EVALUATING THE DIFFUSIVE CAPABILITIES OF POLYMERIC NANOPARTICLES IN MUCOSAL ENVIRONMENTS

By

HENA HAQUE

BS, SIMMONS UNIVERSITY, 2022

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Graduate Program of Biomedical Engineering at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2025

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

As the sole author of this thesis, I authorize Brown University to lend it to other institutions for the purpose of scholarly research.

Date 05/01/2025 Hena Haque

HENA HAQUE, Author

I further authorize Brown University to reproduce this thesis by photocopying or other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Date 05/01/2025 Hena Haque

HENA HAQUE, Author

This thesis by Hena Haque is accepted in its present form by the Center for Biomedical Engineering as satisfying the thesis requirements for the degree of Master of Science

Date_____ Signature: _____

Dr. Edith Mathiowitz, Advisor

Date_____ Signature: _____

Dr. Anita Shukla, Reader

Date_____ Signature: _____

Dr. Eric Darling, Reader

Approved by the Graduate Council

Date_____ Signature: _____

Dr. Marissa Gray, Biomedical Engineering
Master's Program Director

Acknowledgements

Firstly, I would like to thank my advisor Dr. Edith Mathiowitz for being an amazing source of support and guidance throughout the past two years. She has always encouraged me to think outside of the box and conduct research based on passion and curiosity. Her mentorship has shaped me into a more confident version of myself, both inside and outside of the lab.

I would like to thank Kosta Milankovic and Vera Fonseca for their assistance with the diffusion and cell culture studies. Their patience and willingness to teach has made me a better scientist. I would also like to recognize Jiaying Yan and Will Borges for their previous work in the Mathiowitz Lab, they built a strong foundation with endless opportunities for future research with polymeric nanoparticles.

I would like to thank Dr. Anita Shukla and Dr. Eric Darling for their participation on my thesis committee, and Dr. Marissa Gray who has been supportive and helpful since my first day on campus. I would like to thank the previous and current members of the Mathiowitz Lab for always creating a safe and welcoming environment and providing guidance when needed.

I would like to thank my parents who have sacrificed more than I will know to get me to this point in my education and for continuously believing in me. I would like to thank my sister and brother-in-law for their unwavering support and love. Lastly, a special thank you to my fiancé who has been a constant source of encouragement, who listens to me talk endlessly about my research, and has been my biggest cheerleader these past two years

1.0 Introduction **1**
1.1 Oral Drug Delivery ***1***
1.2 Polymeric Nanoparticles ***1***
1.3 Gastrointestinal (GI) System and Cellular Uptake ***2***
1.4 Bio-coating ***4***
2.0 Materials and Methods **5**
2.1 Preparation of Coated Particles ***5***
2.2 Diffusion Coefficient ***7***
2.2.1 Fluorescence Microscopy 8
2.2.2 Particle Tracking Analysis 9
2.3 Nanoparticle Characterization ***10***
2.3.1 Zeta Potential 10
2.3.2 Dynamic Light Scattering 11
2.4 Cell Absorption Studies ***12***
3.0 Results and Discussion **15**
3.1 Diffusion Coefficient ***15***
3.1.2 Polystyrene 15
3.1.2.1 Polystyrene/Hyaluronic Acid *15*
3.1.2.2 Polystyrene/Mucin *18*
3.1.2.3 Polystyrene/Hyaluronic Acid/0.1% Mucin *20*
3.1.2.4 Polystyrene/35 KDa PEG *23*
3.1.2.5 Polystyrene/100 KDa PEG *25*
3.1.2.6 Polystyrene/300 KDa PEG *27*
3.1.3 Silica 29
3.1.3.1 Silica/Hyaluronic Acid *31*
3.1.3.2 Silica/Mucin *32*
3.1.3.3 Silica/35 KDa PEG *33*
3.1.3.4 Silica/100 KDa PEG *35*
3.1.3.5 Silica/300 KDa PEG *36*
3.1.4 PMMA 37
3.2 Nanoparticle Characterization: Zeta Potential & Dynamic Light Scattering (DLS) ***38***
3.2.1 Hyaluronic Acid 38
3.2.2 Polystyrene 40
3.2.2.1 Polystyrene/Hyaluronic Acid *40*
3.2.2.2 Polystyrene/Mucin *42*
3.2.2.3 Polystyrene/Hyaluronic Acid/0.1% Mucin *43*

3.2.2.4 Polystyrene/35 KDa PEG *45*
3.2.2.5 Polystyrene/100 KDa PEG *46*
3.2.2.6 Polystyrene/300 KDa PEG *47*
3.2.3 Silica 49
3.2.3.1 Silica/Hyaluronic Acid *49*
3.2.3.2 Silica/Mucin *50*
3.2.3.3 Silica/35 KDa PEG *52*
3.2.3.4 Silica/100 KDa PEG *53*
3.2.3.5 Silica/300 KDa PEG *54*
3.3 Cell Absorption Studies ***56***
3.3.1 0.1% Mucin Coated Polystyrene Nanoparticles 56
3.3.2 0.1% 100KDa PEG Coated Polystyrene Nanoparticles 61
3.3.3 0.1% 300KDa PEG Coated Polystyrene Nanoparticles 66
4.0 Conclusion **71**
5.0 Design Optimization and Future Directions **73**
5.1 Significance Testing ***74***
6.0 References **75**
7.0 Supplementary Information **77**
7.1 Nikon Ti2-E Widefield Microscope Settings ***77***
7.2 Image J Particle Tracker 2D/3D Code ***78***
7.3 Python Statistical Analysis Code ***80***
7.4 Malvern Zetasizer Settings ***84***
7.5 Significance Testing ***85***

Table of Figures

Figure 1: Diagram of Polymeric Nanoparticles

Figure 2: Diagram of Muco-adhesive vs. Mucus-Penetrating Nanoparticles

Figure 3: TEM Images of Polystyrene Particles In Vivo

Figure 4: Diagram Demonstrating Bio-coating

Figure 5: Coated Nanoparticle Preparation Schematic

Figure 6: Image of Fluorescent Polystyrene Nanoparticles in GI Mucin

Figure 7: Cuvettes Used in Nanoparticle Characterization

Figure 8: Setup of 12-well Transwell Plate for Cellular Uptake Studies

Figure 9: Mean Diffusion Coefficient of HA Coated PS NPs

Figure 10: Mean Diffusion Coefficient of Mucin Coated PS NPs

Figure 11: Mean Diffusion Coefficient of HA and 0.1% Mucin Coated PS NPs

Figure 12: Mean Diffusion Coefficient of 35KDa PEG Coated PS NPs

Figure 13: Mean Diffusion Coefficient of 100KDa PEG Coated PS NPs

Figure 14: Mean Diffusion Coefficient of 300KDa PEG Coated PS NPs

Figure 15: Mean Diffusion Coefficient of Uncoated Coated Silica NPs

Figure 16: Mean Diffusion Coefficient of HA Coated Silica NPs

Figure 17: Mean Diffusion Coefficient of Mucin Coated Silica NPs

Figure 18: Mean Diffusion Coefficient of 35KDa PEG Coated Silica NPs

Figure 19: Mean Diffusion Coefficient of 100KDa PEG Coated Silica NPs

Figure 20: Mean Diffusion Coefficient of 300KDa PEG Coated Silica NPs

Figure 21: Zeta Potential of HA Coated NPs

Figure 22: Zeta Potential and Size of HA Coated PS NPs

Figure 23: Zeta Potential and Size of Mucin Coated PS NPs

Figure 24: Zeta Potential and Size of HA and 0.1% Mucin Coated PS NPs

Figure 25: Zeta Potential and Size of 35KDa PEG Coated PS NPs

Figure 26: Zeta Potential and Size of 100KDa PEG Coated PS NPs

Figure 27: Zeta Potential and Size of 300KDa PEG Coated PS NPs

Figure 28: Zeta Potential and Size of HA Coated Silica NPs

Figure 29: Zeta Potential and Size of Mucin Coated Silica NPs

Figure 30: Zeta Potential and Size of 35KDa PEG Coated Silica NPs

Figure 31: Zeta Potential and Size of 100KDa PEG Coated Silica NPs

Figure 32: Zeta Potential and Size of 300KDa PEG Coated Silica NPs

Figure 33: Transcytosis of 0.1% Mucin Coated PS NPs

Figure 34: Cumulative Transcytosis of 0.1% Mucin Coated PS NPs

Figure 35: 0.1% Mucin Coated PS NPs Confined Within Caco-2 Cells

Figure 36: Transcytosis of 0.1% 100KDa PEG Coated PS NPs

Figure 37: Cumulative Transcytosis of 0.1% 100KDa PEG Coated PS NPs

Figure 38: 0.1% 100KDa PEG Coated PS NPs Confined Within Caco-2 Cells

Figure 39: Transcytosis of 0.1% 300KDa PEG Coated PS NPs

Figure 40: Cumulative Transcytosis of 0.1% 300KDa PEG Coated PS NPs

Figure 41: 0.1% 300KDa PEG Coated PS NPs Confined Within Caco-2 Cells

Table of Tables

Table 1: Coating Fabrication Concentrations

Table 2: Experimental Conditions for Cellular Uptake Studies

Table 3: Mean Diffusion Coefficient of HA Coated PS NPs

Table 4: Standard Deviations of HA Coated PS NPs

Table 5: Mean Diffusion Coefficient of Mucin Coated PS NPs

Table 6: Standard Deviations of Mucin Coated PS NPs

Table 7: Mean Diffusion Coefficient of HA and 0.1% Mucin Coated PS NPs

Table 8: Standard Deviations of HA and 0.1% Mucin Coated PS NPs

Table 9: Mean Diffusion Coefficient of 35KDa PEG Coated PS NPs

Table 10: Standard Deviations of 35KDa PEG Coated PS NPs

Table 11: Mean Diffusion Coefficient of 100KDa PEG Coated PS NPs

Table 12: Standard Deviations of 100KDa PEG Coated PS NPs

Table 13: Mean Diffusion Coefficient of 300KDa PEG Coated PS NPs

Table 14: Standard Deviations of 300KDa PEG Coated PS NPs

Table 15: Zeta Potential of HA Coated NPs

Table 16: Standard Deviations of Mean Zeta Potential of HA Coated NPs

Table 17: Zeta Potential and Size of HA Coated PS NPs

Table 18: Zeta Potential and Size of Mucin Coated PS NPs

Table 19: Zeta Potential and Size of HA and 0.1% Mucin Coated PS NPs

Table 20: Zeta Potential and Size of 35KDa PEG Coated PS NPs

Table 21: Zeta Potential and Size of 100KDa PEG Coated PS NPs

Table 22: Zeta Potential and Size of 300KDa PEG Coated PS NPs

Table 23: Zeta Potential and Size of HA Coated Silica NPs

Table 24: Zeta Potential and Size of Mucin Coated Silica NPs

Table 25: Zeta Potential and Size of 35KDa PEG Coated Silica NPs

Table 26: Zeta Potential and Size of 100KDa PEG Coated Silica NPs

Table 27: Zeta Potential and Size of 300KDa PEG Coated Silica NPs

Table 28: Transcytosis of 0.1% Mucin Coated PS NPs

Table 29: Standard Deviations of Mean Transcytosis of 0.1% Mucin Coated PS NPs

Table 30: Cumulative Transcytosis of 0.1% Mucin Coated PS NPs

Table 31: 0.1% Mucin Coated PS NPs Confined Within Caco-2 Cells

Table 32: Transcytosis of 0.1% 100KDa PEG Coated PS NPs

Table 33: Standard Deviations of Mean Transcytosis of 0.1% 100KDa PEG Coated PS NPs

Table 34: Cumulative Transcytosis of 0.1% 100KDa PEG Coated PS NPs

Table 35: 0.1% 100KDa PEG Coated PS NPs Confined Within Caco-2 Cells

Table 36: Transcytosis of 0.1% 300KDa PEG Coated PS NPs

Table 37: Standard Deviations of Mean Transcytosis of 0.1% 300KDa PEG Coated PS NPs

Table 38: Cumulative Transcytosis of 0.1% 300KDa PEG Coated PS NPs

Table 39: 0.1% 300KDa PEG Coated PS NPs Confined Within Caco-2 Cells

Abstract of Evaluating the Diffusive Capabilities of Polymeric Nanoparticles in Mucosal Environments, by Hena Haque, ScM., Brown University, May 2025

Oral drug delivery is a preferred route of administration for medication due to convenience and accessibility. However, due to enzymatic degradation in the gastrointestinal (GI) tract and poor diffusion through the mucosal barrier, oral drugs have low bioavailability. Polymeric nanoparticles (PNPs) can protect oral drugs from the harsh environment of our digestive tract, and their physicochemical properties can be tailored to enhance targeted drug delivery. One way to enhance the diffusion and cellular uptake of PNPs is through bio-coating – a physical coating of the particles with GI mucin[8]. Bio-coating can alter their surface charge and size – two factors that influence diffusion through GI mucin. 5 different polymeric coatings were evaluated to determine the effect that bio-coating has on diffusion, surface charge, and hydrodynamic diameter. Diffusion coefficients were determined through fluorescent particle tracking. Changes in zeta potential and hydrodynamic diameter as a result of bio-coating were monitored to evaluate the interactions between the coating, nanoparticle, and GI mucin. Finally, Caco-2 cells were utilized to evaluate the transcytosis and uptake of bio-coated nanoparticles while mimicking the human GI environment. By coating PNPs with extremely negatively charged solutions, such as hyaluronic acid, their diffusion in GI mucin can be enhanced and aggregation can be reduced. Coating them with slightly negatively charged solutions, such as 100KDa polyethylene glycol, also improves diffusion compared to nearly neutral charged solutions, and significantly improves cellular transcytosis.

1.0 Introduction

1.1 Oral Drug Delivery

One of the most important factors to consider when designing drug delivery systems is the route of administration. Oral drug delivery is the most preferred by those seeking medical care. Patients are more likely to comply with their dosage regimen if their drugs are administered orally because they do not have to be in a sterile environment; patients can administer the drugs themselves at their own convenience; and they reduce financial burden by avoiding trips to the hospital or a doctor's office, especially for chronic illnesses[1,2]. For these reasons, oral drug delivery has been an attractive field for therapeutic research. Although there are many benefits to oral drug delivery, this route of administration comes with its own unique set of obstacles. Oral drugs often have low bioavailability compared to intravenous injections due to the first pass effect[1], enzymatic degradation in the gastrointestinal (GI) tract[1,2], and the inability to diffuse through the mucosal lining of the epithelium[3]. One way to address these obstacles is the use of polymeric nanoparticles[2].

1.2 Polymeric Nanoparticles

Polymeric nanoparticles allow for sustained drug release while also protecting the encapsulated drug from harsh or unstable environments[2]. Polymeric nanoparticles have been used in the field of oral drug delivery due to their ability to protect biologics that are susceptible to enzymatic degradation in the GI tract and instability in the low pH environment of the stomach[2,4]. Polymeric nanoparticles are also able to safely and effectively transport hydrophobic or low solubility drugs[2]. They do so by encapsulating the active drug within the core of the particle; the polymer can either make up the solid core of the particle or the external shell as seen in Figure 1. Polymeric nanoparticles are customizable. It is easy to modulate their surface

properties such as their size, surface charge, hydrophobicity, stiffness, etc[2,4]. Their small size allows for an increased surface area of drug exposure and easy transcytosis through cells into the bloodstream[2]. However, there is still one challenge that lies between the particles' ability to enter the bloodstream: the mucosal lining of the GI tract[1].

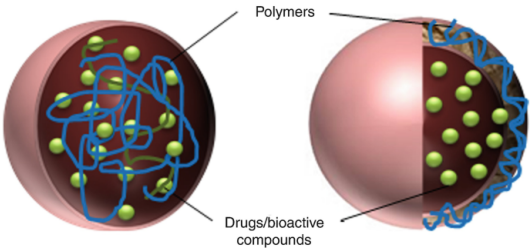


Figure 1. Diagram of polymeric nanoparticles encapsulating the drug within the core.[5]

1.3 Gastrointestinal (GI) System and Cellular Uptake

The mucosal barrier of the human GI tract lines the small and large intestine. The small intestine has one unattached mucus layer, while the large intestine has one attached and one unattached mucus layer. The unattached mucus layer of the small and large intestine is loose and easy to remove. The attached mucus layer of the large intestine is several microns thick and renews almost every hour. GI mucus is made up of highly glycosylated proteins and has both hydrophobic and hydrophilic regions that can interact with oral drugs[6]. This poses an obstacle for oral drugs as they can adhere to the mucus and form aggregations instead of diffusing through to reach the epithelial cells lining the GI tract and entering the bloodstream demonstrated by Figure 2. Polymeric nanoparticles have been shown to penetrate the mucosal lining of the GI tract and appear in the bloodstream after oral administration as shown by the transmission electron microscope images in Figure 3[7,8]. However, there are several contradicting findings regarding the ideal characteristics of nanoparticles for mucus penetration[8]. Some suggest that neutral charged polymeric nanoparticles have enhanced mucus penetrating abilities[9], while others report that

positively charged particles have greater diffusion[10]. To better explain the mechanism of action behind mucus penetrative polymeric nanoparticles, researchers at the Mathiowitz lab proposed a concept called bio-coating[8].

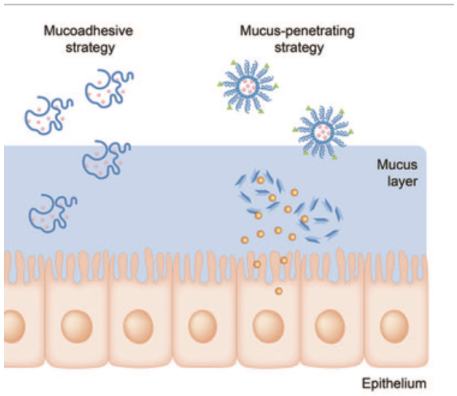


Figure 2. Diagram highlighting the differences between muco-adhesive and mucus-penetrating nanoparticles[,11].

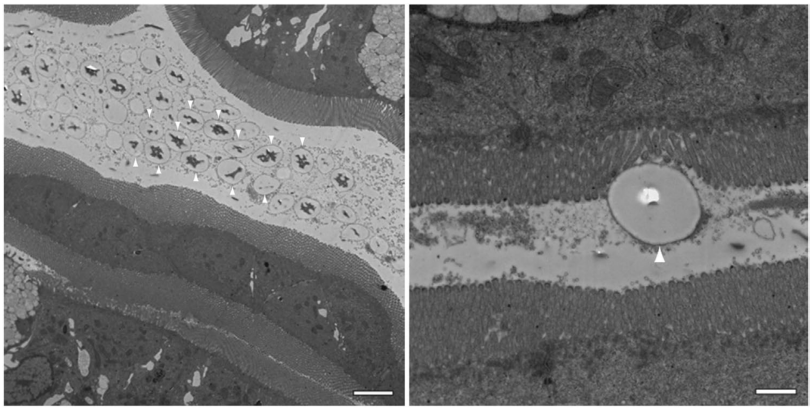


Figure 3. Transmission electron microscope images of polystyrene particles in the intestinal lumen after oral administration[8].

1.4 Bio-coating

Bio-coating is a naturally occurring phenomenon that occurs in the body when particles interact with the GI mucus. Bio-coating is when particles enter the mucosal lining and undergo spontaneous coating in mucus[8]. This mucus coating neutralizes the charge of the particles and enhances the diffusion across the mucosal lining as shown in Figure 4. Bio-coating alters the physicochemical properties of the particles (size, charge, hydrophobicity, etc.) by physically covering the particles and thus improving movement through the mucus and reducing aggregation[8]. By altering the surface properties of the particles through a physical coating, the particles' diffusive capabilities are also altered.

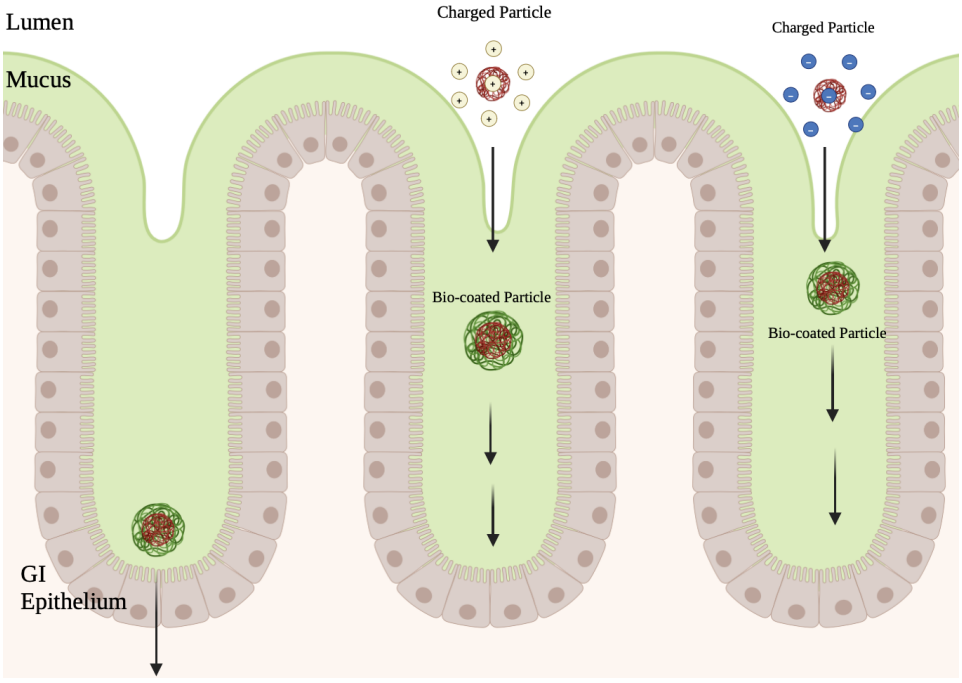


Figure 4. Diagram showing positively and negatively charged nanoparticles undergoing bio-coating in the GI mucosal environment. The bio-coated particle is theorized to have enhanced diffusion and cellular uptake.

The diffusion coefficient is a metric that is used to measure the movement of particles over time and can be used as an indicator of mucus penetration or adhesion. Determining the ideal chemical properties (size and surface charge) needed for mucus diffusion can improve polymeric nanoparticles' ability to penetrate the mucosal lining and cellular uptake. Thus, these properties can be applied to polymeric nanoparticles used in drug delivery to enhance the bioavailability of oral drugs. The goal of this research is to evaluate the mucus penetrative capabilities of polystyrene nanoparticles coated with various glycoprotein solutions (hyaluronic acid, porcine mucin, 35KDa PEG, 100KDaPEG, and 300KDaPEG) by measuring their diffusion coefficient, characterizing their physicochemical properties, and measuring uptake in Caco-2 cells.

2.0 Materials and Methods

2.1 Preparation of Coated Particles

The red fluorescent nanoparticles used were 0.52 μm polystyrene (PS) (CAT# 19507-5, Polysciences Inc.), 0.50 μm silica (CAT# ASFR-21-0050, Abvigen Inc.), and 0.50 μm polymethylmethacrylate (PMMA) (CAT# APFR-21-0050, Abvigen Inc.). The nanoparticle stock solutions were diluted to a concentration suitable for imaging. For PS particles, 10 μL of stock solution (2.5% aqueous solution, 3.64×10^{11} particles/ml) was added to 20 mL of deionized water for a final diluted stock solution concentration of about 0.0135 mg/mL. For silica, PE, and PMMA particles, 15 μL of stock solution (10 mg/mL) was added to 10 mL of deionized water for a final diluted stock concentration of about 0.015 mg/mL.

