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Ionically Complexed Nanoparticles for Heparin Oral Delivery[#]

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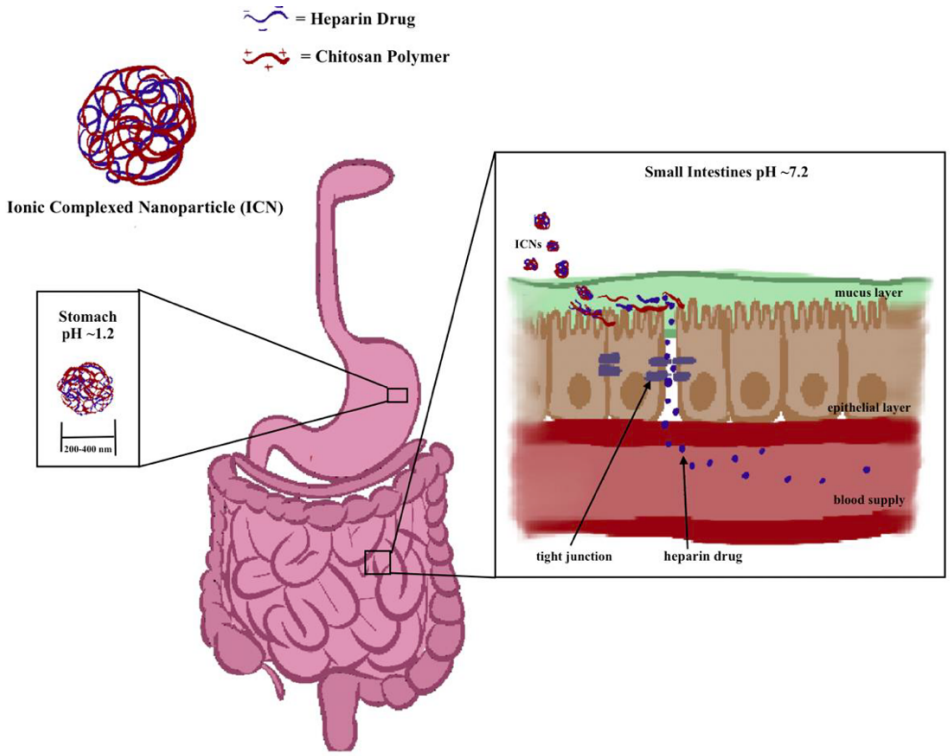
Graphical Abstract



Abstract

Ionically complexed nanoparticles were prepared from an anionic polysaccharide drug, heparin, entrapped by a positively charged chitosan polysaccharide. In this study, the encapsulation of heparin was studied to optimize the properties needed for its oral drug delivery. Chitosan, used in various biomedical applications, was selected as a cationic polymer for heparin encapsulation. These particles were prepared with a slightly positive charge and an appropriate size for oral drug delivery. In addition, the release profiles of these ionically complexed nanoparticles were improved by using FDA-approved stabilizers, such as pluronic non-ionic surfactant and polyvinyl alcohol. These results obtained *in vitro* suggest that these stabilized, ionically complexed nanoparticles may be well-suited for the oral drug delivery of heparin into the gastrointestinal tract.

Keywords

Heparin, Chitosan, Ionic Complex, Oral Drug Delivery

[#] This article is the peer-reviewed version of the preprint at https://assets.researchsquare.com/files/rs-1631202/v1/0f2a6de6-c7a2-43cd-98ba-3262ce29139b.pdf?c=1654550166

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Purpose and Rationale

Patient compliance, in which a patient accurately and consistently follows a prescriber's orders, is important for promoting drug efficacy and patient health [1]. Heparin is a widely used anticoagulant drug administered *intravenously* as part of inpatient treatment. Outpatients also self-administered it through *subcutaneous* injections one to six times a day. Unfortunately, irritation, redness, sores, and pain are often associated with multiple heparin injections [2]. As a result of these expensive and painful injections, parenteral administration of heparin can lead to low patient compliance. This research aims to develop a formulation that could improve the oral delivery of heparin and mitigate the problem of patient compliance.

Introduction

Heparin is a polydisperse polysaccharide with an average molecular weight (M_W) of ~17 kDa [3]. It is a heterocopolymer of disaccharides comprised of sulfated D-glucosamine and iduronic or glucuronic acid residues [4]. Because of the variable *N*-acetylation/sulfation and *O*-sulfation of these disaccharide repeating units, heparin has extensive structural or sequence heterogeneity (Figure 1) [5]. Moreover, heparin remains anionic over a wide range of pH values (pH 0.5 to 14) due to the presence of sulfo- and carboxyl groups [6]. On average, each heparin disaccharide has ~2.7 sulfate groups resulting in a total negative net charge of approximately −75 [7]. Since heparin has an average Mw of 15 kDa, this property gives heparin the highest negative charge density of any known naturally derived biomolecule [7,8]. Thus, it is not surprising that, because of its high Mw and high negative charge, heparin has an extremely low oral bioavailability that has been estimated at <1% [9].

