Double Wall Polymeric Nanoparticle Carrier for Sustained Release of Biologics in the Gastrointestinal Tract

By

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Thesis

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1. Abstract

Inflammatory bowel disease (IBD) consists of a group of intestinal disorders that cause prolonged inflammation along the gastrointestinal (GI) tract, specifically within the intestines. Biologics are used to treat severe cases of IBD, however they are currently only given through IV/infusion which is cumbersome for the patient. By creating a delivery vehicle for these biologics, the treatment can be delivered orally to create an easier treatment method for the patients as well as target the lower GI tract. There are a few challenges with oral drug delivery, the biggest one being the harsh pH environment of the stomach. In order for the biologics to successfully reach and treat the bowels, they must remain intact throughout the harsh upper GI tract. In this study, double wall polymeric nanoparticle carriers were designed as an oral delivery vehicle that can target the intestines using a pH-sensitive system.

Biologics were encapsulated within polymeric nanoparticles comprised of polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA). These nanoparticles were then coated with a bioadhesive and pH sensitive polymer, Poly(butadiene maleic anhydride-co-DOPA) (PBMAD). By coating the nanoparticle with PBMAD, the core was shielded from an acidic environment similar to that in the upper GI tract. When the double-walled particles were placed in more basic pH environments, similar to that in the lower GI tract, the PBMAD dissolved and detached from the nanoparticles leading to a release of the biologics. These studies show a potential a localized release in the intestines. Once optimized, these double-walled nanoparticles could enhance localized delivery and payload to the disease site.

2. Background

2.1 The Gastrointestinal Tract

The human gastrointestinal (GI) tract is the organ system that digests substances consumed orally. The GI tract is composed of four main parts: the esophagus, stomach, small intestines, and colon as shown in Figure 1. The esophagus is the point of entry for the GI tract. It transports orally-ingested items from the mouth to the stomach with a quick transit time of 10-14 seconds [1]. The stomach acts as a basin for the solids that are ingested. As the solids sit in the stomach, they are broken down by a combination of acid and enzymatic degradation to turn everything into chyme. In order for the stomach to successfully turn solids into chyme, it secretes hydrochloric acid (HCl) to maintain the stomach's acidity within a range of pH values from 1 to 3.5. From there, the stomach delivers the chyme at a controlled rate to the small intestines. On average, ingested material remains in the stomach for anywhere from 5 minutes to 4 hours, depending on the person's fasted state [1]. In the small intestines, the enzymatic degradation is completed and nutrients from the broken-down substances are absorbed. The intestinal epithelium is composed of villi and microvilli that increase the absorptive surface area in the intestines [1]. The pH here neutralizes to a range between 6 and 7.5, as HCl is no longer being secreted. The

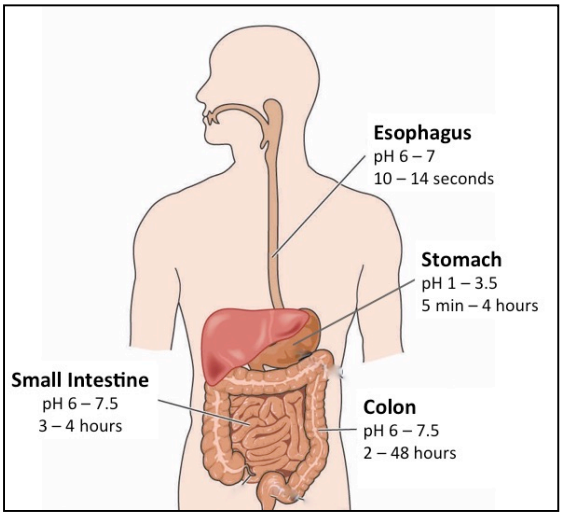

Figure 1. This diagram shows the average time food spends in each part of the digestive system along with the average pH.

average transit time for particles through the small intestines is 3-4 hours. The next and last stop in the GI tract is the colon. The colon has a homeostatic role in the GI tract where it absorbs sodium ions, chloride ions, and water while giving off bicarbonate and potassium ions. The pH of the colon is similar to that of the small intestines with a pH in the range of 6 to 7.5. The ingested food is stored and compacted into feces over the course of 2 to 48 hours.

All along the GI tract is a multi-layered mucus membrane that both protects the epithelial lining of the GI tract and helps the ingested solids move down. The two main layers of mucus consist of a firmly adherent layer and a loosely adherent layer, shown in Figure 2 [2]. The firmly adherent mucus is attached to the epithelial lining and protects it from pathogens and large, undigested boluses of food. Studies have found that not even suction could remove the adherent layer in rat intestines [3]. The loosely adherent layer resides between the firmly adherent layer and the lumen. Between the loose and firm layers, there is a slipping plane that allows the loose mucus to pass through the digestive tract. Its main role is to help undigested food move through the system without damaging the epithelium as well as to remove harmful compounds and organisms

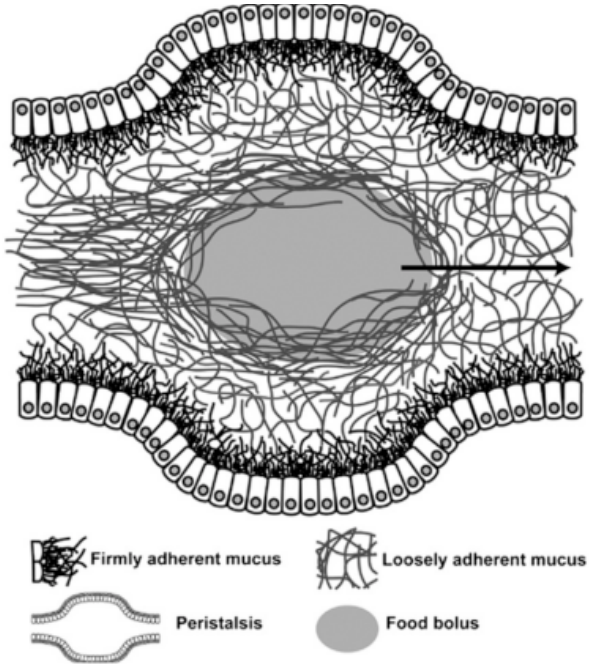

Figure 2. This illustration shows the fate of an ingested bolus of food. Mucin in the loosely adherent layer adheres to the food to help it pass without perturbing the firmly adherent layer and the epithelium [2].

introduced through diet [2].

2.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a lifelong condition that consists of constant inflammation along various parts of the GI tract. There are no known reasons for the occurrence of this disease, but researchers believe it is due to a mixture of environmental factors and genetics [4]. There are two types of IBD, Crohn's Disease and Ulcerative Colitis (UC). In UC, the inflammation only occurs within the colon. In Crohn's, the inflammation can present itself anywhere from the mouth to the anus, but it is most commonly located in the small intestines and colon [5]. The inflammation location for both diseases along the GI tract can be seen in Figure 3. The inflammation in both of these diseases is due to constant attack by the immune system. The immune system usually protects the body from foreign bodies, but in Crohn's and UC, something causes the immune system to launch an attack within the GI tract that won't shut off [5]. This constant attack leads to constant inflammation, which hinders the GI tract's ability to function properly. When the small intestines and colon are inflamed, they become unable to properly absorb the water and nutrients that the body needs. This results in diarrhea, weight loss, vitamin deficiencies, and growth delays in children [5].

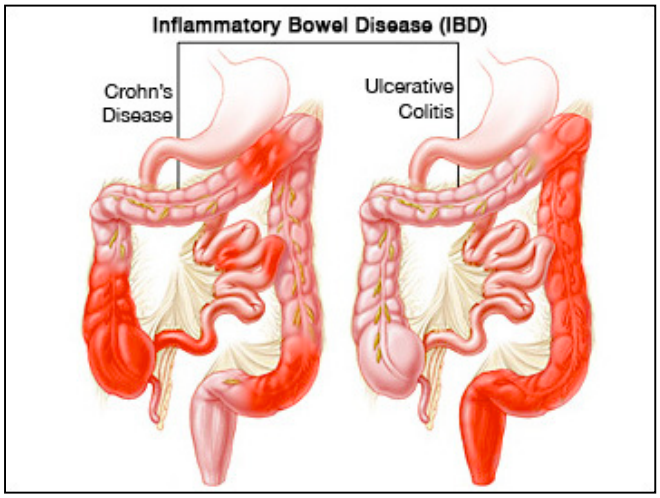

Figure 3. This image highlights the different inflammation sites that flare up in Inflammatory Bowel Disease. In Crohn's, the inflammation occurs in "on and off" regions throughout the lower GI. In UC, the inflammation occurs within the colon [24].

In severe cases, the constant immune attack leads to destruction of the healthy cells and causes ulcers to form throughout the lower GI. Ulcers are the erosion of the mucus and epithelial layers of the GI tract. Despite the ulcerous environment of the intestines and colon, the pH of those regions remains around the normal range of pH 6-7.5, or slightly higher [6].