Next, the coating solutions were prepared. Each coating was prepared in 4 concentrations, 0%, 0.01%, 0.1%, and 1% coating. 5 different coatings were evaluated: 1.5-1.8

MDa hyaluronic acid (CAT# 53747-1G, Sigma-Aldrich), Type II Porcine mucin (CAT# M2378-100G, Sigma-Aldrich), 300KDa polyethylene glycol (PEG) (CAT#06105, Polysciences, Inc.), 100KDa PEG (CAT# 181986-250G, Sigma-Aldrich), and 35KDa PEG (CAT# 81310-1KG, Sigma-Aldrich). Because the concentration of the coating solutions was halved when combining with the particles, the concentrations of the coating solutions were doubled during fabrication. Table 1 summarizes the amount of polymer added to 10 mL of deionized water to prepare the coated solutions of varying concentrations. To maintain accuracy and avoid trying to weigh out small masses of polymer for the low concentration coatings, the 1% coating solution was made first and following concentrations were prepared through serial dilutions.

For the 1% coating solution, 200 mg of polymer was weighed using an analytical balance and then added to 10 mL of deionized water. This solution was then sonicated and vortexed for about 15 minutes. For the serial dilutions, 1 mL of the 1% coating solution was added to 9 mL of deionized water, yielding the 0.1% solution. The 0.1% coating solution was then sonicated and vortexed for about 15 minutes. Then, 1 mL of the 0.1% coating solution was added to 9 mL of deionized water to create the 0.01% coating solution. The 0.01% coating solution was also sonicated and vortexed for about 15 minutes. 0.5 mL of coating solution and 0.5 mL of diluted particle stock solution were combined in a microtube to create a 50/50 v/v coated particle solution. Once the coating solution and particle solutions were combined in the microtube, the sample was then vortexed and sonicated for at least 15 minutes. The samples were then left overnight in a fridge to ensure complete coating of the particles.

% Coating (g/mL)	% Coating (After Halving) (g/mL)	Mass of Polymer (mg)
5	2.5	500
2	1	200
0.2	0.1	20
0.02	0.01	4
0	0	0

Table 1. The mass of polymer (HA, mucin, 35KDa-300KDa PEG) (mg) needed to fabricate various concentrations (g/mL) of polymeric coating before and after halving.

2.2 Diffusion Coefficient

The movement of the nanoparticles in the mucosal environment was measured by observing their Brownian motion under fluorescent microscopy. A series of time lapse videos was recorded and then processed using particle tracking software. Prior to conducting the diffusion studies, glass slides with the coated and uncoated nanoparticle solutions were prepared. To model the GI system, 5% mucin solution was prepared using Type II Porcine mucin powder (CAT# M2378-100G, Sigma-Aldrich). This was done by mixing 500 mg of mucin with 10 mL of deionized water and sonicating for 20-25 minutes. The coated and uncoated nanoparticle solutions were also vortexed and sonicated for 15 minutes prior to slide preparation. A standard microscope glass slide was used, and a rectangular double sided iSpacer sticker (CAT#IS211, SunJin Lab) was placed on top of the glass slide. The iSpacer sticker acted as a very thin well, ensuring that the liquids dispensed onto the glass slide were evenly distributed in a closed environment. Within the iSpacer chamber, 25 μL of the 5% mucin solution was dispensed. On

top of the dispensed mucin, 5 µL of nanoparticle solution (coated or uncoated) was dispensed. Immediately following that, a glass coverslip was placed directly on top of the iSpacer chamber parallel to the glass slide, effectively sealing the mucin and nanoparticles within the chamber. Figure 5 is a schematic showing the entire sample preparation protocol starting with coating of the fluorescent nanoparticles. In the diffusion studies, time lapse videos were taken at different time points (0, 15, 30, and 60 minutes) following the dispensation of the nanoparticles.

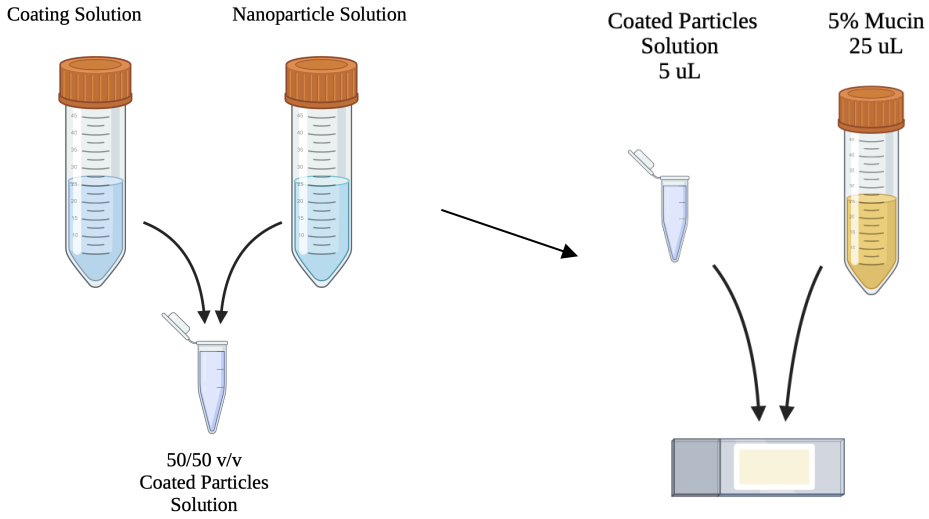


Figure 5. A schematic of the glass slide preparation starting with coating of the fluorescent nanoparticles in a 50/50 v/v solution, followed by preparation of the glass slide used in microscopy.

2.2.1 Fluorescence Microscopy

To conduct the diffusion study, the Nikon Ti2-E Widefield Microscope was used to observe the fluorescent nanoparticles. A 20x objective was used with the CY3 filter. The prepared glass slides were placed upside down on the viewing stage, and the nanoparticles were brought into view by adjusting the z-plane typically to around 1800-2000 µm. An example of

what the fluorescent polystyrene nanoparticles look like in the GI mucus under the microscope is shown in Figure 6. The exposure of the time-lapse videos was set to 100 ms, with 100 loops of images per video. The images were taken at 10 different and random areas of the sample chamber, yielding 10 individual time-lapse videos. The sets of images were taken at different time points, so each concentration of coated particles and uncoated particles has 40 images total. The images were then saved onto a hard drive and were subsequently ready to be processed. Exact settings of the microscope and imaging conditions can be found in the supplementary information.

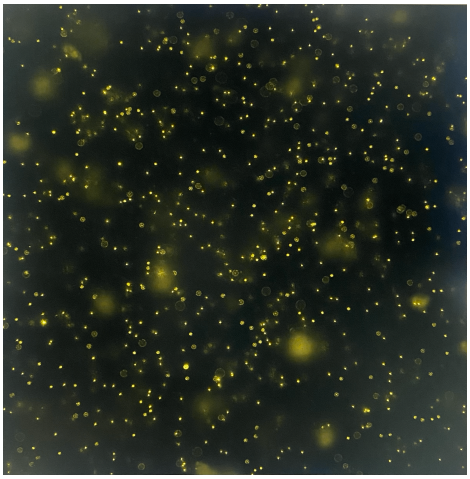


Figure 6. An image taken using the Nikon Ti2-E Widefield Microscope of 0.52 μm polystyrene nanoparticles in 5% GI mucin. The small fluorescent yellow dots are the polystyrene particles and the blurry, larger chunks of fluorescence seen in the background of the 5% mucin.

2.2.2 Particle Tracking Analysis

The time-lapse videos were then analyzed using the ImageJ software, specifically the Particle Tracker 2D/3D package. Each frame of each time-lapse video was stitched together, and each particle's trajectory was modeled throughout the video. This was done for all 10 videos per

sample. A Python script was used to calculate the mean squared displacement over time of each particle in the video. Using this calculation, the script was able to provide a csv file containing the mean diffusion coefficient (m^2/s) for each particle, for each video. Another Python script was used to process all 10 trajectory files and conduct statistical analysis on the findings. Key results of this second script include the mean and median diffusion coefficient (m^2/s) for each sample as well as the standard deviation, and graphical representation of the population. This allowed for the determination of the accuracy of the dataset and the elimination of outliers. Program codes can be found in the supplementary information.

2.3 Nanoparticle Characterization

Nanoparticle characterization allowed for the examination of the interactions between the coating solutions and the nanoparticles themselves. Previous findings showed that neutralization of nanoparticle charge can enhance their diffusive properties in 5% mucin[8]. Thus, the zeta potential of each sample was determined to monitor changes in surface charge of the particles and evaluate the effects on their diffusive properties. The size of the nanoparticles were also determined in relation to the concentration of glycoprotein solution to monitor the interaction between the coating and particles.

2.3.1 Zeta Potential

The Malvern Panalytical Zetasizer Nano was used to determine the Zeta potential (mV) readings of each sample. A disposable capillary zeta cell was used to hold the sample solutions, shown in Figure 7. About 750 μL of coated or uncoated nanoparticle solution was dispensed using a micropipette in one opening of the capillary cell. Once the solution had filled both sides of the U-shaped tube inside the cell, any trapped air bubbles were extracted by gently tapping the

cuvette against the lab bench. Two plastic stoppers were then placed simultaneously on both openings to ensure that the sample liquid level was maintained at an appropriate height on both sides of the U-shaped tube. The cuvette was wiped down with a Kimwipe prior to being inserted into the Zetasizer with the welding line of the capillary cell facing the user. Using the Malvern Zetasizer software, 3 Zeta potential readings were taken of each sample. Each reading consisted of 10 runs. The temperature of the cuvette was maintained at 25℃. The average zeta potential values of the nanoparticles were determined using the 3 readings. Exact software and instrument settings can be found in the supplementary information.

2.3.2 Dynamic Light Scattering

The Malvern Panalytical Zetasizer Nano was used to determine the hydrodynamic diameters (nm) of each sample using Dynamic Light Scattering (DLS). A disposable sizing cuvette was used to hold the sample solutions, shown in Figure 7. At least 1 mL of coated or uncoated nanoparticle solution was dispensed into the sizing cuvette. Any trapped air bubbles were extracted by gently tapping the cuvette against the lab bench. The cuvette was then wiped down with a Kimwipe prior to being inserted into the Zetasizer, ensuring that the arrow indicating the direction of the cuvette was facing the user. Using the Malvern Zetasizer software, 3 DLS readings were taken of each sample, each reading consisting of 10 runs. The temperature of the cuvette was maintained at 25℃. The average hydrodynamic diameters of the nanoparticles were determined using the 3 readings. Exact software and instrument settings can be found in the supplementary information.

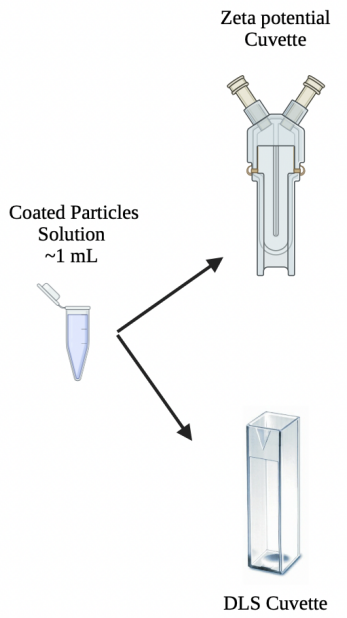


Figure 7. An example of the two different types of cuvettes used during nanoparticle characterization.

2.4 Cell Absorption Studies

Cell absorption studies were performed to determine the absorption capabilities of coated fluorescent polystyrene nanoparticles. 0.45 μm polystyrene nanoparticles were used because in previous findings, they showed promising uptake during in vivo studies[12]. To model the GI environment, Caco-2 cells were used in this study. Caco-2 cells were seeded onto transwell meshes with a pore size of 3 μm and cultured for 3 weeks to ensure proper gap junction formation. Following the growth of the cells, 4 test conditions were studied as described by Table 2.

Condition (coating/mucin layer)	
Condition A (+/+)	0.1% coating & 5% mucin layer
Condition B (+/-)	0.1% coating & no mucin layer
Condition C (-/+)	No coating & 5% mucin layer
Condition D (-/-)	No coating & no mucin layer

Table 2. Identifying the four conditions used in the cellular uptake studies. The symbol to the right of the hash indicates presence of a 0.1% polymeric coating and the symbol to the left of the hash indicates presence of a 5% mucin layer.

Within Conditions A and B, the particles were coated with either 0.1% mucin, 0.1% 100 KDa PEG, or 0.1% 300 KDa PEG. Within Conditions C and D, the particles were left uncoated. Within Conditions A and C, 5% mucin was dispensed on top of the monolayer of cells first, the nanoparticles were dispensed on top of the mucin layer after. Within Conditions B and D, there was no 5% mucin layer dispensed on top of the monolayer of cells, instead the nanoparticles were dispensed directly on top of the cells. The setup of the transwell plate is shown in Figure 8. The solution under the transwell mesh was collected every 24 hours for 4 days. The collected solution was then centrifuged, and the pellet, containing the nanoparticles that had passed through the cells, was collected by removing the entire volume of supernatant. The supernatant was discarded. The pellet was then resuspended in 100 uL of HBSS to ensure the volume of each sample was kept constant. The resuspended solution was added to a separate 96-well plate for imaging. After the resuspended solution had allowed the particles to settle, the BioTeK Cytation

machine was used to visualize and take 2-D images of the collected nanoparticles. A single z-plane was selected as the focal plane for imaging, this z-plane was at the bottom of the well after the particles had settled. 12 images were taken of each well in randomly selected areas. Using the threshold method in ImageJ, the collected nanoparticles were quantified as percent area per field of view. First, the total area of the image was calculated. Then, the area of the fluorescent regions of the image were calculated. By dividing the fluorescent area over the total area, a percent area per field of view was calculated. This allows us to determine transcytosis since the fluorescent regions were only due to the presence of nanoparticles in the solution.

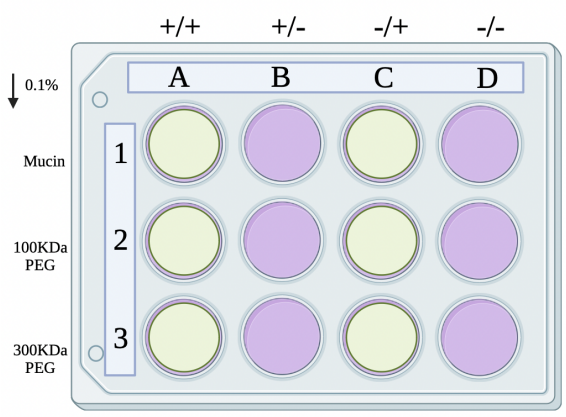


Figure 8. A diagram of the setup of the 12-well transwell plate for the transcytosis and cellular uptake studies. From top to bottom, the particles were coated with 1 of the 3 polymeric coatings (mucin, 100KDa PEG, or 300KDa PEG). From left to right, the cells were subjected to 1 of the 4 experimental conditions (Condition A, B, C, or D).

After the 4-day study was concluded, the Caco-2 cells were fixed and stained to allow for 3-D imaging. Any remaining nanoparticles were washed off the cells, and the cell membranes were stained using a WGA stain. The cells were then fixed in formalin. Using the confocal

microscope, images were taken of each sample starting from the top of the cells to the bottom at every 0.4 μm, yielding a z-stack of images. The top of the cells was identified by the green staining of the cell membrane, and the bottom of the cells was identified by the lack of green fluorescence indicating the end of the cell. Therefore the NPs internalized within the cells were readily identified. NPs stuck to the surface of the cell were not included. 2 fluorescent channels were used (red and green) to distinguish between the cells (green) and the nanoparticles contained within the cells (red). ImageJ was then used to stitch the z-stack of images together to create one field of view, and the threshold method was again used to determine the nanoparticles internalized within the cells as percent area per field of view.

3.0 Results and Discussion

3.1 Diffusion Coefficient

3.1.2 Polystyrene

0.5 μm polystyrene nanoparticles were used because previous findings show that this nanoparticle has good cellular uptake, enhanced diffusion in GI mucin, and applications to oral drug delivery.[8,12]

3.1.2.1 Polystyrene/Hyaluronic Acid

Hyaluronic acid coatings were prepared over a range of concentrations. Red fluorescent polystyrene particles (0.52 μm) were coated with 0%-1% hyaluronic acid for at least 24 hours prior to conducting diffusion studies. The diffusion study for this formulation was repeated 3 times. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration can be seen in Figure 9. Table 3 shows the numerical values of the diffusion coefficient, and Table 4 reports the associated standard deviations of the results. From Figure 9 it can be seen that there is

a positive correlation between the concentration of hyaluronic acid coating and the mean diffusion coefficient, with the exception of the 0.05% coating within each time point. The control in this study was uncoated polystyrene nanoparticles shown in the leftmost bar. Over time, the lower concentration coatings and uncoated samples showed little change in diffusion. However, at higher concentrations of HA, it can be seen that over time, the diffusion coefficient declines and increases again. This could be due to the fact that initially, the coating was interacting well with the nanoparticle and enhancing the diffusion capabilities through the GI mucus. Around the 30-minute mark, the GI mucus has effectively bio-coated the coated particles, a concept described as “spontaneous bio-coating”. This interaction may slightly hinder the particle's ability to diffuse. Despite these changes, compared to the bare polystyrene nanoparticles, the coated particles with any concentration of HA (except 0.05%) show an increased diffusion coefficient at every time point.

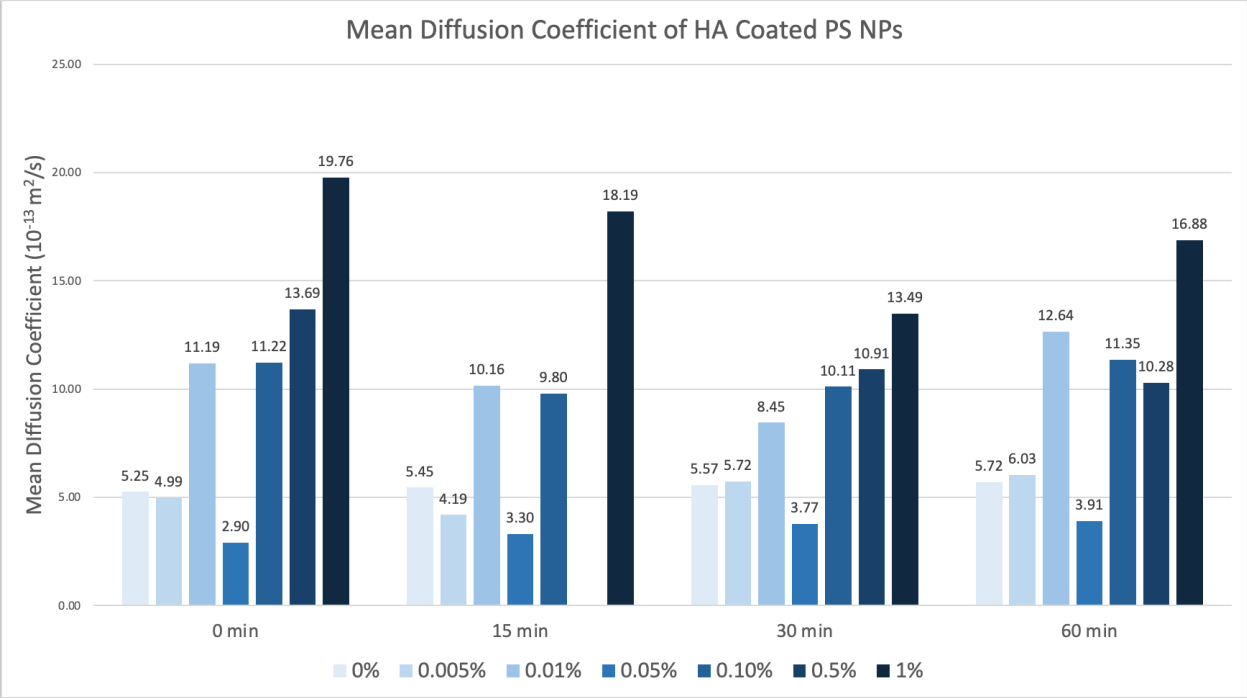


Figure 9. Mean Diffusion Coefficient of HA Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficient of HA Coated PS NPs (10^{-13} m^2/s)				
	0 minutes	15 minutes	30 minutes	60 minutes
0% HA	5.25	5.45	5.57	5.72
0.005% HA	4.99	4.19	5.72	6.03
0.01% HA	11.19	10.16	8.45	12.64
0.05% HA	2.90	3.30	3.77	3.91
0.10% HA	11.22	9.80	10.11	11.35
0.5% HA	13.69	N/A	10.91	10.28
1.0% HA	19.76	18.19	13.49	16.88

Table 3. Mean diffusion coefficients of HA coated PS NPs

Std. Deviations of Diffusion Coefficient of HA Coated PS NPs +/- (10^{-13} m^2/s)				
	0 minutes	15 minutes	30 minutes	60 minutes
0% HA	11.18	12.27	13.57	13.27
0.005% HA	9.11	8.78	11.92	14.86
0.01% HA	43.30	48.94	39.59	62.56
0.05% HA	4.74	6.22	6.89	7.52
0.10% HA	53.66	41.58	39.22	44.37
0.5% HA	71.87	0.00	50.39	51.58
1.0% HA	137.70	117.79	91.53	96.35

Table 4. Standard deviations of diffusion coefficients of HA coated PS NPs

3.1.2.2 Polystyrene/Mucin

The mucin coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The diffusion study for this formulation was

performed 1 time as a repeat study since this formulation has been studied by other members of the Mathiowitz lab in previous years. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration can be seen in Figure 10. Table 5 shows the numerical values of the diffusion coefficient, and Table 6 reports the associated standard deviations of the results. Shown in Figure 10, the diffusion coefficient of the polystyrene nanoparticles was slightly enhanced over time. The uncoated particles serve as a control and are shown with the blue bar. Initially, the uncoated particles displayed the highest diffusion, with the coated particles showing lower diffusion. However, starting at the 15-minute time point, the opposite results were shown.