Successful oral transmucosal drug delivery depends on nanoparticle durability throughout the mucus layers of the gastrointestinal (GI) tract. This protective membrane is the first line of defense against pathogenic invasion and epithelial cell damage to GI organs, including the mouth, esophagus, stomach, small and large intestines, and anus. Mucin fibers rich in cysteine are crosslinked in a mesh network with hydrophobic globular non-glycosylated regions that allow for disulfide bond formation [10]. Sialic acid, carboxyl groups, and sulfate residues in these crosslinked mucin fibers contribute to the negative surface charge of this mucus, leading to the trapping of cationic nanoparticles [11]. To overcome clearance from the mucus, nanoparticles are engineered with a specific surface, chemical adhesive, and muco-penetrating properties that enhance interaction with the mesh network. For example, chitosan and other thiomer-coated nanoparticles can travel through the acidic stomach without forming disulfide bonds; they endure the GI pH gradient until absorption at the small intestine. As these nanoparticles leave the gastric mucosa layer and approach epithelium with a higher pH, they demonstrate mucoadhesive properties with thiol-group disulfide bond formation among the mucus glycoproteins [12]. Previous diffusivity studies of the intestinal mucosa have concurred that small molecules exhibit better muco-penetration when compared with larger macromolecules, so manipulating the size of nanoparticles is another consideration for drug delivery [13]. However, while decreasing the size of nanoparticles can boost mucus penetration, smaller nanoparticles could suffer from poor cellular uptake [14]. Therefore, the complex structure and functionality of the mucus cause continued difficulties for drug delivery systems. Nanoparticle design must overcome all mucosa, cellular absorbance, and additional physiological obstacles.

Summary of Relevant Literature

There have been many studies aimed at enhancing the oral bioavailability of heparin. While low Mw heparins (~6 kDa) [15], prepared through the controlled chemical or enzymatic depolymerization of heparin [16], show improved *subcutaneous* bioavailability, they show only slightly improved oral bioavailability [17]. Covalently-linked or ion-paired small hydrophobic molecules have been used to marginally improve oral bioavailability [18]. Surfactants, such as saponins [19] or sodium N-[8(-2-hydroxybenzoyl) amino] caprylate (SNAC) [9] can enhance heparin's oral bioavailability but are believed to damage the intestinal barrier.

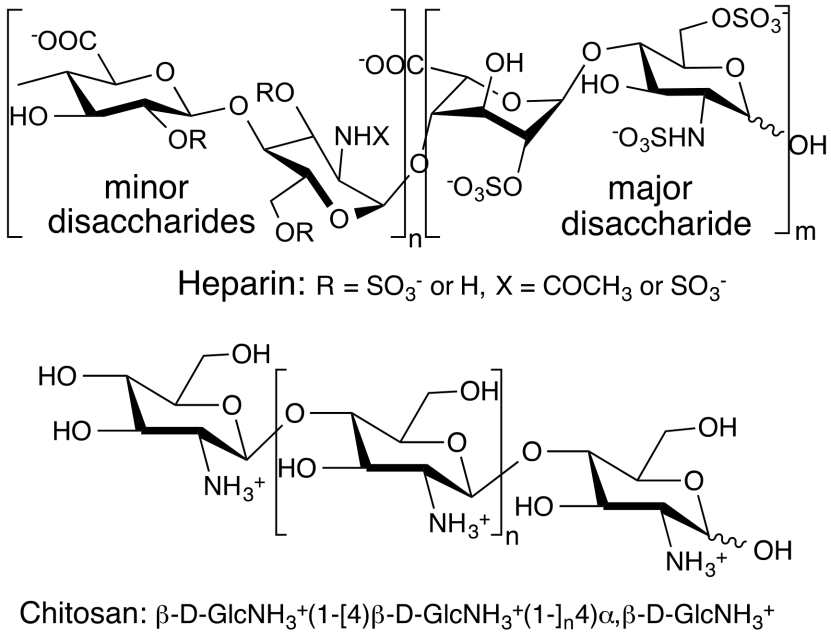

Figure 1. Structure of heparin on the top and the structure of chitosan on the bottom.

The success in enhancing gene delivery by complexing DNA or RNA polyanions with polycations [20, 21] has motivated similar approaches for delivering heparin [22]. Recently, nanoparticles have been explored for the enhanced oral delivery of biopolymers [23], including heparin [24,25].