The treatments available for IBD vary depending on the severity of the disease. Patients are first started on the mildest treatment then given progressively stronger medications depending on their response [4]. Figure 4 shows a pyramid diagram of the current treatment standards. For mild cases, patients are given aminosalicylates, which prevent inflammation and carry no risk of major side effects. If a patient has mild IBD with complications, like a fistula or abscess, they are given antibiotics to treat infection and inflammation. If these medications do not relieve symptoms, patients are then given corticosteroids, which lower the activity of the immune system. By lowering the activity of the immune system, the patient is suspect to a higher risk of side effects and infection as it hinders the body from fighting off foreign bodies such as bacteria [5]. Because of this, corticosteroids are only prescribed for short periods at a time. If corticosteroids do not work, the patients are given immunomodulators, which modify and quiet down the

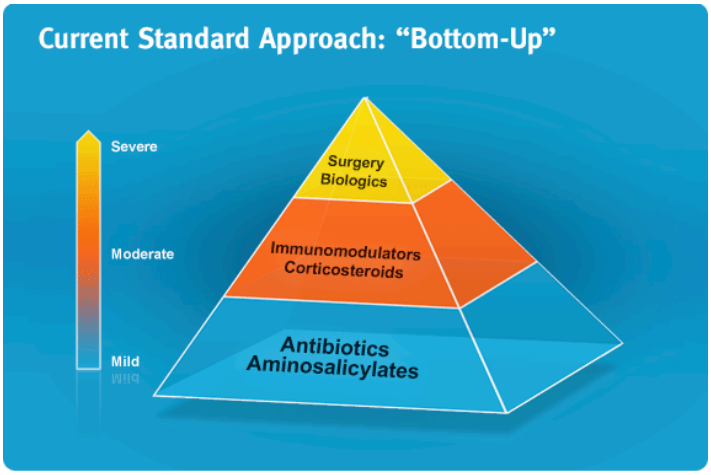

Figure 4. IBD treatments are prescribed based on symptoms and improvements. Patients are started on the mildest form of treatment, then progressively given stronger medication. The only medication not available in oral form is biologics.

immune system. These four types medications come in pill, liquid, or IV form [4]. If none of those medications work, patients are either given biologics for treatment or undergo surgery to remove necrotic or ulcerous tissue.

Biologic therapies aim to stop the immune system from attacking healthy GI tissue. This is currently being done in two different ways. One method stops the overexpression of a protein which triggers the immune system to constantly attack the intestinal wall [7]. By inhibiting this protein overexpression, the immune system reduces its attack, and the inflammation dies down. The second method inhibits a receptor from recruiting white blood cells into the intestines [8]. By impeding this rush of immune cells into the intestines, inflammation is reduced and the symptoms of IBD decrease as the intestines heal. Biologic therapies are currently only administered through IV/infusion since an oral delivery method has yet to be developed. Patients must visit the hospital for 30 minute dosing sessions at 0, 2, and 6 weeks when they first start the treatment and then return every 8 weeks thereafter [9]. Since IBD is a lifelong disease that is never cured, only treated, this dosing regimen can be very cumbersome for the patient. For this reason, the goal of this project is to create a potential oral delivery method for IBD biologic therapies. By enabling patients to take the biologic therapy orally, they would no longer be confined to hospital visits and IV/infusion administration every two months.

2.3 Challenges of Oral Drug Delivery

In order to deliver a therapeutic orally, three main obstacles must be overcome. The first challenge is that larger molecules, such as biologics, have poor stability, bioavailability, and retention time within the body. As soon as such drugs are administered, they are either rapidly cleared from the system, or enzymatically degraded

before it can reach the intended disease site [2]. In order to combat this premature denaturing and clearance of biologics, they can be encapsulated within polymeric nanoparticles [10]. By encapsulating the therapeutic load within nanoparticles, the biologics will be shielded from enzyme degradation, resulting in a higher payload to the disease site.

The second challenge with oral delivery is the mucus membrane that coats the GI tract. The rapid secretion and shedding of the GI tract mucus considerably limits the effectiveness of nanoparticle drug delivery systems [2]. Particles are typically trapped within the loose mucus and removed from the system before any drug absorption can take place. In order to increase the drug residence time, particles must penetrate the loosely adherent layer of the mucus and release the drug load closer to the epithelial surface [11]. This can be achieved by incorporating a bioadhesive, or mucoadhesive, polymer in the nanoparticle composition. Bioadhesion refers to the interactive forces between a synthetic material and a mucosal surface [11]. Incorporating bioadhesive polymers to nanoparticles for oral delivery allows for an enhanced interaction with the absorptive epithelium, and therefore an increase in GI tract transit time.

The third challenge is keeping the particles intact through the harsh pH environment of the upper GI tract in order to release biologics in the small intestines and colon. Due to the very acidic and enzymatic environment of the stomach, most substances that enter the GI tract through the esophagus get broken down into chyme before reaching the intestines. Commonly used biodegradable nanoparticles would get broken down in the stomach, leading to an early release of the biologics. If the biologics are released prematurely in the stomach, they will become unstable and denature before

reaching the disease site. To combat this degradation, polymeric nanoparticles can include a pH-sensitive system, similar to an enteric coating. Integrating an enteric coating would allow particles to remain intact in low pH environments, such as the stomach, and dissolve in higher pH environments, such as the intestines. However, an enteric coating alone will not produce the most efficient and effective system of oral delivery. As mentioned before, the loose mucus layer would likely remove the drug load before it can be absorbed into the epithelium. For this reason, the optimal way to deliver biologic therapeutics to the lower GI tract is using a pH sensitive, bioadhesive polymeric nanoparticle.

2.4 Polymeric Nanoparticles

Nanoparticles have been explored as a means for drug delivery for numerous types of applications. Their small size and variable compositions make them ideal candidates to facilitate the transport of drugs. As an application for oral drug delivery, nanoparticles have an easier entry through the intestinal lumen to reach the lymphatic system than microparticles. Particles that are micron size and above usually get trapped within the loose mucus layer and cannot successfully deliver drugs to the intestinal lumen. Nanoparticles, however, are small enough to disseminate further through the mucus to better deliver the payload to the intestinal epithelia [12].

Polymeric nanoparticles have been shown to provide robust stability and a promising ability to create a controlled release compared to other types of nanoparticles [12]. They can be used to deliver hydrophilic drugs, hydrophobic drugs, proteins, vaccines, and biological macromolecules [12]. There are numerous polymers that are used to create nanoparticles, but the two that are most commonly used are poly(lactic

acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA). These two polymers are known for their biocompatibility and resorbability through natural pathways [12]. PLA gets broken down into lactic acid and PLGA into lactic acid and glycolic acid, both of which are naturally metabolized in the body. The Food and Drug Administration (FDA) has already approved both of these polymers for human use and nanomedicines [10].

There are a several methods of synthesizing polymeric nanoparticles including solvent evaporation, double emulsion, spray drying, and phase inversion nanoencapsulation (PIN) [10], [13], [14]. Each method results in slightly varying methods of drug encapsulation as well as the shape and size of nanoparticles formed. For the purpose of encapsulating proteins, such as biologic therapeutics, into spherical nanoparticles, the PIN method provides the optimal formulation [14].

There are three main types of bowel-targeting delivery systems currently being explored. There are time controlled systems, enzyme based systems, and pH-dependent systems [15]. Time controlled systems focus on the average transit time it takes for the body to process items ingested orally. They aim to accomplish a sustained release until the time point that substances typically reach the bowels. The issue with this method of targeting is that the transit time can vary depending on the patients' fasted state as well as the onset of IBD. IBD patients have irregular onsets of symptoms like diarrhea, which effect the transit time of ingestion [15]. Because of the irregularity of transit time with IBD patients, temporally controlled systems are not ideal for treatment.

Enzymatically controlled systems, or prodrugs, are delivery systems that are degraded by enzymes found in the bowels. This degradation leads to the triggered release

of the drug. However, in Crohn's Disease patients, enzyme activity is reduced so this type of delivery system is not as effective for severe IBD treatment [15].

Drug delivery systems that are pH-dependent often have an enteric coating. Enteric polymers are insoluble in the stomach's pH environment and prevent drug dissolution until they reach a more alkaline environment, where the system solubilizes and releases the drug. The biggest challenge with enteric coatings is that it doesn't accurately release drugs to specific sites [15]. The drug dissolution is quite variable and has no method of ensuring entry into the intestinal epithelia.

2.5 Double-Walled Nanoparticle Carrier

In order to optimize the oral delivery of biologics to the lower GI tract, a new nanoparticle system was designed. The effective use of biodegradable and biocompatible PLA and PLGA as a means of protein and biological delivery has been proven many times [16–19]. Incorporating biologics into the matrix of PLA/PLGA nanoparticles can shield the drugs from immediate degradation and destabilization, but when administered orally, those carriers are not able to remain intact past the harsh gastric environment. To effectively treat severe IBD and deliver biologics purely to the small intestines and colon, additional components must be added to this PLA/PLGA nanoparticle system. First would be the incorporation of an enteric coating to create localized delivery in the lower GI tract. An enteric coating would keep the particles intact through the acidic stomach region, and dissolve to release its contents within the more alkaline intestines. Once the coating dissolves in the intestines, the new issue becomes the inaccuracy and inefficiency of delivering the payload to the intestinal epithelia due to the mucus membrane lining the lumen. This can be overcome with the incorporation of a bioadhesive polymer.

Integrating a bioadhesive polymer would provide an interaction between the particles and the mucus membrane of the lower GI, allowing the particles to enter the firmly adherent layer, shown in Figure 5. This would lead to a prolonged particle residence time at the disease site and allow for the biologics to achieve high enough levels of uptake to reach therapeutic concentrations specifically within the intestines and colon [11].