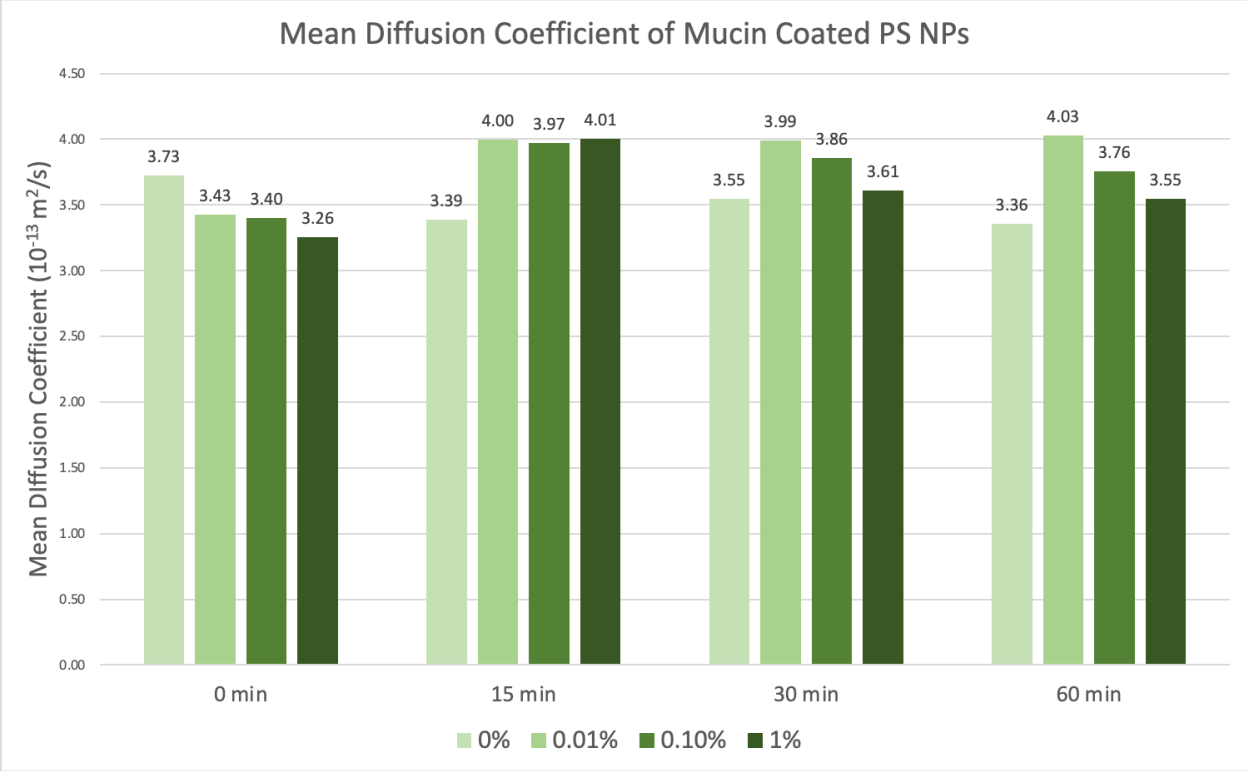


Figure 10. Mean Diffusion Coefficient of Mucin Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficients of Mucin Coated PS NPs (10^{-13} m^2/s)				
	0% Mucin	0.01% Mucin	0.1% Mucin	1.0% Mucin
0 minutes	3.73	3.43	3.40	3.26
15 minutes	3.39	4.00	3.97	4.01
30 minutes	3.55	3.99	3.86	3.61
60 minutes	3.36	4.03	3.76	3.55

Table 5. Mean diffusion coefficients of mucin coated PS NPs

Std. Deviations of Mucin Coated PS NPs +/- (10^{-13} m^2/s)				
	0% Mucin	0.01% Mucin	0.1% Mucin	1.0% Mucin
0 minutes	7.52	6.59	7.09	5.53
15 minutes	6.44	7.94	8.38	7.83
30 minutes	7.40	7.72	7.96	7.23
60 minutes	6.91	8.34	8.05	7.42

Table 6. Standard deviations of diffusion coefficients of mucin coated PS NPs

3.1.2.3 Polystyrene/Hyaluronic Acid/0.1% Mucin

Hyaluronic acid coatings were prepared over a range of concentrations. Red fluorescent polystyrene particles (0.52 μm) were coated with 0%-0.5% hyaluronic acid for at least 24 hours. The coated particles were then coated again with 0.1% porcine mucin for 24 hours prior to conducting the diffusion studies. The diffusion study for this formulation was repeated 3 times. The average diffusion coefficient (10^{-13} m^2/s) can be seen in Figure 11 as a function of coating concentration. Table 7 shows the numerical values of the diffusion coefficient, and Table 8 reports the associated standard deviations of the results. Figure 11 shows that with increasing concentration of hyaluronic acid with the addition of the 0.1% mucin coating, the diffusion

coefficient of the PS nanoparticles declines. The uncoated particles show the highest diffusion at every time point. This is the opposite pattern compared to Figure 9 in which increasing concentration of the HA coating led to increased diffusion coefficient.

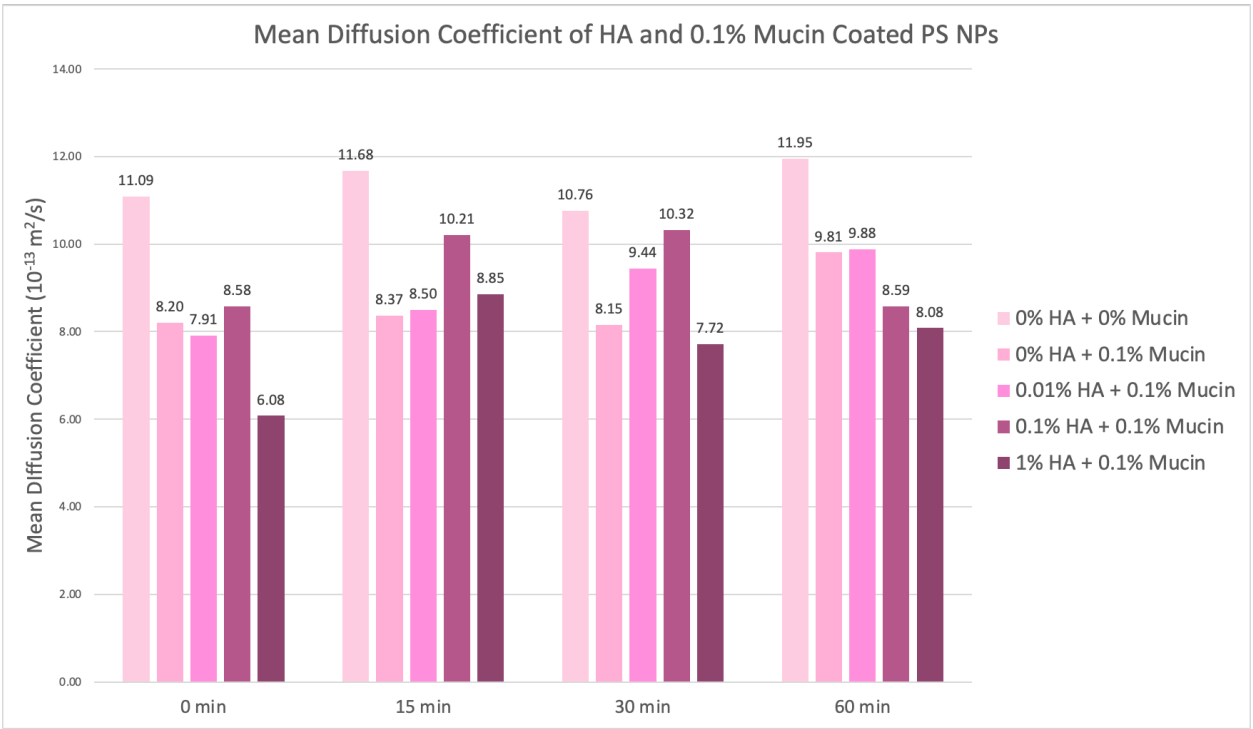


Figure 11. Mean Diffusion Coefficient of HA and 0.1% Mucin double Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficients of HA + 0.1% Mucin Coated PS NPs (10^{-13} m^2/s)				
	0 minutes	15 minutes	30 minutes	60 minutes
0% HA + 0% Mucin	11.09	11.68	10.76	11.95
0% HA + 0.1% Mucin	8.20	8.37	8.15	9.81
0.01% HA+ 0.1% Mucin	7.91	8.50	9.44	9.88
0.1% HA+ 0.1% Mucin	8.58	10.21	10.32	8.59
1.0% HA+ 0.1% Mucin	6.08	8.85	7.72	8.08

Table 7. Mean diffusion coefficients of HA and 0.1% mucin coated PS NPs

Std. Deviations of HA + 0.1% Mucin Coated PS NPs +/- (10^{-13} m^2/s)				
	0 minutes	15 minutes	30 minutes	60 minutes
0% HA + 0% Mucin	0.01	0.23	0.68	1.97
0% HA + 0.1% Mucin	2.00	2.47	3.41	3.21
0.01% HA+ 0.1% Mucin	3.71	3.40	3.01	2.63
0.1% HA+ 0.1% Mucin	3.63	4.78	3.43	1.54
1.0% HA+ 0.1% Mucin	1.37	2.04	3.83	0.74

Table 8. Standard deviations of diffusion coefficients of HA and 0.1% mucin coated PS NPs

3.1.2.4 Polystyrene/35 KDa PEG

The 35KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The diffusion study for this formulation was performed 2 times. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration can be seen in Figure 12. Table 9 shows the numerical values of the diffusion coefficient, and Table 10 reports the associated standard deviations of the results. From Figure 12, it can be seen that apart from the 0-minute and 60-minute time point, the diffusion coefficients of the different concentration coatings of 35KDa PEG on polystyrene did not differ significantly over time. From 15 to 30 minutes, the uncoated and 0.1% 35KDa PEG coated samples showed similar diffusion. At 0 minutes, the 0.1% coated particles showed enhanced diffusion compared to the control, but at 60 minutes that pattern is reversed. At 60 minutes, the 0.01% coated particles showed enhanced diffusion compared to the other experimental groups. From these results, there is an unclear pattern of diffusion throughout time and repeated studies of this formulation would be beneficial.

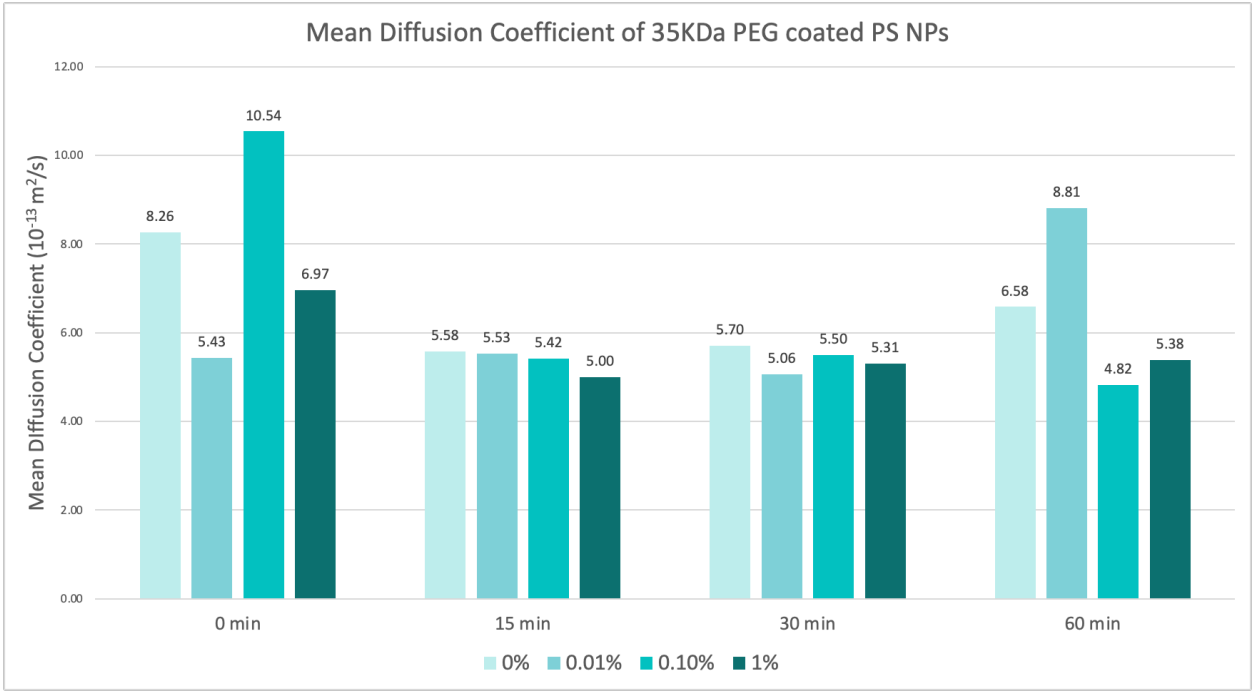


Figure 12. Mean Diffusion Coefficient of 35KDa PEG Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficients of 35KDa PEG Coated PS NPs (10^{-13} m^2/s)				
	0% 35KDa PEG	0.01% 35KDa PEG	0.1% 35KDa PEG	1.0% 35KDa PEG
0 minutes	8.26	5.43	10.54	6.97
15 minutes	5.58	5.53	5.42	5.00
30 minutes	5.70	5.06	5.50	5.31
60 minutes	6.58	8.81	4.82	5.38

Table 9. Mean diffusion coefficients of 35KDa PEG coated PS NPs

Std. Deviations of 35KDa PEG Coated PS NPs +/- (10^{-13} m^2/s)				
	0% 35KDa PEG	0.01% 35KDa PEG	0.1% 35KDa PEG	1.0% 35KDa PEG
0 minutes	0.02	1.73	7.51	1.54
15 minutes	0.32	0.10	0.76	0.80
30 minutes	0.16	0.68	1.04	0.74
60 minutes	0.31	3.85	0.83	0.14

Table 10. Standard deviations of diffusion coefficients of 35KDa PEG coated PS NPs

3.1.2.5 Polystyrene/100 KDa PEG

The 100KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The diffusion study for this formulation was performed 2 times. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration can be seen in Figure 13. Table 11 shows the numerical values of the diffusion coefficient, and Table 12 reports the associated standard deviations of the results. From Figure 13, it can be seen that in general, there is no clear pattern or significant difference between the concentrations of 100KDa PEG coating and time. At 0 minutes, the 0.1% coated particles showed the highest diffusion, greater than the uncoated control particles. At almost every time point after, except 15 minutes, the coated particles showed enhanced diffusion, time 30 minutes having the most difference. The 0.01% 100KDa PEG coating showed a steady incline in diffusion coefficient over time.

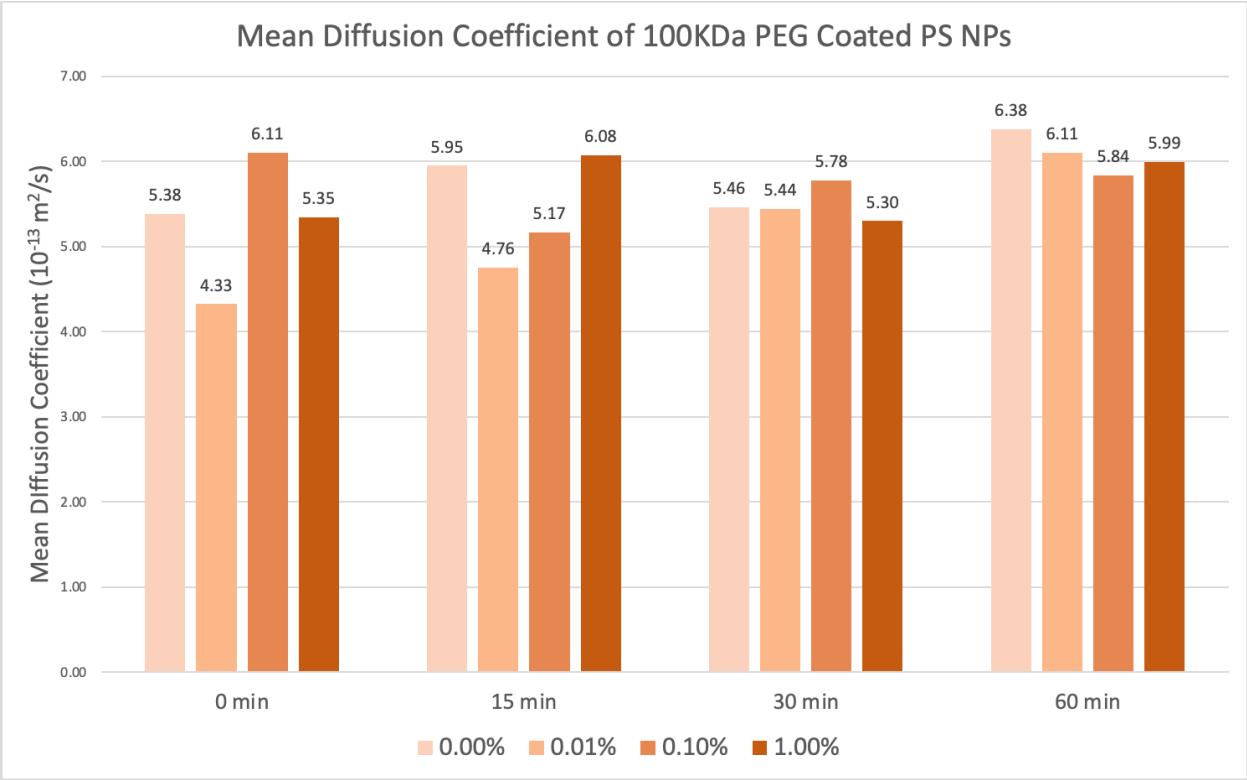


Figure 13. Mean Diffusion Coefficient of 100KDa PEG Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficients of 100KDa PEG Coated PS NPs (10^{-13} m^2/s)				
	0% 100KDa PEG	0.01% 100KDa PEG	0.1% 100KDa PEG	1.0% 100KDa PEG
0 minutes	5.38	4.33	6.11	5.35
15 minutes	5.95	4.76	5.17	6.08
30 minutes	5.46	5.44	5.78	5.30
60 minutes	6.38	6.11	5.84	5.99

Table 11. Mean diffusion coefficients of 100KDa PEG coated PS NPs

Std. Deviations of 100KDa PEG Coated PS NPs +/- (10^{-13} m^2/s)				
	0% 100KDa PEG	0.01% 100KDa PEG	0.1% 100KDa PEG	1.0% 100KDa PEG
0 minutes	0.35	1.46	0.08	0.45
15 minutes	0.08	0.62	0.25	0.35
30 minutes	1.50	0.24	1.53	0.20
60 minutes	1.42	0.50	0.08	0.92

Table 12. Standard deviations of diffusion coefficients of 100KDa PEG coated PS NPs

3.1.2.6 Polystyrene/300 KDa PEG

The 300KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The diffusion study for this formulation was performed 1 time as a repeat study since this formulation has been studied by other members of the lab in previous years. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration can be seen in Figure 14. Table 13 shows the numerical values of the diffusion coefficient, and Table 14 reports the associated standard deviations of the results. From Figure 14, it can be seen that there is no clear pattern between the concentrations of 300KDa PEG coatings and time. The only time point in which the coated particles, 0.1% 300KDa PEG, showed significantly enhanced diffusion compared to the control was at 15 minutes. The diffusion coefficients of all the groups remained relatively unchanged, with the exception of the control group. The uncoated particles showed highest diffusion at 0 minutes. It is important to note that the highest molecular weight of PEG, the 300KDa PEG, showed higher diffusion compared to the lower molecular weights. However, because the uncoated control particles showed a significantly higher diffusion coefficient than is normally seen, it can be

concluded that this experiment was an outlier, and that doing a repeat study would improve the accuracy of the findings.

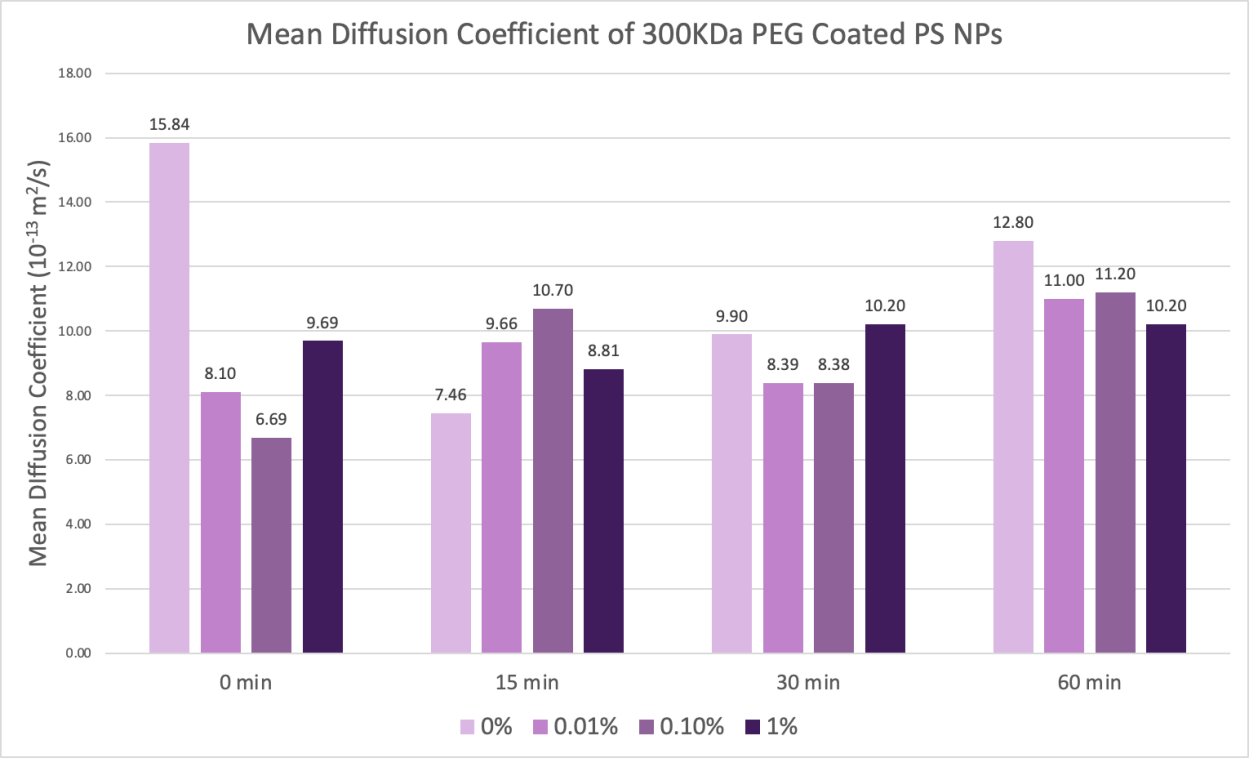


Figure 14. Mean Diffusion Coefficient of 300KDa PEG Coated PS NPs (10^{-13} m^2/s) as a function of time and concentration. The lightest shade bars represent lowest concentration, and the darkest shade bars represent the highest concentration.

Mean Diffusion Coefficients of 300KDa PEG Coated PS NPs (10^{-13} m^2/s)				
	0% 300KDa PEG	0.01% 300KDa PEG	0.1% 300KDa PEG	1.0% 300KDa PEG
0 minutes	15.84	8.10	6.69	9.69
15 minutes	7.46	9.66	10.70	8.81
30 minutes	9.90	8.39	8.38	10.20
60 minutes	12.80	11.00	11.20	10.20

Table 13. Mean diffusion coefficients of 300KDa PEG coated PS NPs

Std. Deviations of 300KDa PEG Coated PS NPs +/- (10^{-13} m^2/s)				
	0% 300KDa PEG	0.01% 300KDa PEG	0.1% 300KDa PEG	1.0% 300KDa PEG
0 minutes	49.35	23.61	17.85	39.84
15 minutes	21.77	37.24	51.33	28.56
30 minutes	36.95	27.26	22.06	39.65
60 minutes	58.09	43.08	47.71	35.45

Table 14. Standard deviations of diffusion coefficients of 300KDa PEG coated PS NPs

3.1.3 Silica

Red fluorescent silica particles (0.5 μm) were used to conduct the diffusion studies. The particles were coated with various glycoprotein and polymeric solutions. The diffusion studies derived inconclusive results. Upon examining bare silica particles dispensed on top of 5% mucin under the microscope, they exhibited a great amount of aggregation, as shown by the decline in diffusion in 5% mucin in Figure 15. This was determined by visibly comparing the amount of particles that were “free floating” (free to diffuse in the mucin) and the amount of particles that were clumped and aggregating in the mucin chunks. 5% mucin itself is prone to aggregation,

especially over time. However, when the fluorescent particles are aggregating in the mucin, these chunks can appear more visibly under the microscope. The experimental design of the diffusion studies relies on the stark contrast between the 5% mucin layer (no fluorescence) and the nanoparticles (very brightly fluorescent). Thus, when there is a high level of particle aggregation, it can be impossible to distinguish between the mucin layer and the particles. When processing these images, ImageJ may take up to 7 days to run its program since it cannot distinguish between what is a particle and what is not. For this reason, a series of studies was conducted to further examine the interactions between silica particles, the polymeric coatings, and 5% mucin to determine what could be causing such instability. These studies were conducted in almost identical procedure. However, due to the challenging nature of imaging these samples, only 3 images were taken as opposed to the normal 10. The uncoated and coated particles were observed both in 5% mucin as well as just deionized water. These studies were performed only once.

