Interactions of silica nanoparticles with therapeutics for oxidative stress attenuation in neurons

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ABSTRACT

Oxidative stress plays a major role in many disease pathologies, notably in the central nervous system (CNS). For instance, after initial spinal cord injury, the injury site tends to increase during a secondary chemical injury process based on oxidative stress from necrotic cells and the inflammatory response. Prevention of this secondary chemical injury would represent a major advance in the treatment of people with spinal cord injuries. Few therapeutics are useful in combating such stress in the CNS due to side effects, low efficacy, or half-life. Mesoporous silica nanoparticles show promise for delivering therapeutics based on the formation of a porous network during synthesis. Ideally, they increase the circulation time of loaded therapeutics to increase the half-life while reducing overall concentrations to avoid side effects. The current study explored the use of silica nanoparticles for therapeutic delivery of anti-oxidants, in particular, the neutralization of acrolein which can lead to extensive tissue damage due to its ability to generate more and more copies of itself when it interacts with normal tissue.

Both an FDA-approved therapeutic, hydralazine, and natural product, epigallocatechin gallate, were explored as anti-oxidants for acrolein with nanoparticles for increased efficacy and stability in neuronal cell cultures. Not only were the nanoparticles explored in neuronal cells, but also in a co-cultured \textit{in vitro} model with microglial cells to study potential immune responses to near-infrared (NIRF)-labeled nanoparticles and uptake. Studies included nanoparticle toxicity, uptake, and therapeutic response using fluorescence-based techniques with both dormant and activated immune microglia co-cultured with neuronal cells.

\textbf{Keywords:} Silica nanoparticles, NIRF, nanomedicine, nanotoxicity, ROS, inflammation, spinal cord injury

1. INTRODUCTION

1.1 Neuronal Injury

Inflammation and oxidative stress are standard characteristics found in neuronal injury, such as multiple sclerosis (MS) and spinal cord injury (SCI). Part of the oxidative stress cascade is the presence of highly reactive aldehydes that react not only with proteins but also with lipids. In the central nervous system, the presence of lipids is abundant both in cell membrane and the myelin sheaths, which are critical for sending electrical signals back and forth.

Acrolein is a reactive aldehyde of particular interest in both MS and SCI. \textit{In vivo} models have found that acrolein can persist after SCI, worsening the initial injury.\textsuperscript{1} Acrolein levels are also increased in mouse models of MS.\textsuperscript{2} Such oxidative stress is produced by various means. In an initial injury, necrotic cell death releases proteins and molecules haphazardly. Enzymes from lysosomes and free radicals from the mitochondria can cause damage to cells that may be either mildly injured or unharmed. Upon exposure, the initial injury site grows as the surrounding areas attempt to mitigate the effects of such release. Beyond that, the immune system releases factors and creates an area of inflammation.

Clinically, the immunomodulator methylprednisolone is used to reduce the inflammation and pain caused by such trauma. Researchers continue to explore potential treatments or find ways to deliver methylprednisolone. These treatments vary from small molecules to complex natural products. Hydralazine, an anti-hypertensive drug, has been explored for its effects against acrolein both \textit{in vitro} and \textit{in vivo}.\textsuperscript{2–4} The azine group (N-N) actively undergoes a Michael
addition with the carbonyl of acrolein to form a Schiff base. Even when acrolein reacts with amino acids in proteins, the carbonyl is free to react with hydralazine, making it rather promising. Other molecules include natural products, notably polyphenols.\textsuperscript{5–7} They react and scavenge acrolein at ratios higher than small molecules like hydralazine. Unfortunately, though, natural products are limited by their bioavailability and their anti-oxidant mechanisms are poorly understood. In the case of small molecules, they have other mechanisms of action that may cause side effects.

1.2 Nanomedicine

Due to the limited capacity of many therapeutics, the field of nanomedicine is flooded with researchers attempting to improve therapeutic delivery through various means. Broadly, nanoparticles can be classified into two categories, inorganic and organic, but researchers have also begun to combine them for their various properties.

Inorganic nanoparticles are quantum dots and those made of gold, silver, and iron oxide. They have properties mostly used for imaging (PET, MRI, EM) and diagnostics but often require some type of "biocompatibility" coating after the initial formation, which tends to require simpler chemistry than that of organic nanoparticles. Therapeutics must be tethered to the nanoparticles, which is not necessarily ideal for small molecules. Another factor that affects their use for nanomedical purposes is cytotoxicity, and heavy metal leaching in quantum dots have been a particular cause for toxicity concerns.