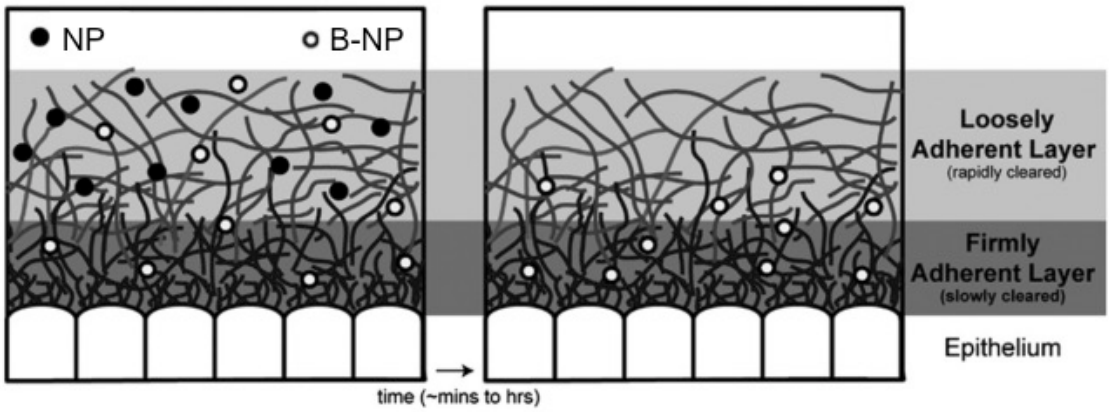

Figure 5. This schematic illustration shows the fate of regular nanoparticles (NP) and bioadhesive nanoparticles (B-NP) when administered to the GI mucosal surface. Because B-NP's can enter the firmly adherent layer and thus are in closer proximity to cells, cells will be exposed to a greater dose of drug released from B-NP compared to drug released from NP. As the loosely adherent layer is cleared, NP's are removed, whereas B-NP are retained longer within the firmly adherent layer and continue to release drugs to cells. Figure adapted from [2].

There are a variety of bioadhesive polymers available for this type of application, but according to a study previously conducted in the Mathiowitz lab, the most effective polymer is poly(butadiene-maleic anhydride-co-L-DOPA) (PBMAD) [11]. This study compared the *in vivo* uptake of various bioadhesive polymer nanoparticles to see which polymer enhances uptake the most. It was proven that PBMAD above all others shows the most effective uptake into the intestinal mucosa. PBMAD is a polymer that was synthesized by the Mathiowitz lab to have a hydrophobic backbone and high carboxylic end groups [11]. This polymer construction provides increased hydrogen bonding which results in the physical entanglement with mucin strands. In addition to PBMAD's

heightened ability to interact with mucin, it also has a pH-sensitive quality. It was found that in acidic environments, PBMAD becomes insoluble and rigid, while in more alkaline environments, it dissolves and becomes adhesive [11]. Since PBMAD has both enteric and bioadhesive properties, incorporating it into the PLA/PLGA nanoparticles would ideally target the delivery of biologics to treat IBD.

To construct a viable double-walled polymeric drug delivery vehicle that has a biodegradable core and an enteric and bioadhesive coating, two types of nanoparticle formulation techniques were explored: sequential phase inversion nanoencapsulation (sPIN) and single step double-walled nanoencapsulation (SSDN), both developed by the Mathiowitz lab. sPIN uses the PIN method to create double-walled nanoparticles in two separate steps, first creating the core followed by the coating. To fabricate the core by PIN, diluted polymer and drug solutions were added to an excess of non-solvent for the polymer. As the solvent leaves the polymer solution, the polymer instantaneously precipitates in the form of micro or nanospheres in a self-assembly system [14], [20]. The size of the particles is dictated by the ratio and molecular weight of polymers and drugs used. In order to create the second wall or coating, the PIN process was repeated using the core nanoparticles and PBMAD as the polymer solution.

SSDN, as its name implies, creates double-walled nanospheres all in one process. To fabricate the particles using SSDN, shell polymer solution is added to core polymer and drug solution, and then rapidly added to a non-solvent. This results in spontaneously self-assembling double-walled nanoparticles with homogeneous coatings.

The nanospheres created with sPIN and SSDN were characterized using dynamic light scattering (DLS) for size and zeta potential, scanning electron microscopy (SEM) to

confirm size and morphology, and atomic force microscopy to observe surface characterizations. The release kinetics of each particle formulation were observed in varying pH solutions and simulated gastric and intestinal fluid to verify and observe the effects of the enteric coating.

3. Materials and Methods

3.1 Materials

Poly(L-Lactic Acid) 2 kDa (i.v. 0.1-0.2 dL/g in $CHCl_3$) was purchased from Polysciences, Inc. (Warrington, PA). PLGA 95 kDa (75:25; i.v. 0.67 dL/g in $CHCl_3$) and D,L-PLA 18 kDa (i.v. 0.21 dL/g in $CHCl_3$) were purchased from Birmingham Polymers, Inc. (Pelham, AL). PLGA 6 kDa (85:15) was received from Takeda Pharmaceuticals (Cambridge, MA). These polymers were stored at -15°C until use. The bioadhesive PBMAD was synthesized in the Mathiowitz lab via ring-opening, side-chain conjugation reaction in dimethyl sulfoxide (DMSO) as described in detail in reference [21]. This was stored at room temperature. Ethanol, Dichloromethane (DCM), Tetrahydrofuran (THF), petroleum ether, and 0.22 μm Fluoropore Polytetrafluoroethylene (PTFE) filters were all purchased from Fisher Scientific. Simulated gastric fluid without pepsin (0.2% (w/v) Sodium Chloride in 0.7% (v/v) Hydrochloric Acid) and simulated intestinal fluid without pancreatin (USP XXII Formulation which contains water, potassium dihydrogen phosphate, and sodium hydroxide) are both produced by Ricca Chemical Company (Arlington, TX) and bought from Fisher Scientific. All solvents used in this study were of the highest commercial grade available.

3.2 Sequential Phase Inversion Nanoencapsulation (sPIN)

To make the core nanoparticle, biologics, PLA, and PLGA (1:1 mass ratio of PLA to PLGA) were dissolved in dichloromethane (DCM) at 1.5% (w/v). This polymer solution was then added dropwise to petroleum ether, a non-solvent for both polymers, at a 1:125 ratio while stirring. This mixture was left to stir for 2-5 minutes to allow for DCM to escape the emulsion, leading to spontaneous formation of the nanoparticles. The resulting nanoparticles were collected using a 0.22 μm Fluoropore PTFE membrane filter under a positive pressure filtration column (Millipore, Inc.; Billerica, MA). Constituents retained on the membrane were scraped, flash frozen in liquid nitrogen and lyophilized for 24 hours. Once fully lyophilized, the PLA/PLGA core particles were mixed with PBMAD in an ethanol solution at a 20:1 mass ratio. This polymer solution was added dropwise to petroleum ether at a 1:100 ratio while stirring. The same filtration, collection, and lyophilization techniques were used to complete the double-wall nanoencapsulation process. A diagram of this process is shown in Figure 6.

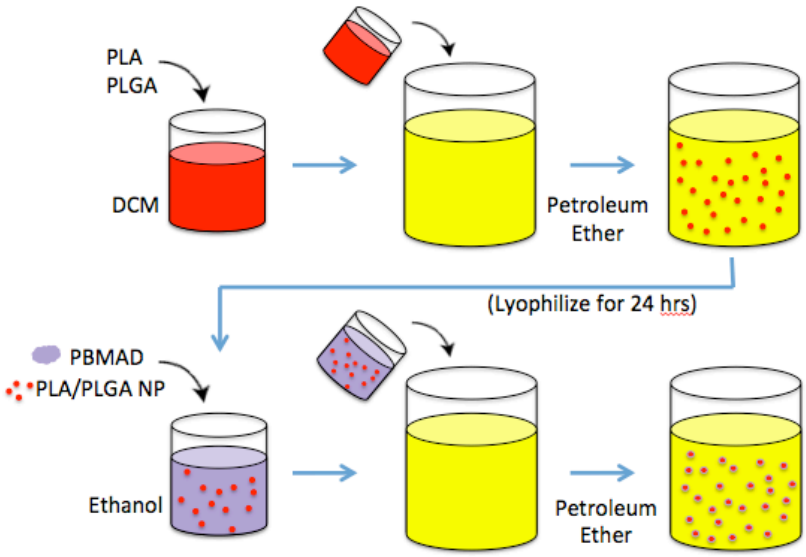

Figure 6. Schematic of the sequential phase inversion nanoencapsulation (sPIN) process.

3.3 Single Step Double-Walled Nanoencapsulation (SSDN)

To construct double-walled nanospheres all in one step, a volume ratio of 2% (w/v) PBMAD in ethanol was added to a 2% (w/v) core polymer solution (either PLA 18 kDa or PLGA 95 kDa mixed with the drug) in tetrahydrofuran (THF). The volume ratio of core polymer solution to shell polymer solution for the PLA-PBMAD particles is 1.28, while the volume ratio for PLGA-PBMAD is 1.56 [21]. The resultant mixture was added quickly to petroleum ether at a polymer solution to non-solvent ratio of 1:75. This final mixture was continuously stirred for an additional 2-5 minutes to fully allow all of the solvents to be replaced with non-solvent, resulting in the spontaneous formation of the double-walled nanoparticles. The particles were collected using the same 0.22 μm Fluoropore PTFE membrane filter under a positive pressure filtration column (Millipore, Inc.; Billerica, MA). The contents of the membrane were then scraped, flash frozen in

liquid nitrogen, and lyophilized for 24 hours. A diagram showing this process is shown in Figure 7.

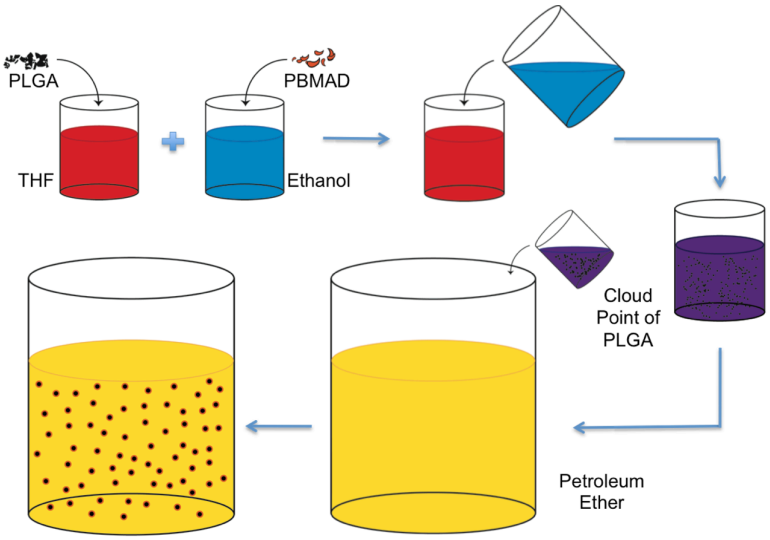

Figure 7. Schematic of the Single Step Double-walled Nanoencapsulation (SSDN).

