OPTIMIZING LYOPHILIZATION TECHNIQUES FOR ENHANCED STABILITY AND FUNCTIONALITY OF HYPER-COMPLIANT MICROPARTICLES

By

STEPHANIE CALDERON

B.A. Biology, Amherst College, 2021

Thesis

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

PROVIDENCE, RHODE ISLAND

MAY 2025

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

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

Date May 1, 2025

Signed by: Stephanie Calderon AB92FBA144C8459...

Stephanie Calderon, Author

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

Date May 1, 2025

Signed by: Stephanie Calderon AB92FBA144C8459...

Stephanie Calderon, Author

This thesis by Stephanie Calderon is accepted in its present form by the Graduate Program in Biotechnology as satisfying the thesis requirements for the degree of Master of Science

Date 04/28/2025 | 11:06 AM EDT Signature: DocuSigned by: Eric Darling 46DB889E981A404...

Dr. Eric Darling, Advisor

Date 04/28/2025 | 10:07 AM EDT Signature: DocuSigned by: EDITH MATHIOWITZ E063C09A6AB6428...

Dr. Edith Mathiowitz, Reader

Date 04/28/2025 | 9:51 AM EDT Signature: Signed by: 7209E7BD8D21466...

Dr. Eric Huang, Reader

Approved by the Graduate Council

Date _____ Signature: _____

Dr. Thomas Lewis, Dean of the Graduate School

Acknowledgements

I'm extremely grateful to my advisor, Professor Eric Darling, for his constant guidance and support throughout this project. Our bi-weekly meetings were invaluable to my professional growth and provided essential direction for advancing the work presented in this paper.

I would also like to express my thanks to Dr. Edith Mathiowitz and Dr. Eric Huang for their invaluable experience. Their insights were instrumental in shaping certain experiments, especially during times when I felt uncertain about the next steps.

A huge thank you to everyone in the Darling Lab, especially Vera Fonseca, for patiently answering my many questions about lab protocols and equipment. Your training and support were critical, could not have taken my pictures without you!

Additionally, I would like Jacquelyn Schell for her constant administrative and career support. You made my time at Brown feel comfortable and supported, which I am really grateful for. Thank you for being my very first professor here!

Table of Contents

Abstract..... 7

Introduction..... 8

Methods..... 12

Hyper-compliant microparticle formation.....12

Double-encapsulated hyper-compliant microparticle formulation 13

Lyophilization Process.....13

In-vitro dissolution studies.....15

Results..... 17

Effects of Lyophilization.....17

Dissolution Study.....23

Discussion and Conclusions 25

References 34

Table of Figures

Figure 1..... *18*

Figure 2..... *19*

Figure 3..... *20*

Figure 4..... *21*

Figure 5..... *22*

Figure 6..... *23*

Abstract of Optimizing Lyophilization Techniques for Enhanced Stability and Functionality of Hyper-Compliant Microparticles, by Stephanie Calderon, ScM, Brown University, May 2025.

Microparticles have been researched for their use as a drug delivery system due to their non-toxicity and flexibility. Recently, advancements have been made to formulate microparticles with the elasticity and size of mammalian cells, allowing for a wider breadth of use. The goal of this study was to evaluate different lyophilization techniques and how they would affect the size and remaining count of hyper-compliant microparticles. We also observed how lyophilization would affect the drug release profile of a double encapsulated hyper-compliant microparticle with curcumin inside. All formulated microparticles were visualized using microscopy and measured using ImageJ. Aliquots from H2O sinks were taken at predetermined time points and analyzed over 96-192 hours for drug concentration. Regardless of freezing method, it was found that hyper-compliant microparticles post-lyophilization were always smaller than the control, and a significant loss of microparticles was also associated with lyophilization. With the addition of a surfactant, and sugar, lyophilization did not have a significant effect on size, but less microparticles were still counted. Lyophilized double encapsulated microparticles were found to have a slightly faster dissolution than non-lyophilized double encapsulated microparticles. However, even by the end of the 192 hours, not all the drug had been release. It was also found that lyophilization changes the drug release profile of the microparticle from zero order transport to Fickian diffusion. These findings show that lyophilization of hyper compliant microparticles can be done in a way that preserves the size of the microparticles without changing their mechanical properties significantly. Furthermore, it shows that double encapsulated microparticles may be a viable system as a microcarrier.