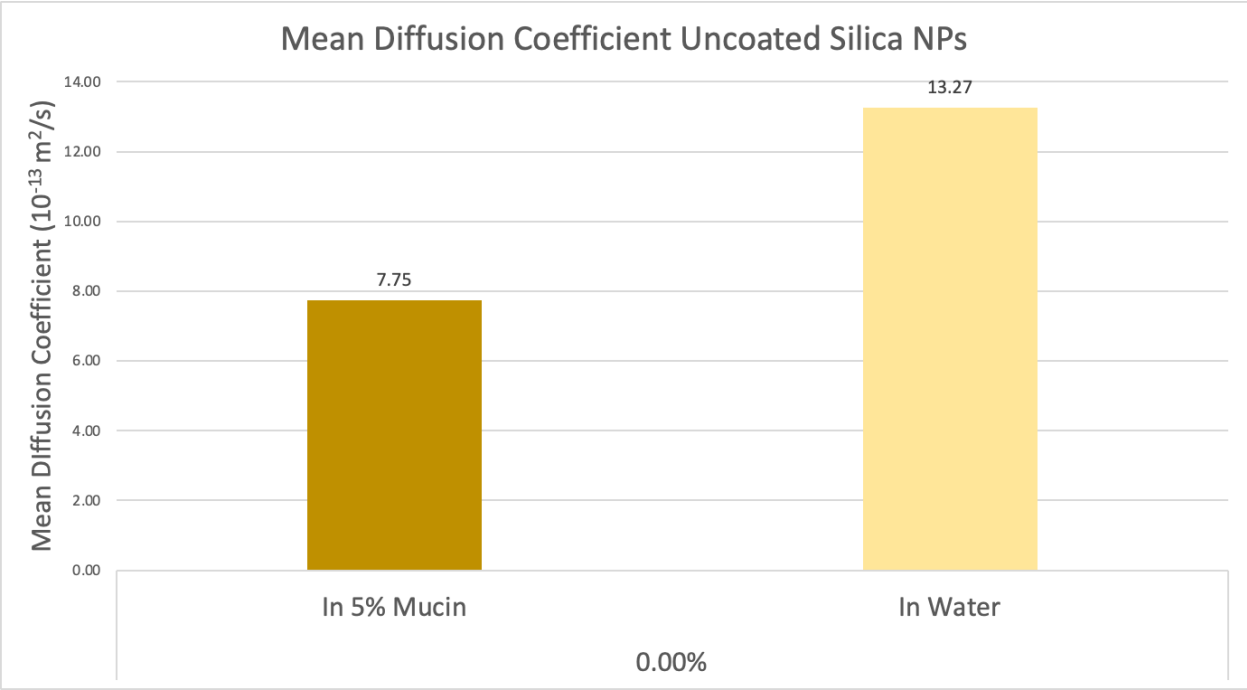


Figure 15. Mean diffusion coefficient of uncoated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water.

3.1.3.1 Silica/Hyaluronic Acid

The hyaluronic acid coatings were prepared in concentrations of 0%, 0.005%, 0.05%. The coated samples were left overnight in the fridge. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration and whether a 5% mucin layer was present can be seen in Figure 16. The "In Water" represents just the coated particles in water. From Figure 16, it can be seen that in both concentrations of HA, the silica particles showed enhanced diffusion when coated with HA and introduced to the 5% mucin versus in just water. HA effectively reduced the aggregation of silica and enhanced the diffusion of silica particles in the GI mucin. The drastic difference in diffusion coeffcient between the silica particles and polystyrene

particles could be attribute to a significantly smaller sample size. A larger sample size and multiple replicates for each experiment would provide more accurate results. For this reason, in these studies the diffusion coefficient is mostly used to make qualitative comparisons (more or less aggregation) rather than quantitative comparisons.

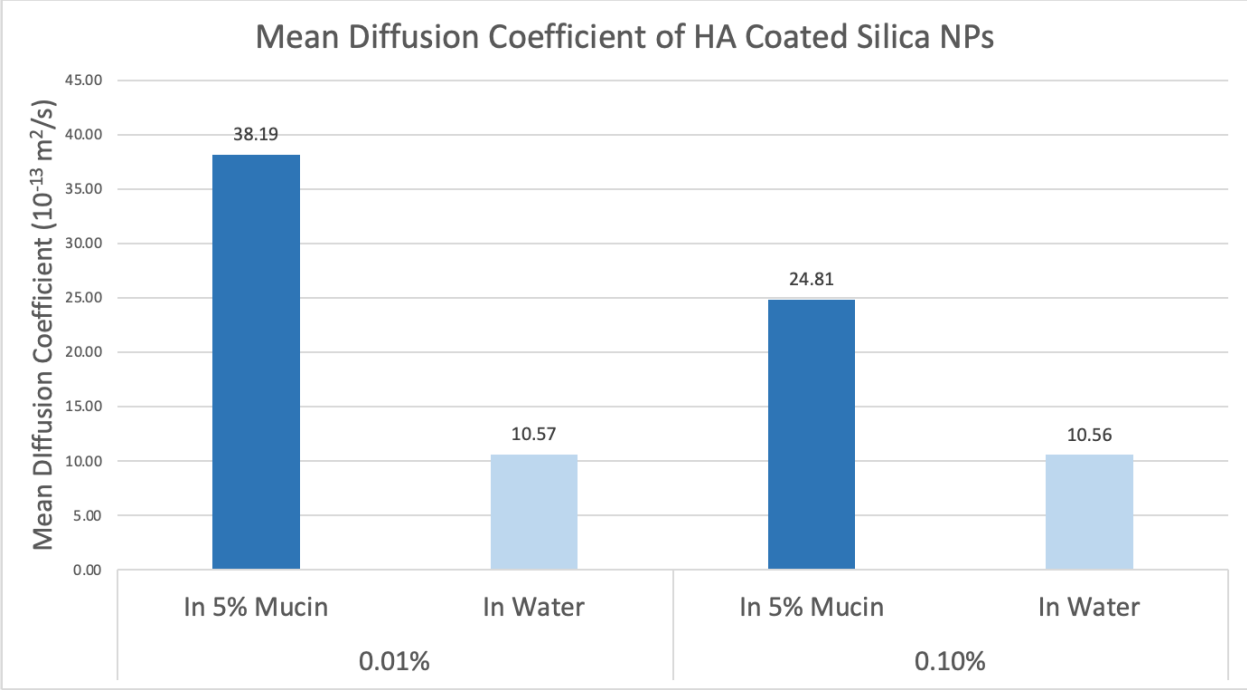


Figure 16. Mean diffusion coefficient of 0.01% or 0.1% HA coated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water.

3.1.3.2 Silica/Mucin

The mucin coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration and whether a 5% mucin layer was present can be seen in Figure 17. From Figure 17, it can be seen that when the coated particles were introduced to the

GI mucin, the diffusion coefficients of these particles declined significantly. It is also important to note that the values for the diffusion coefficients of these samples are extremely high. This indicates that there was some error in the experiment and must be further researched in the future. Qualitatively speaking, when observed under the microscope, the coated particles showed enhanced aggregation in the 5% mucin.

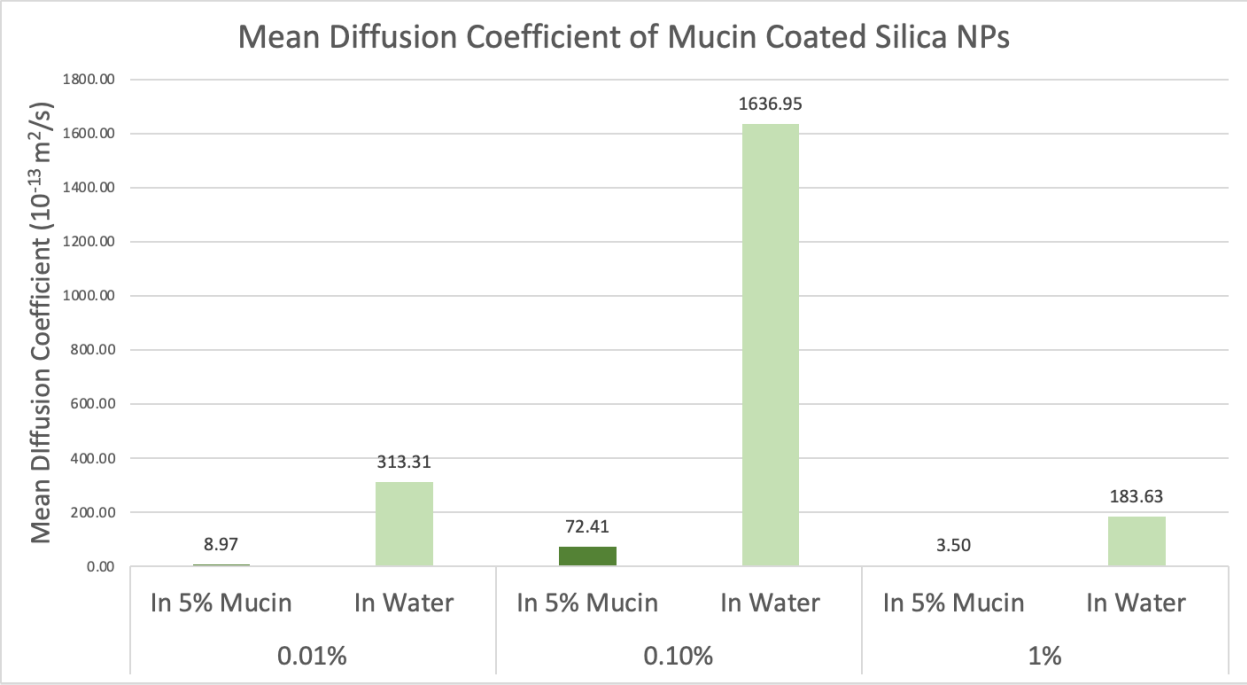


Figure 17. Mean diffusion coefficient of 0.01%, 0.1%, or 1% mucin coated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water.

3.1.3.3 Silica/35 KDa PEG

The 35KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration and whether a 5% mucin layer was present can be

seen in Figure 18. From Figure 18, it can be seen that similarly to the mucin coatings, the particles showed diminished diffusion when introduced to the 5% mucin. However, when comparing these results to the results for polystyrene coated with mucin, the mean diffusion coefficients of the particles with and without the 5% mucin layer are more similar. The difference between the 5% mucin layer and no mucin layer is much smaller than that of the mucin coated silica particles. When observing under the microscope, these samples showed reduced aggregation compared to the mucin coated silica particles, but more aggregation compared to the HA coated silica particles.

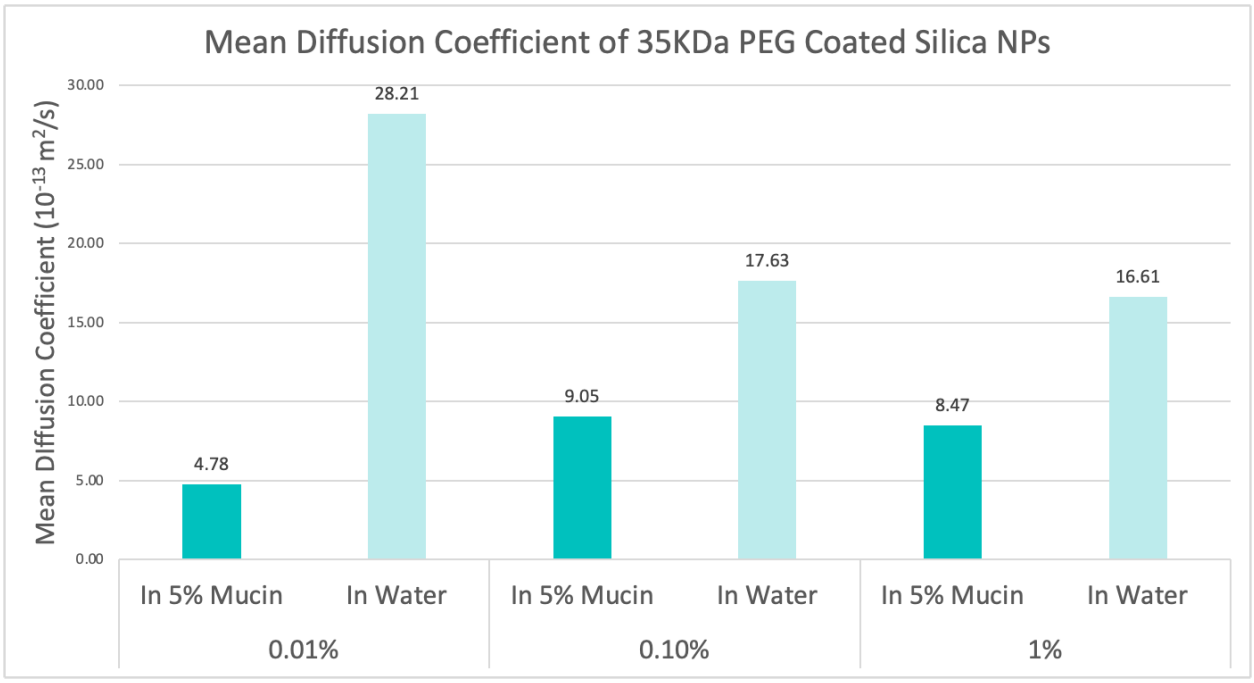


Figure 18. Mean diffusion coefficient of 0.01%, 0.1%, or 1% 35KDa PEG coated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water.

3.1.3.4 Silica/100 KDa PEG

The 100KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration and whether a 5% mucin layer was present can be seen in Figure 19. From Figure 19, it can be seen that the particles with and without the 5% mucin layer show similar diffusion coefficients. The difference between the 5% mucin layer and no mucin layer is even smaller than that of the 35KDa PEG coated particles. In fact, the 1% 100KDa PEG coated particles showed enhanced diffusion when interacting with the 5% mucin. When observed under the microscope, these samples showed reduced aggregation compared to the 35KDa PEG coated silica particles (with the exception of the 1% 100KDa PEG coated particles), but more aggregation compared to the HA coated silica particles.

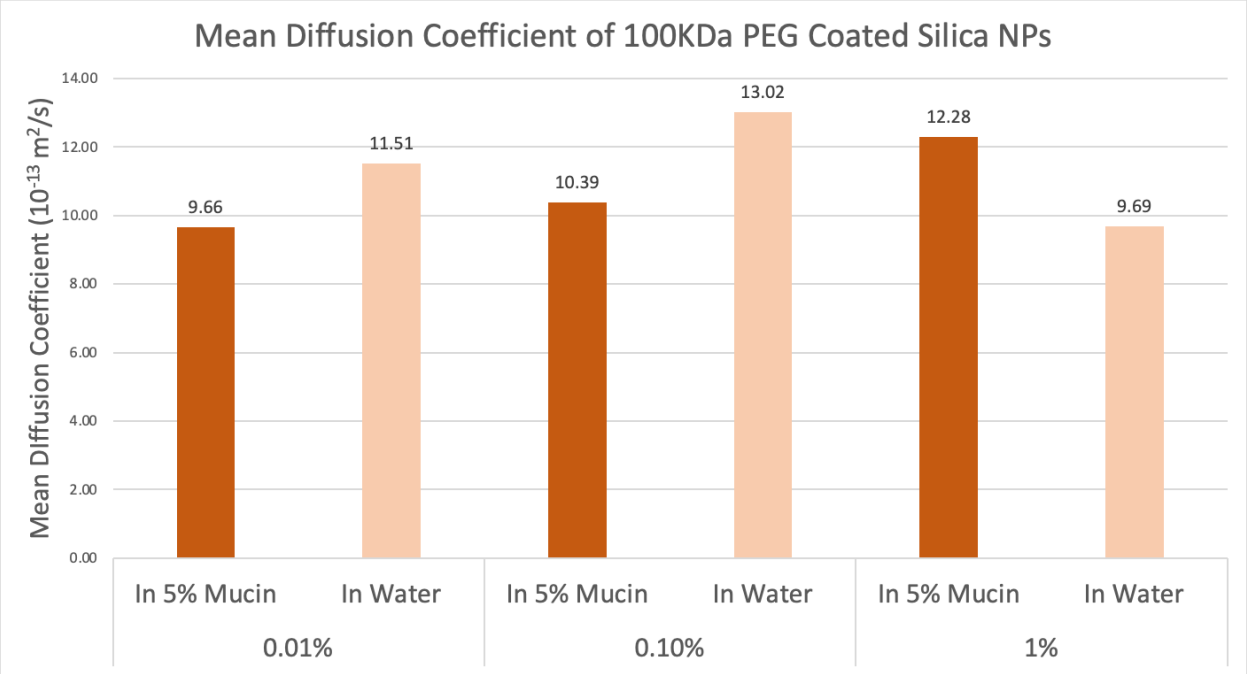


Figure 19. Mean diffusion coefficient of 0.01%, 0.1%, or 1% 100KDa PEG coated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water.

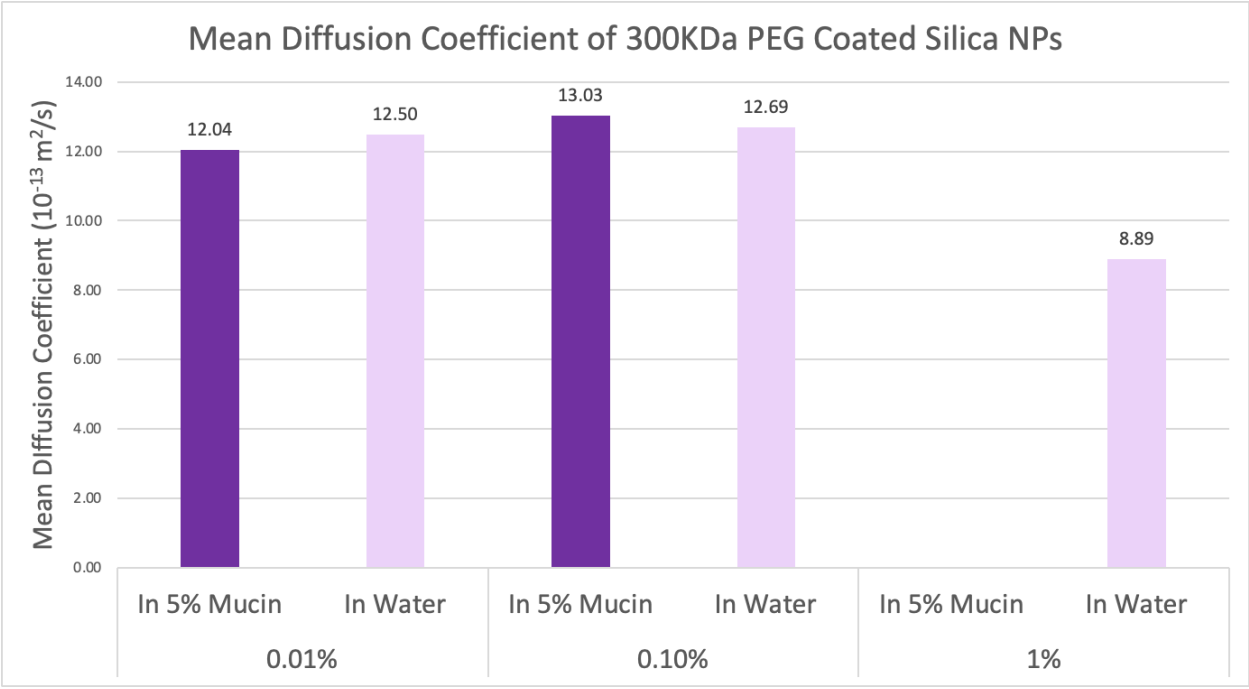
3.1.3.5 Silica/300 KDa PEG

The 300KDa PEG coatings were prepared in concentrations of 0%, 0.01%, 0.1%, and 1%. The coated samples were left overnight in the fridge. The average diffusion coefficient (10^{-13} m^2/s) as a function of coating concentration and whether a 5% mucin layer was present can be seen in Figure 20. From Figure 20, it can be seen that the particles with and without the 5% mucin layer show similar diffusion coefficients. The difference between the 5% mucin layer and no mucin layer is even smaller than that of the 100KDa PEG coated particles. The 0.1% 300KDa PEG coated particles showed enhanced diffusion when interacting with the 5% mucin. The diffusion for the 1% 300KDa PEG coated particles in 5% mucin was not able to be observed due

to time constraints, further research would be required to examine the results from that category. However, it is interesting to note that the "In Coated Media" value of the 1% 300KDa PEG coated particles compared to the other diffusion coefficients is diminished. When observed under the microscope, these samples showed reduced aggregation compared to the 100KDa PEG coated silica particles, but more aggregation compared to the HA coated silica particles.

Figure 20. Mean diffusion coefficient of 0.01%, 0.1%, or 1% 300KDa PEG coated silica NPs (10^{-13} m^2/s) with or without a 5% mucin layer. The dark shade bar indicates diffusion in 5% GI mucin and the light shade bar indicates diffusion in water. The 1% 300KDa PEG coated silica particles diffusion in 5% mucin is not applicable due to time constraints.

3.1.4 PMMA



Uncoated PMMA particles were observed under the microscope in 5% mucin, however due to significant aggregation no imaging was possible. Similar studies done for the silica

particles would be required to determine the possible causes of such, but due to time constraints could not be done.

3.2 Nanoparticle Characterization: Zeta Potential & Dynamic Light Scattering (DLS)

3.2.1 Hyaluronic Acid

To get an understanding of the general effect that the hyaluronic acid coating has on zeta potential of the nanoparticles, each type of particle (PS, silica, PE, and PMMA) was coated with 0%-1% hyaluronic acid and their surface charges were measured using the Malvern Zetasizer, as shown by the results in Figure 21. Table 15 shows the numerical mean values of the zeta potentials that were measured as well as the associated standard deviations in Table 16. The blue bars show the zeta potentials of the uncoated particles. From Figure 21, it can be seen that with each particle, the coating of hyaluronic acid makes their surface charge become more negative. The 0.1% hyaluronic acid in particular has the largest effect on the zeta potential and decreases the charge of each particle significantly and the most out of the other concentrations. Looking at just hyaluronic acid without any particles, it can be seen that in general, HA is a very negative solution. The 0.1% HA solution has the most negative charge of about -75 mV. It can be concluded that the HA coating has a significant effect on the zeta potential of the nanoparticles.