3.4 Nanoparticle Characterization

3.4.1 Particle Size and Zeta Potential Analysis

All formulations were reconstituted from powder form into purified deionized water with vortex and bath sonication. A Malvern Zetasizer Nanoseries was used to evaluate the size distribution and zeta potential of the core and double-walled nanoparticles formulations.

3.4.2 Scanning Electron Microscopy (SEM)

SEM was used to analyze the morphology of the core and double-walled nanoparticle formulations. Formulations were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700

Scanning Electron Microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) with an accelerating voltage of 8 kV.

3.4.3 Atomic Force Microscopy (AFM)

The surfaces of the double-walled nanoparticles were evaluated in acidic and alkaline solutions using AFM (MFP-3D-BIO, Asylum Research) in AC (tapping) mode. AFM samples were made by adding 30μL of 0.02% (w/v) double-walled particles in hydrochloric acid (HCl) or double-distilled water and placed on a square cover slip until the liquid evaporated. The square cover slips were then adhered to a slide using double-sided tape. AFM scans used a sharp, silicon cantilever (tip diameter < 20 nm) with a nominal stiffness value of 40 N/m (Budget Sensors). Topographical height and phase shift scans were acquired to determine surface and polymer composition. In order to ensure that the data gathered were real and not imaging artifacts, height and phase data were captured for both directions (trace and retrace), different scan sizes were analyzed (ranging from 0.1 - 10 μm), different scan speeds were used on the same sample (1 Hz and 0.8 Hz), and multiple spots per sample were imaged [21].

3.4.4 Drug Release Study

Double-walled nanoparticle batches were placed in 1.5 mL eppendorf tubes for each release study. Either dispersion buffer, T buffer, simulated gastric fluid (SGF), or simulated intestinal fluid (SIF) was added to dissolve the particles in 1mL of solution. At various time points, the eppendorf tubes full of dissolved particles would be spun down using the ThermoForma microcentrifuge (5522 MicroMax RF) for 8 minutes at 10,000 G

and 10°C. The supernatant was collected for future HPLC analysis and the solution was replaced at the same volume for particle re-suspension.

3.4.5 High Performance Liquid Chromatography (HPLC)

The samples from the release study were analyzed by size exclusion chromatography (SEC) using a Tosoh Biosep, TSK Gel G3000 SWXL with detection at 280 nm. A Waters 600 controller maintained a flow rate of 0.5 ml/min; the mobile phase consisted of sodium phosphate, monobasic and dibasic, sodium chloride, and 10% acetonitrile (v/v). The amount of released protein was determined using a calibration curve constructed using a series of standard protein solutions. ImageJ data analysis was performed to estimate the release of biologics when PBMAD overlapped in release.

4. Results and Discussion

Biologics for the treatment of IBD are unable to be given in oral form due to the rapid degradation that would occur in the acidic pH environment of the stomach. To combat this degradation, they can be encapsulated within polymeric nanoparticles. Once they are successfully encapsulated, measures need to be taken to effectively target therapeutic delivery to the small intestines and colon. By adding a second wall of PBMAD to the nanoparticles, they are able to remain intact through the acidic stomach and dissolve in the small intestines/colon. The bioadhesive PBMAD will also help the nanoparticles penetrate through the loose mucus into the firmly-adherent mucus layer and increase the drug payload to the intestinal epithelial cells. In order to combine all the useful qualities of PBMAD with the biodegradable properties of PLA and PLGA, two

double-wall nanoencapsulation methods were tested to determine the best yield, drug loading, encapsulation, and release kinetics.

Each particle formulation tested for this project was made with different biodegradable polymer to PBMAD ratios. The sPIN double-walled particles were made with a 17:1 mass ratio of PLA/PLGA to PBMAD. One formulation of SSDN particles was made with a 6:1 mass ratio of PLGA to PBMAD, while the other formulation was made with a 1:1 mass ratio of PLA to PBMAD. The ratio of polymers used for the SSDN method were chosen based on the cloud point ratio for each polymer [21]. All particle assemblies aimed for 1.2% drug loading.

4.1 Particle Size and Zeta Potential Analysis

Analysis of the hydrodynamic diameter and estimated surface charge of the particles were performed using a dynamic light scattering instrument (DLS). DLS determines the distribution of particle size based on the Brownian motion of the particles in water. The zeta potential is an estimation of the surface charge of particles. It determines the electrophoretic mobility of the particles as they are moved from one electrode to another in the instrument. Particles that have zeta potential greater than +25 mV or below -25 mV are typically stable [22]. Figures 8 and 9 show the DLS results for the hydrodynamic diameter and zeta potential of the nanospheres made using each method of encapsulation. The yield and theoretical drug loading achieved with each particle preparation can be seen in Table 1.

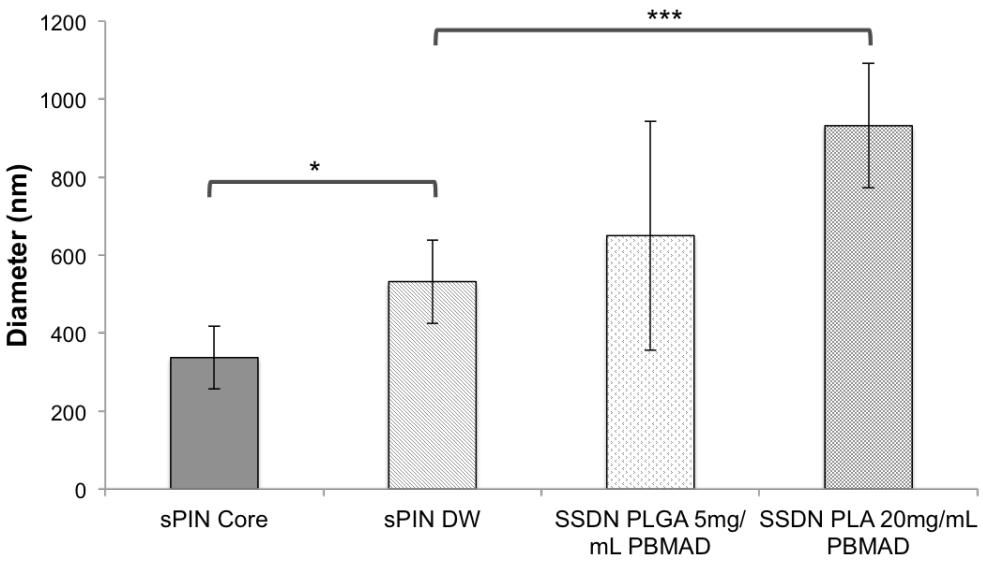

Figure 8. Nanoparticles were made using two different encapsulation methods, sPIN and SSDN. The hydrodynamic diameter of the particles was measured using DLS. The addition of PBMAD to the sPIN core particles increased the diameter of the particles, indicating a successful encapsulation. The SSDN particles with greater PBMAD concentrations range from nano to micron in size.

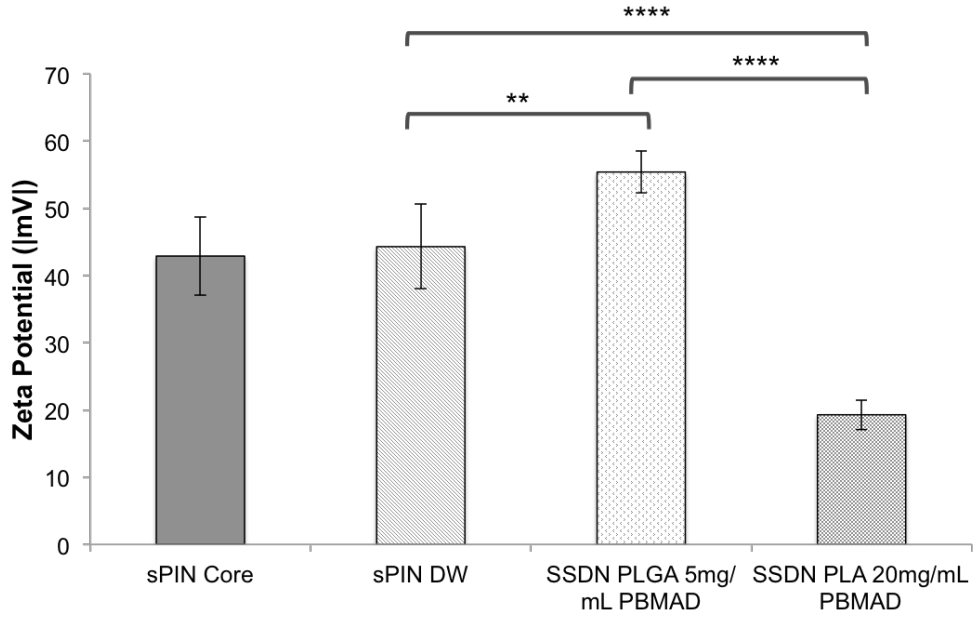

Figure 9. The zeta potential of each particle formulation was determined a DLS instrument. The core and double-walled sPIN nanoparticles along with the PLGA SSDN particles all have zeta potential below -25 mV indicating they are stable [22]. The PLA SSDN with greater amounts of PBMAD has a more neutral zeta potential of -19 mV.

