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Polyhexamethylene Biguanide Release by Chitosan-Heparin Nanoparticles

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Honors Research Project

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Contents

Abstract

Chitosan and heparin were used to synthesize nanoparticles using polyelectrolyte complexation. The optimal mixing ratio was determined to be 1:4 by studying the particle characteristics of zeta potential and dynamic light scattering of the ratios 1:2, 1:3, 1:4, 1:5 and 1:6 (chitosan: heparin). The particles were loaded with the antibiotic polyhexamethylene biguanide (PHMB) and the release was measured over 335 hours using the dialysis membrane incubation method[1] and detected via ultraviolet-visible (UV-Vis) spectroscopy. Around 80% of the encapsulated PHMB was released over the measured period. The goal of this part of the study was to determine the drug release behavior of chitosan-heparin nanoparticles and their viability as release agents for future wound healing applications. This study concluded that the performance of the particles exhibits sustained release over prolonged periods to be further investigated as transport agents. Antibacterial studies are needed to evaluate the effectiveness of the particles *in vitro* against gram-positive and gram-negative bacterial species.

Executive Summary:

Introduction:

Bacterial infection can occur during the process of wound healing which presents a significant obstacle for treating and covering open wounds.[2] Transdermal delivery of antibiotic agents to the wound site allows for the continued healing via combating infection[3]. The application of nanotechnology in drug delivery has increasingly gained prevalence in the past decade.[7] This study focuses first on the design of heparin-chitosan based nanoparticles with an optimum morphology and polydispersity and then on the delivery of polyhexamethylene biguanide (PHMB) by means of heparin-chitosan nanoparticles (hep-cs NPs). The formation of particles occurs via polyelectrolyte complexation of chitosan and heparin, where the positively charged PHMB is encapsulated within the negatively charged particle.

Results:

The optimal ratio of chitosan to heparin was determined to be 1:4 (chitosan: heparin volume ratio) among all the ratios tested (1:2, 1:3, 1:4, 1:5 and 1:6 (chitosan: heparin)). Hep-cs NPs synthesized, using the previously stated volume ratio, exhibit structural stability and uniformity with an average charge of -32.0±1.0 mV and average size of 198.2±34.0 nm in their plain state. After loading the particle with PHMB, the average charge was measured to be +56.9±1.1 mV with the average size of 531.8±18.6 nm. The encapsulate PHMB within the hep-cs NPs was found to have a controlled and sustained release over the period of 14 days, allowing most of the drug (79.8%) to be released.

Conclusions:

Positively charged chitosan was added to negatively charged heparin in a ratio of 1:4 (chitosan: heparin) to produce a negatively charged nanoparticle that encapsulated the positively

charged antibiotic PHMB. The polysaccharide-based, hep-cs NPs are stable, well dispersed particles capable of encapsulating and releasing the antibiotic PHMB. The successful release activity exhibited by the particles indicates possible viability for use in wound dressings to prevent bacterial infection and promote regenerative behavior of dermal tissue. Using hep-cs NPs to deliver PHMB in wound healing can be a more cost-effective method than the more expensive, silver-based technologies.

Skills and Experience Gained:

I have gained skills in laboratory techniques, experimental design, data analysis, and technical report writing while working on this project. I have become familiar with zeta potential testing, dynamic light scattering measurements, ultraviolet-visible spectroscopy, drug release study techniques, particle synthesis, and literature review. Working directly with a graduate student on this project has helped me gain insight on my own career path and affirmed my choice of continuing my education and pursuing research. I have increased my confidence in lab work and independence as I was given the opportunities to grow in these areas throughout the project.

Recommendations:

Further research is needed to assess whether this solution is safe and effective both *in-vitro* and *in-vivo*. Cytotoxicity assays should be conducted to assess the safety of the loaded NPs against human dermal fibroblasts. Bacterial studies are needed to determine the concentrations at which PHMB kills bacterial cells. These data will determine whether the concentrations of PHMB released by hep-cs NPs, as shown in this study, will prevent bacterial infection while maintaining human dermal cell activity.