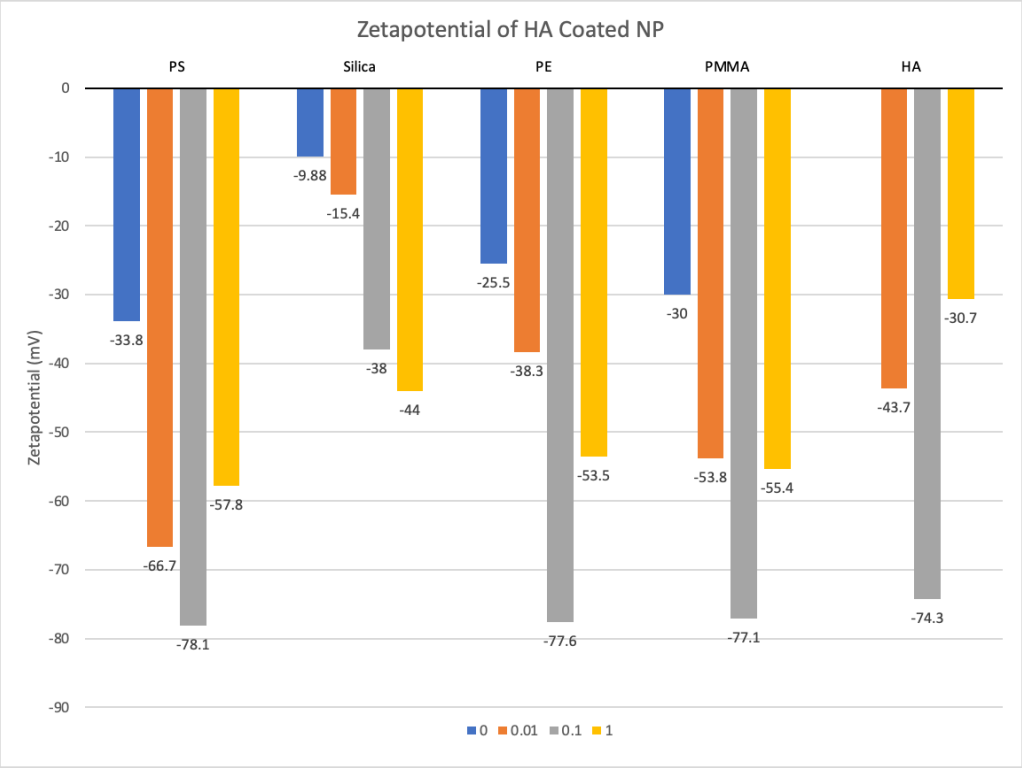


Figure 21. Average zeta potentials of HA coated PS, Silica, PE, PMMA and pure HA from left to right as a function of coating concentration (0%-1%).

Zeta Potentials of HA Coated NPs (mV)					
	Silica	PS	PMMA	PE	Pure HA
0% HA	-9.88	-33.8	-30	-25.5	N/A
0.01% HA	-15.4	-66.7	-53.8	-38.3	-43.7
0.1% HA	-38	-78.1	-77.1	-77.6	-74.3
1% HA	-44	-57.8	-55.4	-53.5	-30.7

Table 15. Zeta potentials (mV) of HA coated NPs as well as pure HA with no particles.

Std. Deviations of Zeta Potentials of HA Coated NPs +/- (mV)					
	Silica	PS	PMMA	PE	Pure HA
0% HA	2.48	0.473	0.115	0.586	N/A
0.01% HA	7.54	0.551	1.04	1.46	17.1
0.1% HA	8.85	3.11	2.07	2.19	1.8
1% HA	2.42	2.69	5.64	2.57	2.83

Table 16. Standard deviations of HA coated NPs as well as pure HA with no particles.

3.2.2 Polystyrene

3.2.2.1 Polystyrene/Hyaluronic Acid

Polystyrene nanoparticles coated with 0%-0.5% hyaluronic acid were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 22A and Figure 22B respectively and the associated numerical values are shown in Table 17. Figure 22A shows that with increasing concentration of HA coating, the PS nanoparticles become significantly more negative, reaching peak negative charge at 0.05% coating ($p<0.000001$). The zeta potential of the nanoparticles then becomes less negative at higher concentrations from 0.1% to 1% coating. The 0.5% and 1% coating show no significant difference compared to the uncoated particles. This shows that at higher concentrations of HA, the coating's ability to alter the surface charge of the particle is diminishing. Figure 22B shows that with increasing concentration of HA coating, the size of the PS nanoparticles becomes larger up to 0.1% coating. The size of the particle decreases after reaching this 0.1% effective concentration. The decrease in size could be due to high levels of aggregation, or the coating and particle are no longer interacting well.

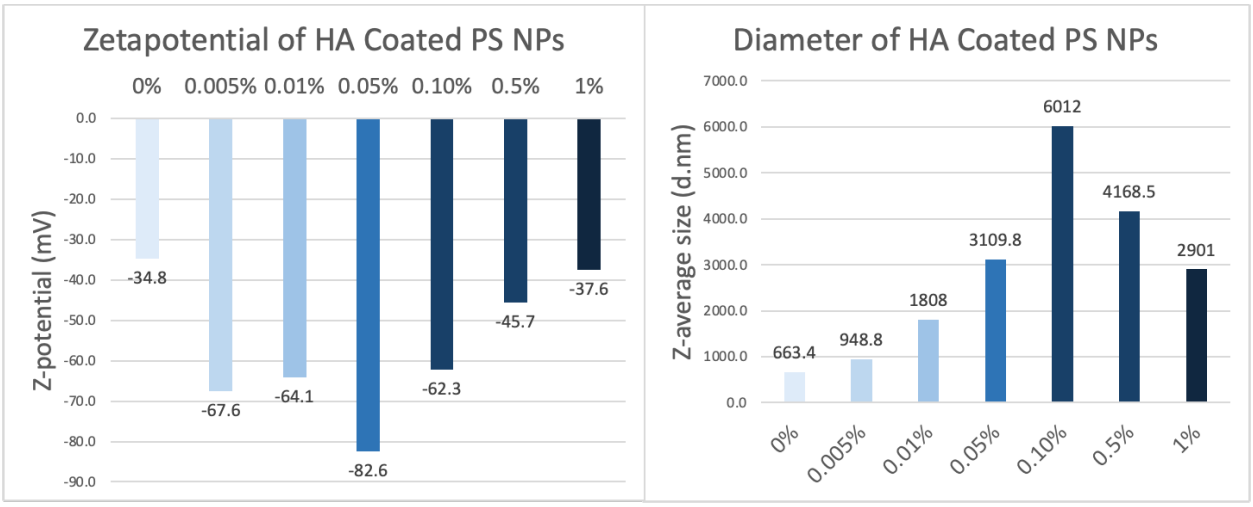


Figure 22: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of HA coated PS particles as a function of coating concentration.

HA Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% HA	-36.9 +/- 6.96	676.7 +/- 203.4
0.005 HA	-67.6 +/- 13.69	948.8 +/- 159.7
0.05% HA	-82.6 +/- 19.66	3109.8 +/- 664
0.5% HA	-45.7 +/- 5.57	4168.5 +/- 2416

Table 17. Zeta potentials (mV) and hydrodynamic diameters (nm) of HA coated PS NPs.

3.2.2.2 Polystyrene/Mucin

Polystyrene nanoparticles coated with 0%-1% mucin was characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 23A and Figure 23B respectively and the associated numerical values are shown in Table 18. Figure 23A shows that with increasing concentration of mucin coating, the PS nanoparticles become more neutral. At the highest concentration of coating, the nanoparticles are almost neutral at -2.63 mV. Figure 23B shows that from 0% to 0.1% coating, the size of the PS nanoparticles increases. There is no significant difference in size between 0.1% and 1%. This data gives us information about the interaction between the coating and particle. This data tells us that the coating is effectively covering the nanoparticle.

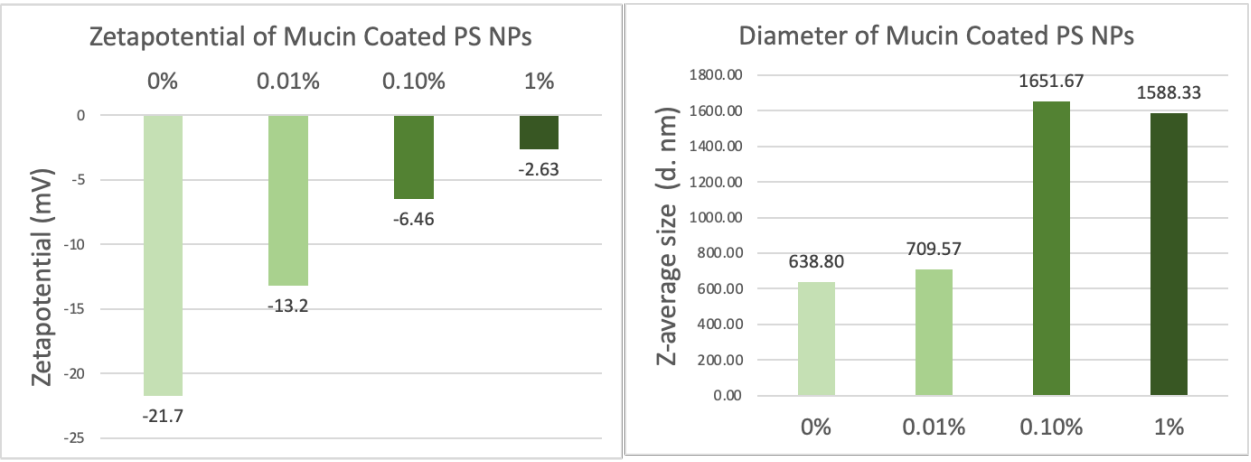


Figure 23: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of mucin coated PS particles as a function of coating concentration.

Mucin Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% Mucin	-21.7 +/- 6.61	638.80 +/- 56.39
0.01% Mucin	-13.2 +/- 1.37	709.57 +/- 39.12
0.1% Mucin	-6.46 +/- 0.78	1651.67 +/- 184.02
1.0% Mucin	-2.63 +/- 0.45	1588.33 +/- 255.57

Table 18. Zeta potentials (mV) and hydrodynamic diameters (nm) of mucin coated PS NPs.

3.2.2.3 Polystyrene/Hyaluronic Acid/0.1% Mucin

Polystyrene nanoparticles coated with 0%-1% HA and 0.1% mucin were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 24A and Figure 24B respectively and the associated numerical values are shown in Table 19. Figure 24A shows that with increasing concentration of HA with the 0.1% mucin coating from 0% HA to 0.1% HA, there is no significant change in zeta potential compared to the uncoated particles. However at 1% HA + 0.1% mucin the charge declines significantly to -49.30 mV ($p=0.00002$). Figure 24B shows that the 0% HA + 0.1% mucin coated particles and uncoaed particles have no significant difference in size. However, the sizes of the 0.01% HA and 0.1% HA with the 0.1% mucin particles are significantly larger than the uncoated control particles, indicating that the double coatings are effectively covering the particles. The 1% HA + 0.1% mucin coating had the largest size, however due to the high standard deviations of this sample the findings are not significant. At low concentrations of HA, the mucin is neutralizing the inherent negative charge of the HA. The interaction between the mucin and HA could also explain why the hydrodynamic diameter of the particles is closer to the values seen with

polystyrene particles coated solely with mucin seen in Figure 23B. It could be that at lower concentrations of HA, the mucin is interacting more with the polystyrene. However, at high concentration of HA, the HA has reached an effective concentration that causes more interaction with the polystyrene. This concept is demonstrated by the sudden decrease in charge and sudden increase in size, closer to values seen by polystyrene particles coated with just HA as seen in Figure 22B.

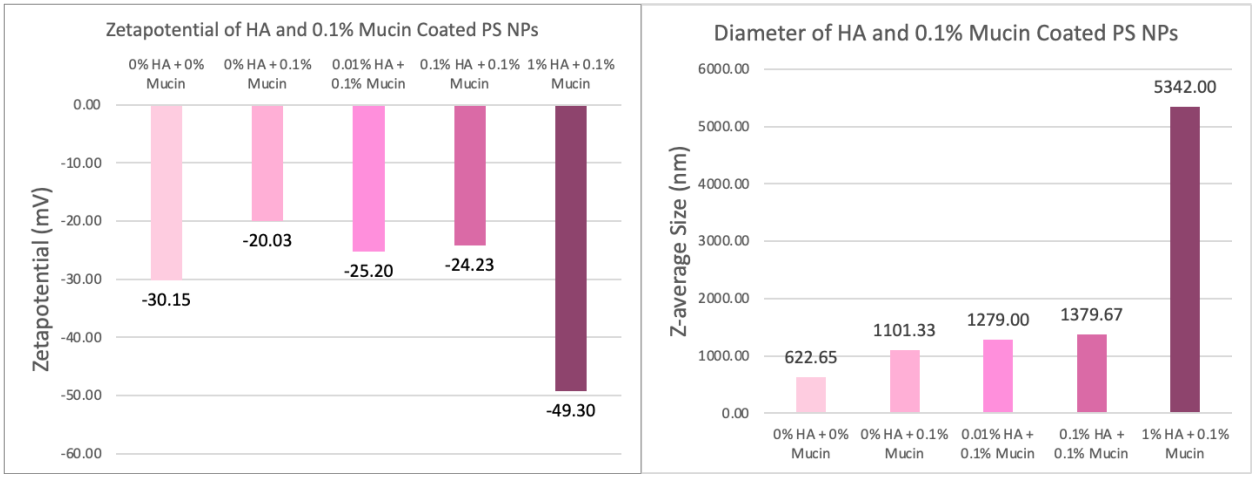


Figure 24: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of HA + 0.1% mucin double coated PS particles as a function of coating concentration.

HA + 0.1%Mucin Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% HA + 0% Mucin	-30.15 +/- 5.1	622.65 +/- 77.00
0% HA + 0.1% Mucin	-20.03 +/- 10.14	1101.33 +/- 436.20
0.01% HA+ 0.1% Mucin	-25.20 +/- 8.87	1279.00 +/- 313.00
0.1% HA+ 0.1% Mucin	-24.23 +/- 3.18	1379.67 +/- 122.66

1.0% HA+ 0.1% Mucin	-49.30 +/- 5.15	5342.00 +/- 4004.29

Table 19. Zeta potentials (mV) and hydrodynamic diameters (nm) of HA and 0.1% mucin double coated PS NPs.

3.2.2.4 Polystyrene/35 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 35KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 25A and Figure 25B respectively and the associated numerical values are shown in Table 20. Figure 25A shows that initially, the 0.01% 35KDa PEG coating made the surface charge of the polystyrene more negative at -27.17 mV compared to the uncoated particles. With increasing concentration of 35KDa PEG coating following the 0.01% coating, the PS nanoparticles become less negative. However, there is little change between the different concentrations of coating. Between 0% and 0.1% 35KDa PEG coating there is no significant difference in zeta potential. However, there is a significant difference between the uncoated particles compared to 0.01% ($p=0.0021$) and 1% (0.0023). Figure 25B shows that from 0% to 1% coating there is no significant difference in size. This could indicate that the 35KDaPEG coating is not interacting well with the polystyrene particles and they are not being coated sufficiently.

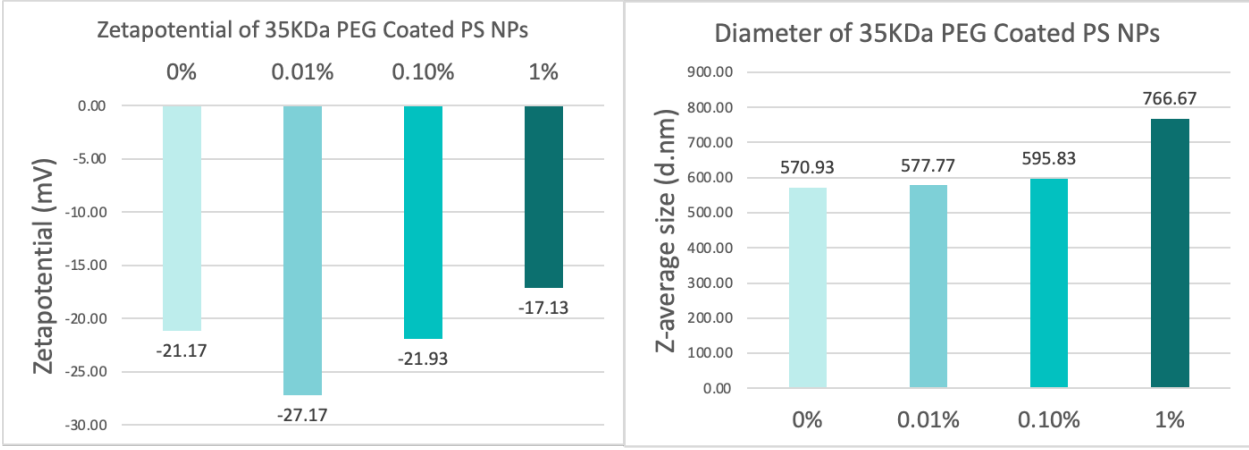


Figure 25: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 35KDa PEG coated PS particles as a function of coating concentration.

35KDa PEG Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% 35KDa PEG	-21.17 +/- 0.75	570.93 +/- 10.77
0.01% 35KDa PEG	-27.17 +/- 0.29	577.77 +/- 24.56
0.1% 35KDa PEG	-21.93 +/- 0.15	595.83 +/- 11.16
1.0% 35KDa PEG	-17.13 +/- 0.61	766.67 +/- 42.47

Table 20. Zeta potentials (mV) and hydrodynamic diameters (nm) of 35KDa PEG coated PSNPs.

3.2.2.5 Polystyrene/100 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 100KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 26A and Figure 26B respectively and the associated numerical values are shown in Table 21. Figure 26A shows that with increasing concentration of 100KDa PEG coating, the PS nanoparticles become more neutral. Between 0.01% and 0.1% coating, the zeta potential changes drastically ($p=0.0054$). There is no significant difference in zeta potential between 0% and 0.01% coating. Figure 26B shows that with increasing concentration of 100KDa PEG coating, the size of the PS nanoparticles becomes larger. However, the changes in size as a function of coating concentration are not significant. This tells us that the coating is not effectively covering the nanoparticles.

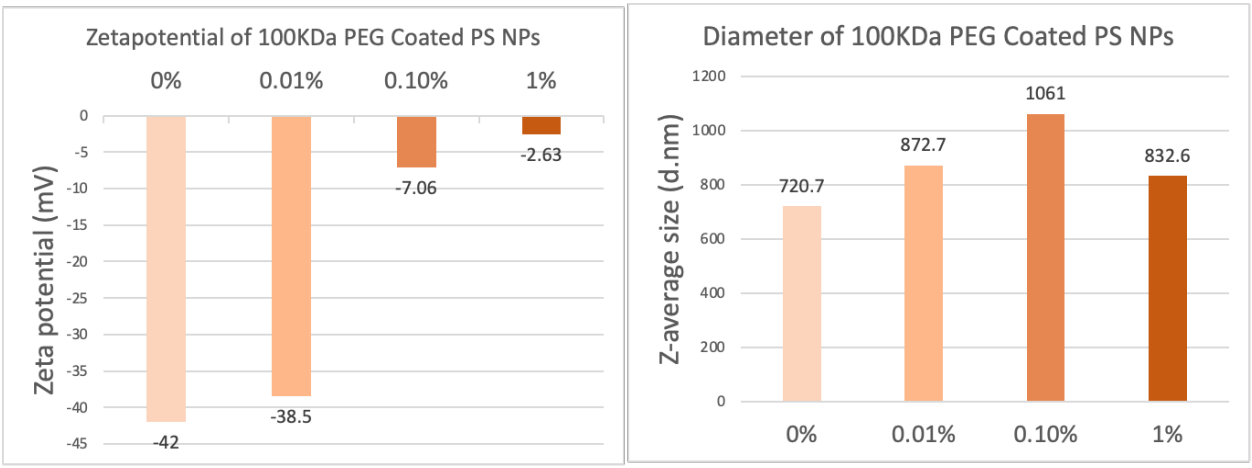


Figure 26: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 100KDa PEG coated PS particles as a function of coating concentration.

100KDa PEG Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% 100KDa PEG	-42 +/- 2.55	720.7 +/- 40.67
0.01% 100KDa PEG	-38.5 +/- 4.18	872.7 +/- 21.99
0.1% 100KDa PEG	-7.06 +/- 0.456	1061 +/- 130.4
1.0% 100KDa PEG	-2.63 +/- 0.0153	832.6 +/- 12.59

Table 21. Zeta potentials (mV) and hydrodynamic diameters (nm) of 100KDa PEG coated PS NPs.

3.2.2.6 Polystyrene/300 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 300KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 27A and Figure 27B respectively and the associated numerical values are shown in Table 22. Figure 27A shows that with increasing concentration of 300KDa PEG coating, the PS nanoparticles become more neutral, with the exception of the 0.1% 300KDa PEG coating. The 0.1% coating led to a more negative zeta potential compared to the uncoated sample. The 1% coating led to a drastic change in zeta potential nearing almost neutral very suddenly. Figure 27B shows that with increasing concentration of 300KDa PEG coating, the size of the PS nanoparticles becomes larger. The largest size is seen at 1% 300KDa PEG which is significantly greater than the uncoated particles ($p=0.003509$), which makes sense since it is the highest concentration of polymer coating. The steady increase of size over concentration also indicates that the coating is effectively covering the nanoparticles. The neutral charge at 1% coating paired with the large diameter indicates a prospective formulation for enhanced diffusion.

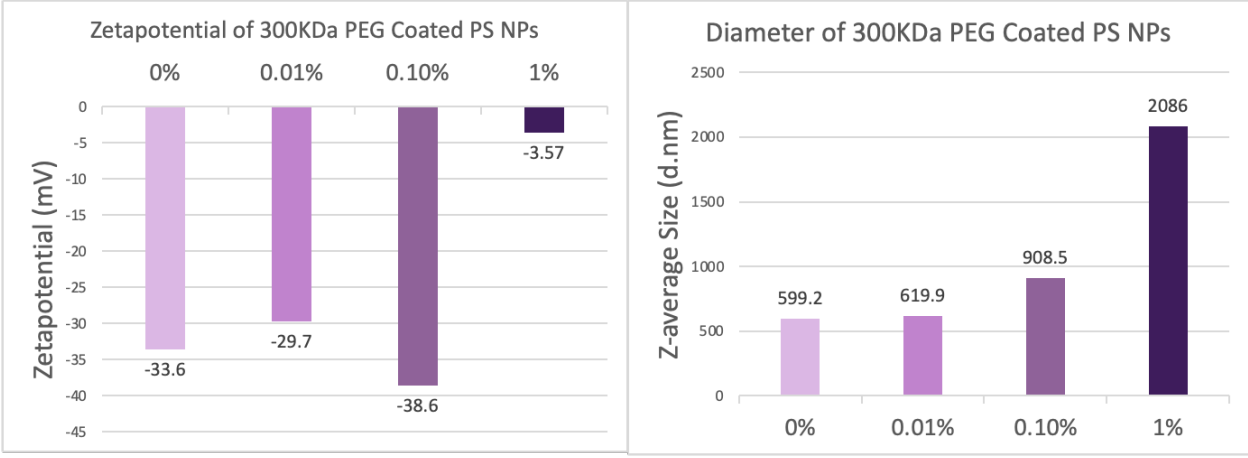


Figure 27: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 300KDa PEG PS particles as a function of coating concentration.

300KDa PEG Coated PS NPs		
	Zeta Potential (mV)	Diameter (nm)
0% 300KDa PEG	-33.6 +/- 0.36	599.2 +/- 37.13
0.01% 300KDa PEG	-29.7 +/- 0.08	619.9 +/- 22.90
0.1% 300KDa PEG	-38.6 +/- 0.22	908.5 +/- 159.3
1.0% 300KDa PEG	-3.57 +/- 0.02	2086 +/- 178.0

Table 22. Zeta potentials (mV) and hydrodynamic diameters (nm) of 300KDa PEG coated PS NPs.