This paper examines a nanoparticle formulation of heparin as a targeted oral delivery approach to increase patient compliance by providing a safe, painless, and easy means of anticoagulant administration [26]. Heparin, an anticoagulant, works through the formation of a serine protease inhibitor complex with antithrombin III and is used to prevent common postsurgical complications such as deep vein thrombosis and peripheral arterial embolisms [27]. Heparin is a polyanionic drug due to its many carboxyl and sulfo groups [27]. Therefore, it is an ideal candidate to be used in ionic complexed nanoparticles (ICNs) (Figure 2) [28]. ICNs consists of a cationic polymer entrapping an anionic drug. This method of creating nanoparticles has been used to load charged therapeutics such as DNA, drugs, or probes for various biomedical applications such as delivery and sensory purposes [29]. ICNs are suitable for drug delivery due to their small size and easier entrance into cells, improved bioavailability, and ability to control the release of medication. ICNs are pH sensitive, which is beneficial for oral drug delivery into pH-sensitive environments such as the gastrointestinal (GI) tract [30]. A previous study used chitosan to encapsulate heparin for oral delivery [30]. Our study builds on this by looking at the effect that pH has on nanoparticle size, zeta potential, and the use of stabilizers.

Chitosan is a polysaccharide derivative of the natural product, chitin, obtained from crustaceans. Chitosan is prepared through de-N-acetylation and is an excellent candidate for heparin complexation because of its cationic nature. Chitosan also has therapeutic properties such as antioxidant activity, cholesterol trapping, and antibacterial effects [31]. Chitosan is approved by the FDA and is generally regarded as safe (GRAS) because it is biodegradable and non-toxic. Due to its mucoadhesive character, Chitosan has been used in previous drug delivery applications. It adheres to the mucosal epithelial layer of the GI tract and enhances the penetration of large molecules or ions through the mucosal surfaces by opening tight junctions [32,33].

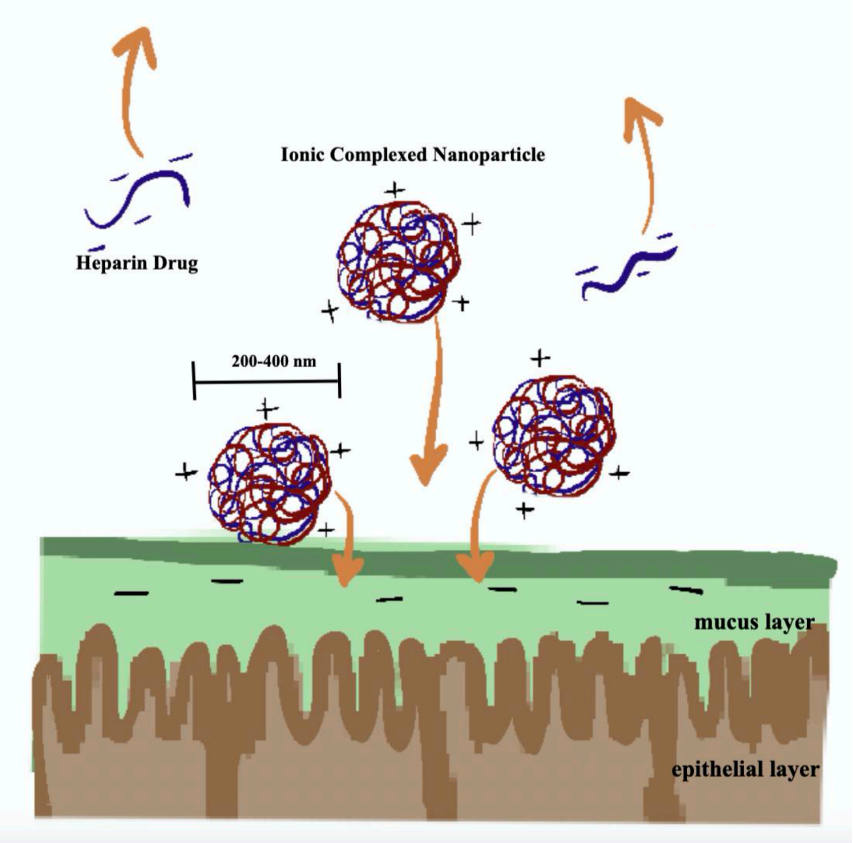

Figure 2. ICNs as they pass through the gastrointestinal tract.

We have prepared ionicallty complexed nanoparticles using chitosan to encapsulate heparin for oral drug delivery applications. For these particles to be successful in drug delivery, they need to have optimal size, charge, pH responsiveness, and drug loading leading to oral bioavailability. These nanoparticles maintain the stability of the stomach's very acidic conditions and the small intestines' neutral pH. Oral drug delivery relies on absorption in the small intestines, where the epithelial layer is the most permeable [34]. Ideally, nanoparticles should be less than 200 nm in diameter with a slightly positive zeta potential, between 1 and 10 mV. These properties allow ICNs to interact with the negatively-charged epithelial layer and move through this cell layer and into the circulation, giving a relatively high oral bioavailability [35]. Moreover, an ideal drug delivery system should have a drug loading of at least 50% [28]. These parameters, if met, should result in anticoagulation with an oral heparin dose of 100 mg.