Acknowledgements:

I would like to thank Dr. Leipzig for allowing me to use his laboratory and laboratory resources to complete this research and project. Special thanks to Shahrzad Fathollahipour for her guidance, time, effort, and willingness to assist me in this project.

Introduction:

In the process of wound healing, bacterial infection presents a significant issue, delaying and in some cases preventing the wound from healing.[2] Preventing bacterial infection requires antibiotic treatment. Polyhexamethylene biguanide (PHMB) is an antibiotic that has been shown to perform effectively with regenerative benefit.[4] Dermal tissue toxicity and biocide resistance are both risks associated with high concentrations of antiseptics which impair the wound healing process.

Nanostructure use, notably in colloidal nanoparticles, has increased noticeably in the field of drug delivery within the last decade. Nanoparticles easily interact with biological molecules due to their sub-microscopic size.[5] Chitosan has become prevalent in particulate drug delivery systems due to its positive charge and ability to bind to biological materials. Chitosan has also proved to be a safe and efficient for use in the pharmaceutical industry.[67]

We have shown the ability of heparin-chitosan nanoparticles (hep-cs NPs) loaded with PHMB to act as a reliable antiseptic delivery agent to release PHMB over a prolonged amount of time in a controlled manner. The positively charged chitosan molecules bind to the negatively charged heparin and forms a nanoparticle structure with tailorable surface charge. Due to a 1:4 mixture of chitosan: heparin, the resulting particle charge was negative allowing the positively charged PHMB to be encapsulated by the particle. Wound dressings can be created with a greater cost effectiveness without sacrificing efficacy, using PHMB in place of costlier silver-based particles. In this study, the composition ratio of hep-cs NPs is tailored to achieve best surface charge, size and polydispersity, and the release of PHMB is measured and evaluated.

Background:

Using nanoparticles in drug release applications appears to be a viable area of research imperative to multiples fields of study including biomedical and pharmaceutical industries.[6] For instance, nanoparticles synthesized by chitosan and hyaluronic acid have shown to be effective carriers and release agents of heparin for the treatment of asthma.[8] Using this mechanism of polyelectrolyte complexation, we desire to investigate a nanoparticle (PCN) that can maintain a controlled release of antiseptic of wound healing applications.

Formation of polysaccharide-based PCNs has been shown to be affected by the charge mixing ratios, charge densities, and the way the two polyelectrolytes are added together. Formation of negatively charged hep-cs PCN can be from charge mixing ratios below 0.50.[9] Negatively charged, the PCN will be able to encapsulate the positively charged PHMB molecules. Drug delivery by nanoparticles with chitosan has been shown to be an effective transporter of micro- and nanoparticles due to its mucoadhesive properties and ability to entrap molecules.[4] Nanoparticles based on heparin have been shown to be biodegradable, anticoagulant, and effective for antitumor therapy[10].

Experimental Methods:

Preparing the acetate buffer solution:

0.1 M acetic acid was made by adding 2.86 ml of glacial acetic acid supplied by EMD Millipore (EMD Millipore, Cleveland, Ohio, United States of America) to 497.14 ml of nanowater. 0.1 M sodium acetate solution was made by dissolving 6.8 g sodium acetate trihydrate (ARMRESCO, Solon, Ohio, United States of America) in 500 ml nanowater. pH 5 acetate buffer solution was made by mixing the acetic acid solution and the sodium acetate solution by a volume

ratio of 59:141 acetic acid solution: sodium acetate solution. The pH was verified to be 5 using Oakton Ion 510 series pH probe (Cole-Palmer, Vernon Hills, Illinois, United States of America).

PHMB Calibration Curve:

Ultraviolet-visible spectroscopy (UV-Vis) calibration of PHMB (BOC Sciences, Shirley, New York, United States of America) was created using concentrations of 600, 300, 200, 150, 100, 50, 10, and 5 µg/ml in filtered nanowater. 600 µg/ml solution was made by dissolving 0.012 g of PHMB in 20 ml of filtered nanowater. The other concentrations of the calibration trend were created using serial dilutions of 600 µg/ml.

