Ionically Complexed Nanoparticles for Heparin Oral Delivery

Bhagyashree Subramaniam
Rensselaer Polytechnic Institute

Nicole Leonick
Rensselaer Polytechnic Institute

Verenya Gade
Rensselaer Polytechnic Institute

David Frey
Rensselaer Polytechnic Institute

Fuming Zhang
Rensselaer Polytechnic Institute

Robert J Linhardt
Rensselaer Polytechnic Institute

Richard Gross (grossr@rpi.edu)
Rensselaer Polytechnic Institute  https://orcid.org/0000-0002-5050-3162

Research Article

Keywords: Heparin, Chitosan, Ionic Complex, Oral Drug Delivery

Posted Date: June 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1631202/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
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 properties needed for its oral drug delivery. Chitosan, used in a variety of 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. 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.

1.0 Introduction

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 that is administered intravenously as part of inpatient treatment. It is also self-administered by outpatients through subcutaneous injections one to six times a day. Irritation, redness, sores, and pain are often associated with multiple injections of heparin [2]. As a result of these expensive and painful injections, parenteral administration of heparin can lead to low patient compliance. The goal of this research is to develop a formulation that could improve the oral delivery of heparin and mitigate the problem of patient compliance.

Heparin is a polydisperse polysaccharide having a weight 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 (Fig. 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, heparin has 2.7 sulfate groups per disaccharide monomer resulting in a total negative net charge of approximately −75 [7]. Since heparin has an average molecular weight 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 molecular weight and high negative charge, heparin has an extremely low oral bioavailability that has been estimated at <1% [9].

Successful oral transmucosal drug delivery is dependent upon nanoparticle durability throughout the gastrointestinal (GI) tract mucus layers. This protective membrane serves as 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 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].
overcome clearance from the mucus, nanoparticles are engineered with specific surface and 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.

There have been many studies aimed at enhancing heparins oral bioavailability. While low molecular weight heparins ($M_w \sim 6 \text{kDa}$) [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. The success in enhancing gene delivery by complexing DNA or RNA polyanions with polycations [20] has motivated similar approaches for the delivery of heparin [21]. Recently, nanoparticles have been explored for the enhanced oral delivery of biopolymers [22], including heparin [23, 24].

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 [25]. 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 [26]. Heparin is a polyanionic drug due to its many carboxyl and sulfo groups [26]. Therefore, it is an ideal candidate to be used in ionic complexed nanoparticles (ICNs) (Fig. 2) [27]. Ionic complexed nanoparticles consist 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 a variety of biomedical applications such as delivery and sensory purposes [28]. 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 [29]. A previous study was done using chitosan to encapsulate heparin for oral delivery purposes [29]. Our study builds on this by looking at the effect 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. Prepared through the de-N-acetylation of chitin, chitosan represents 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 [30]. Chitosan is approved by the Food and Drug administration (FDA) and is generally regarded as safe (GRAS) because it is biodegradable and non-toxic. Chitosan has been used in previous drug delivery applications due to its mucoadhesive character, as 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 [31, 32].

The goal of this research is to prepare ICNs 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, drug loading leading to oral bioavailability. These nanoparticles maintain stability of the very acidic conditions of the stomach and the neutral pH at the small intestines. Oral drug delivery relies on absorption in the small intestines where the epithelial layer is the most permeable [33]. 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 [34]. Moreover, an ideal drug delivery system should have a drug loading of at least 50% [27]. These parameters, if met, should result in anticoagulation with an oral heparin dose of 100 mg.

2.0 Materials & Methods

2.1 Materials

Low molecular weight chitosan (chitosan, Mw 50-190 kDa, 85% deacetylated) was obtained from Sigma Aldrich (St. Louis, MO, USA). Unfractionated heparin (Mw 15 kDa) was obtained from Celsus laboratories (Cincinnati, OH). Concentrated glacial acetic acid, phosphate buffered saline (PBS, pH 7.4), poly(vinyl alcohol) (PVOH, Mw), and Pluronic F-127 (Mw 12,600 g/mol) were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2 Preparation of nanoparticles

Samples were prepared by dissolving UFH in deionized (DI) water at a concentration of 1 mg/mL and, separately, dissolving chitosan in 1% glacial acetic acid. After both were completely dissolved, 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.