Liposomes, polymeric nanoparticles, and silica-based nanoparticles could be classified under organic nanoparticles in that they do not have an inherent imaging property and require some type of "label." The molecules used are generally designed to be inherently biocompatible to reduce the need for an extra step or prevent cytotoxicity. With that, the need for extensive chemical synthesis often becomes a major factor in creating the nanoparticles. Therapeutics undergo encapsulation in liposomes and polymeric nanoparticles and adsorption for silica nanoparticles, but their size can then extend into the micron range.

With a proper combination of properties and characteristics, nanomedicine may help to improve various properties of therapeutics with unrealized potential. Preventing metabolism, aiding in uptake, increasing specificity, and increasing the half life of therapeutics are all goals in the field with applications in a huge range of medical problems and disease.

1.3 Using silica-PEG nanoparticles for neurological injury

The current study highlights the use of PEGylated silica nanoparticles for their use in therapeutic delivery specifically for neurological injury. Silica nanoparticles allow the small molecule hydralazine and natural product epigallocatechin gallate (EGCG) to adsorb to the nanoparticle surface with the intention of prolonging their activity against acrolein and other oxidative species. The nanoparticles were studied in both a single-cell and co-culture system using neurons and microglial cells. The interactions of the nanoparticles and their response in vitro were studied.

2. MATERIALS AND METHODS

2.1 Synthesis of PEGylated silica nanoparticles

The silica nanoparticles were synthesized using a modified Stöber method.\textsuperscript{8,9} 20 mg of hydrazide-modified methoxy poly(ethylene) glycol (mPEG-Hz) MW5000 (0.004 mmol) (Laysan Bio LLC, Auburn, AL) was dissolved in 6 mL of methanol. Upon dissolution, 750 µL of 2 M NaOH (1.5 mmol) was added to the mixture and vortexed. After 1 hr of mixing, 64 µL of tetramethylorthosilicate (0.434 mmol) (TMOS, Sigma, St. Louis) was added to the mixture and vortexed. The solution was vortexed at 25°C for an additional hour. To remove most of the solvent, the milky solution was added to a 30 kDa Amicon membrane filter (Millipore, Billerica, MA) and centrifuged for 15 minutes at 2800 g. The filtrate was removed, and 5 mL of nanograde water was added to the filter, suspending the white precipitate, and spun again for 10 minutes. The solution was aged for 15 days before 3 mL of nanograde water was added to the filter and spun for 15 minutes. Residual methanol was removed via evaporation. Concentration of the nanoparticle solution was obtained gravimetrically by solvent evaporation under vacuum. For use in cell culture, the nanoparticle samples were sterile filtered using a 0.2 µm syringe filter and diluted to the appropriate concentrations in phosphate buffered saline (PBS).

2.2 Characterization of loaded nanoparticles

Solutions of 1 mg/mL of nanoparticles were loaded with hydralazine (1600 µg/mL, 25% EtOH) at concentrations of 0 ug/mL, 100 ug/mL, 250 ug/mL, and 500 ug/mL, corresponding to 0, 10, 25 and 50-percent loading by mass. The hydrodynamic diameter of the nanoparticle population was measured using a Malvern Zetasizer ZS. An average of three measurements was used to determine the average hydrodynamic diameter.
2.3 Cell culture of B35 neuroblastoma cells

B35 rat neuroblastoma cells were obtained from American Tissue Culture Collection (ATCC, Manassas, Virginia) and cultured in T-25 or T-75 flasks with DMEM (Sigma) media containing 10% fetal bovine serum (FBS). The cells were split twice per week and seeded in concentrations from 1:5 to 1:10.

2.4 Cell culture of C8-B4 microglial cells

C8B4 mouse glioblastoma cells were obtained from American Tissue Culture Collection (ATCC, Manassas, Virginia) and cultured in T-25 or T-75 flasks with DMEM (ATCC) media containing 10% fetal bovine serum (FBS). The cells were split upon reaching at least 80% confluency (7-14 days) and seeded to maintain at least 1x10^5 cells/mL.

2.5 Cellular responses to loaded nanoparticles

B35 cells were seeded overnight in a Cyntellect LEAP plate at 4 x 10^5 cells/mL (20,000 cells/well) in DMEM with 10% FBS. Varying concentrations of hydralazine, EGCG, and nanoparticles loaded with hydralazine and EGCG were added to the cells and incubated for 24 hours. After 24 hours, the cells were washed with DPBS twice, and they were incubated with 2 uM calcein am and 10 uM DHE for 30 minutes. The excess dye was washed away, and the cells were analyzed in DMEM with 10% FBS and 10 ug/mL Hoechst 33342 using LEAP. The analysis settings were optimized using the positive and negative controls before obtaining data from all samples. The data was exported for post-processing in Excel.

