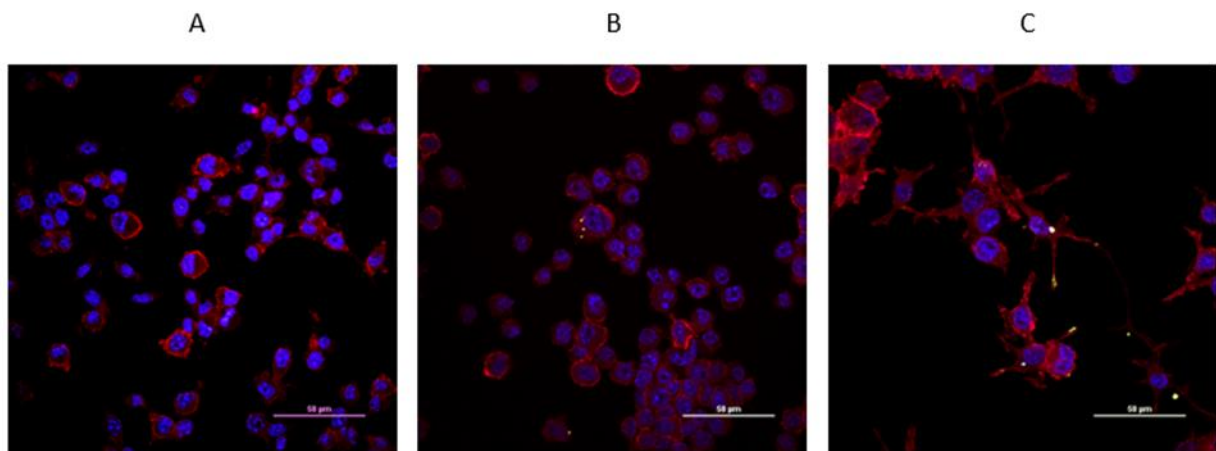


Figure 3: SIMA9 cells internalize polymeric nanoparticles. A) control SIMA9 containing no nanoparticle, no LPS). Blue stain is DAPI (nucleus) and red stain is anti F480 (SIMA9/microglia surface marker). B) SIMA9 cells treated with BSA-FITC nanoparticle (20 µg/mL for 24 hours).. The yellow dots are BSA-FITC nanoparticles. C) SIMA9 cells treated with BSA-FITC nanoparticle (20 µg/mL for 24 hours) after treatment with LPS (100 ng/mL for 24 hours). Magnification is 60X.

To determine potential cytotoxicity of the nanoparticle formulations, MTT assays were performed. MTT is a common assay used to determine cell viability. In this assay, MTT will react and undergo a color change (reduction) when it encounters oxidoreductase enzymes that are NADH dependent. Because the presence of these enzymes indicates NADH levels, the MTT reduction can be used to determine metabolic activity.¹⁴ The results of the two MTT experiments are shown in **Figures 4** and **5**. SIMA9 cells were treated with increasing concentrations of two different nanoparticle formulations for 24 hours followed by viability measurements.

In the first MTT assay, it appears that there is no correlation between the amount of viable cells and the concentration of nanoparticle in the treatment (**Figure 4A, B**). In addition, some of the treatment groups had over 100% viability. We performed a second assay to determine the reproducibility. However, the second MTT assay (**Figure 5A, B**) not only shows no correlation between treatment concentration and percent viability but also yielded different values. In the second MTT there were also several values for percent viability for the Gd-DTPA nanoparticle that were over 100%. However, this could indicate that the cells proliferated during the assay. Because these two assays yielded different results, it cannot be determined if there is or is not a correlation between viability and concentration. However, it appears that the nanoparticles do not cause significant cytotoxic effects as almost all the treatments across the two trials show % viability over 80%. This is consistent with previous research on the biocompatibility of these nanoparticles.^{6,7}

One possible explanation for the variability of these MTT assays is the protocol of the assay calls for the tissue culture medium to be removed and samples are washed for several wash steps. As SIMA9 cells are semi-adherent, these methods likely disturb the cells, which may be

the reason that there was no consistent trend of viability as some wells had more cells undisturbed than others.