	Particle Type	Yield (%)	Theoretical Loading (%)
sPIN	**Core:** Drug + PLA + PLGA	53.0	0.986
	DW: Core + PBMAD	57.6	0.953
SSDN	Drug + PLGA + PBMAD	26.4	0.991
	Drug + PLA + PBMAD	40.1	0.648

Table 1. This shows the varying percent yields and loading of each encapsulation method. The SSDN method seems to result in lower yield percentage. The PLA + 20mg/mL PBMAD formulation was not able to encapsulate as much of the drug as the other encapsulation approaches.

The double-walled sPIN particles are manufactured in two separate steps. First, the core, made of a 1:1 ratio of PLA and PLGA plus the drug, is assembled. These core nanoparticles had an average hydrodynamic diameter of 337 nm. When the PBMAD layer was added to the core in the second step of sPIN, the average hydrodynamic diameter increased to 532 nm. This increase in size verifies the successful addition of PBMAD to the nanoparticles. The zeta potential of both the core and double-walled sPIN particles remained around -43 mV, indicating the particle formulations are stable. The similarity in the sPIN particles' zeta potential could indicate that the low ratio of PBMAD used did not coat the core nanoparticles completely. It is possible that the PBMAD attached to the particles in a non-uniform manner so that PLA/PLGA was still present on the surface along with patches of PBMAD. The sPIN formulations had around 55% yield, which is common due to loss of material during manufacturing. Specifically during particle production, a great deal of polymer and drug are lost due to nucleation on the sides of beakers and filter columns. Product also can get lost during the positive pressure filtration phase due to leaks in the sides of the columns. The theoretical

drug loading in the double-wall sPIN particles was 0.953% when the production was aiming for 1.2% loading.

The SSDN particles are made in a single step. The first SSDN particle assembly made was with biologics, PLGA (75:25), and 5 mg/mL PBMAD. This production had a large loss of material, signified by a low yield of 26.4%. At the same time, this production exhibited the highest theoretical drug loading at 0.991%. According to the zeta potential, which was measured to be -55 mV, the particles that were able to form were stable. The particle sizes averaged around 650 nm, but seemed to have two main populations, as indicated by a high polydispersity index of 1. There was one distribution of particles in the range of 200-400 nm and a second distribution ranging from 750-900 nm. The varying particle sizes indicate a non-uniform particle formation, with majority of the particles on the lower size range while some particles were closer to micron sized. It seems that with this ratio of PLGA: PBMAD, the double-walled particles were not able to form as uniformly as with the sPIN method. Further studies, described in the following sections, were performed to more deeply analyze the PLGA-PBMAD particles.

The SSDN PLA-20 mg/mL PBMAD particles had 40% yield and a low theoretical drug loading of 0.648%. Zeta potential analysis indicated an unstable suspension with a value of -19 mV, and the particles formed were around 930 nm in size, indicating a production closer to microparticles rather than nanoparticles. The high ratio of PBMAD in these particles caused the DLS measurements, performed in water to, to provide skewed data. The water, with a more alkaline pH, caused the particles to swell and dissolve, leading to incorrect measurements. For this reason, particle diameter measurements were performed using ImageJ analysis on SEM images of the particles.

The large particle sizes imply that the high ratio of PBMAD to PLA (almost 1:1) is not conducive to forming particles on the nano scale.

4.2 Scanning Electron Microscopy

To observe particle formations and verify particle size, SEM images of the sPIN and SSDN particle formulations were taken and are shown in Figure 10.

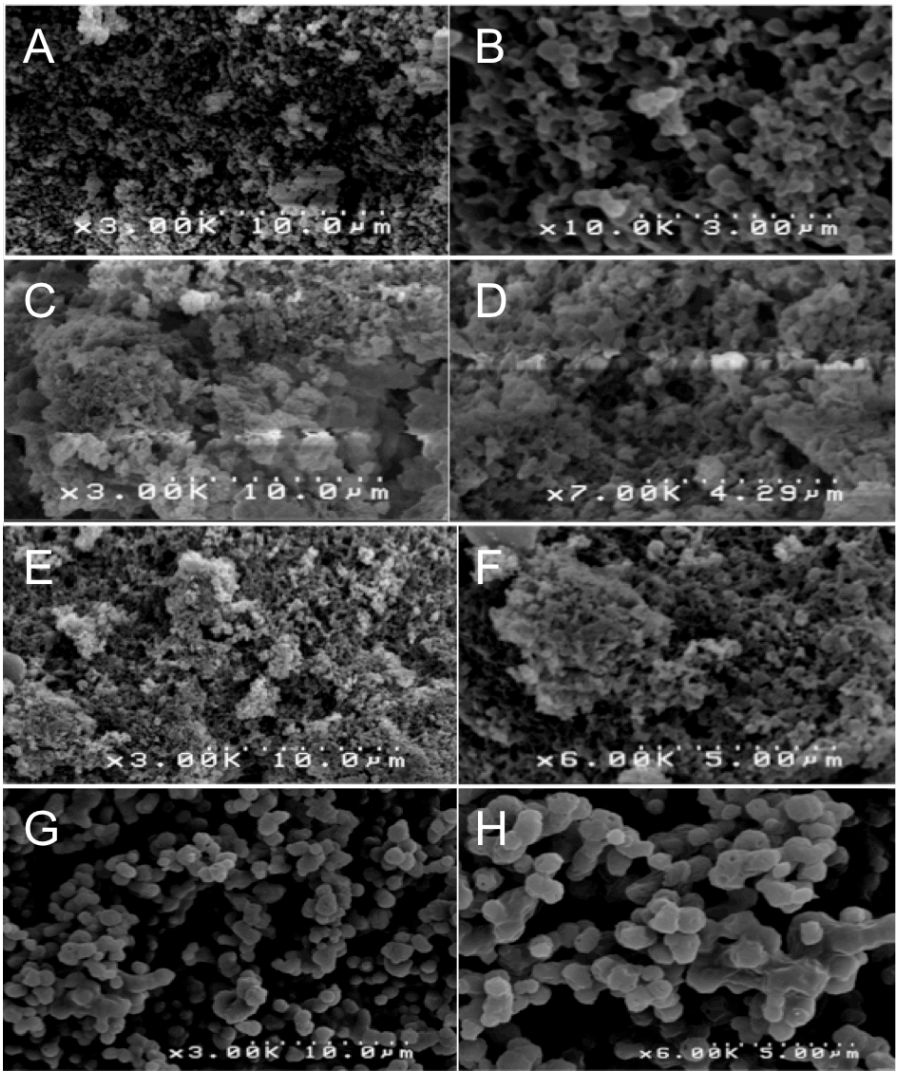

Figure 10. SEM images of various formulation particles. The images on the left are at a magnification of 3K and the images on the right are at a magnification of 6-10k. Images **A** and **B** are the sPIN core particles, **C** and **D** are sPIN double-walled particles, **E** and **F** are SSDN PLGA-5mg/mL PBMAD double-walled particles, and **G** and **H** are SSDN PLA-20mg/mL PBMAD double-walled particles.

SEM images of PLA/PLGA particles often show a network of spherical particles rather than discernable individual particles, as can be seen in Figures 10A and 10B. They appear this way because the SEM images are of dry samples on an adhesive sticker. When the particles are within solution, they can be dispersed into individual particles. The addition of PBMAD (Figures 10C and 10D) seems to cluster networks of particles even closer together than before. Instead of smooth, spherical nanoparticles, the sPIN double-walled nanoparticles have more of a jagged, uneven surface. This change in surface morphology confirms the addition of an outer coating of PBMAD to the core PLA/PLGA particles.

SSDN PLGA/PBMAD particles show similar particle clustering to the sPIN double-walled particles. These SEM images (Figures 10E and 10F) revealed that aggregates and some micron-sized particles were formed, confirming the results of the DLS size analysis. These aggregates and large particles explain the bimodal distribution of particle sizes.

The SEM images of the SSDN PLA/PBMAD (Figures 10G and 10H) show that the particles are micron in size, rather than nano, and seem uniform and spherical in shape.

4.3 AFM Analysis

To characterize the surface composition of the sPIN and SSDN particles, AFM phase imaging was employed. AFM uses a cantilever tip to oscillate over a sample and the difference in phase angle between the cantilever oscillating in air and during scanning is recorded as the phase shift [23]. The stiffness of the materials in the samples will induce different phase angle shifts due to the contact interaction between the cantilever

and the surface of the sample. In order to accurately determine which polymers are present on the surface of the double-walled particles, controls of pure PLA/PLGA and PBMAD nanoparticles were imaged first. Figures 11 and 12 show AFM phase angle shift measurements for sPIN and SSDN particles with their controls.