Synthesis of hep-cs NPs:

0.9 mg/ml solution of chitosan was made by dissolving 0.09 g of chitosan (90KDa, 20% DDA, supplied by Mycodev) in 100 ml of pH 5 acetate buffer. 0.95 mg/ml heparin solution was made by dissolving 0.285 g heparin sodium salt from porcine intestinal mucosa (Sigma Aldrich, St. Louis, Missouri, united States of America) in 300 ml of acetate buffer. Solutions of 25 ml total volume were made with volume ratios of 1:2, 1:3, 1:4, 1:5, and 1:6 (chitosan: heparin) for determination of optimal mixing ratio. The solutions were washed by centrifugation with a speed of 5,000 × G for 20 minutes and the supernatant was replaced with equal volume of filtered nanowater. Particle solutions were frozen at -80ºC and freeze-dried for storage.

Particle Loading:

A solution of 1 mg/ml PHMB was made by dissolving 0.50 g of PHMB in 50 ml filtered nanowater. 14 ml of solution was added dropwise to each nanoparticle suspension after washing while maintaining 800 rpm stirring speed. The solutions remained under 800 rpm stirring for 24 hours. The solutions were decanted into 50 ml centrifuge tubes and centrifuged for 20 minutes at 5,000 × G. 30 ml of supernatant was stored in the freezer and replaced with filtered nanowater. The solutions were centrifuged again for 20 minutes at a speed of 5,000 × G. The supernatant was removed and stored in the freezer and freeze dried for storage. The particles were re-suspended in 20 ml of filtered nanowater.

PHMB Release:

5 ml of PHMB loaded hep-cs NP solution was placed into 12 - 14 kDa MWCO-cut off dialysis membrane (Spectrum Labs, Rancho Dominguez, California, USA) and submerged into 100 ml of phosphate-buffered-saline (PBS) in a light protected vessel. This was repeated for a total of 3 replicates. The samples were kept in a thermostatically controlled water bath set at a temperature of 37ºC. 300 µl samples of solution were removed and replaced with fresh PBS at time intervals between 0.5 hours and 335 hours as indicated in **Figure 4**.

PHMB Concentration Measurement for Release and Calibration:

Concentration measurements were made were made by measuring absorbance with ultraviolet-visible spectroscopy using Tecan Infinite® M200 and 16-well Tecan NanoQuant

Plate™. A 2 µl drop of PHMB solution were placed on the NanoQuant plate and placed into the UV-vis spectrometer. The Absorbance was measured and concentration was calculated using **Equation 1**.

Size and Zeta Potential Measurement Before and After PHMB Loading:

Size and zeta potential measurements were made using dynamic light scattering and zeta potential readings by Zetasizer Nano Z analyzer and Malvern Zetasizer Nano Series Disposable Folded Capillary Cells (Malvern Analytical, Almelo, Netherlands).

Data and Results:

Chitosan-heparin nanoparticles were successfully synthesized by polyelectrolyte complexation. Size and PDI results indicate that the particles were stable and uniformly distributed. Particles were synthesized using volume ratios of 1:2, 1:3, 1:4, 1:5, and 1:6