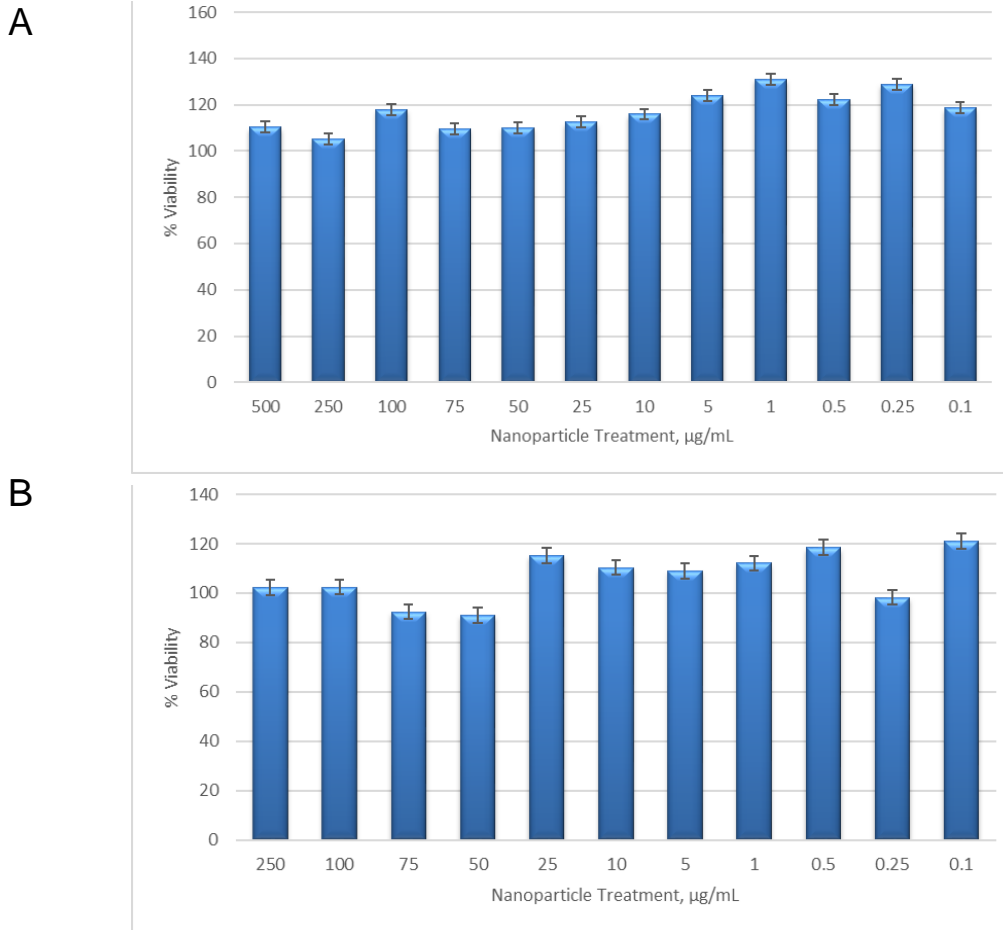


Figure 4: Viability of SIMA9 cells treated with polymeric nanoparticles. Results from the first MT with MTT assay. Cells were treated with increasing concentrations of Gd-DTPA loaded LTP nanoparticles (A) or BSA-FITC LTP nanoparticles (B) for 24 hours followed by MTT assay. N = 3 per treatment.