3.2.3 Silica

3.2.3.1 Silica/Hyaluronic Acid

Polystyrene nanoparticles coated with 0%-0.5% hyaluronic acid were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 28A and Figure 28B respectively and the associated numerical values are shown in Table 23. Figure 28A shows that with increasing concentration of HA coating, the PS nanoparticles become more negative reaching peak negative charge at 0.05%, followed by more neutral charge at 0.5% coating. Figure 28B shows that with increasing concentration of HA coating, the size of the PS nanoparticles becomes significantly larger up to 0.05% coating ($p=0.0018$) followed by a steep decline at 0.5% coating ($p=0.0004$). This indicates that the 0.05% coating is more effective at covering the nanoparticles and is associated with the most negative charge zeta potential at -74.6 mV.

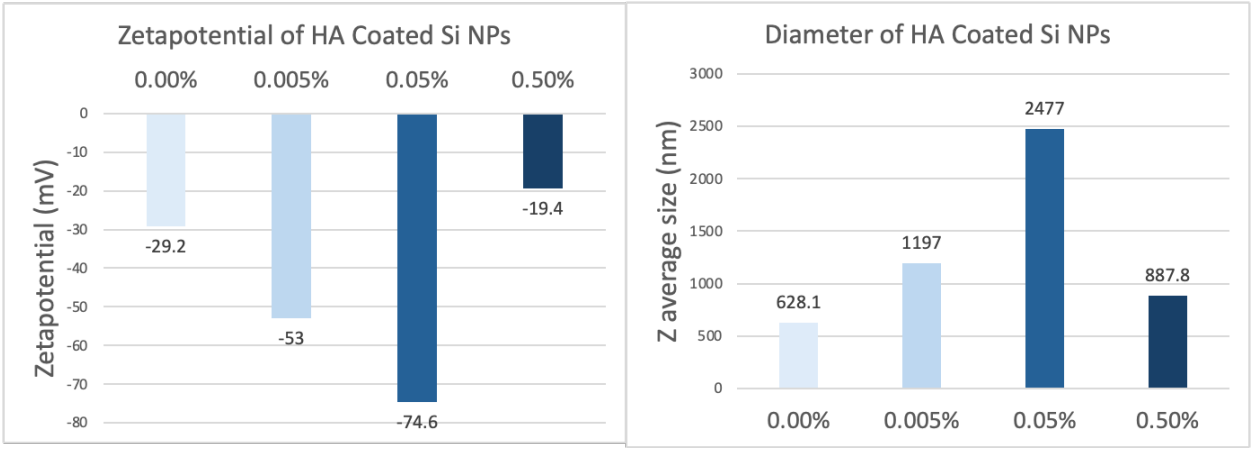


Figure 28: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of HA coated silica particles as a function of coating concentration.

HA Coated Silica NPs		
	Zeta Potential (mV)	Diameter (nm)
0.005% HA	-53 +/- 1.61	1197 +/- 105.1
0.05% HA	-74.6 +/- 1.11	2477 +/- 137.6
0.5% HA	-19.4 +/- 1.71	887.8 +/- 185.1

Table 23. Zeta potentials (mV) and hydrodynamic diameters (nm) of HA coated silica NPs.

3.2.3.2 Silica/Mucin

Polystyrene nanoparticles coated with 0%-1% mucin were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 29A and Figure 29B respectively and the associated numerical values are shown in Table 24. Figure 29A shows that with increasing concentration of mucin coating, the PS nanoparticles become more neutral. Figure 29B shows that with increasing concentration of mucin coating, the size of the PS nanoparticles becomes larger, with the largest size being at 0.1% mucin coating followed by a decline in size at 1% coating. This could indicate that the 1% mucin coating is not interacting well with the silica nanoparticle.

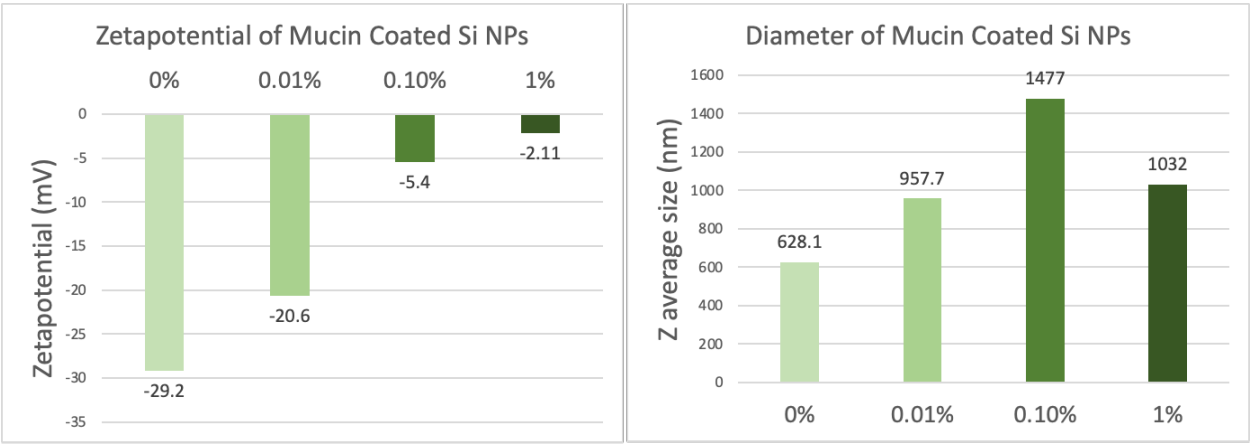


Figure 29: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of mucin coated silica particles as a function of coating concentration.

Mucin Coated Silica NPs		
	Zeta Potential (mV)	Diameter (nm)
0.01% Mucin	-20.6 +/- 1.04	957.7 +/- 11.4
0.1% Mucin	-5.4 +/- 0.20	1477 +/- 137.40
1.0% Mucin	-2.11 +/- 0.16	1032 +/- 51.20

Table 24. Zeta potentials (mV) and hydrodynamic diameters (nm) of mucin coated silica NPs.

3.2.3.3 Silica/35 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 35KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 30A and Figure 30B respectively and the associated numerical values are shown in Table 25. Figure 30B shows that with increasing concentration of 35KDa PEG coating, the PS nanoparticles become less negative, but with no set pattern. The particles are not near neutral but are less negative compared to the control. The most negative particles are the uncoated ones. The

only significant difference was seen between the 0% and 1% coating (p=0.0049). Figure 30B shows that with increasing concentration of 35KDa PEG coating, there is no significant difference in size when comparing the uncoated and coated particles, with increasing concentration of coating. The relatively unchanged size of nanoparticles with increasing concentration may indicate that the polymeric coating is not effectively covering the nanoparticles.

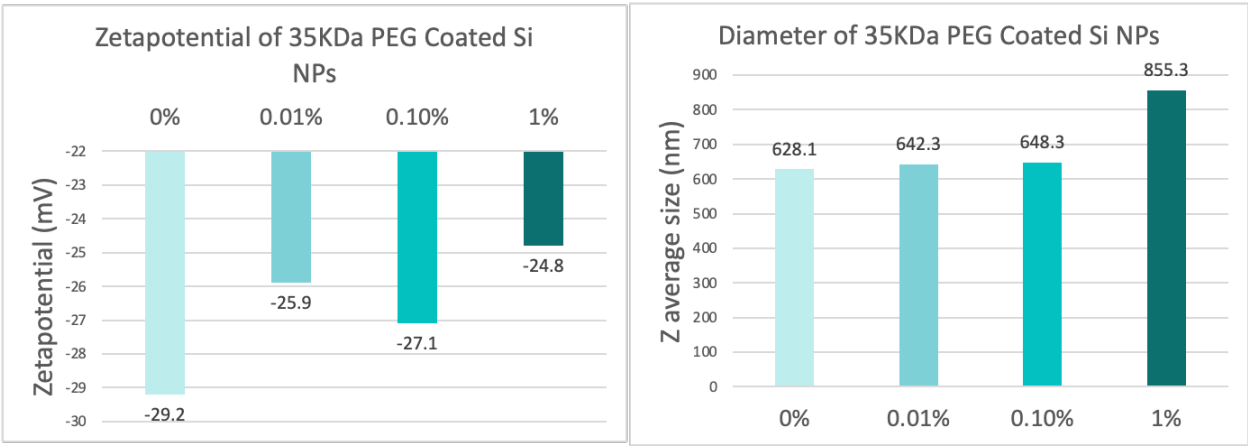


Figure 30: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 35KDa PEG coated silica particles as a function of coating concentration.

35KDa PEG Coated Silica NPs		
	Zeta Potential (mV)	Diameter (nm)
0.01% 35KDa PEG	-25.9 +/- 0.12	642.3 +/- 22.96
0.1% 35KDa PEG	-27.1 +/- 1.24	648.3 +/- 36.00
1.0% 35KDa PEG	-24.8 +/- 1.00	855.3 +/- 42.10

Table 25. Zeta potentials (mV) and hydrodynamic diameters (nm) of 35KDa PEG coated silica NPs.

3.2.3.4 Silica/100 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 100KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 31A and Figure 31B respectively and the associated numerical values are shown in Table 26. Figure 31A shows that with increasing concentration of 100KDa PEG coating, the PS nanoparticles become more neutral. The most neutral charge was seen with the 1% coating. Figure 31B shows that with increasing concentration of 100KDa PEG coating, the size of the PS remains relatively unchanged. The only significant difference in size was seen when comparing the 1% coating to 0% and 0.1% coatings. The unchanged size of the nanoparticles with increasing concentration may indicate that the coating and particles are not interacting well.

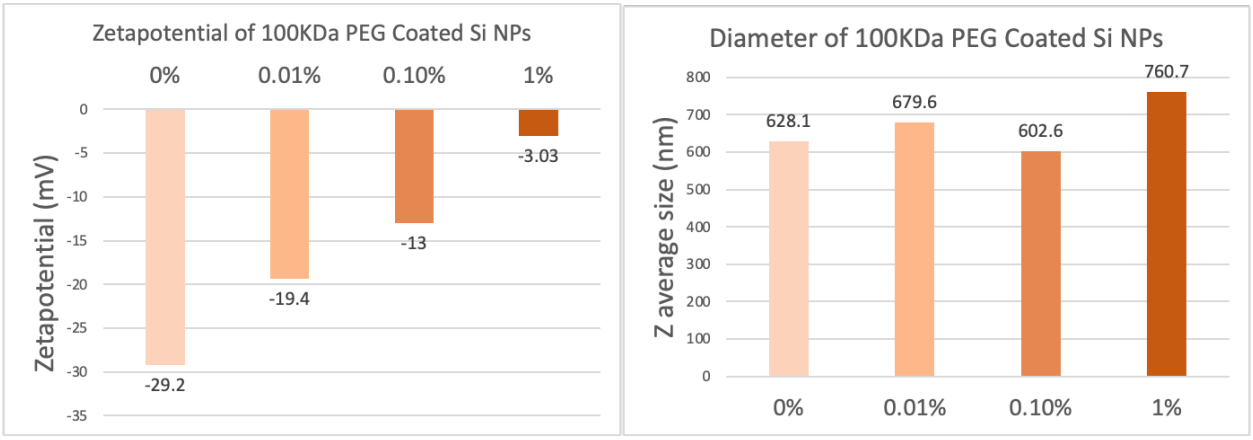


Figure 31: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 100KDa PEG coated silica particles as a function of coating concentration.

100KDa PEG Coated Silica NPs		
	Zeta Potential (mV)	Diameter (nm)
0.01% 100KDa PEG	-19.4 +/- 0.31	679.6 +/- 60.29
0.1% 100KDa PEG	-13 +/- 0.60	602.6 +/- 22.24
1.0% 100KDa PEG	-3.03 +/- 0.13	760.7 +/- 6.93

Table 26. Zeta potentials (mV) and hydrodynamic diameters (nm) of 100KDa PEG coated silica NPs.

3.2.3.5 Silica/300 KDa PEG

Polystyrene nanoparticles coated with 0%-1% 300KDa PEG were characterized using their zeta potential and hydrodynamic diameter. The results from these studies are shown in Figure 32A and Figure 32B respectively and the associated numerical values are shown in Table 27. Figure 32A shows that with increasing concentration of 300KDa PEG coating, the PS nanoparticles become more neutral. The most neutral charge is seen at the highest concentration of coating. Figure 32B shows that with increasing concentration of HA coating, the size of the PS nanoparticles becomes significantly larger at 1% coating ($p=0.0040$). The highest size is seen at 1% 300KDa PEG. The steady increase of size over concentration also indicates that the coating is effectively covering the nanoparticles at concentrations above 0.01%.

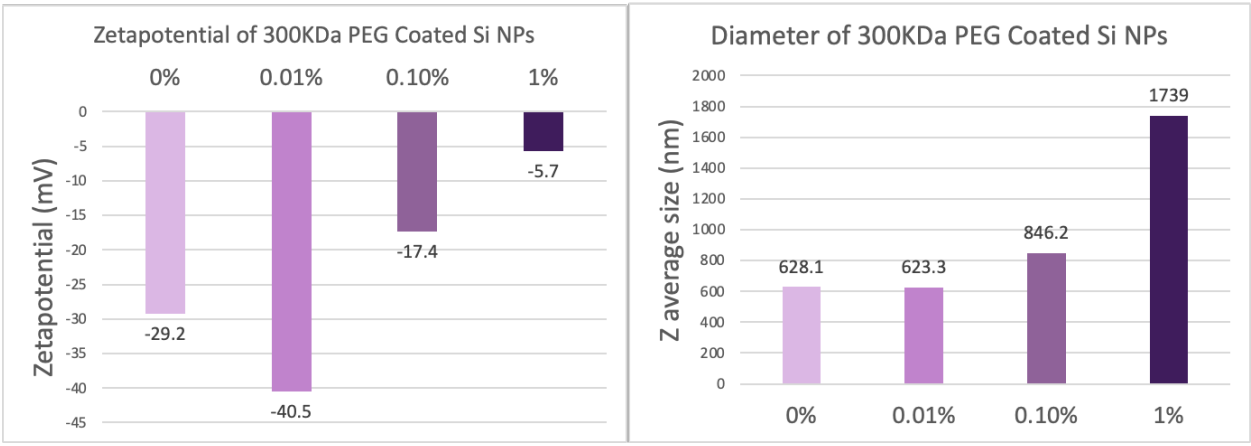


Figure 32: (A) Average zeta potentials (mV) and (B) average hydrodynamic diameters (nm) of 300KDa PEG coated silica particles as a function of coating concentration.

300KDa PEG Coated Silica NPs		
	Zeta Potential (mV)	Diameter (nm)
0.01% 300KDa PEG	-40.5 +/- 0.80	623.2 +/- 22.43
0.1% 300KDa PEG	-17.4 +/- 0.21	846.2 +/- 146.5
1.0% 300KDa PEG	-5.7 +/- 1.48	1739 +/- 194.7

Table 27. Zeta potentials (mV) and hydrodynamic diameters (nm) of 300KDa PEG coated silica NPs.

3.3 Cell Absorption Studies

Caco-2 cells were used to mimic the cellular lining of the GI system and monitor the uptake of uncoated and coated polystyrene nanoparticles. 3 different coatings were observed in these studies: mucin, 100KDa PEG, and 300KDa PEG each with 4 sets of experimental conditions. The conditions were labeled as follows: A (+/+): Coated nanoparticles and 5% mucin

layer; B (+/-): Coated nanoparticles and no 5% mucin layer; C (-/+): uncoated nanoparticles and 5% mucin layer; and D (-/-): uncoated nanoparticles and no 5% mucin layer. Firstly, the percent of nanoparticles per field of view that underwent transcytosis for each condition and coating was determined. Then, the percent of nanoparticles per field of view that were internalized was determined after the 96-hour time point. These nanoparticles diffused into the cells through the apical side, but did not diffuse out of the basal side. For this reason, these particles were confined to the interior of the cells and using 3D fluorescent imaging these particles were able to be visualized and quantified. It is important to note that the units of these studies are percent nanoparticles per field of view. This is a ratio of the fluorescent area of an image to the entire area of the image. The fluorescence is from the nanoparticles, thus these studies allow us to make comparisons between different experimental conditions on transcytosis and internalization but do not quantify the amount of nanoparticles that participated.

3.3.1 0.1% Mucin Coated Polystyrene Nanoparticles

Figure 33 depicts the percent of uncoated or 0.1% mucin coated nanoparticles per field of view that diffused into and out of the Caco-2 cells and the associated numerical values and standard deviations can be seen in Tables 28 and 29, respectively. From Figure 33 it can be seen Condition A (+/+) showed a rapid increase in nanoparticle diffusion over time. At each time point, Condition A (+/+) showed significantly higher nanoparticle diffusion compared to every other condition. This indicates the 0.1% mucin coating, and the presence of a 5% mucin layer enhanced the diffusion of these particles through the Caco-2 cells. The second highest diffusion of nanoparticles was seen in Condition C (-/+), where the particles were uncoated, but there was a 5% mucin layer present. This could indicate that the spontaneous bio-coating of the nanoparticles by the 5% mucin layer led to the enhanced diffusion of the particles. Condition B

(+/-) showed negligible uptake until the final time point at 96 hours post deposition, which shows that the 0.1% mucin coating did help the nanoparticles diffuse through the cells compared to Condition D (-/-), but the absence of the 5% mucin layer significantly hindered diffusion through the cells.

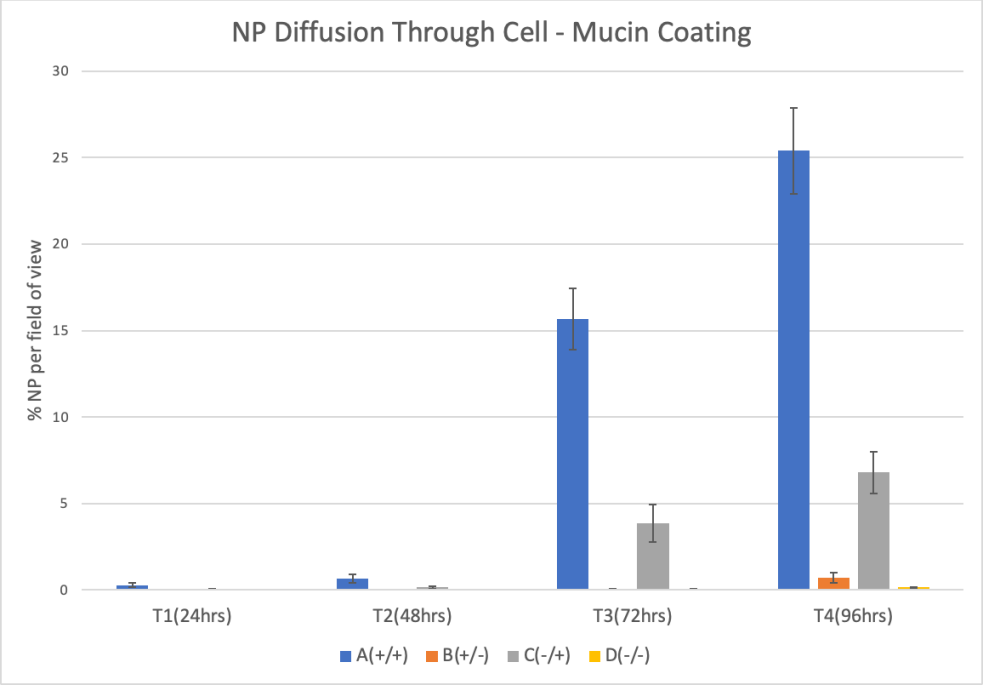


Figure 33. Amount of uncoated or 0.1% mucin coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

% NP Diffusion Through the Cell - Mucin Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.29	0.666	15.67	25.39
B(+/-)	0.020	0.028	0.035	0.715
C(-/+)	0.035	0.151	3.877	6.792
D(-/-)	0.011	0.027	0.038	0.145

Table 28. Mean percent mucin coated or uncoated NP diffusion through the cell per field of view.

Std. Deviations of % NP Diffusion Through the Cell - Mucin Coating				
	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.133	0.241	1.772	2.479
B(+/-)	0.007	0.011	0.0166	0.296
C(-/+)	0.022	0.084	1.086	1.222
D(-/-)	0.006	0.017	0.011	0.030

Table 29. Standard deviations of the mean percent mucin coated or uncoated NP diffusion through the cell per field of view.

Figure 34 shows a cumulative report of the nanoparticle's diffusion over time and the associated numerical values can be seen in Table 30. From Figure 34, it is apparent that Condition A (+/+) had the highest transcytosis by the end of the 96 hours. This further supports the idea that both the polymeric coating, in this case 0.1% mucin, coupled with the 5% mucin layer on top of the Caco-2 cells are responsible for rapid and efficient diffusion of the polystyrene nanoparticles through the cells. It is also important to note that the control condition, Condition D (-/-), had the lowest amount of overall cumulative nanoparticle diffusion almost to a negligible degree.

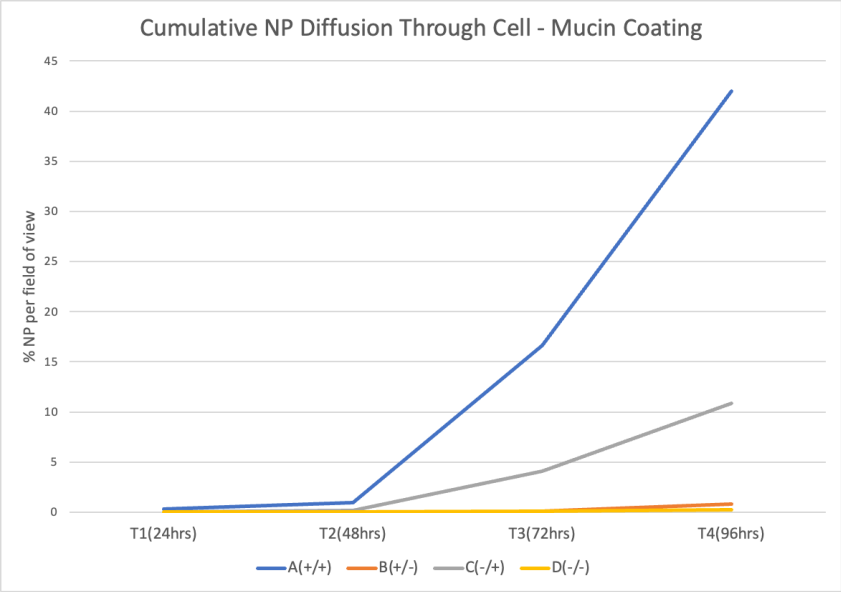


Figure 34. Cumulative uptake of uncoated or 0.1% mucin coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

Cumulative % NP Diffusion Through the Cell - Mucin Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.287	0.9528	16.62	42.01
B(+/-)	0.019	0.0477	0.083	0.799
C(-/+)	0.035	0.1863	4.0635	10.86
D(-/-)	0.0111	0.0385	0.0766	0.2212

Table 30. Cumulative mean percent mucin coated or uncoated NP diffusion through the cell per field of view.