Introduction

Drug Delivery Systems

As the field of medicine advances, the complexity of therapeutic agents has grown significantly. Modern treatments increasingly rely on biologics, such as proteins and peptides, which offer targeted and highly specific mechanisms of action. The administration of medication, however, faces a self-made roadblock with the proteins/peptides that the drug is made of. The delivery of certain proteins may be impeded by their hydrophilic or hydrophobic nature, protein structure, or how they interact with the body.[1] Many therapeutic peptides also have very short in-vivo half-lives (t½ 2 - 30 minutes) because of enzymatic degradation and fast renal clearance.[2] This results in poor bioavailability and in order to obtain the necessary therapeutic effect, higher doses or more frequent administration is given which increases the risk of adverse effects.[1] As such researchers developed a variety of drug delivery systems (DDSs) to address this issue of drugs being delivered to the wrong site or at the incorrect dosage. Drug delivery systems are formulations or devices that allow the introduction of a drug into the body and streamline the delivery to the target site.[3] Drug delivery systems can be advantageous for the treatment of patients via a variety of ways including: controlled release of drug or peptides, precise targeting of the damaged/diseased tissue, enhancing protein solubility, sustained release instead of burst release to avoid side effects, and improve distribution of protein/peptides in

the body.[3] Drug delivery systems are also capable of decreasing the rate of drug clearance from the body, such that lower concentrations of the drug can be given, decreasing the chances of an overdose.[1] Lower concentrations would also be useful for certain drugs that are difficult to synthesize, purify or become toxic at higher doses. Treatment duration would also be lengthened, decreasing treatment frequency which would increase patient compliance.[4] In addition, the DDSs must be able to bind the therapeutic protein/peptide in a fashion that does not affect its bioactivity but hold it sufficiently until the DDS reaches its site of action and releases the protein/peptide in a controlled, sustained manner. In this instance, DDSs formulated with bio-compatible and biodegradable materials would certainly be advantageous to minimize any adverse host response to the DDSs.[1] Using drug delivery systems has been shown to drastically improve patient outcomes via reducing toxicity, being able to bypass certain bodily barriers such as the blood brain barrier, enhancing therapy efficacy, and increasing patient compliance.[5] There are a variety of novel DDSs that have been developed over the years, with one of the most well-known being microparticles.

Microparticles

Microparticles are micrometer-sized particles that are made from synthetic or natural biomaterials.[6] Microparticles come in a variety of structures, depending on their use and intended drug release profile. They can be a single, double or even multi-shelled hollow microparticle that surrounds the drug-filled core, or a matrix system, where the drug is dispersed throughout the polymer.[7] Their small size enables administration via inhalers or

needles, which is advantageous for the non-intrusive delivery of drugs.[8] Microparticles also fit various drug release methods, such as osmosis, or dissolution depending on the drug's and the polymer's properties.[7] Micro encapsulation as a drug delivery system provides solutions to the issues that arise in hospital settings that lead to adverse events. They protect the encapsulated drug against degradation, are made from safe and biodegradable materials for a low risk of toxicity and can accurately control the release rate of the incorporated drug.[9] Additionally, they have flexible dosage and drug combinations and as such, may be used with a variety of drug regimens.