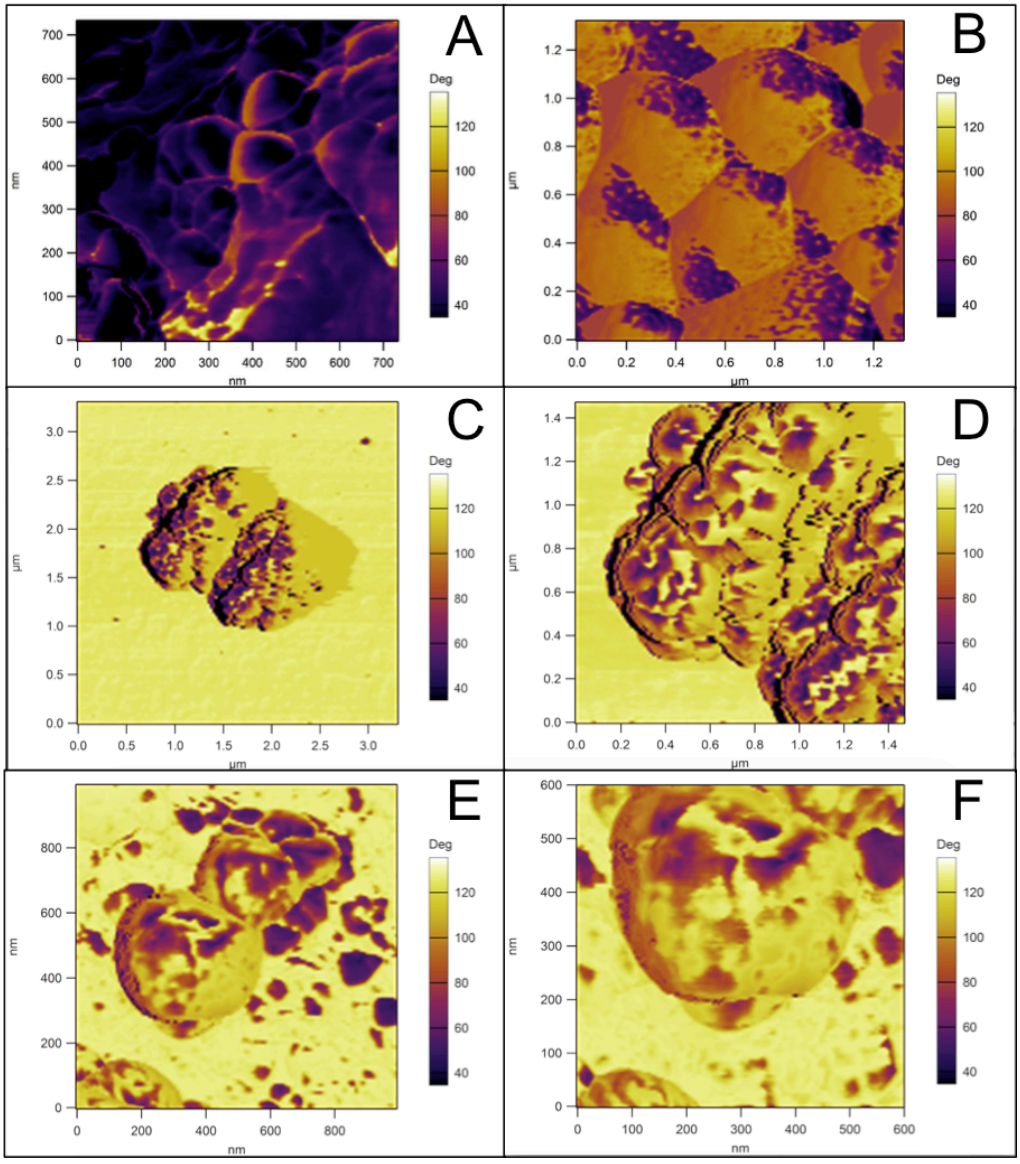

Figure 11. AFM phase angle shift images of pure PBMAD nanoparticles (**A**), pure PLA/PLGA nanoparticles (**B**), sPIN double-walled nanoparticles in acidic solution (**C and D**), and sPIN double-walled nanoparticles in alkaline solution (**E and F**) are shown in this figure. The purple to orange scale bar represents the phase angle shift magnitude. Note that purple PBMAD dissolves off of the particles in alkaline environments in contrast to the compact particles in acidic solution.

The AFM images shown in Figures 11A and 11B are used as controls to help distinguish the polymers present on the surface of the double-walled particles. Figure 11A shows PBMAD-only nanoparticles made using PIN method. Figure 11B shows PLA/PLGA nanoparticles also made using the PIN method. Between the two images, it is easy to distinguish between the PBMAD and PLA/PLGA polymers that make up the nanoparticles. PBMAD induces a phase angle shift of about 50°, represented as purple, while PLA/PLGA induces a higher phase angle shift around 100°, represented as yellow/orange. Those phase angle values were used to identify the polymers present on the surface of the double-walled nanoparticles shown in Figures 11 C-F.

Figures 11C and 11D show the particles in hydrochloric acid (HCl), pH 0.26, to represent how they would hold up in an acidic environment similar to the stomach. In HCl, the particles look to be intact with spots of PBMAD, represented in purple, scattered along their surface. This confirms the hypothesis made from DLS zeta potential analysis that the ratio of PBMAD to PLA/PLGA used for sPIN wasn't sufficient enough to coat the surface of the core particles, but rather attach to the particles in spots instead, leading to no change in zeta potential.

Figures 11E and 11F show the double-walled nanoparticles submerged in double-distilled water (pH 7) before AFM imaging to view how they change in more alkaline environments, similar to the intestines. It appears that some of the PBMAD, represented in purple, dissolved off the core particles, leaving the smoother PLA/PLGA particle surface void of the PBMAD shell. This shows promising results that indicate the sPIN particles are, in fact, pH-sensitive.

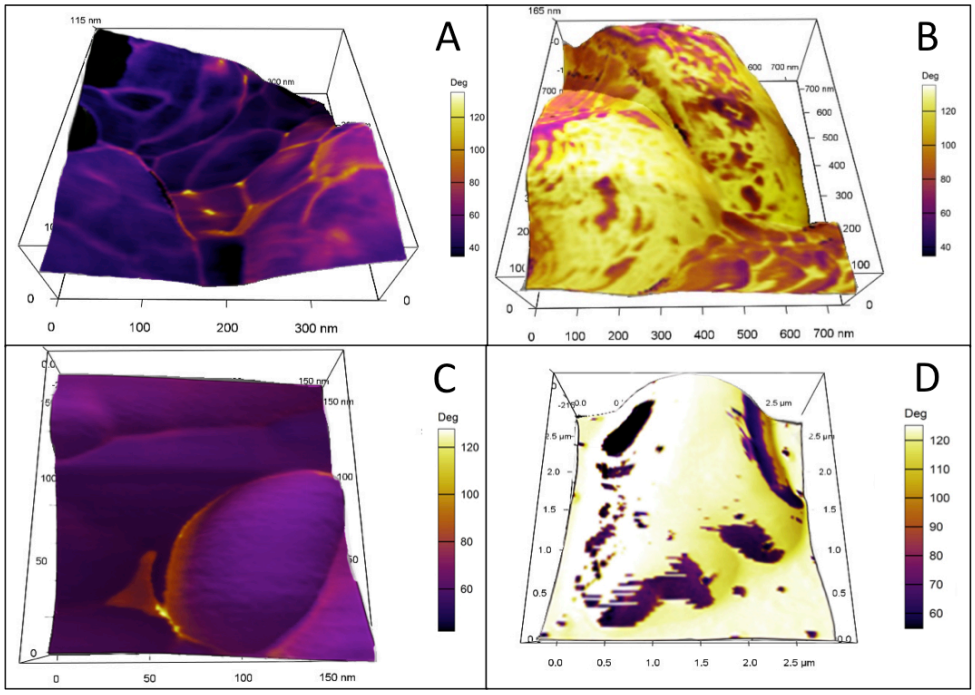

Figure 12. This figure shows 3D phase angle shift images of pure PBMAD particles made by the SSDN method (**A**), PLGA 75:25 nanoparticles made by SSDN (**B**), SSDN PLGA/PBMAD double-walled particles in acidic solution (**C**), and SSDN PLGA/PBMAD double-walled particles in alkaline solution (**D**). The yellow to purple scale bar represents varying phase angle shifts to distinguish polymers. This 3D image superimposes the phase angle shift image onto a 3D height measurement of the particles.

The AFM images shown in Figures 12A and 12B show PBMAD and PLGA 75:25 nanoparticles made by SSDN, respectively. Similar to the phase angle shifts of the controls in Figure 11, PBMAD induces a phase angle shift of around 50°, while PLGA induces a phase angle shift closer to 100°. The double-walled nanoparticles shown in Figure 12C induce a phase angle around 60°, indicating a phase shift that is in between the PBMAD and PLGA controls. These SSDN double-walled nanoparticles, unlike the sPIN particles shown in Figure 11, seem to have full PBMAD coating on the surface of the core PLGA particle rather than a spotted shell. This is shown by the smooth and uniform surface that predominantly has a phase angle shift similar to that of the pure PBMAD particles.

Similar to the sPIN particles, the SSDN particles seem to keep their PBMAD coating intact in acidic environments, shown in Figure 12C, then shed that coating in alkaline environments, shown in Figure 12D. By exposing the particles to an alkaline environment, a buffer of pH 10, the surface composition changes from purple, indicative of PBMAD, to yellow, indicative of PLGA. From this, it can be deduced that the particles made by SSDN were able to form a complete core and shell structure. These images also confirm that the SSDN particles are pH-sensitive, similar to the sPIN particles.

4.4 Release Study in Varying pH

The drug release profiles of sPIN and SSDN particles were next assessed. For one release study, the particles were dissolved in acidic dispersion buffer (DB), pH 4, for six hours to mimic digestive transit time through the stomach, then transferred to T-buffer (TB), pH 6.3, for the remainder of the study to mimic intestinal and colon transit time. For a second release study, the particles were dissolved in TB, pH 6.3, for the entire duration to observe if there is a difference in release profile compared to when the particles are put in acidic solution first. The purpose of observing drug release in DB to TB and also TB alone is to observe whether or not the pH-sensitive PBMAD hinders the release of drugs in acidic solution.

Samples taken at various time points throughout the study were centrifuged and supernatants were collected and analyzed using HPLC size exclusion chromatography (SEC). SEC is used to sort molecules in a sample by size. It separates the molecules by using a column filled with porous material. As dissolved molecules of various sizes flow through the column, the smaller molecules get caught between the pores, causing them to

go through a lengthier path, while larger molecules pass through the porous material quickly. Using this technique, the goal was to separate the high molecular weight biologics from PBMAD and other excipients present in the buffer solutions. However, the adhesive nature of PBMAD ended up sticking to the porous material and drug, causing an extended elusion curve that overlaps with the elution of biologics, shown in Figure 13.