2.6 Co-culture model: measuring oxidative stress with dihydroethidium (DHE)

Dihydroethidium (Cayman Chemical, Ann Arbor, MI) is a compound that, once activated by reactive oxygen species, integrates with DNA and fluoresces red. B35 and C8-B4 cells were seeded overnight in a Cyntellect LEAP plate at 1.5 x 10^5 cells/mL and 5 x 10^4 cells/mL (1500 cells/well and 500 cells/well in 384-well plate), respectively, in DMEM with 10% FBS. Therefore, about 30% of the original population of each sample was C8-B4 cells, optimized before the experiment. Additional wells using only B35 cells and C8-B4 cells were also seeded at 2x10^5 cells/mL (2000 cells/well). Varying concentrations of nanoparticles and native mPEG-Hydrazide in DPBS were added to the cells and incubated for 24 hours. 200 uM hydrogen peroxide was used as positive control for oxidative stress. After 24 hours, the cells were washed with DPBS twice, and they were incubated in 10 uM DHE for 30 minutes. The excess dye was washed away, and the cells were analyzed in DMEM with 10% FBS and 10 ug/mL Hoechst 33342 using LEAP. The analysis settings were optimized using the positive and negative controls before obtaining data from all samples. The data was exported for post-processing in Excel.

2.7 Co-culture: Cell viability

To determine specific viability of the cells after nanoparticle incubation, B35 and C8-B4 cells were seeded overnight in a Cyntellect LEAP plate at 1.5 x 10^5 cells/mL and 5 x 10^4 cells/mL (1500 B35 cells/well and 500 C8-B4 cells/well in 384-well plate) in DMEM with 10% FBS. Therefore, about 30% of the original population of each sample was C8-B4 cells, optimized before the experiment. Additional wells using only B35 cells and C8-B4 cells were also seeded at 2x10^5 cells/mL (2000 cells/well). Varying concentrations of nanoparticles and mPEG-Hydrazide in DPBS were added to the cells and incubated for 24 hours. 200 uM hydrogen peroxide was used as a positive control. Before running the experiment, the optimal dye concentrations were determined using untreated cells and cells treated with the positive control. After 24 hours, the cells were washed with DPBS twice, and they were incubated in 2 uM calcein am green for 30 minutes. The excess dye was washed away, and the cells were analyzed in DMEM with 10% FBS and 10 ug/mL Hoechst 33342 using LEAP. The analysis settings were optimized using the positive and negative controls before obtaining data from all samples. The data was exported for post-processing in Excel.

2.8 Statistical analysis

To determine statistical significance, ANOVA was performed on each data set. For pair wise comparisons, a post hoc test using the Least Significant Difference (LSD) was used. P-values less than 0.01 were considered significant.
3. RESULTS AND DISCUSSION

3.1 Characterization of mPEG-Hz modified nanoparticles

The silica nanoparticles were prepared to incorporate the polymer into the silica network (Figure 1), which eliminates an additional PEGylation step that is common in many other synthesis schemes. In the current study, the PEG serves a dual purpose. PEG is nanomedicine's "stealth" molecule, as it has been shown to prevent opsonization. Specifically for spinal cord injury, PEG serves as a target for injured neurons and associates with them, aiding in membrane repair. Additionally, the hydrazide moiety on the end of the PEG is free for labeling with a fluorescent probe for detection in later experiments.

Nanoparticles were diluted to a concentration of 1 mg/mL and loaded with a stock solution of hydralazine. The average hydrodynamic diameter and PDI of each sample is shown in Table 1. As the concentration of hydralazine increased, the size of the nanoparticles increased. Based on how the average diameter is calculated, the 25% loaded sample shows an average of 34.4 nm, but the sample contained two populations of similar intensity: 18 nm and 56 nm. It is possible the concentration was similar to that in a buffered titration curve, and at 50% loading, it has overcome a threshold to have a more uniform distribution of nanoparticles with hydralazine. The PDI increases between the 10% and 25%-loaded nanoparticles but decreases again in the 50%-loaded sample.