However, while microparticles have great therapeutic potential, they are far from perfect. Microparticles are especially limited due to their rapid circulation times in the human body, which can often lead to unsuccessful delivery of the therapeutic agent to the correct site.[1] Furthermore, due to the stiffness of their outer shells, which is higher than that of mammalian cells, microparticles have a real risk of blocking blood flow.[10] Unlike microparticles or microbeads, mammalian cells have elastic properties that allow them to readily pass through small capillaries, the same of which can be applied to microparticles to overcome the risk of blockage.[11] In collaboration, the Darling and Mathiowitz Laboratories at Brown University formulated the first microparticles with similar properties to that of mammalian cells.[12] These particles were deemed hyper-compliant microparticles (HCMPs) and can mimic the elasticity (0.1–5 kPa), size and shape of the softer mammalian cells.[12] HCMPs are made from polyacrylamide through inverse emulsion, and their elastic properties can be tuned from 0.25 - 10 kPa.[12]

Lyophilization

The industry standard for storage is to lyophilize micro and nanoparticles, but this is the case for microbeads that have a stiffer outer shell compared to HCMPs. Additionally, lyophilization has been found to cause stress to nanoparticles, decreasing the stability of these particles.[13] Freeze-drying is also known to cause several stresses leading to physical instability, such as aggregation, fusion, or leakage.[14] Although it has been found that addition of sugars such as glucose, aids in the prevention of stress that leads to physical instability that is commonly seen.

This study was inspired by previous work which investigated the drug dissolution properties and rates of hyper-compliant microparticles loaded with dexamethasone. Sowers found that HCMPs loaded with dexamethasone released all of the drug within 24 hours due to their high swelling ratio that causes their pores to quickly fill with water and release the drug.[15] Sowers proposed for future studies to entrap drug into nanoparticles and then encapsulate those nanoparticles into HCMPs for long-term release. In this manner, the HCMPs would act as a carrier, and drug release would mainly be affected by the properties of the microparticle that is encapsulated within itself. This study will expand upon this idea posed by Sowers' work, studying the drug release mechanisms of a microparticle encapsulated in HCMPs, as well as researching the effects of lyophilization in HCMPs. Due to hyper-compliant microparticles being a novel type of particle, lyophilization in these particles has not been adequately investigated. This is crucial information that is missing, as it would be ideal to store HCMPs for long-term use. This would allow manufacturers to

produce drug-loaded HCMPs in large quantities and store them for months or potentially years at a time. In this way, thousands of HCMPs can be used once rehydrated. This would save both time and money, as it would be cheaper and faster to produce and store large batches rather than make them as needed.

The aim of this study is to both evaluate how different lyophilization techniques affect hyper-compliant microparticles' properties, and double-encapsulating HCMPs to observe how lyophilization affects drug dissolution rates.

Methods

Hyper-compliant microparticle formation

The aqueous, or dispersed phase was created in a 1.7 mL conical tube that consisted of 2% bis-acrylamide (Bio-Rad), 4% acrylamide (Bio-Rad), 8% dye (Sharpie), 0.1% initiator which was ammonium persulfate (APS) and 1% catalyst, TEMED (Fisher) (116g/mol). The rest of the aqueous phase was DI H2O which was added until the dispersed phase equated to 400uL in volume. After TEMED was added to the dispersed phase, it was vortexed and quickly added to 3-5x volume of the oil, or continuous phase, which consisted of 1% Hypermer 1031 (Croda). Particles were then left for 45 minutes to an hour at room temperature in the dark to ensure cross-linking and particle formation. After this time, contents were centrifuged at 15,000 rpm (Eppendorf 5418 Benchtop Microcentrifuge Fixed Angle Rotor) for 10 seconds to separate the continuous and dispersed phase. The continuous layer on top was removed, and particles were washed three times with a 0.1% triton X-100 solution followed by three DI H2O washes. After washing, particles were resuspended in

1mL DI H2O and stored at room temperature in the dark. The particles made via this method had an elasticity of 4 kPa.

In order to make stiffer (10 kPa) microparticles, the percentage of acrylamide was increased to 8% and bisacrylamide was increased to 0.3% of the total volume of the dispersed phase. The percentage of the remaining reagents were unchanged, and methodology is as described above. Additionally, for experiments with the addition of sucrose or triton, prior to lyophilization either 1% triton x-100 or 1% sucrose was added to the particles suspended in 1mL H2O.