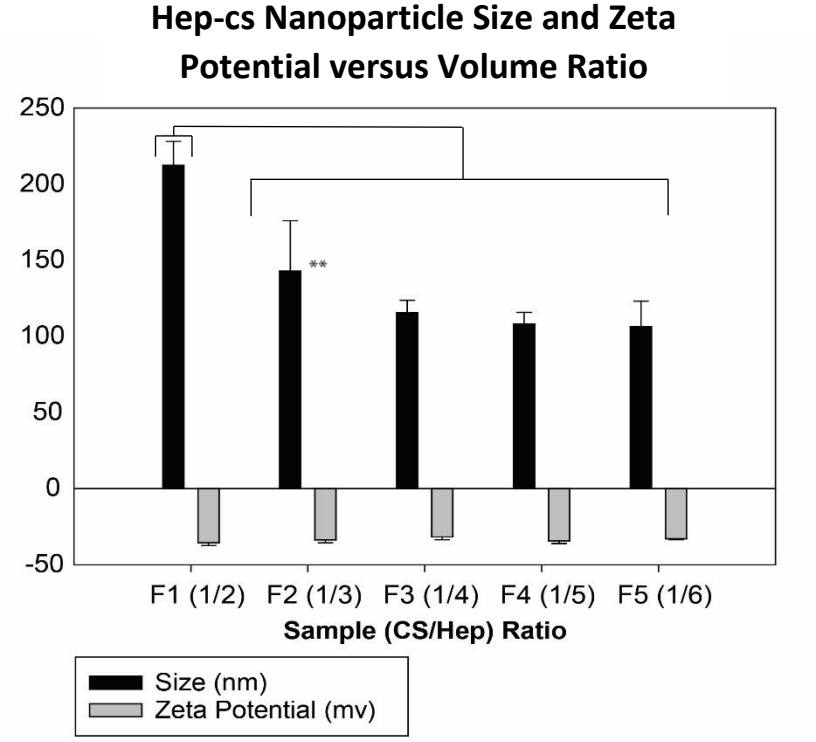

*Figure 1. Shows the charge and size measurements of plain particles synthesized using the ratios of 1:2, 1:3, 1:4, 1:5, and 1:6 (chitosan: heparin). (Data all n=3, mean±S.D. ** indicates One-Way ANOVA, Tukey's Post Hoc, P<0.0002)*

(chitosan/heparin). The ratio of 1/4 was selected as the optimal ratio due to particle size of 136.5 nm and a charge of -42.4 mV.

Table 1. Polydispersity index of each nanoparticle formulation

Group	Chitosan Conc. (mg/ml)	Heparin Conc. (mg/ml)	Chitosan to Heparin ratio	PDI
1	0.9	0.95	1/2	0.209±0.02
2	0.9	0.95	1/3	0.193±0.017
3	0.9	0.95	1/4	0.201±0.014
4	0.9	0.95	1/5	0.200±0.027
5	0.9	0.95	1/6	0.199±0.031

Using 1/4 chitosan/heparin ratio, particles were synthesized and loaded with PHMB. Size and charge measurements were made before and after loading. The particle size before loading. The particle zeta potential measured was -32.0±1.0 mV. The zeta potential of the PHMB loaded

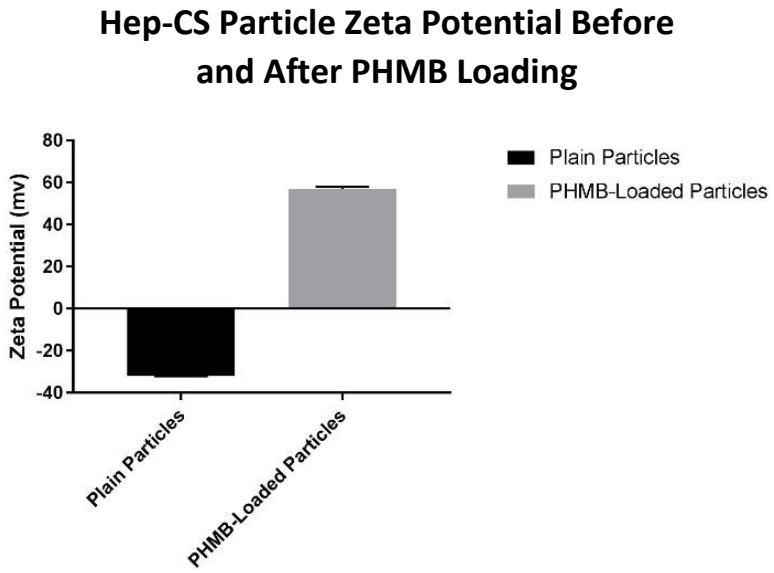

Figure 2. The zeta potential of chitosan-heparin nanoparticles before (plain) and after PHMB loading. (Data all n=3, mean±S.D.)