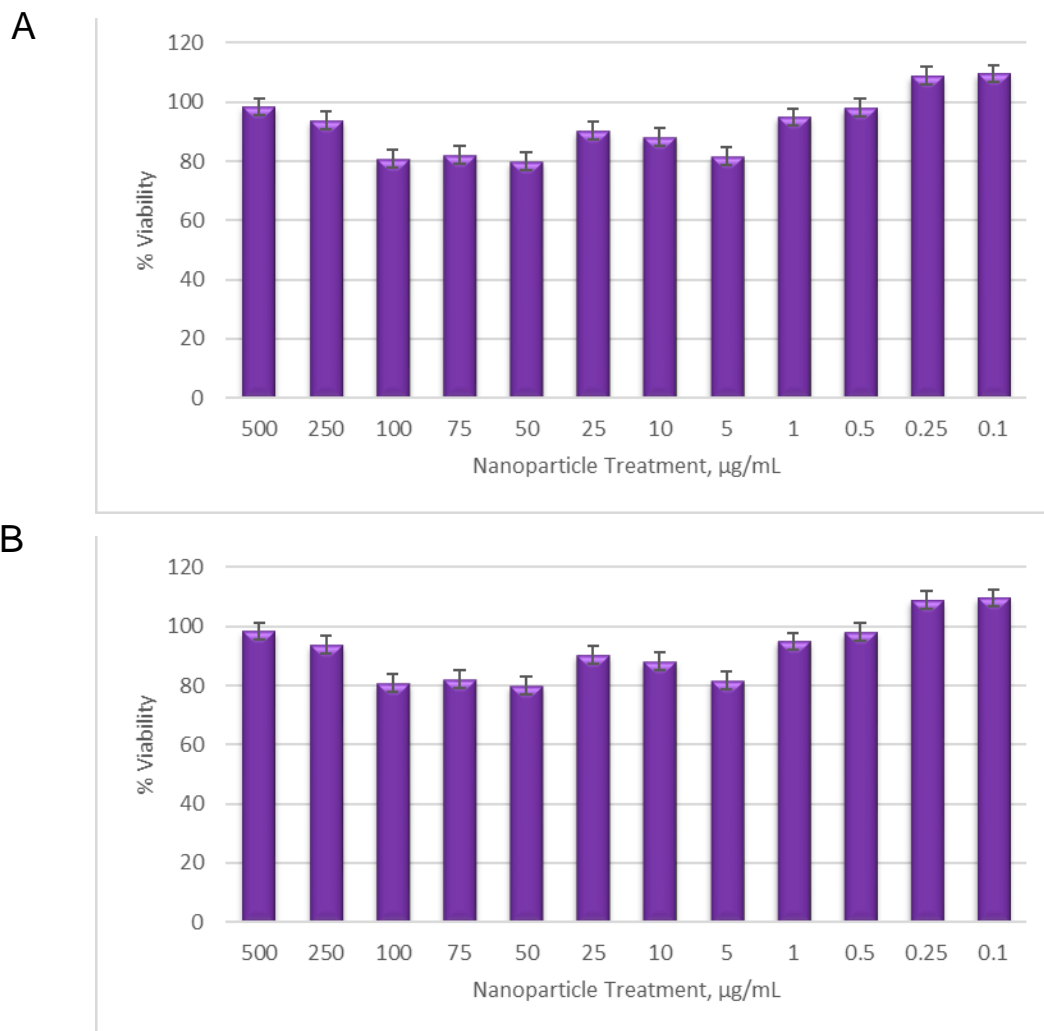


Figure 5: Viability of SIMA9 cells after LTP nanoparticle treatment. MTT was repeated to measure toxicity of Gd-loaded nanoparticles. Cultures of SIMA9 cells were treated with increasing concentrations of Gd-DPTA particles (A) or BSA-FITC LTP nanoparticles (B) and viability was measured after a total of 24 hours of treatment by MTT. N = 3 per treatment.

To obtain more reliable results, a resazurin-based cytotoxicity assay was used. In this assay, a reduction of resazurin causes a color change from blue to red, which indicates metabolically active cells.¹⁵ The fluorescence of the plate was read at 590 nm and the resulting values were corrected and converted to % viability. The results of this assay are shown in **Figure 6**. Based on the results of the resazurin-based assay, it is difficult to determine a pattern between concentration of nanoparticle in the treatment and cell viability. In the PLGA treatment, the % viability did not correlate with amount of treatment. However, it also does not appear that PLGA

has a significant effect on the overall viability of the cells as the lowest % viability value was near 77%, which corresponded to a treatment of .005 $\mu\text{g/mL}$. These results are similar to our LTP nanoparticle formulations. Because this treatment is nearly negligible, it is likely that this low viability can be attributed to an inaccurate cell concentration (cells/well). This could also explain the % viability values over 100%. While the PLGA-Rol nanoparticles still did not display a correlation between cell viability and treatment concentration, the results were more consistent than those of PLGA. They also showed higher % viability values overall, so based on these data the PGLA-Rol nanoparticle do not affect cell viability. Overall, these results indicate that these two nanoparticle formulations are likely not cytotoxic to microglia-like cells, which again confirms what has been shown in previous studies.^{6,7}