MATERIALS & METHODS

3.1 materials

Low Mw chitosan (chitosan, Mw 50–190 kDa, 85% deacetylated) was obtained from Sigma Aldrich (St. Louis, MO, USA). Unfractionated heparin (UFH) (Mw 15 kDa, with ~2.7 sulfate groups) was obtained from Celsus laboratories (Cincinnati, OH). In addition, concentrated glacial acetic acid, phosphate-buffered saline (PBS, pH 7.4), poly(vinyl alcohol) (PVOH, Mw 89-98 kDa), and Pluronic F-127 (Mw 12,600 g/mol) were obtained from Sigma Aldrich (St. Louis, MO, USA).

3.2 Preparation of nanoparticles

Samples were prepared by separately dissolving UFH in deionized water at a concentration of 1 mg/mL and dissolving chitosan in 1% glacial acetic acid. After completely dissolving both samples, the heparin solution was added dropwise with a 1 mL pipette at a rate of two drops per second into the chitosan solution under continuous stirring (400 rpm) at room temperature.

3.3 Size analysis and zeta potential

3.3.1 Mean nanoparticle size, polydispersity index, and zeta potential were determined by DLS at 25°C with a Malvern Zetasizer ZSP (Worcestershire, UK) using a 173° backscattering angle. Sample preparation for DLS measurements was performed by transferring 100 mL of the nanoparticle solution into 10 mL of MiliQ water. An optical microscope (Motic BA210, with a 100× oil immersion objective lens) was used for visual observations of nanoparticles at varying pH for changes in NP size.

3.3.2. Scanning Electron Microscopy (SEM). For sample preparation, 2%v/v of sorbitol was added to the nanoparticle solution to reduce or eliminate particle aggregation during drying. SEM images on dried particles were obtained using a Supra 55 SEM. Images were acquired at 2.5 mV with an aperture of 10 mm at room temperature and a minimum vacuum of 4×10^{-6} bar. These dried particles were platinum sputter coated using a Denton Vacuum Desk IV Sputter Coater at 25 mA for 90 s under a vacuum of 0.03 bar. This was done to enhance the contrast and reduce the charging.

3.4 Encapsulation efficiency

Encapsulation efficiency was measured by determining the amount of free heparin in solution. Triplicate samples were taken at the start of each time measurement at varying pHs, centrifuged, and the supernatant with free heparin was measured using a micro-carbazole assay [36,37]. The concentration of the free heparin was calculated using Eq. 1 below.

$$\text{Encapsulation Efficiency } (EE)\ \% = \frac{total\ amount\ of\ heparin\ added - free\ heparin}{total\ amount\ of\ heparin\ added} \times 100$$

Eq 1. Definition of encapsulation efficiency

3.5 In vitro drug release

In vitro drug release studies were performed in a 20 mL volume while incubating at 37°C, 150 rpm, and varying pH values to simulate different environments within the GI tract. The media used was without enzymes. The initial pH values of 3, 5, and 7 were diluted with varying concentrations of glacial acetic acid and phosphate buffered solutions. Simulated intestinal fluid at pH 7.2 and simulated gastric fluid at pH 1.2 were also used as media during NP incubations following a literature protocol for a study that similarly focused on oral delivery [38]. At predetermined time points, 1 mL aliquots were taken and centrifuged. The free heparin that remained in the solution was then measured using a micro-carbazole assay, and the equation below was used to measure the percentage release [36,37].

$$Drug\ Release\ \% = 100 - \left(\frac{total\ amount\ of\ heparin\ added - free\ heparin}{total\ amount\ of\ heparin\ added} \times 100\right)$$

Eq 2. Definition of drug Release

3.6 Stabilizers

FDA-approved stabilizers such as pluronic-127 and PVOH were used to prevent the premature release of the heparin into the surrounding solution. These stabilizers have a neutral charge, are soluble in water, and are biocompatible. The stabilizers, diluted to a concentration of 1 mg/mL, were added to nanoparticle solutions at 1, 2, and 5%, where the percentage corresponds to the nanoparticle solution. NP size, zeta potential, and heparin release measurements determined the stabilizers' effects.

RESULTS & DISCUSSION

This investigation focused on the ionic complexation of chitosan and UFH as a vehicle for the oral delivery of UFH. The hypothesis studied herein is that stable nanoparticles of chitosan and UFH could be designed and prepared to withstand varying pH in the GI tract to enable adherence of the nanoparticles to the epithelial layer in the small intestines and infiltrate into the circulatory system.