Table 1. Size of nanoparticles loaded with hydralazine

<table>
<thead>
<tr>
<th>Loading (%)</th>
<th>Diameter (nm)</th>
<th>St. Dev. (nm)</th>
<th>PDI</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>14.18</td>
<td>2.23</td>
<td>0.262</td>
</tr>
<tr>
<td>10</td>
<td>15.68</td>
<td>0.95</td>
<td>0.285</td>
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<td>25</td>
<td>34.40</td>
<td>2.41</td>
<td>0.302</td>
</tr>
<tr>
<td>50</td>
<td>275.9</td>
<td>13.91</td>
<td>0.295</td>
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</table>

3.2 Therapeutic interactions and activity

In attempts to create an appropriate in vitro model for SCI, the nanoparticles showed no benefit over free therapeutics. Various problems may account for the lack of efficacy:

1) The interaction of the nanoparticles and the therapeutic negatively affects the cell behavior through a change in either a physical or chemical characteristic.

2) The nanoparticles may be releasing the treatment too slowly to overcome the bolus dose of acrolein in the models, resulting in an increase in cell death before the concentration becomes effective. In the case of the natural product epigallocatechin gallate (EGCG), it prolongs the activity of EGCG, which can become a pro-oxidant and pro-apoptotic compound at high enough concentrations, negatively affecting the cells even further.

3) The cell model is not ideal for the type of in vivo problem that is being simulated.

In an attempt to test first of these hypotheses, the cells were incubated with the treatments and treatment-loaded nanoparticles and toxicity assays were performed after 24 hours. The results for hydralazine are shown in the Figure 2. The cells incubated with hydralazine-loaded nanoparticles behaved no differently than those exposed only to hydralazine.
Nothing showed any differences that were statistically significant.

The interaction was not detrimental to the cells, but it does not address the hypothesis that the nanoparticles may be releasing the treatments slowly, both extending their activity and keeping the concentration too low to be beneficial against acrolein. On the other hand, the experiment with EGCG proved slightly different (Figures 3 and 4). At high concentrations, EGCG can become a pro-oxidant rather than an antioxidant. Additionally, in solution, EGCG will polymerize or undergo modifications. For most of the concentrations, the EGCG was not problematic. With EGCG alone, 50 uM was detrimental to cells in both ROS production and cell number. The next lowest concentration, 37.5 uM was detrimental only to the cell number. On the other hand, at both 37.5 uM and 50 uM with the nanoparticles, the ROS was increased, which suggests a higher pro-oxidant activity when loaded into the nanoparticles, and the cell number was decreased, which was similar to the free EGCG.
The nanoparticles may be protecting the EGCG from degradation. Additionally, it is more difficult to predict the response of a therapeutic when the cells are likely taking up the nanoparticles. With cells "eating" the nanoparticles, the effective concentration may be higher in some of the cells than in others. It would increase the EGCG concentration in certain cells that uptake the nanoparticles, making the EGCG more effective in those cells.

Figure 4. ROS production of EGCG-loaded nanoparticles. ROS was increased in the 50 uM, EGCG-only sample, but the ROS was increased in both the 37.5 uM and 50 uM samples with EGCG-loaded nanoparticles. ** indicates a significant difference compared to the corresponding control at P < 0.01.

3.3 Nanotoxicity in microglial cells

For clinical translation, nanoparticles would have to interact with immune cells within the central nervous system. C8-B4 cells are a microglial cell line with macrophage markers and characteristics. They were obtained from mice and have been shown to have phagocytic properties.18 Determining how the macrophage-like cells react to nanoparticles is important for clinical translation. Upon activation, such cells release inflammatory factors, ROS, and RNS.19
C8-B4 cells, immortal glioblastoma cells, were exposed to mPEG-Hz nanoparticles for 24 hours and stained with DHE and Hoechst 33342 (Figure 5). The cells reacted negatively to the nanoparticles, despite the PEG coating. The amount of ROS increased significantly as the amount of nanoparticles increased. The PEG used in the synthesis of the nanoparticles was incubated with the different cells types both alone and together. In that experiment, the PEG did not produce a response seen with the nanoparticles (Figure 6). Therefore, it is likely that the cells are reacting to the nanoparticles.

It was also critical to look at viability of C8-B4 cells after nanoparticle exposure (Figure 7). The viability of the cells decreased significantly as the nanoparticle concentration increased. The cell number was mostly consistent, with the exception of the largest concentration. Contrary to the response from the B35 cells, there is a larger amount of dead cells within the population rather than demonstrating a high viability and low cell number. The concentrations affecting the C8-B4 cells were much lower than those that affected the B35 cells, showing a increased sensitivity to the nanoparticles by cells involved in an immune response.
Figure 7. Viability and cell number of C8-B4 cells after exposure to nanoparticles. "*" indicates a significant difference compared to the corresponding control at $P < 0.01$.