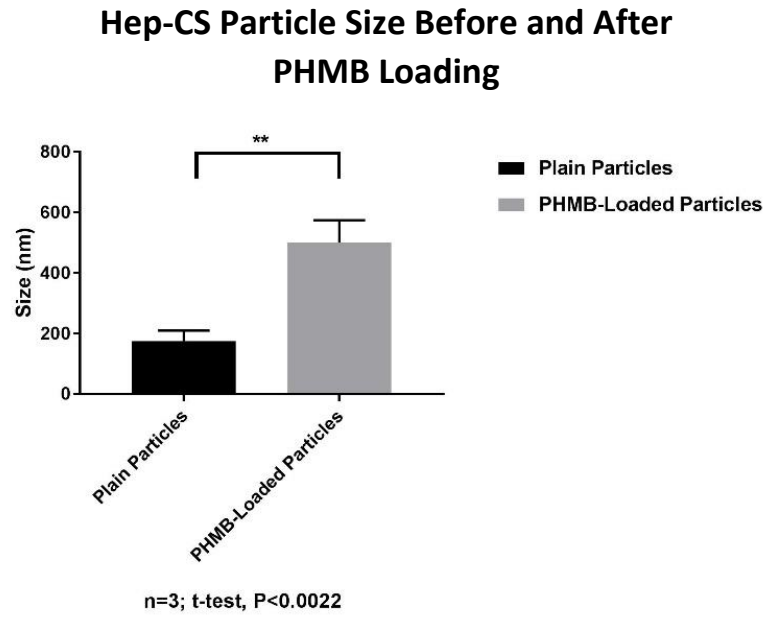

*Figure 3. Size measurements of particles before (plain) and after PHMB loading. Error bars represent standard deviation. (Data all n=3, ** P<0.0022, mean±S.D.)*

nanoparticle was +56.9±1.1 mV. The particle size was found to be 198.2±34.0 nm before PHMB loading and 531.8±18.6 nm after loading.

Calibration curve of PHMB using UV-Vis spectroscopy resulted in a linear curve with the following equation:

$$Absorbance = 0.00352 \times Concentration - 0.0443 \qquad \textbf{\textit{Equation 1}}$$

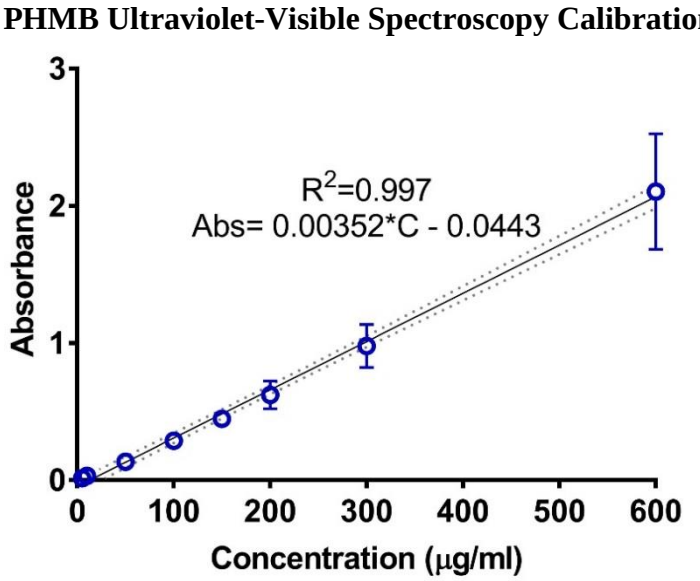

Figure 4. The calibration curve for Absorption of PHMB. Error bars represent standard deviation (n=3; mean±S.D.).

The calibration curve in **Figure 1** was used to calculate concentrations of PHMB in release samples and cumulative release percentage shown in **Figure 4** using **Equation 1**. The calibration trend shown in the figure was created using a linear regression of absorption data with an R-squared value of 0.997.