4.1 Preparation of nanoparticles

Chitosan with a relatively low Mw (50 to 190 kDa) was selected instead of higher chitosan Mw grades (> 1 million kDa) to circumvent problems of high viscosity and poor solubility.

Table 1. The table below shows the effect of varied heparin loading on the NP size (nm) and the encapsulation efficiency (%). This data was used to move forward with 50% heparin loading

UFH loading[a]	25%	35%	50%	65%	75%
NP size (nm):	489.5±36.5	456.0±75.5	351.2±70.6	609.6±49.1	630.0±65.7
Encapsulation efficiency (%):	39.5±16.4%	53.7±14.3%	95.7±3.7%	71.0±11.9%	60.5±13.8%

[a]UFH wt% = (100 – chitosan wt%) ; UFH = unfractionated heparin.

Table 1 depicts how UFH loading (total of UFH and chitosan wt% equals 100) influences the NP size and encapsulation efficiency. Particle sizes ranged from 332.2 nm to 667.9 nm, and an encapsulation percentage of 39.5 to 95.7%. At 50% UFH, its encapsulation efficiency is highest, and the NP size is relatively low (363.9 nm). The polydispersity index (PDI) was used to accept or reject the data. Values exceeding 0.5 were rejected as this indicated that the solution was non-homogenous. Furthermore, the zeta potential (ZP) at 50% UFH loading is +19 ± 3 mV. This slightly positive ZP is preferred since mucin glycoproteins have high sulfate and sialic acid contents, leading to attractive ionic interactions with the nanoparticles [12,39]. Furthermore, based on previous work, slightly positive 200–300 nm range nanoparticles are expected to readily pass through the epithelial surface of mucosal tissues in the GI tract. Furthermore, at 50% heparin loading, the encapsulation efficiency is 95.7 ± 3.5%. Hence, subsequent studies were performed at 50% loading.

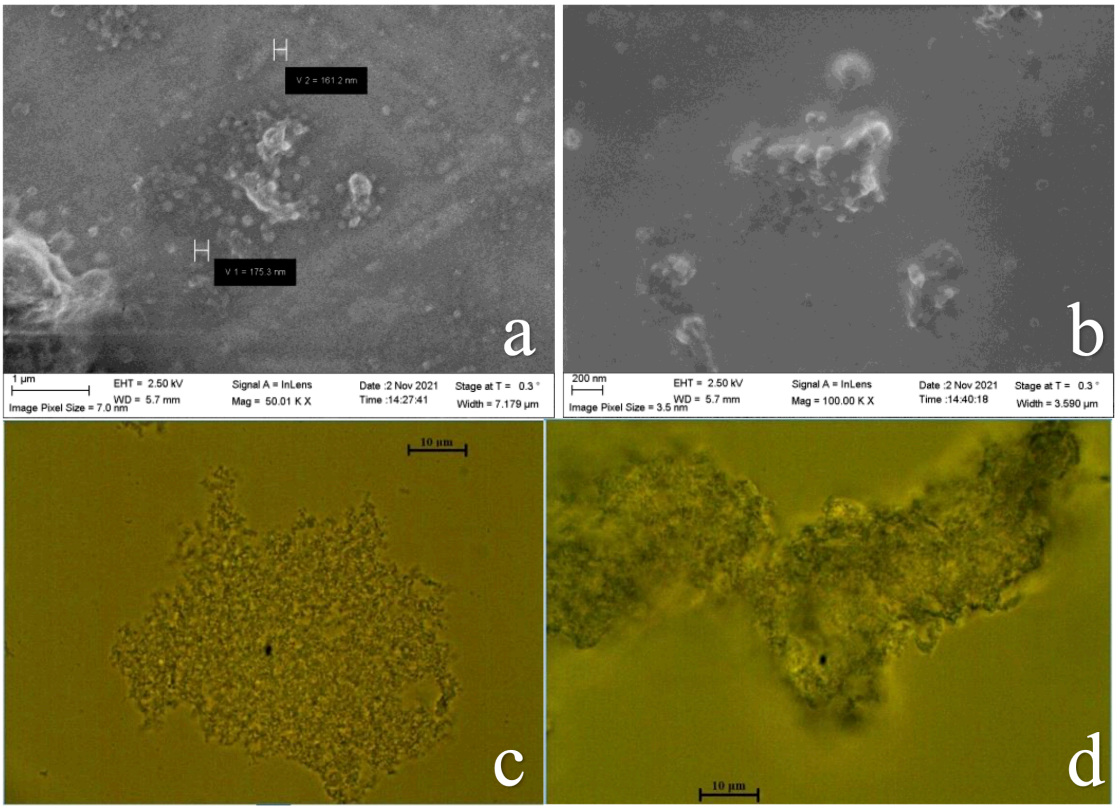

Figure 3. 3a and 3b. Scanning electron microscope images of the nanoparticles (NPs) in simulated gastric fluid solution (pH 1.2). The NPs size was observed to be about 200 nm at lower and higher magnifications (top left and right images, respectively. The aggregation is due to the drying, which was combated with sorbitol additive. 3c and 3d. Bottom. The optical microscopy images of what were NPs after incubation in simulated intestinal fluid solution (pH 7.2). The NPs started to aggregate (3c) and swelled in size (3d) such that the average NP size increased to 30–40 mm.