Double-encapsulated hyper-compliant microparticle formulation

1-2 um curcumin-encapsulated PLGA microparticles were obtained from the Mathiowitz lab. These microparticles were encapsulated in our hyper-compliant microparticles utilizing similar methodology as described above. In order to make these double-encapsulated microparticles, the dispersed phase was created in a 1.7 mL conical tube with 4% acrylamide (Bio-Rad), 0.08% bis-acrylamide (Bio-Rad), 0.1% ammonium persulfate, 1% TEMED, and 0.37% curcumin-encapsulated PLGA microparticles. Similar to HCMPs, the rest of the dispersed phase was DI H2O until the dispersed phase was 400ul and methodology is as described above.

Lyophilization Process

Prior to lyophilization, particles were frozen using two different methods for separate experiments. The first method was flash-freezing via submergence in liquid nitrogen for 15 seconds. The second method was slowly freezing overnight in a Mr. Frosty Freezing

Container (Nalgene) with 100% isopropyl alcohol. Regardless of the freezing method, particles were vortexed briefly before freezing and frozen particles were put in the lyophilizer and sealed. Lyophilizer settings were 84 mT and temperature was kept at -51° C. Particles were left overnight and removed from the lyophilizer the following day. After lyophilization, particles were either stored at room temperature or in a -20° C freezer for seven days. At the end of this time period, particles were resuspended in 1mL DI H2O and stored at room temperature for at least 72 hours to ensure proper rehydration of the microparticles. Imaging was done by using a microscope, and particle counting was conducted after this time using a hemocytometer.

Imaging

After complete rehydration, particles were vortexed briefly before imaging. For each sample, 50ul of the HCMPs suspension was pipetted onto a glass slide with a cover slip gently placed over as to cause minimal disturbance to the HCMPs. Imaging was performed with a microscope at either 10x or 4x magnification. Once there was a view of interest, the HCMPs were used as a focus for the microscope to zoom in on. The amount of light was adjusted to appropriately view the particles. At least 50 pictures were taken of each sample and analysis of all images were done in ImageJ. To investigate if lyophilization changes the size of the particles, the size of the particles was calculated in ImageJ. This was done by calibrating the scale bar in ImageJ based on the pixels/um for each camera and different magnification settings.

In-vitro dissolution studies

Double curcumin-encapsulated microparticles were produced, washed, and lyophilized as described previously, and then placed in a 50mL H2O sink. Simultaneously, curcumin-encapsulated PLGA microparticles of matched concentration were placed in another H2O sink. After placing samples in the sink, tubes were gently inverted and allowed to settle for 10 minutes before taking the first time point.

Time points were then taken at t = 30 minutes, and every 24 hours until 96 hours, and 192 hours for lyophilized and non-lyophilized samples, respectively. Aliquots were taken in triplicate from the top half of the sink to ensure only dissolved curcumin was being read and placed in a quartz 96 well plate. A BioTek Cytation 3 Cell Imaging Multi-Mode Reader was used to measure UV-Vis at 300 nm.

A standard curve of curcumin v.s absorbance was calculated using serial dilutions of known curcumin concentrations. A stock solution of 14 mg was dissolved in a 50:50 ethanol and H2O solution in 50mL (0.28 mg/mL) (solubility limit = 10 mg/mL).[16] A mixture of ethanol and H2O was used due to the fact that curcumin has extremely low solubility in water (solubility limit = 0.6 μg/mL).[17] Curcumin's poor solubility in water necessitates the use of an ethanol-water mixture to achieve concentrations suitable for analysis.