The release of PHMB from hep-cs nanoparticles is shown in **Figure 5**. Controlled release was observed over the measured period. After a total of 335 hours, 79.89% of PHMB loaded into the hep-cs NPs was released. Cumulative release was determined using the initial amount of PHMB of 140 mg. **Appendix 1** shows the cumulative release percentage calculated at each time point. Cumulative release percentage at time *t* (hours) was determined by the equation:

$$\left(\sum_0^t \frac{Concentration \times PBS\ volume \times Sample\ Volume}{total\ mg\ PHMB\ loaded\ into\ particles}\right) \times 100 = Cumulative\ Release\ \% \qquad \textbf{\textit{Equation 2}}$$

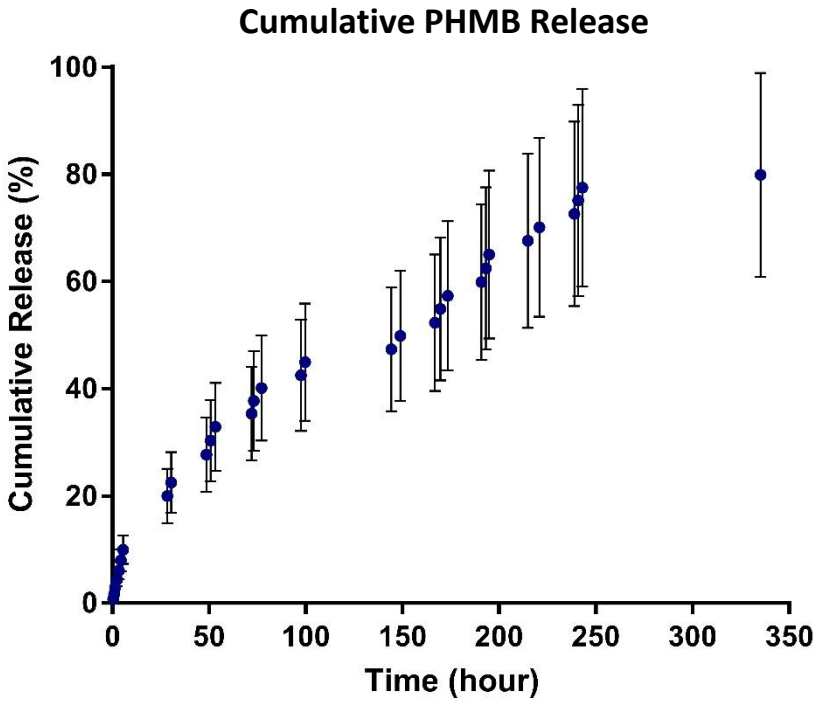

Figure 5. The Cumulative release percentage of PHMB measured over time. Error bars represent standard deviation (Data all n=3, mean±S.D).

Discussion/Analysis:

Polyelectrolyte complexation of chitosan and heparin form stable nanoparticle structures and provide an effective release delivery system for PHMB. Mixing using a ratio of 1:4 (Chitosan: Heparin) results in a stable particle sufficient in encapsulating PHMB, confirming methods used by Boddohi et al [3]. Using four times more heparin that chitosan creates a negatively charged particle due to the excess of negative charges from heparin, as seen by **Figure 3**. The loading of PHMB into the particle increases the zeta potential drastically due to the encapsulating of the positively charged molecule. Packing the particle with the large polymer also increases the particle size significantly, which is shown in **Figure 4**. The release study shown in **Figure 5** suggests that using hep-CS NPs to deliver PHMB will effectively provide a less-expensive, yet effective method of preventing bacterial infection in wound healing applications over prolonged periods.

Errors present in this study are shown in the graphs and figures as standard deviation values. Additional errors present include human measurement errors due to pipetting, dry weight transfer loss, and instrument error. All measurements taken were made in triplicate with no prominent outliers.

Conclusion:

Heparin-chitosan nanoparticles formed by polyelectrolyte complexation exhibit stability in charge, size and drug release over prolonged periods. Further study is required before implementation of this technology. Cytotoxicity of PHMB released from nanoparticles should be studied to show that the amount of antibiotic released is able to kill bacteria without damaging the dermal tissue of the wound area. Additional research is recommended to evaluate the effectiveness of hep-CS NPs for delivery of additional molecules such as growth factors.