2.3 Size analysis and zeta potential

2.3.1 Mean nanoparticle size, polydispersity index and zeta potential were determined by DLS at 25 °C with a Malvern Zetasizer ZSP (Worcestershire, U.K.) 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 100x oil immersion objective lens) was used for visual observations of nanoparticles at varying pH for changes in NP size.

2.3.2. Scanning Electron Microscopy (SEM). For sample preparation, 2% v/v 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 a 10 mm at room temperature and a minimum vacuum of 4 x 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.

2.4 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 in varying pHs, centrifuged, and the supernatant with free heparin was measured using a micro-carbazole assay [35,36]. The concentration of the free heparin was calculated by Eq. 1 below.

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

Eq 1. Encapsulation efficiency

2.5 In vitro drug release

In vitro drug release studies were performed in a 20 mL volume while under incubation 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 [37]. At predetermined time points, 1 mL aliquots were taken and centrifuged. The free heparin that remained in solution was then measured using a micro-carbazole assay and the following equation was used to measure the percentage release [35,36].

2.6 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 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 correspond to the nanoparticle solution. The effects of stabilizers were determined by measurements of NP size, zeta potential and heparin release.

3.0 Results & Discussion
This investigation focused on the ionic complexation of chitosan and unfractionated heparin (UFH) as a vehicle for 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.

### 3.1 Preparation of nanoparticles

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

#### Table 1

| 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%)

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 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). 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, 38]. 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.

### 3.2 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 its way to the small intestines for uptake into the circulation. These NPs are pH sensitive due to the impact of pKa on protonation. The amine group in chitosan (Fig. 1) has a pKa of ~6.5. As such, it is soluble under acidic conditions where amine moieties are protonated, leading to an increased binding area [39]. 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 (Fig. 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 the course of 2–4 hours under varying pH values of the simulated fluids. The NP size remained relatively constant at about 200–400 nm (Figs. 3A and 3B). However, in simulated intestinal fluid, at pH 7.2, the particles swelled (Fig. 3C) and aggregated (Fig. 3D). These events resulted in increased PDI that exceeded 0.5, indicating a non-homogenous particle distribution.

The ZP was observed over the course of four hours under varying pH solutions. As seen in Fig. 4a, the ZP 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, indicating the breaking apart of the NP, with the anionic heparin being released to the surface. This indicates 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, 38].

Figure 4b shows the release profiles of heparin from the NPs over the course of 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, thereafter, little, or no further heparin release occurs to 4 h. 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 zeta potential to interact with the negatively charged layer [40].

### 3.3 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. Figure 1s shows the effect of the concentration of stabilizer was studied on NP size, ZP and release profiles. The concentration of 5% stabilizer showed a decrease in NP size while also keeping the ZP slightly positive. The release profiles were similar in the varying concentrations of stabilizer with the 5% being slightly more stable. Figure 4 shows the release profile with 5% stabilizer added to solution, over the course of 2 h was 60% lower than that of the ICNs with no stabilizer.

### 4. Conclusion
Ionic 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. The studies herein describe 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 the developed heparin oral delivery system in animal models.

Declarations

*Ethics approval and consent to participate:* Informed consent was obtained from all individual participants included in the study.

*Consent to Publish:* A statement confirming consent to publish has been received from all participants.

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

*Competing interests:* The authors have no relevant financial or non-financial interests to disclose.

*Funding:* This work was supported by GlycoMIP, a National Science Foundation Materials Innovation Platform funded through Cooperative Agreement DMR-1933525 (to F.Z. and R.J.L.). We also acknowledge funds received from NIH grant: DK111958 (R.J.L, G.S.).