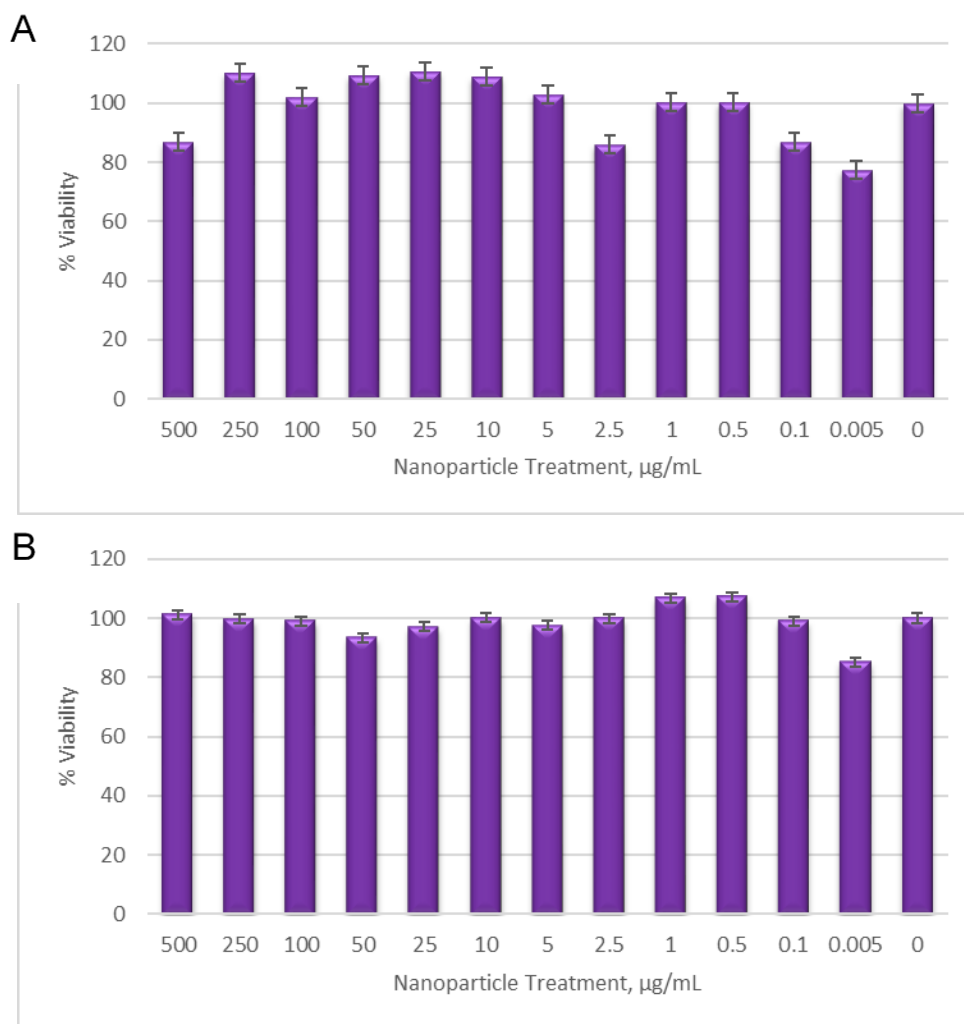


Figure 6: Viability of SIMA9 cells after PLGA-Rol nanoparticle treatment. Resazurin-based assay was used to measure toxicity of Rolipram-loaded nanoparticles. Cultures of SIMA9 cells were treated with increasing concentrations of PLGA particles (A) or PLGA-Rol nanoparticles (B) and viability was measured after a total of 24 hours of treatment by resazurin. N = 3 per treatment.

To determine if the nanoparticle causes activation of the microglia, an ELISA was performed to measure release of the pro-inflammatory cytokine, tumor necrosis factor (TNF). TNF can be released by microglia upon activation to a pro-inflammatory M1 phenotype and will result in activation of other immune cells in to promote inflammation.⁴ To use these

nanoparticles to treat inflammatory CNS diseases, the formulation should not induce an inflammatory response by itself. This would indicate that the microglia are activated by the nanoparticle and it cannot be used as a delivery system for contrast agent detect early activation of microglia.

The optical density, measured at 450 nm, was subsequently used to determine the concentration of TNF- α in the samples, by constructing a standard curve using known concentrations of TNF- α stock solutions (see materials and methods and **Figure 7**). Using this curve, the average absorbance readings of standards were converted to concentrations (see Table 3) by using the Beer-Lambert Law.¹⁸ In addition to averages for each treatment, the standard deviation was also calculated to determine the coefficient of variation percentage, CV%. The higher this value is, the more variation has occurred in the data, meaning there is greater inconstancy.