4.2 In-vitro analysis

The effect of varying pH, simulating the GI tract, was studied on the UFH-chitosan ICNs. The ICNs, which are pH sensitive, need to survive varying pH before making their way to the small intestines for uptake into the circulation. These NPs are pH sensitive due to the impact of pKa on protonation. For example, the amine group in chitosan (Figure 1) has a pKa of ~6.5. Therefore, it is soluble under acidic conditions where amine moieties are protonated, leading to an increased binding area [40]. This results in higher ionic complexation between chitosan and UFH at lower pH with a spherical shape. However, at higher pHs of around 7.2, mimicking what occurs within the small intestines, chitosan begins to deprotonate and lose the electrostatic strength to ionically complex with UFH, which in turn leads to swelling and deformation of the NPs.

Three different pH values and two simulated fluids (gastric fluid, pH 1.2; intestinal fluid, pH 7.2)) were used to evaluate nanoparticle stability (Figure 3). The particles were maintained at physiological temperatures of 37°C while shaking at 150 rpm to simulate *in vivo* parameters. Particle sizes were observed over 2–4 hours under varying pH values of the simulated fluids. The NP size remained relatively constant at about 200–400 nm (Figures 3A and 3B). However, in simulated intestinal fluid, at pH 7.2, the particles swelled (Figure 3C) and aggregated (Figure 3D). These events resulted in increased PDI that exceeded 0.5, indicating a non-homogenous particle distribution.

The ZP was observed over four hours under varying pH solutions. As seen in Figure 4a, the zeta potential did not substantially change when incubated at pH 1.2 in simulated gastric fluid. However, incubations at pH 7.2 in simulated intestinal fluid resulted in an abrupt decrease in ZP to almost −20 mV, indicating the breaking apart of the NP, with the anionic heparin being released to the surface. This suggests that the formulated UHF/chitosan nanoparticles are not stable at higher pHs that occur within the small intestines. This instability is unfavorable to heparin delivery in the small intestines, where low partially positive ZP values and nanoparticle sizes around 200 are favored for transport across the mucosal epithelial membrane [12, 39].

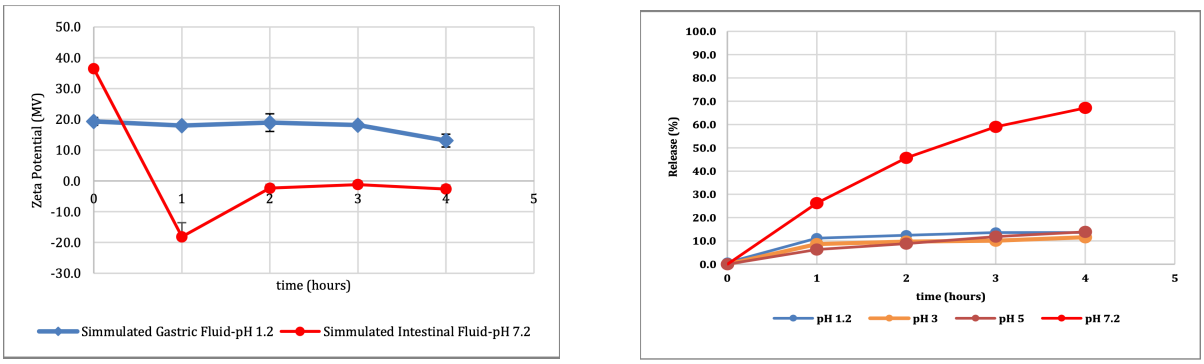

Figure 4. Plot 4a (left) displays ZP values as a function of incubation time in both simulated gastric and simulated intestinal fluids. Plot 4b (right) shows the effect of incubation pH as a function of time on UFH release in varying pH conditions and in the simulated gastric (pH 1.2) and intestinal (pH 7.2) fluids. The error bars were smaller than the data point symbols.