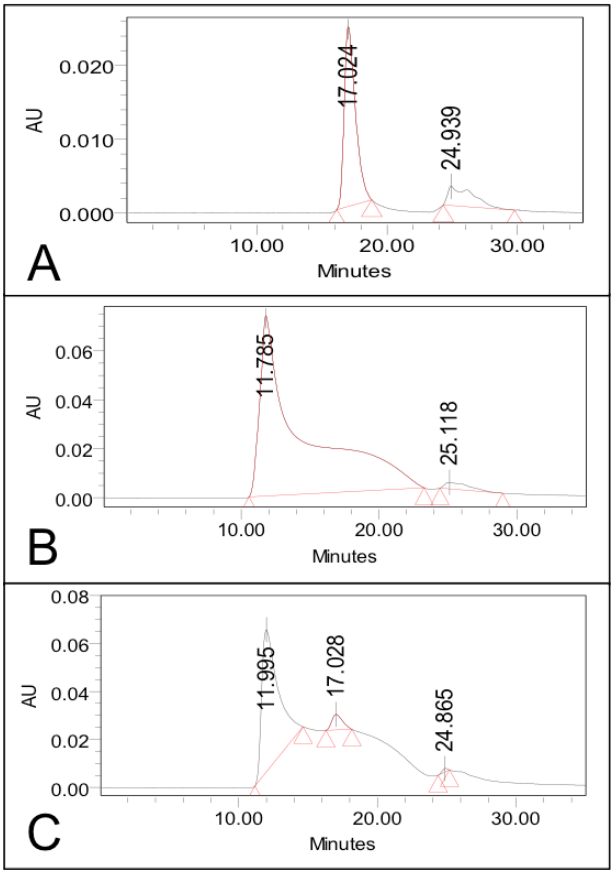

Figure 13. This figure shows three HPLC chromatograms. The chromatogram in (**A**) shows the elution curve of the biologics used for encapsulation. The drug comes out around 17 minutes. Chromatogram (**B**) shows the extended elution curve of PBMAD. The drug and PBMAD peaks overlap with each other when dissolved double-walled nanoparticles are examined (**C**). The peaks around the 25-minute mark denote excipients in the buffers used.

As can be seen in Figure 13B, PBMAD's elution curve has a high peak around 12 minutes and extends past the 20 minute mark. Meanwhile, the biologics elude at 17

minutes, shown in Figure 13A. This overlap in elution curves, shown in Figure 13C, made analyzing the exact amount of biologics released difficult. The chromatogram produces a superposition of multiple curves, so depending on the concentration of drug being released, its peak sometimes does not show through the PBMAD curve. In an attempt to separate the drug from PBMAD, centrifugal filters were used in an additional step before HPLC analysis. The filters used were 0.22 micron PTFE that should have been large enough for PBMAD to pass through while collecting the high molecular weight drug. However, some of the adhesive PBMAD ended up sticking to and blocking the filter, in effect, preventing any filtration from occurring. This blockage did not go away in spite of multiple attempts to alter the centrifuge parameters.

To work around this masked drug release curve, ImageJ analysis was performed on each chromatogram to manually calculate the area under the curve (AUC) at the 17-minute mark. The AUC's from the release study chromatograms were compared to a calibration curve made from the AUC's of various standards with known concentrations of drug. From the concentration of drug released, an estimated mass of drug released was calculated based on the volumes of samples taken. This method of analysis provides an upper bound for the amount of drug released as it may include a portion of the PBMAD curve that overlaps with it. Although the amount of calculated drug release might be an overestimation due to an inadequate method of drug release measurement, it is still possible to observe the type of releases that occur with different methods of nanoencapsulation in varying pH solutions. Figure 14 shows the accumulated drug release profiles of sPIN and the two SSDN particles in DB to TB or TB alone.

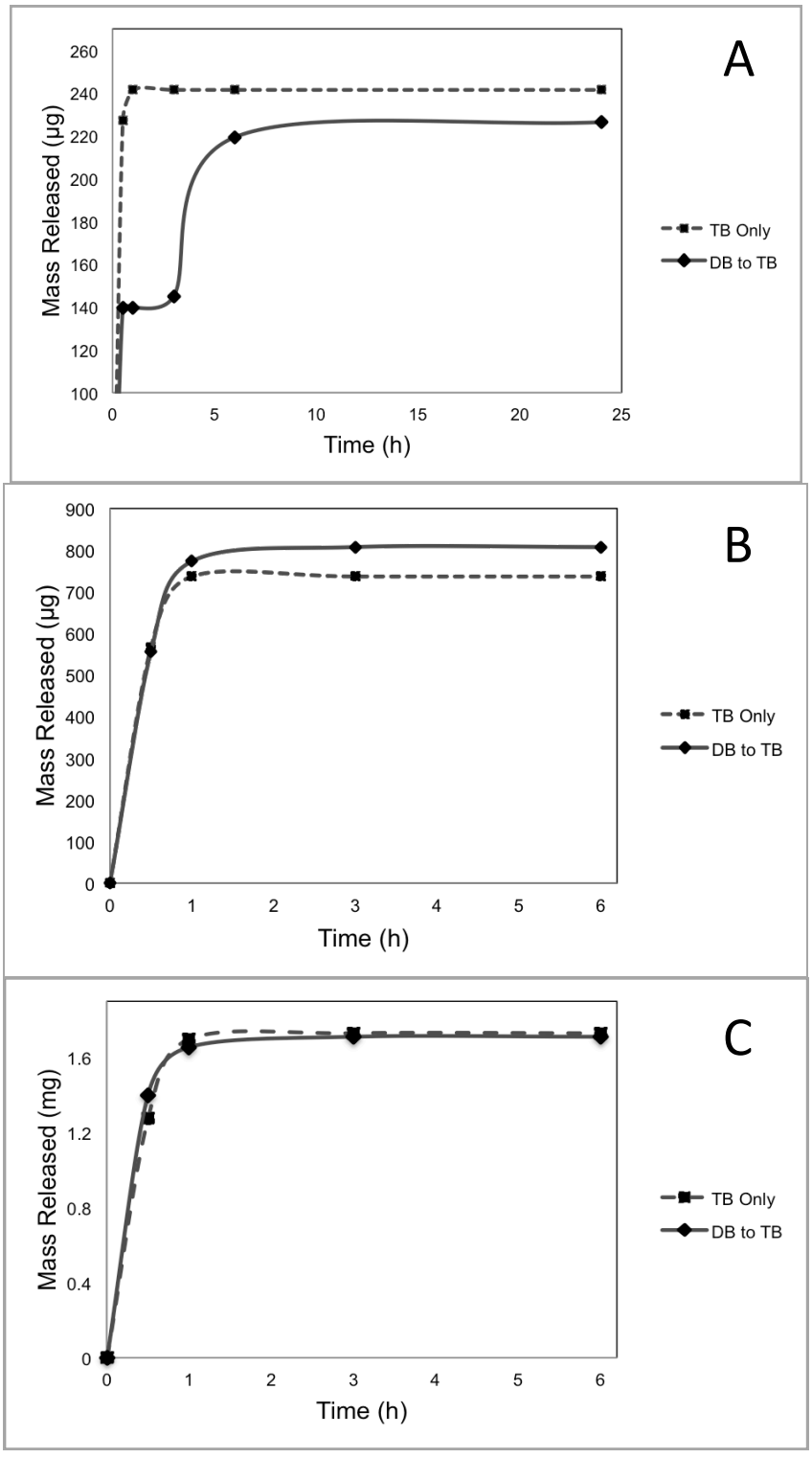

Figure 14. These graphs show the accumulated drug release profiles for double-walled nanoparticles made by sPIN (**A**), SSDN with PLGA 5mg/mL PBMAD (**B**), and SSDN with PLA 20mg/mL PBMAD (**C**). The solid lines are releases in pH 4.0 for the first six hours, then transferred to pH 6.3. The dotted lines are releases in pH 6.3 only.

Figure 14A shows the drug release profiles of the sPIN double-walled nanoparticles in two different pH solutions. The solid line shows the release when they are first put in dispersion buffer, pH 4.0, for the first six hours, and then switched to T-buffer, pH 6.3, for the remainder of the study. The dotted line shows the release when the particles are only put in T-buffer.

When the particles are first dispersed in acidic solution, similar pH to the stomach, a small amount of drug is released. No further drug is then released until after hour six, when the particles are moved to more alkaline pH solution, similar to the intestines. The initial burst release of drug could be due to excess drug present on the surface of the particles. Since no washing step was incorporated between formulating the particles and running the release study, excess drug that was not encapsulated could have been present on the surface of the particles and detatched once dissolved in solution. The second release of biologics that occurred when the nanoparticles were moved to pH 6.3 indicates that the double-walled nanoparticles are pH-sensitive and are capable of containing biologics within the particles in acidic solution and then degrading and releasing the drugs in higher pH environments.

When the sPIN particles are dispersed within T-buffer for the entirety of the study (Figure 14A dotted line), the biologics are completely released within the first hour. This burst release indicates that the PBMAD shell dissolves and release the drug in alkaline pH solutions. This burst release in TB verifies that the second release seen in the DB to TB study was not due to a time dependent system, but driven by the change in pH.

The SSDN double-walled nanoparticle drug release profiles, made with PLGA and PLA respectively, can be seen in Figures 14B and 14C. Both of these particle

preparations result in a burst release in both DB to TB release studies as well as TB only releases. Even though the AFM images show a full coating of PBMAD around the particles, it appears the SSDN particles were not assembled well enough to effectively encapsulate the biologics. Perhaps during the SSDN procedure, the system was not given enough time to allow for the particles to precipitate and form completely.