Figure 8 contains the LEAP images obtained for the C8-B4 cells. The increase in ROS production at 100 ug/mL of nanoparticles is clearly evident, as is the significant decrease in cell number. Morphologically, the C8-B4 cells become more rounded and larger, likely from "eating" large numbers of nanoparticles. Additionally, if the cells are dying from exposure to the nanoparticles, they may be "eating" the dead cells. Additional studies, such as confocal microscopy, may give an indication of what is causing activation: the nanoparticles or nearby cell death from the nanoparticles.
3.4 Nanotoxicity in neuronal co-culture model

ROS was produced in C8-B4 cells upon incubation with the nanoparticles. A co-culture model was devised with 30% of seeded cells containing the C8-B4 cells, and the remaining cells were the B35 neurons. Similar concentrations were used at those used with only C8-B4 cells: 25 ug/mL-100 ug/mL. Similar to the results with C8-B4 cells, all of the concentrations produced some ROS, with the exception of the lowest concentration of 25 ug/mL (Figure 9). The ROS level was much lower than that seen in just the C8-B4 cells, but the production of ROS could lead to problems with the normal cells.
Beyond looking at the ROS production, the viability and cell number were also studied in the co-culture model. The C8-B4 cells had a decrease in both, but in the co-culture model, no significant effects were observed (Figure 10). In the case of the co-culture model, two factors are likely involved in the reduced response. First, the amount of microglial cells is significantly lower. Additionally, the neurons are likely taking in the nanoparticles, reducing the amount for the microglial cells to uptake and react to negatively. The nanoparticle numbers used in the assays are still lower than most of those needed for therapeutic delivery. The LEAP images in Figure 11 show the slight increase in ROS observed.

Figure 9. ROS response to nanoparticles after 24 hours in co-culture with B35 neurons and C8-B4 microglial cells. Thirty percent of seeded cells were C8-B4 cells. *** indicates a significant difference compared to the corresponding control at P < 0.01.

Figure 10. Viability and cell number in co-culture model after nanoparticle exposure. Neither viability nor cell number was affected.
Toxicity experiments have indicated little toxicity to B35 cells after 24 hours. In similar experiments with microglial cells, the immune cells of the CNS, a significant response was observed at concentrations much less than those used for the B35 cells. High levels of ROS were observed, and both viability and cell number decreased. In a co-culture model with both types of cells, ROS was affected, but cell number and viability were not. Fortunately, the numbers used were lower than those needed for therapeutic delivery.

With the current knowledge, though, any in vivo studies would have to account for a potential immune response that could be elicited by the nanoparticles. The model may also be useful for studying different nanoparticle formulations, as well. Determining a nanoparticle formulation that reduces the negative response would give an indication of efficacy early in a study.

### 3.5 Co-culture inflammation model

Based on previous experiments, an *in vitro* model was devised to simulate inflammation with the goal of simulating neurological injury. Microglial cells are activated by lipopolysaccharide (LPS) from *E. coli*, which can cause damage to the surrounding neurons. As a control experiment, both types of cells were exposed to the LPS for 24 hours (Figure 12). The LPS did not affect the B35 neuroblastoma cells, but it caused a response in the C8-B4 microglial cells. A co-culture of the two cells types with the addition of LPS may be a suitable *in vitro* model for inflammation before moving to *in vivo* studies.
4. CONCLUSIONS

The project at hand will require fine-tuning of the nanoparticles to improve the therapeutic delivery response, which is a major limitation seen in studies up to this point. Because of the synthesis technique of silica nanoparticles, commercial companies have begun to make them available for researchers. Unfortunately, though, most modifications, such as PEGylation, then must be done by the scientist. In the current study, the synthesis relies on the presence of PEG to reduce synthesis steps, but a more sophisticated process may be necessary to achieve sustained release and reduced toxicity. Overall, though, the current experiments demonstrated a change in therapeutic activity, but whether the change is beneficial remains unclear.

Another limitation of the study involved the lack of a valid cellular model for spinal cord injury. Further work should explore and determine a model that exhibits similar characteristics in vitro. Optimization of the inflammation model would provide another avenue of neurological injury to explore, and technologies are available to determine what type of ROS is being produced in such a model.

The response of the microglial cells when exposed to the nanoparticles is another avenue of research. The concentrations of nanoparticles used were higher than those used for therapeutic delivery, but it provides a glimpse of what may be witnessed upon translation into an animal model. More studies with them could explore the rate at which the cells are taking in the nanoparticles and how that would affect the uptake by non-phagocytic cells. On the other hand, if the nanoparticles are preferentially "eaten" by the phagocytic cells, it may provide insight into ways of targeting such cells and modulate the activation without causing cell death.

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