Figure 4b shows the release profiles of heparin from the NPs over time in media diluted with varying concentrations of glacial acetic acid and phosphate buffered solutions such that the initial pH values are 3, 5, and 7. Studies were also done at concentrations of 1.2 and 7.2 within the simulated gastric and intestinal fluids, respectively. At pH 1.2, 3, and 5, less than 15% of the heparin is released within the first hour, and after that, over 4 h, little or no further heparin release occurs. In contrast, at pH. 7.2, heparin release is 26% within 1 h and reaches almost 70% in 4 h. Release at pH 7.2 is desirable. However, swelling of NP in the small intestine to 30–40 mm along with aggregation will not promote particle uptake and passage through the mucosal epithelial membrane. In other words, for heparin uptake and transport through the mucosal epithelial layer within the small intestines, the NPs need to remain intact

for up to 1–2 h, with a slightly positive ZP to interact with the negatively charged layer [41].

4.3 Stabilizers for slower release

To potentially improve the stability of NPs at pH 7.2, the stabilizers poly(vinyl alcohol) and Pluronic 127 (a triblock copolymer that consists of a central hydrophobic block of polypropylene glycol linked to two hydrophilic polyethylene glycol (PEG) blocks) were evaluated. Poly(vinyl) alcohol and Pluronic 127 strongly impacted the release profiles of heparin from the nanoparticles at higher pHs. In Figure 5, the concentration of 5% stabilizer showed a decrease in NP size while keeping the ZP slightly positive at the neutral pH in simulated intestinal fluid. The release profiles were similar in the varying stabilizer concentrations, with the 5% being more stable. Figure 6 shows the release profile with 5% stabilizer added to the solution, over 2 h was 60% lower than that of the ICNs with no stabilizer.

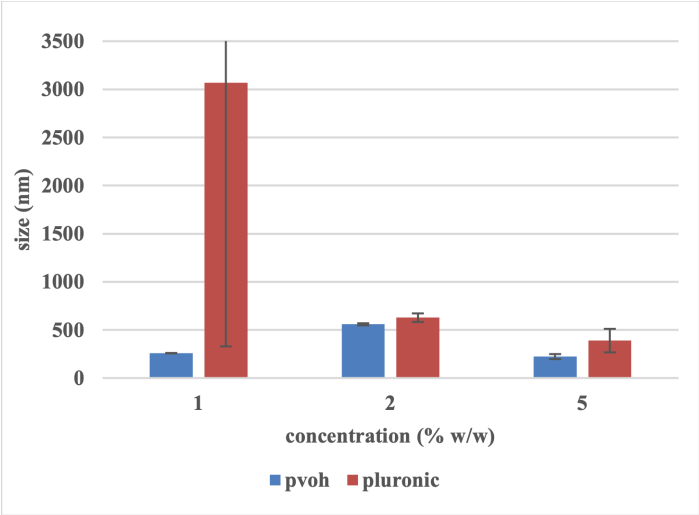

Figure 5. The concentration of stabilizer on initial nanoparticle size.

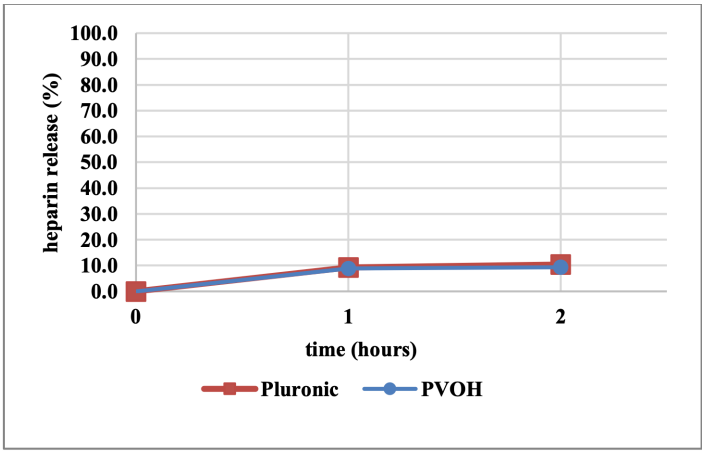

Figure 6. Heparin release in simulated intestinal fluid at pH 7.2 using 5 wt% Pluronic and PVOH stabilizers. The error bars are smaller than the data point symbols.

CONCLUSION

Ionically complexed nanoparticles can release the encapsulated drug based on pH levels. This is beneficial for oral drug delivery, where the drug passing through the gastrointestinal tract encounters varying pH levels. For the heparin to be taken into the circulatory tract, it must pass through low pHs in the stomach cavity and neutral pHs in the small intestines while remaining stable until passing through the epithelial layer within the first 2 hours of entering the intestinal fluid. The epithelial layer within the small intestines is most permeable for molecule and ion uptake. The chitosan can then

enhance the penetration of the heparin through the surface. This work describes how to prepare NPs with 50% heparin loading for oral delivery. In vitro studies indicate that the stabilized heparin-chitosan NP systems meet the criteria to effectively pass through the mucosal epithelial membrane to release heparin for transport in the bloodstream. Future studies will test this heparin oral delivery system in animal models.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose. For signed statements contact the journal office editor@precisionnanomedicine.com.