*Authors' Contributions:* Credit Author statement: 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.

*Acknowledgements:* The authors are grateful to access and training received from the Materials Science and Engineering Core facility in carrying out scanning electron microscopy studies.
Authors information: The authors have provided supplemental information on the effect of concentration of stabilizers on the initial NP size as well as zeta potential (ZP) in gastric and intestinal simulated fluid over time.

References

1. Panesar K. Patient Compliance and Health Behavior Models. Patient Compliance Heal. Behav. Model. 2012. https://www.uspharmacist.com/article/patient-compliance-and-health-behavior-models. Accessed May 5, 2021.

2. Illinois U of. Heparin, Injectable Solution. 2018. https://www.healthline.com/health/drugs/heparin-injectable-solution#other-warnings. Accessed May 5, 2021.

3. United States Pharmacopeia. USP37 Official Monograph. 2013. https://www.uspnf.com/sites/default/files/usp_pdf/EN/USPNF/usp37-nf32_commentary.pdf. Accessed May 5, 2022.

4. Linhardt RJ. Heparin: Structure and Activity. J Med Chem. American Chemical Society; 2003;46:2551–64. Available from: https://doi.org/10.1021/jm030176m.

5. Shriver Z, Capila I, Venkataraman G, Sasisekharan R. Heparin and heparan sulfate: analyzing structure and microheterogeneity. Handb Exp Pharmacol. 2012;159–76. https://pubmed.ncbi.nlm.nih.gov/22566225.

6. Wang HM, Loganathan D, Linhardt RJ. Determination of the pKa of glucuronic acid and the carboxy groups of heparin by $^{13}$C-nuclear-magnetic-resonance spectroscopy. Biochem J. 1991;278(Pt 3):689–95. DOI:10.1042/bj2780689.

7. Paluck SJ, Nguyen TH, Maynard HD. Heparin-Mimicking Polymers: Synthesis and Biological Applications. Biomacromolecules. 2016;17:3417–40. https://doi.org/10.1021/acs.biomac.6b01147.

8. Capila I, Linhardt RJ, Angewandte C. Heparin-Protein I. 2002;41:390–412. https://doi.org/10.1002/1521-3773(20020201)41:3<390::AID-ANIE390>3.0.CO;2-B.

9. Mousa SA, Zhang F, Aljada A, Chaturvedi S, Takieddin M, Zhang H, et al. Pharmacokinetics and pharmacodynamics of oral heparin solid dosage form in healthy human subjects. J Clin Pharmacol. 2007;47(12):1508–20. doi:10.1177/0091270007307242.

10. Liu C, Jiang X, Gan Y, Yu M. Engineering nanoparticles to overcome the mucus barrier for drug delivery: Design, evaluation and state-of-the-art. Med Drug Discov. 2021;12:100110. https://www.sciencedirect.com/science/article/pii/S2590098621000312.

11. Lai SK, Wang Y-Y, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev. 2009;61:158–71. DOI:10.1016/j.addr.2008.11.002.

12. Leal J, Smyth HDC, Ghosh D. Physicochemical properties of mucus and their impact on transmucosal drug delivery. Int J Pharm. 2017;532:555–72. DOI:10.1016/j.ijpharm.2017.09.018.

13. Cone RA. Barrier properties of mucus. Adv Drug Deliv Rev Netherlands. 2009;61:75–85.
14. Pozzi D, Colapicchioni V, Caracciolo G, Piovesana S, Capriotti AL, Palchetti S, Capriotti AL, Palchetti S, Grossi SD, Riccioli A, Amenitsch H, Lagana A. Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells. Nanoscale. 2014;6:2782–92. DOI:10.1039/c3nr05559k.

15. Linhardt RJ, Loganathan D, al-Hakim A, Wang HM, Walenga JM, Hoppensteadt D, Fareed J. Oligosaccharide mapping of low molecular weight heparins: structure and activity differences. J Med Chem. 1990;33:1639–45. DOI:10.1021/jm00168a017.