Figure 35 shows the quantification of the uncoated and 0.1% mucin coated nanoparticles, with or without a 5% mucin layer present that were confined to the interior of the cell and the

associated numerical values can be seen in Table 31. From Figure 35 Conditions B (+/-) and Conditions D (-/-) had the highest percentage of nanoparticles per field of view that were confined to the cell. Conversely, Condition A (+/+) and Condition C (-/+) had the lowest percentage of nanoparticles per field of view confined to the cell. This indicates that the presence of the 5% mucin layer has a significant effect on the nanoparticles' abilities, uncoated or 0.1% mucin coated, to diffuse out of the cells.

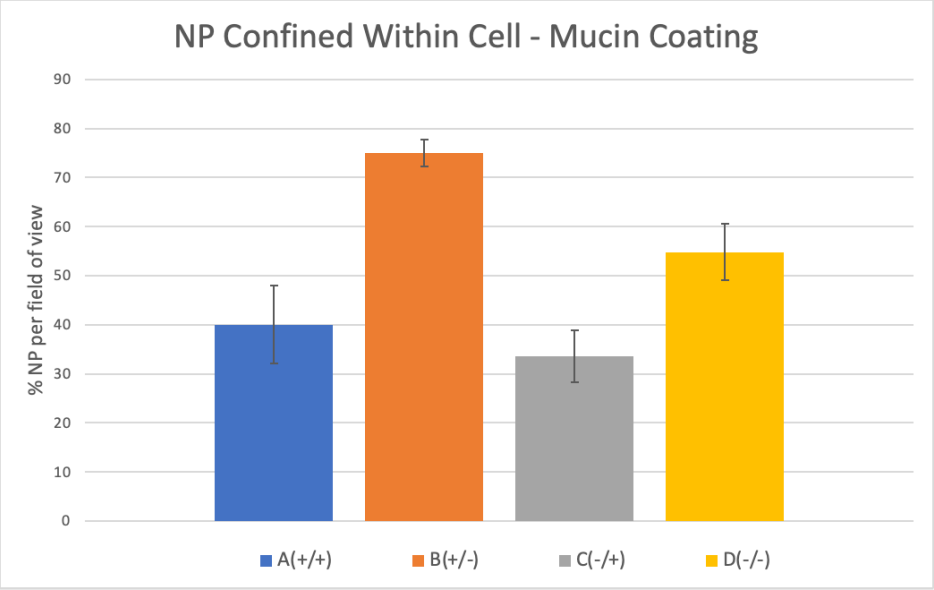


Figure 35. Amount of uncoated or 0.1% mucin coated polystyrene nanoparticles that were able to enter the Caco-2 cells but not exit as a function of 5% mucin layer following the 96-hour time point.

Condition	% NP Confined Within the Cell - Mucin Coating	Std. Deviation
A(+/+)	40.00	+/- 7.962
B(+/-)	75.02	+/- 2.676
C(-/+)	33.56	+/- 5.270
D(-/-)	54.79	+/- 5.71

Table 31. Percent mucin coated or uncoated NP confined within the cell per field of view.

3.3.2 0.1% 100KDa PEG Coated Polystyrene Nanoparticles

Figure 36 depicts the percent of uncoated or 0.1% 100KDa PEG coated nanoparticles that diffused into and out of the Caco-2 cells and the associated numerical values and standard deviations can be seen in Tables 32 and 33, respectively. From Figure 36 Condition A (+/+) showed the highest percentage of nanoparticles per field of view that diffused through the cell at each time point. At a time point of 96 hours, Condition A (+/+) has significantly higher diffusion of the nanoparticles compared to any other experimental group. The diffusion of Condition C (-/+) showed the next highest diffusion, but compared to the 58% diffusion of nanoparticles exhibited by Condition A (+/+) at time point 96 hours, Condition C (-/+) exhibited merely 4.9%. This indicates that the 5% mucin coated has a significant impact on the nanoparticles', coated and uncoated, ability to diffuse through the Caco-2 cells. Condition A (+/+), however, exhibited the highest diffusion, which indicates that the formulation of 0.1% 100KDa PEG with the 5% mucin layer enhanced diffusion significantly.

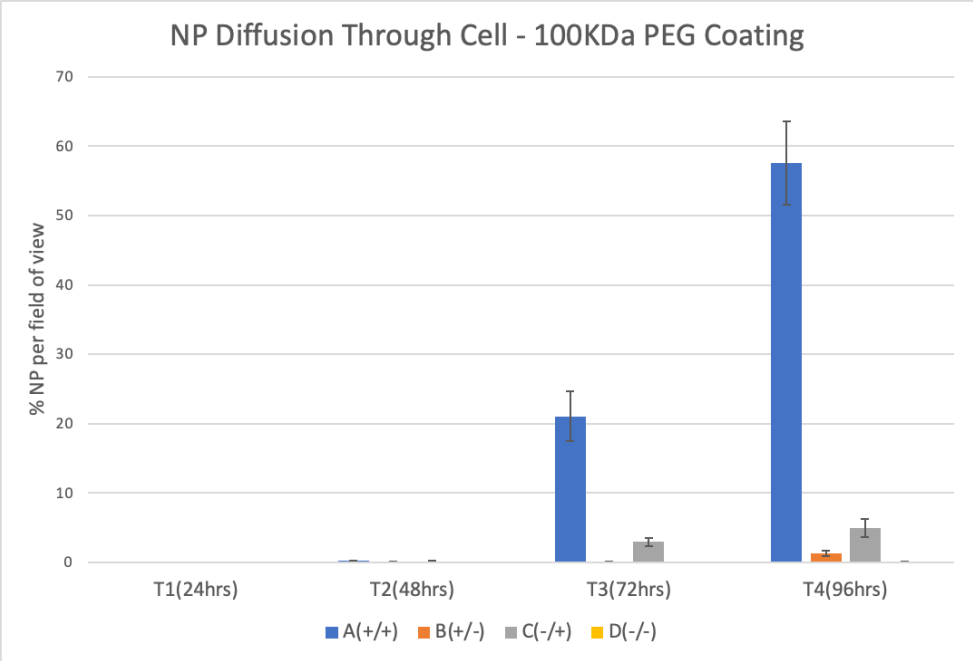


Figure 36. Amount of uncoated or 0.1% 100KDa PEG coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

% NP Diffusion Through the Cell - 100KDa PEG Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.0095	0.2000	21.020	57.595
B(+/-)	0.0062	0.0192	0.0234	1.268
C(-/+)	0.0087	0.133	2.9324	4.901
D(-/-)	0.0047	0.0062	0.0122	0.0180

Table 32. Mean percent 100KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Std. Deviations of % NP Diffusion Through the Cell - 100KDa PEG Coating				
	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.0038	0.0489	3.571	6.0305
B(+/-)	0.0032	0.0105	0.0068	0.3619
C(-/+)	0.0034	0.04056	0.6185	1.303
D(-/-)	0.0021	0.0015	0.0043	0.0062

Table 33. Standard deviations of the mean percent 100KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Figure 37 shows a cumulative report of the nanoparticle's diffusion over time and the associated numerical values can be seen in Table 34. From Figure 37, it is apparent that Condition A (+/+) showed the highest cumulative diffusion of nanoparticles over time, nearing 80% nanoparticles per field, with rapid diffusion occurring between 72 and 96 hours. Condition C (-/+) followed for the next highest cumulative diffusion of nanoparticles over time, nearing 10% nanoparticle diffusion at the 96-hour time point. Condition B (+/-) had less than 5% nanoparticle diffusion over time, and Condition D (-/-) had almost negligible nanoparticle diffusion over time.

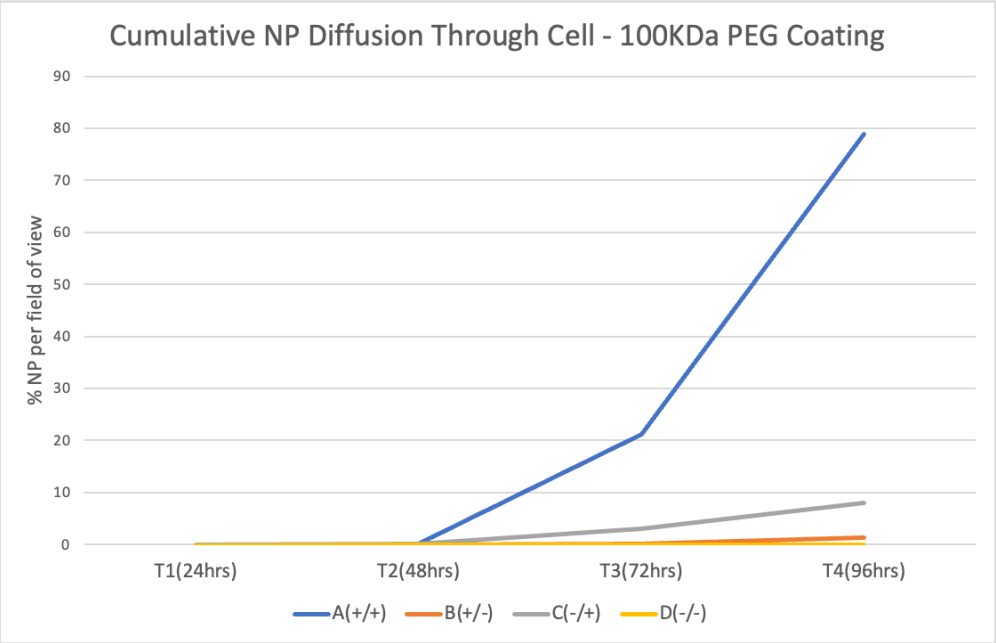


Figure 37. Cumulative uptake of uncoated or 0.1% 100KDa PEG coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

Cumulative % NP Diffusion Through the Cell - 100KDa PEG Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.0100	0.20956	21.23	78.83
B(+/-)	0.0062	0.0253	0.0490	1.317
C(-/+)	0.0087	0.1424	3.075	7.977
D(-/-)	0.0048	0.0110	0.0233	0.0414

Table 34. Cumulative mean percent 100KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Figure 38 shows the quantification of the uncoated and 0.1% 100KDa PEG coated nanoparticles, with or without a 5% mucin layer present that were confined to the interior of the

cell and the associated numerical values can be seen in Table 35. From Figure 38 it can be seen that Condition B (+/-) and Condition A (+/+) exhibited the highest amount of nanoparticles confined to a cell. This indicates that the nanoparticles under this condition were able to enter the cell but were unable to diffuse out of the cell. Condition D (-/-) and Condition C (-/+) also exhibited a proportion of nanoparticles being confined to the cell, but to a lower extent compared to the other two conditions. This indicates that in this formulation, the 0.1% 100KDa PEG coating had a greater impact on the cells being confined to the cell rather than the presence of the 5% mucin layer. One reason for this could be that the 0.1% PEG coating is inhibiting the nanoparticles' expulsion out of the cell. Another could be that when nanoparticles are diffused into the cell through endocytosis, they are not able to escape the vesicle.

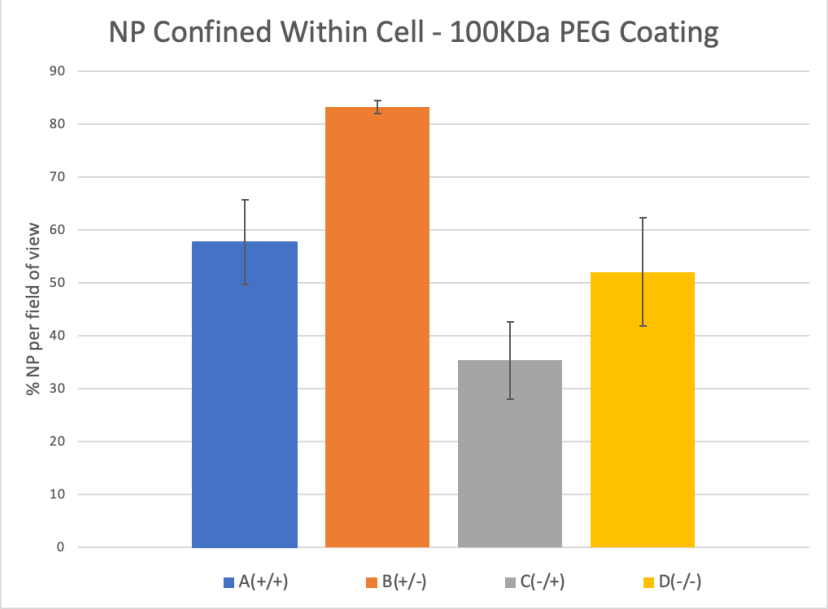


Figure 38. Amount of uncoated or 0.1% 100KDa PEG coated polystyrene nanoparticles that were able to enter the Caco-2 cells but not exit as a function of 5% mucin layer following the 96-hour time point.

Condition	% NP Confined Within the Cell - 100KDa PEG Coating	Std. Deviation
A(+/+)	57.67	+/- 8.0169
B(+/-)	83.22	+/- 1.170
C(-/+)	35.34	+/- 7.300
D(-/-)	52.034	+/- 10.223

Table 35. Percent 100KDa PEG coated or uncoated NP confined within the cell per field of view.

3.3.3 0.1% 300KDa PEG Coated Polystyrene Nanoparticles

Figure 39 depicts the percent of uncoated or 0.1% 300KDa PEG coated nanoparticles that diffused into and out of the Caco-2 cells and the associated numerical values and standard deviations can be seen in Tables 36 and 37, respectively. From Figure 39, Condition C (-/+) exhibited the highest diffusion of nanoparticles at every time point, and had the most significant diffusion at a time point of 96 hours. Condition A (+/+) showed the second highest diffusion of nanoparticles. This pattern is the opposite of what was seen in the other coating formulations. In this formulation, the uncoated particles with the 5% mucin layer had the highest diffusion. Conditions B (+/-) and Conditions D (-/-) had significantly lower diffusion throughout each time point, almost to a negligible degree. It is important to note that the highest diffusion seen throughout the experiment was 7% nanoparticle diffusion which is significantly lower than the two other experiments.

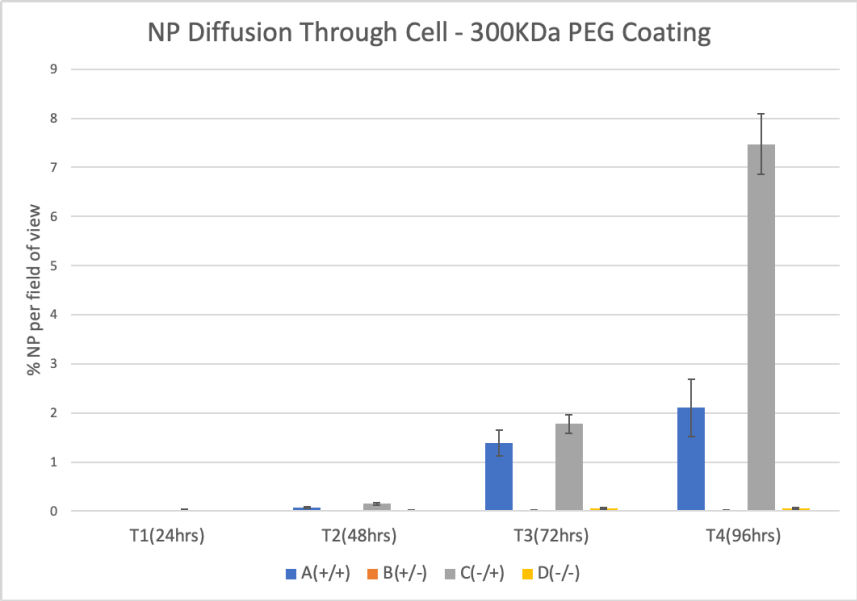


Figure 39. Amount of uncoated or 0.1% 300KDa PEG coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

% NP Diffusion Through the Cell - 300KDa PEG Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.003	0.070	1.3869	2.1034
B(+/-)	0.001	0.0051	0.0125	0.016
C(-/+)	0.029	0.1480	1.775	7.474
D(-/-)	0.006	0.011	0.0569	0.0586

Table 36. Mean percent 300KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Std. Deviations of % NP Diffusion Through the Cell - 300KDa PEG Coating				
	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.0016	0.0147	0.2587	0.5834
B(+/-)	0.00019	0.0021	0.0053	0.004
C(-/+)	0.0099	0.023	0.1921	0.6118
D(-/-)	0.0037	0.005	0.0180	0.0163

Table 37. Standard deviations of the mean percent 300KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Figure 40 shows a cumulative report of the nanoparticle's diffusion over time and the associated numerical values can be seen in Table 38. From Figure 40, it can be seen that Condition C (-/+) had the highest percentage of nanoparticles per field of view over time, with a total of 9% transcytosis followed by Condition A (+/+) at almost 4%. Conditions B (+/-) and D (-/-) had less than 1% diffusion at the end of the 96-hour time point. One reason for the enhanced diffusion of the Condition C (-/+) particles could be that the 5% mucin layer is helping the nanoparticles diffuse into the cells, however with the addition of the 0.1% 300KDa PEG coating as seen in Condition A(+/+), there is less diffusion.

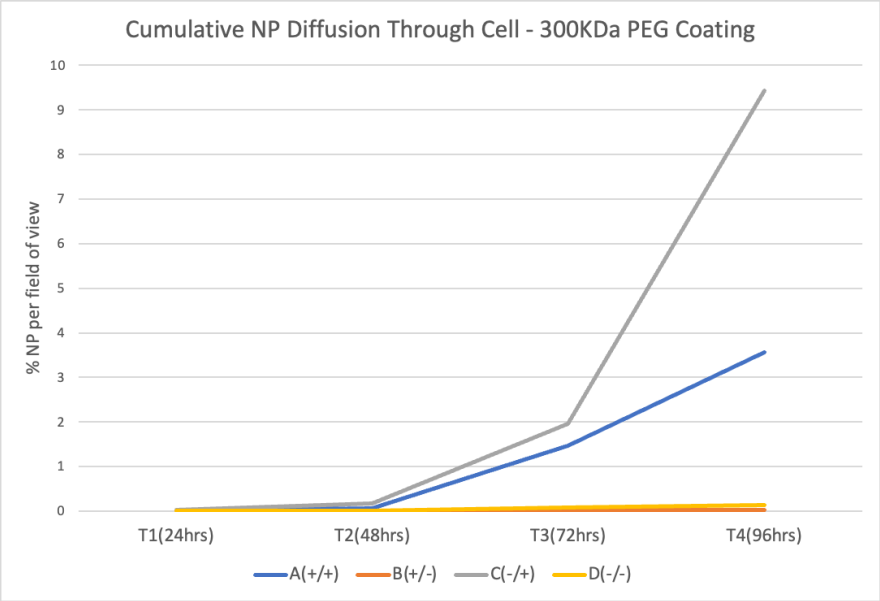


Figure 40. Cumulative uptake of uncoated or 0.1% 300KDa PEG coated polystyrene nanoparticles that underwent transcytosis in Caco-2 cells as a function of 5% mucin layer presence over a period of 96 hours expressed as percentage NP per field of view.

Cumulative % NP Diffusion Through the Cell - 100KDa PEG Coating				
Condition	T1(24hrs)	T2(48hrs)	T3(72hrs)	T4(96hrs)
A(+/+)	0.003	0.0721	1.4591	3.5629
B(+/-)	0.0013	0.0065	0.0189	0.0358
C(-/+)	0.0287	0.177	1.9519	9.4260
D(-/-)	0.0056	0.0169	0.074	0.1324

Table 38. Cumulative mean percent 300KDa PEG coated or uncoated NP diffusion through the cell per field of view.

Figure 41 shows the percentage of nanoparticles per field of view of the uncoated and 0.1% 300KDa PEG coated nanoparticles, with or without a 5% mucin layer present that were confined to the interior of the cell and the associated numerical values can be seen in Table 39.

From Figure 41 it can be seen that this formulation had a significantly higher internalization compared to the mucin and 100KDa PEG formulation. Conditions A (+/+), C (-/+), and D (-/-) all displayed 50-60% confinement of the nanoparticles. This leads us to believe that the particles are able to enter the cell but are not able to exit. Condition A (+/+) and Condition C (-/+) could be trapped due to their size. The low amount of confinement shown by Condition B (+/-) paired with almost negligible diffusion through the cell indicates that these particles are not even entering the cell, they are being blocked outside of the cell. The addition of the 5% mucin layer, seen in Condition A (+/+) helps the nanoparticles enter the cell, but not exit. A few reasons for confinement could be due to their sheer size, or inability to escape the endosome.

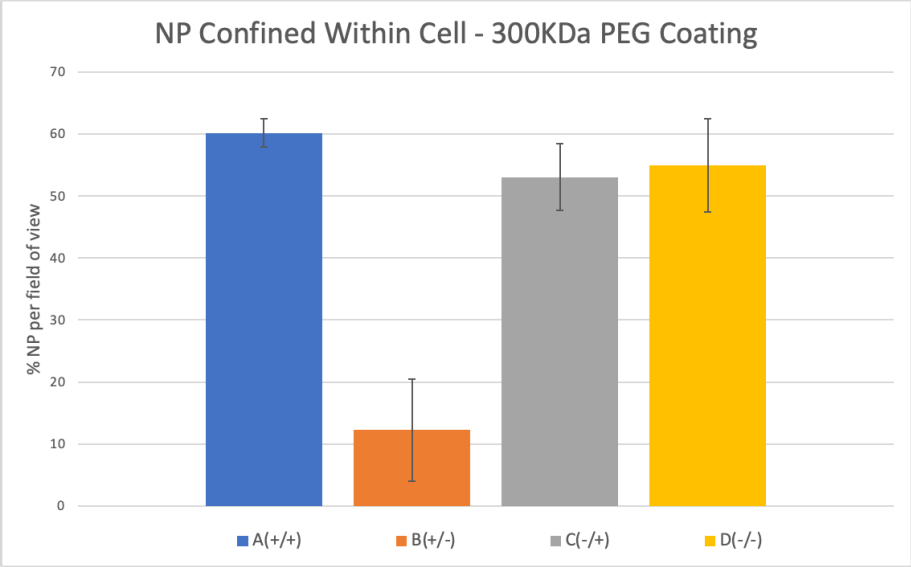


Figure 41. Amount of uncoated or 0.1% 300KDa PEG coated polystyrene nanoparticles that were able to enter the Caco-2 cells but not exit as a function of 5% mucin layer following the 96-hour time point.

Condition	% NP Confined Within the Cell - 300KDa PEG Coating	Std. Deviation
A(+/+)	60.19	+/- 2.214
B(+/-)	12.25	+/- 8.170
C(-/+)	53.03	+/- 5.369
D(-/-)	54.93	+/- 7.522

Table 39. Percent 300KDa PEG coated or uncoated NP confined within the cell per field of view.

4.0 Conclusion

The diffusion studies using particle tracking analysis is a robust technique in predicting the diffusive capabilities of polymeric nanoparticles. Hyaluronic acid coated polystyrene nanoparticles showed significantly enhanced diffusion in GI mucin. The HA coating reduced the charge of the nanoparticles to as low as -82 mV. The steady increase in diameter with increasing concentration of coating also indicates that HA effectively coated the nanoparticle. With silica nanoparticles, the HA coating significantly reduced aggregation in the mucin and even showed enhancement of diffusion in GI mucin compared to the uncoated particles – an observation that was only found with the HA coating.