Acknowledgment and Author Contributions:

Informed consent was obtained from all individual participants included in the study.

The following authors were responsible for Conceptualization (Ideas; formulation or evolution of overarching research goals and aims): Bhagyashree Subramaniam, Robert J. Linhardt and Richard A. Gross; Development of methodology: Bhagyashree Subramaniam, Fuming Zhang, Robert J. Linhardt and Richard A. Gross; Validation (verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs): Bhagyashree Subramaniam, Nicole Leonick, Varenya Gade, David Frey; Investigation (performing experiments): Bhagyashree Subramaniam, Nicole Leonick, Varenya Gade; Resources: Fuming Zhang, Robert J. Linhardt, Richard A. Gross; Writing original draft: Bhagyashree Subramaniam; Writing, review and editing: Robert J. Linhardt, Richard A. Gross, Nicole Leonick; Supervision: Robert J. Linhardt, Richard A. Gross; Project administration: Fuming Zhang, Robert J. Linhardt, Richard A. Gross; All authors read and approved the final manuscript.

Data Availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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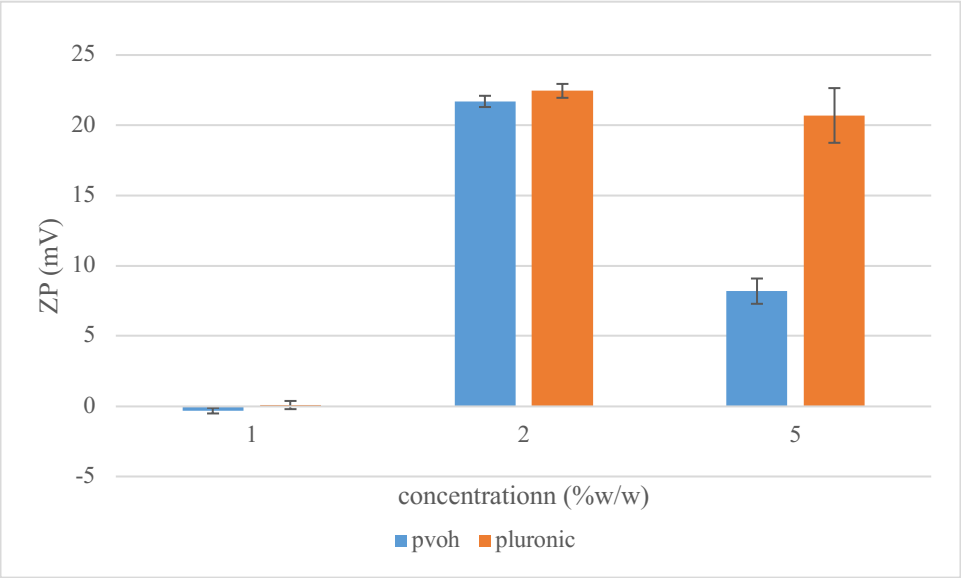
Supplementary Information



Figures 1s. The effect of concentration of stabilizers on the ZP.

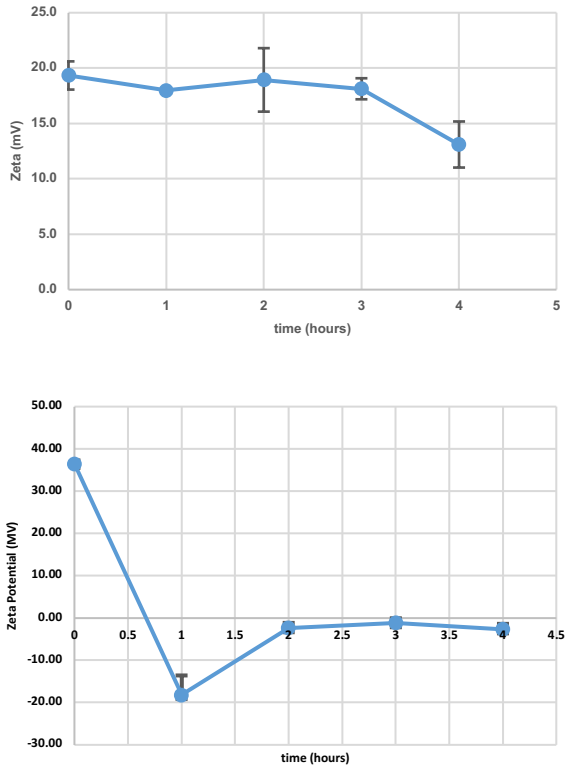


Figure 2s. ZP in gastric (top) and intestinal (bottom) simulated fluid over time.