16. Bhaskar U, Sterner E, Hickey AM, Onishi A, Zhang F, Dordick JS, Linhardt RJ. Engineering of routes to heparin and related polysaccharides. Appl Microbiol Biotechnol. 2012;93:1–16. DOI:10.1007/s00253-011-3641-4.

17. Onishi A, St Ange K, Dordick JS, Linhardt RJ. Heparin and anticoagulation. Front Biosci (Landmark Ed). 2016;21:1372–92. DOI:10.2741/4462.

18. Aamir R, Fyffe C, Korin N, Lawrence DA, Su EJ, Kanapathipillai M. Heparin and Arginine Based Plasmin Nanoformulation for Ischemic Stroke Therapy. Int J Mol Sci. 2021;22(21):11477. https://doi.org/10.3390/ijms222111477.

19. Cho SY, Sim J-S, Kang SS, Jeong C-S, Linhardt RJ, Kim YS. Enhancement of heparin and heparin disaccharide absorption by the Phytolacca americana saponins. Arch Pharm Res. 2003;26:1102–8. DOI:10.1007/BF02994765.

20. Martirosyan A, Olesen MJ, Howard KA. Chapter 11 - Chitosan-based nanoparticles for mucosal delivery of RNAi therapeutics. Adv Genet. 2014;88:325–52. https://doi.org/10.1016/B978-0-12-800148-6.00011-0.

21. Hoffart V, Ubrich N, Lamprecht A, Bachelier K, Vigneront C, Lecompte T, et al. Microencapsulation of Low Molecular Weight Heparin into Polymeric Particles Designed with Biodegradable and Nonbiodegradable Polycationic Polymers. Drug Deliv Taylor & Francis. 2003;10:1–7. https://doi.org/10.1080/713840325.

22. He Z, Hu Y, Nie T, Tang H, Zhu J, Chen K, Liu L, Leong KW, Chen Y, Mao H-Q. Size-controlled lipid nanoparticle production using turbulent mixing to enhance oral DNA delivery. Acta Biomater. 2018;81:195–207. DOI:10.1016/j.actbio.2018.09.047.

23. Patriota YBG, Arruda IES, de Jesus Oliveira AC, de Oliveira TC, de Lemos Vasconcelos Silva E, Chaves LL, Ribeiro FDOS, Da Silva DA, Soares MFDR, Soares-Sobrinho JL. Synthesis of Eudragit® L100-coated chitosan-based nanoparticles for oral enoxaparin delivery. Int J Biol Macromol. 2021;193:450–6. https://doi.org/10.1016/j.ijbiomac.2021.10.111.

24. Maatouk B, Jaffa MA, Karam M, Fahs D, Nour-Eldine W, Hasan A, Jaffa AA, Mhanna R. Sulfated alginate/polycaprolactone double-emulsion nanoparticles for enhanced delivery of heparin-binding growth factors in wound healing applications. Colloids Surf B Biointerfaces. 2021;208:112105. DOI:10.1016/j.colsurfb.2021.112105.

25. Heparin Administration - Drugs. - Merck Manuals Consumer Version. [cited 2021 May 10]. Available from: https://www.merckmanuals.com/home/drugs/administration-and-kinetics-of-drugs/drug-
administration.

26. Heparin -D. 2022. https://diapharma.com/heparin/. Accessed May 5, 2022.

27. Rizvi SAA, Saleh AM. Applications of nanoparticle systems in drug delivery technology. Saudi Pharm J SPJ Off Publ Saudi Pharm Soc. 2017/10/25. Elsevier; 2018;26:64–70. https://pubmed.ncbi.nlm.nih.gov/29379334.

28. Yildiz-Pekoz A, Ozsoy Y. Inhaled Heparin: Therapeutic Efficacy and Recent Formulations. J Aerosol Med Pulm Drug Deliv. 2017;30:143–56. https://doi.org/10.1089/jamp.2015.1273.