Appendix 1: Cumulative PHMB Release from Hep-cs Nanoparticles

Hours	AVG Cumulative Release % of PHMB	Standard Deviation
0.35	0.72	0.20
0.85	1.70	0.43
1.35	2.92	0.88
2.35	4.37	1.24
3.43	6.07	1.65
4.35	7.96	2.14
5.60	9.93	2.63
23.05	12.40	3.23
25.10	14.90	3.83
26.95	17.41	4.46
28.35	19.97	5.08
30.35	22.52	5.72
46.93	25.12	6.34
48.68	27.71	6.95
50.77	30.30	7.58
53.35	32.91	8.18
72.02	35.31	8.74
73.18	37.70	9.31
77.18	40.10	9.81
97.60	42.52	10.39
99.68	44.93	10.96
144.27	47.35	11.54
148.85	49.85	12.14
166.85	52.33	12.73
169.60	54.84	13.34
173.35	57.34	13.94
190.85	59.89	14.51
193.10	62.46	15.10
194.85	65.04	15.67
214.85	67.59	16.22
220.85	70.12	16.71
238.85	72.64	17.26
240.85	75.10	17.81
243.08	77.51	18.39
335.10	79.89	19.00

Appendix 2: Nomenclature

Hep-cs: heparin - chitosan

NPs: nanoparticles

PCN: polyelectrolyte complex nanoparticle

PHMB: polyhexamethylene biguanide

UV-vis: Ultraviolet-visible spectroscopy

Literature Cited:

[1] Singh, Rajesh, and James W. Lillard. "Nanoparticle-Based Targeted Drug Delivery."*Experimental and Molecular Pathology*, vol. 86, no. 3, 2009, pp. 215–223., doi:10.1016/j.yexmp.2008.12.004.

[2] Rojas, Isolde-Gina, et al. "Stress-Induced Susceptibility to Bacterial Infection During Cutaneous Wound Healing." *Brain, Behavior, and Immunity*, vol. 16, no. 1, 2002, pp. 74–84., doi:10.1006/brbi.2000.0619.

[3] Schreier, Hans, and Joke Bouwstra. "Liposomes and Niosomes as Topical Drug Carriers: Dermal and Transdermal Drug Delivery." *Journal of Controlled Release*, vol. 30, no. 1, 1994, pp. 1–15., doi:10.1016/0168-3659(94)90039-6.

[4] Kamaruzzaman, Nor F., et al. "Bactericidal and Anti-Biofilm Effects of Polyhexamethylene Biguanide in Models of Intracellular and Biofilm of Staphylococcus Aureus Isolated from Bovine Mastitis." *Frontiers in Microbiology*, vol. 8, 2017, doi:10.3389/fmicb.2017.01518.

[5] Tissue, Cell and Organ Engineering. Nanotechnologies for the Life Sciences, ed. C. Kumar. Vol. 9. 2006, Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

[6] Agnihotri, Sunil A., et al. "Recent Advances on Chitosan-Based Micro- and Nanoparticles in Drug Delivery." Journal of Controlled Release, vol. 100, no. 1, 2004, pp. 5–28., doi:10.1016/j.jconrel.2004.08.010

[7] P.C. Berscht, B. Nies, A. Liebendorfer, J. Kreuter, Incorporation of basic fibroblast growth factor into methylpyrrolidinone chitosan fleeces and determination of the in vitro release characteristics, Biomaterials 15 (1994) 593 – 600.

[8] Oyarzun-Ampuero, F.a., et al. "Chitosan Hyaluronic Acid Nanoparticles Loaded with Heparin for the Treatment of Asthma." International Journal of Pharmaceutics, vol. 381, no. 2, 2009, pp. 122–129., doi:10.1016/j.ijpharm.2009.04.009.

[9] Boddohi, Soheil, et al. "Polysaccharide-Based Polyelectrolyte Complex Nanoparticles from Chitosan, Heparin, and Hyaluronan." Biomacromolecules, vol. 10, no. 6, 2009, pp. 1402–1409., doi:10.1021/bm801513e.

[10] Kemp, Melissa M., and Robert J. Linhardt. "Heparin-Based Nanoparticles." *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, vol. 2, no. 1, 2009, pp. 77–87., doi:10.1002/wnan.68.