Mucin coated polystyrene nanoparticles showed enhanced diffusion in GI mucin compared to the uncoated particles. The neutralization of the nanoparticles was also confirmed with the coating of the particles. The mucin coated particles also showed enhanced cellular uptake in the Caco-2 cells. These findings confirm the bio-coating theory, that the neutralization and spontaneous coating of particles with mucin enhances their penetrative capabilities in GI mucin. However, with mucin coated silica nanoparticles, there was significant aggregation despite neutralization of charge. The HA and 0.1% mucin double-coated polystyrene

nanoparticles showed a decline in diffusion compared to the uncoated particles. This confirms that the negative charge of the HA when coating particles alone showed an enhancement in diffusion, but the slight neutralization of the charge with the addition of the 0.1% mucin coating slowed down diffusion. The diffusion of the double-coated particles was still higher than that of the only mucin coated polystyrene particles. The only mucin coated particles had a neutralization of charge nearing -2.63 mV with increasing concentration of mucin. From this we can conclude that applying a negative charge to the surface of the polystyrene nanoparticles enhances their diffusion capabilities in GI mucin and reduces aggregation of silica nanoparticles.

PEG, a popular neutralization agent commonly used in drug delivery applications, showed minimal enhancement of diffusion coefficient of the polystyrene particles in GI mucin. From the data, there were no clear patterns that could be observed. The silica particles did not interact well with any of the PEG coatings despite neutralization of charge. However, with increasing molecular weight of PEG, there was less aggregation of the silica particles. The polystyrene particles interact well with the 100KDa PEG and 300KDa PEG at high concentrations. Despite these interactions, the diffusion coefficient remained relatively unchanged over time. However, the 0.1% 100KDa PEG coating significantly enhanced transcytosis and cellular uptake in the Caco-2 cells compared to uncoated particles and all other coated particles. The 0.1% 300KDa PEG coating on the other hand, showed diminished transcytosis and cellular uptake and significantly higher levels of trapped nanoparticles within the Caco-2 cells. It is also important to note that the 5% mucin layer greatly enhances the transcytosis and uptake of the nanoparticles, both coated and uncoated. Both the coating and the 5% mucin layer are beneficial to enhancing the diffusion of polymeric nanoparticles.

From the data it is clear that neutralization of charge can enhance the diffusion coefficient of polymeric nanoparticles in GI mucin, however applying a negative charge to the nanoparticles can enhance the penetrative capabilities even more. The negatively charged particles also reduced aggregation of the particles in the mucin. For bio-adhesive applications, when targeting the cells of the GI system or trying to increase the interacting time of the particles with the epithelial cells, a slightly negative particle would be the ideal candidate. The data from this research showed slightly negative particles had enhanced diffusion compared to uncoated particles, but near neutral charged particles had slower diffusion. This slightly negative charge can be achieved easily through bio-coating with low concentrations of mucin or 100KDa PEG. For mucus penetrating applications, when targeting systemic circulation or other parts of the body, an extremely negatively charged particle would be the ideal candidate. This extreme negative charge can be achieved easily through bio-coating with hyaluronic acid. Based on the drug target, polymeric nanoparticles can be tailored through bio-coating to enhance diffusion and cellular uptake, thus potentially improving the bioavailability of encapsulated oral drugs.

5.0 Design Optimization and Future Directions

Due to time constraints, certain experiments could not be repeated more than once. For accuracy, future experiments would do repeats of the experiments described. Based on the results of this research, a viable candidate for further bio-coating testing would be low molecular weight hyaluronic acid. Unfortunately, high molecular weight HA was not viable for cellular uptake and transcytosis studies. When testing the toxicity of the 0.1% HA coating with the Caco-2 cells, the cells were not able to form tight junctions and replicate properly. It may be that the high molecular weight is not suitable for this type of experiment, therefore a low molecular weight

hyaluronic acid would be interesting to evaluate. Future studies could also evaluate low molecular weight PEG and positively charged coatings. Due to time constraints, only two types of particles were able to be studied extensively. Future studies can focus on examining PMMA and PE particles. One design optimization effort that could be made is to replace the standard microscope slide with a capillary glass slide. This slide would eliminate the need for a coverslip, thus avoiding trapping air bubbles and global shift of particles. This slide would also allow for 3D diffusion studies and minimize human error and experiment variability.

5.1 Significance Testing

Finally, due to time constraints and challenges with the raw data files, robust statistical analysis with the diffusion studies could not be performed. For now, the results depicted in this research are mostly based on comparison testing and examining trends. To improve the accuracy of the data, statistical analysis should be completed in the future. Experimental replicates of at least 3 should also be conducted. Significance testing was completed for the nanoparticle characterization and cellular uptake studies. The results of these tests can be found in the Supplementary Information. Pairwise t-tests were conducted with Bonferroni corrections. Significance testing is an important part of ensuring the accuracy and validity of scientific research, and future studies should prioritize completing these analyses to make definitive conclusions.

6.0 References

1. Date, Abhijit A., et al. “Nanoparticles for oral delivery: Design, evaluation and state-of-the-art.” *Journal of Controlled Release*, vol. 240, Oct. 2016, pp. 504–526, https://doi.org/10.1016/j.jconrel.2016.06.016.
2. Pridgen, Eric M, et al. “Polymeric nanoparticle drug delivery technologies for Oral Delivery Applications.” *Expert Opinion on Drug Delivery*, vol. 12, no. 9, 26 Mar. 2015, pp. 1459–1473, https://doi.org/10.1517/17425247.2015.1018175.
3. Ensign, Laura M., et al. “Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers.” *Advanced Drug Delivery Reviews*, vol. 64, no. 6, May 2012, pp. 557–570, https://doi.org/10.1016/j.addr.2011.12.009.
4. Plapied, Laurence, et al. “Fate of polymeric nanocarriers for oral drug delivery.” *Current Opinion in Colloid & Interface Science*, vol. 16, no. 3, June 2011, pp. 228–237, https://doi.org/10.1016/j.cocis.2010.12.005.
5. Abdul Rahman, Norizah. “Applications of polymeric nanoparticles in food sector.” *Nanotechnology: Applications in Energy, Drug and Food*, 14 Dec. 2018, pp. 345–359, https://doi.org/10.1007/978-3-319-99602-8_17.
6. Johansson, Malin E., et al. “The gastrointestinal mucus system in health and disease.” *Nature Reviews Gastroenterology & Hepatology*, vol. 10, no. 6, 12 Mar. 2013, pp. 352–361, https://doi.org/10.1038/nrgastro.2013.35.
7. LeFevre, M. E., et al. “Accumulation of 2-μm latex particles in mouse Peyer’s patches during chronic latex feeding.” *Experientia*, vol. 34, no. 1, Jan. 1978, pp. 120–122, https://doi.org/10.1007/bf01921939.

8. Azagury, Aharon, et al. “Biocoating—a critical step governing the oral delivery of polymeric nanoparticles.” *Small*, vol. 18, no. 26, 23 May 2022, https://doi.org/10.1002/smll.202107559.
9. Lai, Samuel K., et al. “Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus.” *Proceedings of the National Academy of Sciences*, vol. 104, no. 5, 30 Jan. 2007, pp. 1482–1487, https://doi.org/10.1073/pnas.0608611104.
10. He, Chunbai, et al. “Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles.” *Biomaterials*, vol. 31, no. 13, May 2010, pp. 3657–3666, https://doi.org/10.1016/j.biomaterials.2010.01.065.
11. Rastogi, Harsh, et al. “African Journal of Biological Sciences.” *Mucus-Penetrating Nanoparticles: Overcoming Barriers in Mucosal Drug Delivery*, vol. 6, no. 6, 20 June 2024, pp. 1760–1776, https://doi.org/10.33472/10.48047/AFJBS.6.Si3.2024.1760-1776.
12. Reineke, J., et al. “Can bioadhesive nanoparticles allow for more effective particle uptake from the small intestine?” *Journal of Controlled Release*, vol. 170, no. 3, Sept. 2013, pp. 477–484, https://doi.org/10.1016/j.jconrel.2013.05.043.

7.0 Supplementary Information

7.1 Nikon Ti2-E Widefield Microscope Settings

X-cite: 10%

Light path: L00

Filter: CY3

Exposure: 100 ms

LUTs Scale: 2000-8000 normally, 0 - max depending on aggregation

Analog gain: ~9

Z-scale: 1800 - 2000 μm

Loops: 100

7.2 Image J Particle Tracker 2D/3D Code

```
// Define a function to process a directory
function processDirectory(inputDir) {
   list = getFileList(inputDir);
   for (i = 0; i < list.length; i++) {
      // Update the progress bar
      showProgress(i, list.length);
            fullPath = inputDir + "\\" + list[i];
      // Check if it's a directory
      if (File.isDirectory(fullPath)) {
         processDirectory(fullPath);
      }
      else if (endsWith(list[i], ".nd2")) {
         // Specify Bio-Formats import options for Hyperstack view
         options = "view=Hyperstack stack_order=XYCZT";
         run("Bio-Formats Importer", "open=[" + fullPath + "] " + options);

                        // Enhance contrast
         run("Enhance Contrast", "saturated=0.35");

         // Set the units
         Stack.setXUnit("um");
         Stack.setYUnit("um");

         // Run the Particle Tracker Plugin
         run("Particle Tracker 2D/3D", "radius=3 cutoff=0.001 per/abs=0.500
accelerate_with_clij2 link=2 displacement=10 dynamics=Brownian saveMss");

         close();
      }
   }
```

```
}

// Initial input directory
inputDir = "F:\\04.02.25 PS HA"
processDirectory(inputDir);

// Indicate that the script has finished
showProgress(1);
```

7.3 Python Statistical Analysis Code

```
rom collections import Counter
from sklearn.cluster import KMeans
import pandas as pd
import numpy as np
from openpyxl.chart import BarChart, Reference
import os

def process_csv_file_to_excel_cluster_outliers(file_path, new_file_path):
    print(f"Processing file: {file_path}")
    # Load the CSV data
    df = pd.read_csv(file_path)
    # Check if the required column exists
    if 'Diffusion Coefficient D2 (m^2/s)' not in df.columns:
        print(f"Skipping file: {file_path}. Required column not found.")
        return

    df['Diffusion Coefficient D2 (m^2/s)'] = pd.to_numeric(df['Diffusion Coefficient D2
(m^2/s)'], errors='coerce')
    df = df.dropna(subset=['Diffusion Coefficient D2 (m^2/s)'])

    # Apply log transformation
    df['Log_Diffusion'] = np.log(df['Diffusion Coefficient D2 (m^2/s)'])

    # Perform clustering
    kmeans = KMeans(n_clusters=16, n_init=10, random_state=42)
    df['Cluster'] = kmeans.fit_predict(df[['Log_Diffusion']])

    # Identify the most populated cluster
    cluster_counts = Counter(df['Cluster'])
```

```
        most_populated_cluster = max(cluster_counts, key=cluster_counts.get)
        most_populated_cluster_center = kmeans.cluster_centers_[most_populated_cluster]

        # Calculate distance of each cluster center to the most populated cluster center
        df['Distance_to_Most_Populated'] = df.apply(lambda row:
abs(kmeans.cluster_centers_[int(row['Cluster']), 0] - most_populated_cluster_center), axis=1)

        # Sort by this new distance metric
        df_sorted_by_distance_to_most_populated =
df.sort_values(by=['Distance_to_Most_Populated'], ascending=False)

        # Remove top 15% distant clusters
        unique_clusters = df_sorted_by_distance_to_most_populated['Cluster'].unique()
        n_clusters = len(unique_clusters)
        n_clusters_to_remove = int(n_clusters * 0.15)
        clusters_to_remove = unique_clusters[:n_clusters_to_remove]
        df_filtered =
df_sorted_by_distance_to_most_populated.loc[~df_sorted_by_distance_to_most_populated['Clu
ster'].isin(clusters_to_remove)]

        # Inverse log transformation for other statistics if needed
        df_filtered['Inv_Log_Diffusion'] = np.exp(df_filtered['Log_Diffusion'])

        # Calculate statistics
        statistical_summary = df_filtered['Inv_Log_Diffusion'].describe()

        # Create an Excel writer object
        writer = pd.ExcelWriter(new_file_path, engine='openpyxl')

        # Write the filtered data and statistical summary to Excel sheets
        df_filtered.to_excel(writer, sheet_name='Filtered_Data', index=False)
```

```
        pd.DataFrame(statistical_summary).transpose().to_excel(writer,
sheet_name='Summary', index=False)

        # Create an Excel workbook object
        wb = writer.book

        # The rest of the function remains the same as in the previous version
        ws_freq = wb.create_sheet('Frequency_Distribution')
        hist, bin_edges = np.histogram(df_filtered['Log_Diffusion'], bins=25)
        original_bin_edges = np.exp(bin_edges)

        ws_freq.cell(row=1, column=1, value='Diffusion Coefficient Bin')
        ws_freq.cell(row=1, column=2, value='Frequency')

        next_row = 2  # Start populating data from the second row

        for i in range(len(hist)):
            bin_start = original_bin_edges[i]
            bin_end = original_bin_edges[i + 1]
            ws_freq.cell(row=next_row, column=1, value=f"{bin_start:.2e} to {bin_end:.2e}")
            ws_freq.cell(row=next_row, column=2, value=hist[i])
            next_row += 1

        chart = BarChart()
        chart.type = "col"
        chart.title = "Frequency Distribution of Diffusion Coefficient"
        chart.x_axis.title = 'Diffusion Coefficient'
        chart.y_axis.title = 'Frequency'

        data = Reference(ws_freq, min_col=2, min_row=2, max_row=next_row - 1,
max_col=2)
```

```
            categories = Reference(ws_freq, min_col=1, min_row=2, max_row=next_row - 1)
            chart.add_data(data, titles_from_data=True)
            chart.set_categories(categories)

            ws_freq.add_chart(chart, "E5")

            writer.book.save(new_file_path)
            print(f"File processed and saved as: {new_file_path}")

        def process_all_folders_to_excel_cluster_outliers(folder_path):
            print(f"Starting to process all CSV files in folder: {folder_path}")
            for root, dirs, files in os.walk(folder_path):
                for file in files:
                    if file.endswith('.csv'):
                        file_path = os.path.join(root, file)
                        new_file_path = os.path.splitext(file_path)[0] +
'_cluster_outliers_processed.xlsx'
                        process_csv_file_to_excel_cluster_outliers(file_path, new_file_path)
            print("All files processed.")

        # Uncomment the line below to test the function on a specific folder
        process_all_folders_to_excel_cluster_outliers('D:\\FileName')
```

7.4 Malvern Zetasizer Settings

7.4.1 Zeta Potential

Temperature: 25 ℃

Run time: 60 seconds

Runs: 10

Replicates: 3

Cuvette: Disposable zeta cell

Dispersant: Water

7.4.2 Dynamic Light Scattering

Temperature: 25 ℃

Run time: 60 seconds

Runs: 10

Replicates: 3

Cuvette: Disposable sizing cuvette, RI polystyrene latex

Dispersant: Water

7.5 Significance Testing

Polystyrene

p <0.00238	HA Coated PS NPs Zeta Potential					
	0.005%	0.01%	0.05%	0.1%	0.5%	1%
0%	p=0.000005	p=0.000004	p< 0.000001	p=0.000009	p ≈ 0.022960	p ≈ 0.503864
0.005%		p ≈ 0.400243	≈ 0.042858	p ≈ 0.224409	p=0.000740	p=0.000011
0.01%	-		≈ 0.007746	p ≈ 0.291702	p< 0.000001	p=0.000406
0.05%				p ≈ 0.004529	p=0.000033	p=0.000007
0.1%					p=0.000015	p=0.002375
0.5%						p=0.000392

P <0.00238	HA Coated PS NPs Size					
	0.005%	0.01%	0.05%	0.1%	0.5%	1%
0%	P = 0. .000193	P ≈ 0.000192	p< 0.000001	p ≈ 0.007539	p=0.000383	p ≈ 0.002447
0.005%		p=0.000600	p< 0.000001	p ≈ 0.008418	p=0.000740	p ≈ 0.003378
0.01%	-		p=0.000026	p ≈ 0.011462	p ≈ 0.006119	p ≈ 0.008977
0.05%				p ≈ 0.015107	p ≈ 0.167687	p ≈ 0.416589
0.1%					p ≈ 0.052873	p ≈ 0.015909
0.5%						p ≈ 0.101386

P <0.0083	Mucin Coated PS NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P = 0.0047	P=0.0001	P= 0.00002
0.01%		P ≈ 0.00000	P ≈ 0.0000
0.1%	-		P ≈ 0.0000

P <0.0083	Mucin Coated PS NPs Size		
	0.01%	0.1%	1%
0%	P = 0.0078	P ≈ 0.000000	P= 0.000002
0.01%		P ≈ 0.00000	P = 0.000005
0.1%	-		P = 0.5556

P <0.005	HA + 0.1% Mucin Dual Coated PS NPs Zeta Potential			
	0%HA + 0.1% Mucin	0.01%HA + 0.1% Mucin	0.1%HA + 0.1% Mucin	1%HA + 0.1% Mucin
0%HA +0% Mucin	P = 0.0250	P = 0.1945	P= 0.0365	P = 0.00002
0%HA + 0.1% Mucin		P ≈ 0.2668	P = 0.2664	P = 0.000006
0.01%HA + 0.1% Mucin	-		P = 0.7638	P = 0.000009
0.1%HA + 0.1% Mucin				p ≈ 0.000000

P <0.005	HA + 0.1% Mucin Dual Coated PS NPs Size			
	0%HA + 0.1% Mucin	0.01%HA + 0.1% Mucin	0.1%HA + 0.1% Mucin	1%HA + 0.1% Mucin
0%HA +0% Mucin	P = 0.0109	P = 0.0016	p ≈ 0.000000	p = 0.0077
0%HA + 0.1% Mucin		P ≈ 0.3371	P = 0.0975	P = 0.0130
0.01%HA + 0.1% Mucin	-		P = 0.3893	P = 0.0160
0.1%HA + 0.1% Mucin				p ≈ 0.0179

P <0.0083	35KDa PEG Coated PS NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P = 0.0021	P=0.2182	P= 0.0023
0.01%		P = 0.00009	P=0.00018
0.1%	-		P=0.0035

P <0.0083	35KDa PEG Coated PS NPs Size		
	0.01%	0.1%	1%
0%	P = 0.6912	P=0.0499	P= 0.0114
0.01%		P = 0.3358	P=0.0056
0.1%	-		P=0.0151

P <0.0083	100KDa PEG Coated PS NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P = 0.2963	P=0.0013	P= 0.0014
0.01%		P = 0.0054	P=0.0045
0.1%	-		P=0.0035

P <0.0083	100KDa PEG Coated PS NPs Size		
	0.01%	0.1%	1%
0%	P = 0.0100	P=0.0360	P= 0.0322
0.01%		P = 0.1258	P=0.0667
0.1%	-		P=0.0923

P <0.0083	300KDa PEG Coated PS NPs Zeta Potential		
	0.01%	0.1%	1%
0%	p=0.001913	p=0.000132	p=0.000046
0.01%		p=0.000038	p< 0.000001
0.1%	-		p=0.000011

P <0.0083	300KDa PEG Coated PS NPs Size		
	0.01%	0.1%	1%
0%	p ≈ 0.465897	p ≈ 0.071297	p=0.003509
0.01%		p ≈ 0.085424	p=0.004345
0.1%	-		p=0.001090

Silica

P <0.0083	HA Coated Si NPs Zeta Potential		
	0.005%	0.05%	0.5%
0%	P=0.0003	P=0.000004	P=0.0046
0.005%		P=0.0001	P=0.000016
0.05%			P=0.000006

P <0.0083	HA Coated Si NPs Size		
	0.005%	0.05%	0.5%
0%	P=0.0107	P=0.0018	P=0.1353
0.005%		P=0.0003	P=0.0820
0.05%			P=0.0004

P <0.0083	Mucin Coated Si NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P=0.0006	P=0.00009	P=0.0001
0.01%		P=0.0011	P=0.0008
0.1%			P=0.000035

P <0.0083	Mucin Coated Si NPs Size		
	0.01%	0.1%	1%
0%	P=0.000004	P=0.0084	P=0.0042
0.01%		P=0.0219	P=0.1224
0.1%			P=0.02

P <0.0083	35KDa PEG Coated Si NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P=0.0119	P=0.0796	P=0.0049
0.01%		P=0.2349	P=0.1955
0.1%			P=0.0695

P <0.0083	35KDa PEG Coated Si NPs Size		
	0.01%	0.1%	1%
0%	P=0.4038	P=0.4361	P=0.0086
0.01%		P=0.8217	P=0.0041
0.1%			P=0.0032

P <0.0083	100KDa PEG Coated Si NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P=0.0003	P=0.000008	P=0.0001
0.01%		P=0.0005	P=0.00001
0.1%			P=0.0008

P <0.0083	100KDa PEG Coated Si NPs Size		
	0.01%	0.1%	1%
0%	P=0.2757	P=0.1751	P=0.00009
0.01%		P=0.1461	P=0.1434
0.1%			P=0.0036

P <0.0083	300KDa PEG Coated Si NPs Zeta Potential		
	0.01%	0.1%	1%
0%	P=0.00006	P=0.0004	P=0.0002
0.01%		P=0.0002	P=0.00004
0.1%			P=0.0046

P <0.0083	300KDa PEG Coated Si NPs Size		
	0.01%	0.1%	1%
0%	P=0.7533	P=0.1226	P=0.0099
0.01%		P=0.1156	P=0.0093
0.1%			P=0.0040

Cell Studies - Transcytosis

P <0.0167	Condition A	
	100K PEG	300K PEG
Mucin	P<0.000001	P<0.000001
100K PEG		P<0.00001

P <0.0167	Condition B	
	100K PEG	300K PEG
Mucin	P=0.00095	P=0.000002
100K PEG		P<0.000001

P <0.0167	Condition C	
	100K PEG	300K PEG
Mucin	P=0.000154	P=0.013509
100K PEG		P=0.006071

P <0.0167	Condition D	
	100K PEG	300K PEG
Mucin	P<0.000001	P=0.000001
100K PEG		P<0.000001

P <0.0167	Mucin		
	B	C	D
A	P<0.000001	P<0.000001	P<0.000001
B		P<0.000001	P=0.000029
C			P<0.000001

P <0.0167	100KDa PEG		
	B	C	D
A	P<0.000001	P<0.000001	P<0.000001
B		P<0.000001	P<0.000001
C			P<0.000001

P <0.0167	300KDa PEG		
	B	C	D
A	P<0.000001	P<0.000001	P<0.000001
B		P<0.000001	P<0.000001
C			P<0.000001

Cell Studies - Internalization

P <0.0167	Mucin - Internalization		
	B	C	D
A	P=0.105	P=0.3171	P=0.0653
B		P=0.001266	P=0.0133
C			P=0.0092

P <0.0167	100KDa PEG - Internalization		
	B	C	D
A	P=0.0291	P=0.0238	P=0.4967
B		P=0.006557	P=0.0326
C			P=0.0898

P <0.0167	300KDa PEG - Internalization		
	B	C	D
A	P=0.006408	P=0.1339	P=0.3506
B		P=0.003350	P=0.002709
C			P=0.7405

P <0.0167	Condition A	
	100K PEG	300K PEG
Mucin	P=0.0536	P=0.0399
100K PEG		P=0.6461

P <0.0167	Condition B	
	100K PEG	300K PEG
Mucin	P=0.0205	P=0.002849
100K PEG		P=0.003788

P <0.0167	Condition C	
	100K PEG	300K PEG
Mucin	P=0.7510	P=0.010992
100K PEG		P=0.0317

P <0.0167	Condition D	
	100K PEG	300K PEG
Mucin	P=0.7100	P=0.9798
100K PEG		P=0.7141