This solution was serially diluted by two to produce the following concentrations: 0.28, 0.14, 0.07, 0.035, 0.0175, 0.00875 mg/mL. Absorbance was measured using UV-Vis at 300 nm on the BioTek Cytation machine and plotted against concentration. Using this, the subsequent standard curve equation was used to calculate the unknown concentration of

curcumin in the sink at each time point. Dissolution profiles of all sample groups were fit to the Korsmeyer-Peppas model:

$$M_t / M_\infty = kt^n \quad (1)$$

or the Peppas-Sahlin model:

$$M_t / M_\infty = k_1 t^m + k_2 t^{2m} \quad (2)$$

Where M_t / M_∞ is the fraction of drug released at time t, k is the kinetic constant, and n is the diffusional exponent, used to describe the type of release mechanism.[18] Average dissolution for each time point for control microparticles and double encapsulated HCMPs were plotted against time with their respective standard deviations.

Statistical analysis

A one-way ANOVA, followed by a post-hoc test: the Dunnett's test, was performed on the size and count measurements on the formulated HCMPs to determine whether any significant changes occurred post-lyophilization. The same statistical analysis was done on the double-encapsulated HCMPs to determine if there were any significant differences in % drug dissolution between the lyophilized and non-lyophilized groups.

Results

Liquid Nitrogen

An estimated average of 200,000 non-lyophilized control particles per mL were counted at the end of the flash-freezing experiment. Post-lyophilization, an average of 84,000 particles per mL were counted for the freezer group. For the room temperature group, an average of 91,000 particles per mL were counted. All samples, including controls, were initially flash-frozen using liquid nitrogen. Across all experimental conditions, approximately 600 particles were imaged via microscopy and analyzed using ImageJ software.

Analysis revealed that lyophilized particles were significantly smaller in area compared to their non-lyophilized counterparts, regardless of storage condition (one-way ANOVA: $F(2, 295) = 13.1$, Dunnett's post hoc test: $P < 0.05$; Fig. 1a). Furthermore, both lyophilized groups demonstrated a significant reduction in particle count compared to the non-lyophilized control group (one-way ANOVA: $F(2, 7) = 6.9$, Dunnett's post hoc test: $P < 0.05$; Fig. 1b). Specifically, the room temperature-stored samples had an approximate 57% reduction in particle count, while those stored in the freezer showed a 54% reduction.

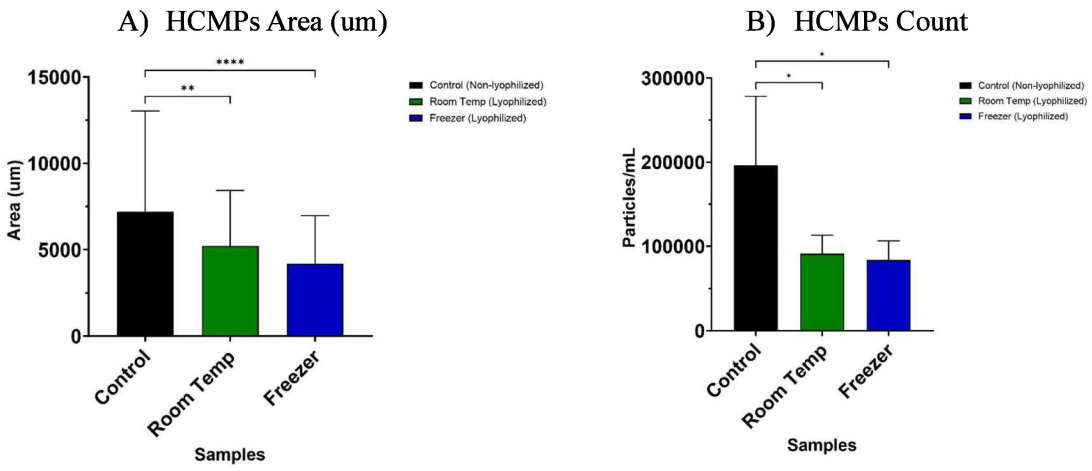

Figure 1. Comparison of area and counts between non-lyophilized and lyophilized HCMPs frozen using liquid nitrogen. Bar graph displaying the average areas and counts of non-lyophilized and lyophilized samples with their respective standard deviations. Non-lyophilized samples are considered the control group and are displayed in black. Lyophilized samples include the samples stored at room temperature in green, and samples stored in a -20° C freezer in blue. Significantly smaller particles were found in both groups of lyophilized samples (A). Significant loss in particle count was found in both groups of lyophilized samples (B).