29. Chen MC, Wong HS, Lin KJ, Chen HL, Wey SP, Sonaje K, Lin Y-H, Chu C-Y, Sung H-W. The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin. Biomaterials Elsevier Ltd. 2009;30:6629–37. http://dx.doi.org/10.1016/j.biomaterials.2009.08.030.

30. Cheung RCF, Ng TB, Wong JH, Chan WY. Chitosan. An Update on Potential Biomedical and Pharmaceutical Applications. Mar Drugs MDPI. 2015;13:5156–86. https://pubmed.ncbi.nlm.nih.gov/26287217.

31. Lin Y-H, Sonaje K, Lin KM, Juang J-H, Mi F-L, Yang H-W, Sun H-W. Multi-ion-crosslinked nanoparticles with pH-responsive characteristics for oral delivery of protein drugs. J Control Release Netherlands. 2008;132:141–9. DOI:10.1016/j.jconrel.2008.08.020.

32. Sonaje K, Lin Y-H, Juang J-H, Wey S-P, Chen C-T, Sung H-W. In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials. 2009;30:2329–39. DOI:10.1016/j.biomaterials.2008.12.066.

33. Surat P In. News Medical Life Sciences pH in the Human Body. 2018. https://www.news-medical.net/health/pH-in-the-Human-Body.aspx.

34. Yang T, Nyiawung D, Silber A, Hao J, Lai L, Bai S. Comparative studies on chitosan and polylactic-co-glycolic acid incorporated nanoparticles of low molecular weight heparin. AAPS PharmSciTech. 2012;13:1309–18. DOI:10.1208/s12249-012-9854-8.

35. Cesaretti M, Luppi E, Maccari F, Volpi N. A 96-well assay for uronic acid carbazole reaction. Carbohydr Polym. 2003;54:59–61. https://doi.org/10.1016/S0144-8617(03)00144-9.

36. Bhaskar U, Hickey AM, Li G, Mundra RV, Zhang F, Fu L, Cai C, Ou Z, Dordick JS, Linhardt RJ. A purification process for heparin and precursor polysaccharides using the pH responsive behavior of chitosan. Biotechnol Prog. 2015;31:1348–59. https://doi.org/10.1002/btpr.2144.

37. Marques MRC, Loebenberg R, Almukainzi M. Simulated Biological Fluids with Possible Application in Dissolution Testing. Dissolution Technologies. 2011. http://dissolutiontech.com/DTresour/201108Articles/DT201108_A02.pdf.

38. Boegh M, Nielsen HM. Mucus as a barrier to drug delivery – understanding and mimicking the barrier properties. Basic Clin Pharmacol Toxicol. 2015;116:179–86. https://doi.org/10.1111/bcpt.12342.

39. Mohammed MA, Syeda JTM, Wasan KM, Wasan EK. An overview of chitosan nanoparticles and its application in non-parenteral drug delivery. Pharmaceutics. 2017;9(4):53. DOI:10.3390/pharmaceutics9040053.
Figures

**Heparin:** \( R = \text{SO}_3^- \text{ or H, } X = \text{COCH}_3 \text{ or SO}_3^- \)

**Chitosan:** \( \beta-D-\text{GlcNH}_3^+ (1\rightarrow4) \beta-D-\text{GlcNH}_3^+ (1\rightarrow4) \alpha,\beta-D-\text{GlcNH}_3^+ \)

*Figure 1*

*Structure of heparin on the top and the structure of chitosan on the bottom.*
Figure 2

ICNs as they pass through the gastrointestinal tract.
Figure 3

3a and 3b. The SEM images of the 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.
Figure 4

Plot 4a (top) displays ZP values as a function of incubation time in both simulated gastric and simulated intestinal fluids. Plot 4b (bottom) displays 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.
Figure 5

*Heparin release in simulated intestinal fluid at pH 7.2 using 5 wt% Pluronic and PVOH stabilizers.*

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- GA.jpg
- SIGayathriHeparinreleasepaper.pdf