When comparing drug release profiles of the two methods of encapsulation, sPIN and SSDN, it is apparent that the sPIN formulation produces the best pH-dependent system for the delivery of biologics. With the SSDN method, the particles are not able to retain the biologics through acidic environments, resulting in a burst release of the drug. Double-walled particles made by sPIN are able to retain some of the biologics in acidic solution, and then release the rest of the therapeutic load when exposed to a more alkaline environment. This proves that sPIN-produced particles are capable of creating an enteric-coated delivery system.

4.5 Release Study in Simulated GI Fluid

To more accurately observe how the sPIN nanoparticles would deliver biologics within the GI tract, a release study was conducted using simulated gastric fluid (SGF), pH 1.36, simulated duodenum (made from a mixture of SGF and SIF), pH 4.64, and simulated intestinal fluid (SIF), pH 7.51. The data was collected and analyzed using the same methods as the buffer release studies in the previous section. Two sets of sPIN double-walled nanoparticles were tested along with a sample of just the core nanoparticles, made of PLA/PLGA before the PBMAD coating was applied. The three samples were dispersed in SGF for the first four hours of the study to mimic stomach transit time, then moved to the simulated duodenum for the next two hours, and finally

transferred to SIF for the remainder of the study to mimic intestinal transit time. The release profiles can be seen in Figure 15.

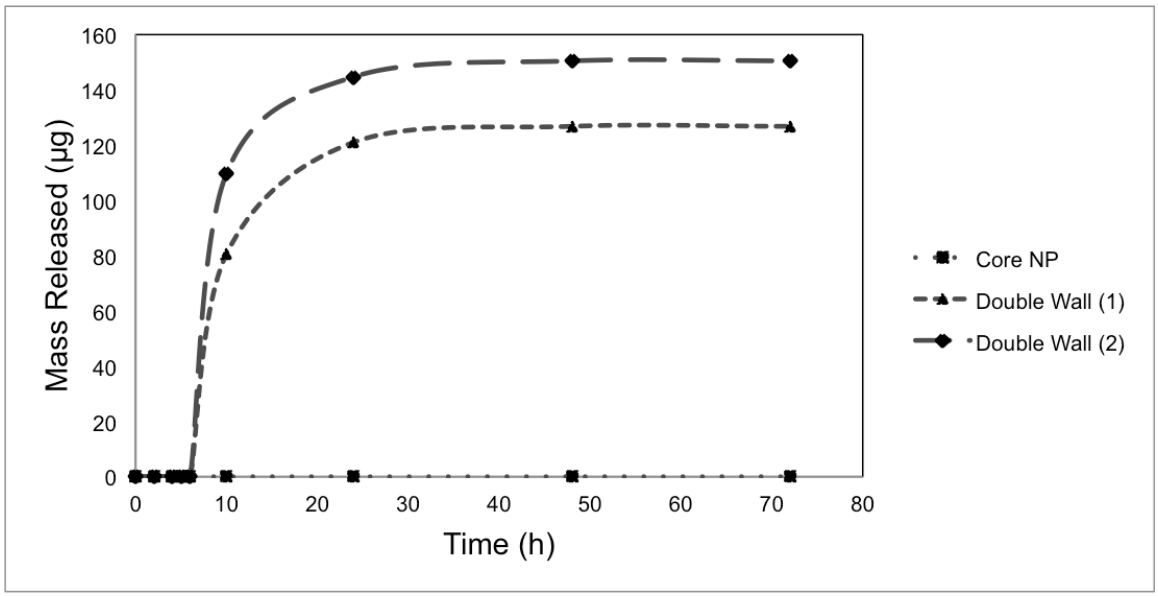

Figure 15. sPIN core and double-walled nanoparticles were exposed to SGF/simulated duodenum fluid for six hours then transferred to SIF to observe their drug release profiles.

The drug release profiles for the double-walled particles show an inhibited release during the first six hours of the experiment, then a gradual release of biologics over the next 24 hours when dispersed in SIF. The core nanoparticles, on the other hand, do not show a release of anything throughout the entire study. To understand this lack of drug release, controls of pure drug and PBMAD were tested in SGF and SIF, shown in Figure 16.

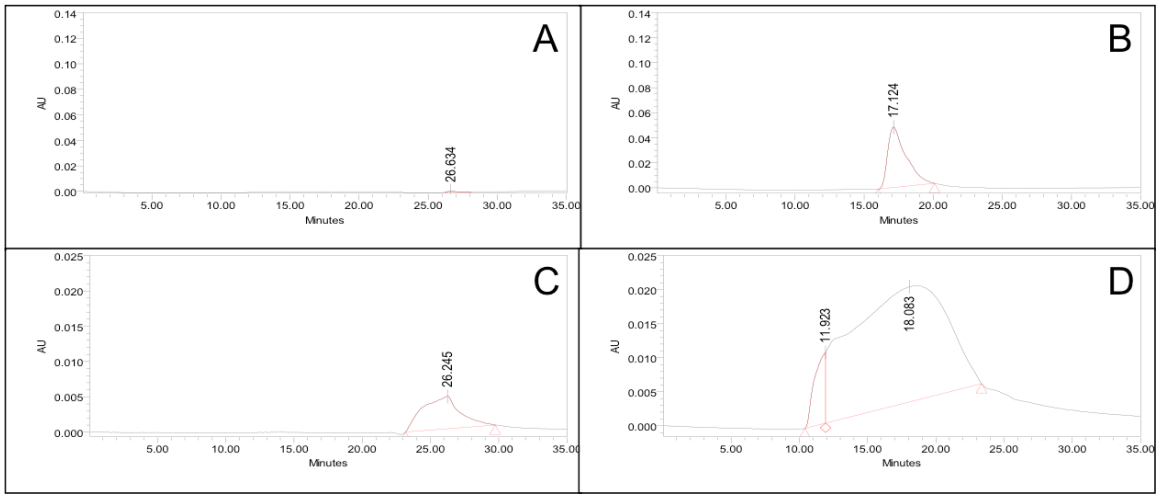

Figure 16. Pure drug was analyzed in SGF (A) and SIF (B). Note the lack of drug seen in SGF compared to SIF. Pure PBMAD was analyzed in SGF (C) and SIF (D). Again, note its lack of presence in SGF compared to SIF.

As shown in Figure 16, there is something in the simulated gastric fluid that denatures or masks both the drug and PBMAD. This lack of drug or PBMAD appearance in SGF causes uncertainty as to how much, if anything, was released during the SGF and duodenum portions of the simulated GI tract release study. For this reason, the amount of drug released cannot be accurately quantified; however, the release study is still beneficial in understanding how the sPIN nanoparticles themselves react in a simulated GI environment. With the double-walled particles, even if some drug and PBMAD were released during the SGF and duodenum phase of the release study and went undetected, there is still a release of drug and PBMAD shown during the SIF phase. With the core nanoparticle release study, absolutely nothing is shown to have a release. This indicates that the biologics were completely released during the SGF and duodenum phase, but went undetected due to the HCl in SGF rapidly denaturing them before the HPLC could confirm its presence. Since the double-walled release profile shows drug being released during the SIF phase, it can be concluded that the nanoparticles were able to retain some

drug during the SGF and duodenum phase. This indicates that the sPIN double-walled delivery vehicle was successfully able to retain some biologics throughout the simulated gastric and duodenum environments, and release them in the simulated intestinal environment.

5. Conclusions

The over-arching goal of oral drug delivery for the treatment of severe IBD is to efficiently target the intestines and colon where the disease state is most prevalent. By creating a delivery method that specifically delivers biologics to the inflamed area, the amount of therapeutic administration would be reduced while achieving an enhanced and localized delivery. The double-walled polymeric nanoparticles designed here have shown the potential to effectively deliver biologics and enhance localized release in the intestines.

Both the sPIN and SSDN particle formulations were able to encapsulate high molecular weight biologics into nanospheres that could be used for oral delivery. The sPIN particle formulation, in particular, showed great promise as a means for localized delivery to the intestinal region. The delayed release achieved in buffer and simulated GI solutions, shown in Figure 14A and 15 respectively, proves that the delivery vehicle made by the sPIN method is a pH-triggered system. Once the sPIN particles were dispersed within a solution of similar pH to the intestines, a release over the course of 24 hours occurred. This long release time would provide ample time for PBMAD to penetrate the mucus layers and deliver the biologics close to the intestinal epithelium. This double-walled nanoparticle formulation could transform the treatment of biologics

for severe IBD from a cumbersome IV/hospital visit to an easy and effective at home/on the go treatment.

6. Future Directions

Though the double-walled nanoparticle formulations tested here show great potential for a unique method of oral delivery, there are aspects of the particle formulations and experiments that can be optimized. Varying the ratio of PBMAD to PLA/PLGA could be tested in order to find the formulation that provides the best drug loading while maintaining an effective pH-triggered release. In addition to that, varying the ratio of biologics to polymer could also provide an enhanced therapeutic loading.

In order to definitively quantify the amount of drug released during release studies, a new method of analysis must be found. Perhaps the use of a liquid chromatography-mass spectrometry instrument could be utilized to differentiate between the PBMAD and drug release. Alternatively, a method of extraction could be implemented to separate PBMAD from the drug for a more efficient HPLC analysis.

Moving forward, the various components of the double-walled nanoparticles should be tested *in vitro* to observe if they are cytotoxic. The formulated nanoparticles should also be tested *ex vivo* with rodent intestinal mucosa and epithelia to examine their bioadhesive quality. Running the *ex vivo* experiment with particles of varying PBMAD ratios would also be beneficial to finding the optimal particle formulation. Finally, *in vivo* experiments using isolated loop and oral gavage administration should be done in order to observe how well the particles penetrate the mucosa over time and effectively deliver biologics.

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