Mr. Frosty Freezing Container

An estimated average of 138,000 non-lyophilized, control particles per mL were counted at the end of the Mr. Frosty freezing container experiment. Post-lyophilization, an average of 65,000 particles per mL were counted for the freezer group. For the room temperature group, an average of 76,000 particles per mL were counted. All samples, including controls, were frozen using the Mr. Frosty container. A total of approximately 110 particles across all groups were imaged using microscopy and analyzed using ImageJ software.

Both groups of lyophilized particles were found to be significantly smaller in area compared to the non-lyophilized control group (one-way ANOVA: $F(2, 84) = 13.65$; Dunnett's post hoc test: $P < 0.05$; Fig. 2a). Moreover, lyophilization led to a significant reduction in particle count relative to the control (one-way ANOVA: $F(2, 5) = 41.48$;

Dunnett's test: $P < 0.05$; Fig. 2b). Samples stored at room temperature had a 53% reduction in particle count, while those stored in the freezer showed a 45% reduction.

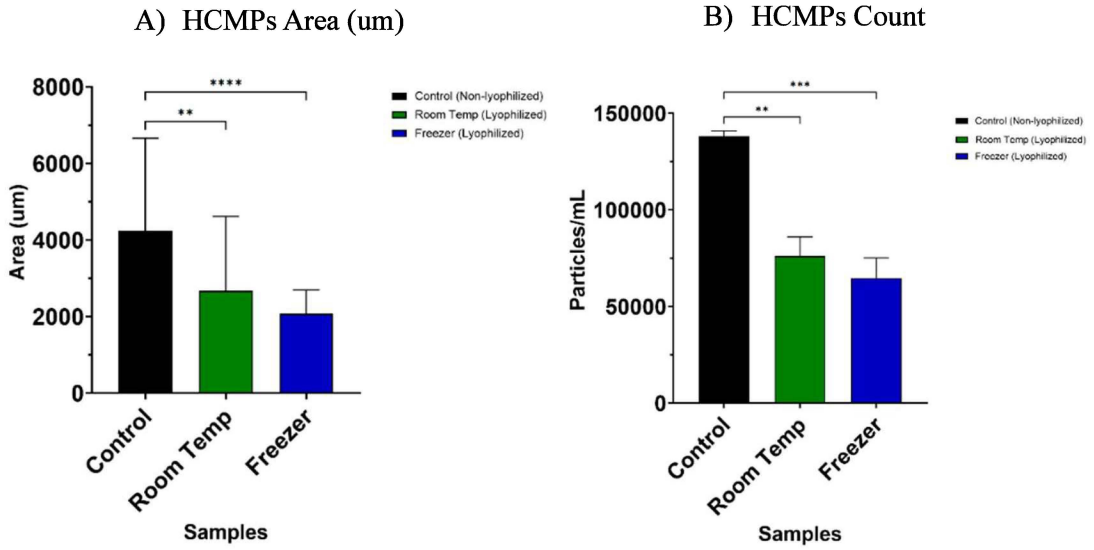

Figure 2. Comparison of area and count between non-lyophilized and lyophilized HCMPs frozen using Mr. Frosty Freezing Container. Bar graph displaying the average areas and counts of non-lyophilized and lyophilized samples with their respective standard deviations as error bars. Non-lyophilized samples are considered the control group and are displayed in black. Lyophilized samples include the samples stored at room temperature in green, and samples stored in a -20 ° C freezer in blue. Significantly smaller particles were found in both groups of lyophilized samples (A). Significant loss in particle count was found in both groups of lyophilized samples (B).

Triton Inclusion

An estimated average of 256,000 non-lyophilized, control particles per mL were counted at the end of the triton experiment. In comparison, lyophilized samples had reduced counts, with an average of 132,000 particles per mL for the freezer-stored group and 143,000 particles per mL for the room temperature-stored group. Prior to freezing, 1% Triton was added to all samples, which were subsequently frozen using a Mr. Frosty freezing container.