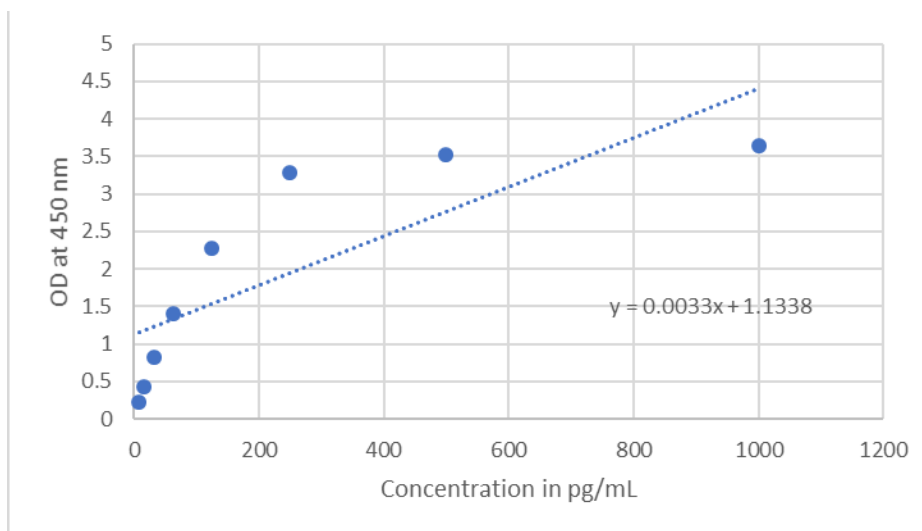


Figure 7: Standard curve of known concentrations of TNF- α . Protein concentrations ranged from 7.8 pg to 1000 pg.

Because the standard curve generated from this ELISA was not linear, the calculated values for concentration of TNF- α cannot be used to make any conclusions about nanoparticle

activation. The error in the standard curve can be attributed to inaccurate pipetting as well as potential contamination during wash steps. While no conclusions about nanoparticle activation of microglia were made from the ELISA, the immunofluorescence images show that the cells treated with only nanoparticles did not display morphology of activation as the cells treated with LPS. This could indicate that the nanoparticle does not cause microglial activation upon uptake. This would further support the role of these nanoparticles as drug delivery agents. A potential future study could look at nanoparticle uptake in primary glia to determine if nanoparticle uptake is specific to microglia when compared to all glial cells.

Conclusions

The goal of this study was to determine the potential use of nanoparticles as a CNS drug delivery system by observing nanoparticle uptake, cytotoxicity and cytokine release upon uptake. Overall, the results support that the nanoparticle is taken up by microglia-like cells and is likely not toxic, which is in support of previous studies.^{5,6,7} These data do not confirm or deny potential activation of microglia due to nanoparticle uptake as no conclusions could be drawn from the ELISA. More conclusions could be drawn if a repeat ELISA was performed. Further experimentation on the effects of different nanoparticle formulations as well as determination of preferential uptake of nanoparticle by microglia could help give a more complete understanding of the effectiveness of nanoparticles as drug delivery systems for the CNS.

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Appendix A- Safety Considerations

Appendix B- Experimental Plate Set-Ups

Appendix A- Safety Considerations

Precautions were taken to ensure safety during all steps of this experiment. Nitrile gloves were worn at all times to avoid contact of any cells/media with the skin. Any piece of equipment that encountered cells and/or media was discarded in a biohazard waste container. All cells/media were handled under a laminar flow hood. With each use of the laminar flow hood, 70% ethanol was used to wipe down the hood surface to maintain a sterile environment. All pipets were placed in a disposal container after use. All work in the lab was done under the supervision of a graduate student.

Appendix B- Experimental Plate Set-Ups

PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA
PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA
PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA	PLGA
PBS	PBS	PBS	cells-no assay								
PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol
PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol
PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol	PLGA-Rol
assay only			PLGA-no cells			media only-no assay			PLGA-Rol- no cells		

Table B1: Plate set up for Resazurin-based cell viability assay. In each of the three-well controls, no assay indicates that no resazurin was used and assay only means resazurin was added.

	Well #	1	2	3	4	5	6	7	8	9	10	11	12
Row Letter	A	1000	500	250	125	62.5	31.25	15.625	7.8125			plate blank	
	B				standards								
	C												
	D												
	E	control						control					
	F	LPS (100 ng/mL)						LPS (100ng/mL)					
	G	LPS+PLGA						LPS+PLGA-Rol					
	H	plga (20µg/mL)						PLGA-Rol (20µL/mL)					

Table B2: Plate set up for ELISA using PLGA and PLGA-Rolipram nanoparticle formulations. The light blue area represents the media from cells treated with the indicated treatments of PLGA and the dark pink area represents the media from cells treated with the indicated treatments of PLGA-Rolipram.