No statistically significant differences in particle area were observed between the control and lyophilized groups (one-way ANOVA: $F(2, 147) = 1.004$; Dunnett's test: $P >$

0.05; Fig. 3a). Despite this, lyophilized samples still had a significant reduction in particle count compared to the non-lyophilized control (one-way ANOVA: $F(2, 3) = 32.14$; Dunnett's test: $P < 0.05$; Fig. 3b). The room temperature-stored samples demonstrated a 44% reduction, while the freezer-stored samples showed a 48% reduction in particle count.

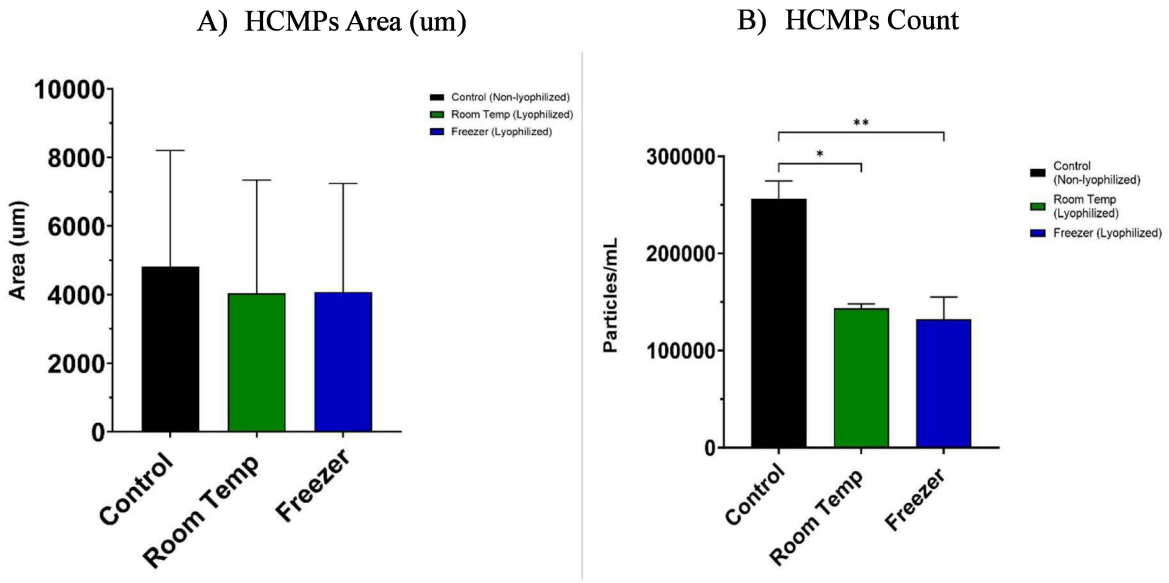

Figure 3. Comparison of area and counts between non-lyophilized and lyophilized HCMPs frozen with 1% triton added. Bar graph displaying the average areas and counts of non-lyophilized and lyophilized samples with their respective standard deviations. Non-lyophilized samples are considered the control group and are displayed in black. Lyophilized samples include the samples stored at room temperature in green, and samples stored in a -20 ° C freezer in blue. Both lyophilized sample groups had 1% triton added prior to lyophilization, which resulted in similar areas between control and experimental groups (A). Significant loss in particle count was still found in both groups of lyophilized samples (B).

Sucrose Inclusion

At the conclusion of the sucrose experiment, the non-lyophilized control group had an average particle concentration of approximately 218,000 particles per mL. Post-lyophilization, an average of 195,000 particles per mL were counted for the freezer group. For the room temperature group, an average of 161,000 particles per mL were counted.

Prior to freezing, 1% sucrose was added to all samples, which were then frozen using a Mr. Frosty freezing container. An estimated total of 220 particles across all groups were imaged using microscopy and subsequently analyzed with ImageJ software.

No statistically significant differences in particle area were observed between the control and lyophilized groups (one-way ANOVA: $F(2, 189) = 1.330$; Dunnett's test: $P > 0.05$; Fig. 4a). However, lyophilization still resulted in a significant reduction in particle count compared to the control group (one-way ANOVA: $F(2, 3) = 200.7$; Dunnett's test: $P < 0.05$; Fig. 4b). Room temperature-stored samples showed a 26% reduction in particle count, while freezer-stored samples showed a 23% reduction.

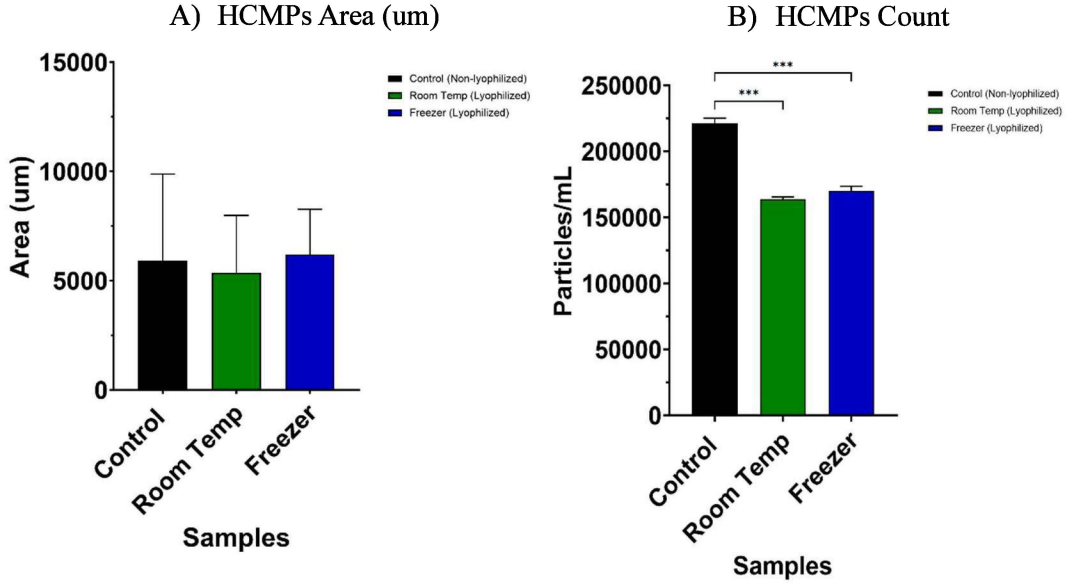

Figure 4. Comparison of area and counts between non-lyophilized and lyophilized HCMPs frozen with 1% sucrose added. Bar graph displaying the average areas and counts of non-lyophilized and lyophilized samples with their respective standard deviations. Non-lyophilized samples are considered the control group and are displayed in black. Lyophilized samples include the samples stored at room temperature in green, and samples stored in a -20 ° C freezer in blue. Both lyophilized sample groups had 1% sucrose added prior to lyophilization, which resulted in similar areas between control and experimental groups (A). Significant loss in particle count was still found in both groups of lyophilized samples (B).

Stiffer (10 kPa) HCMPs

At the end of the experiment, the non-lyophilized 10 kPa particle group had an average concentration of approximately 218,000 particles per mL. Following lyophilization, particle concentrations decreased to 195,000 particles per mL for the freezer-stored group and 161,000 particles per mL for the room temperature-stored group. Across all groups, an estimated total of 220 particles were imaged using microscopy and analyzed with ImageJ software.

No statistically significant differences in particle area were observed between the control and lyophilized groups (one-way ANOVA: $F(2, 215) = 0.6430$; Dunnett's test: $P > 0.05$; Fig. 5a). However, lyophilization resulted in a significant reduction in particle count compared to the non-lyophilized control (one-way ANOVA: $F(2, 4) = 57.81$; Dunnett's test: $P < 0.05$; Fig. 5b). Both the room temperature and freezer-stored samples had an approximate 30% reduction in particle count.

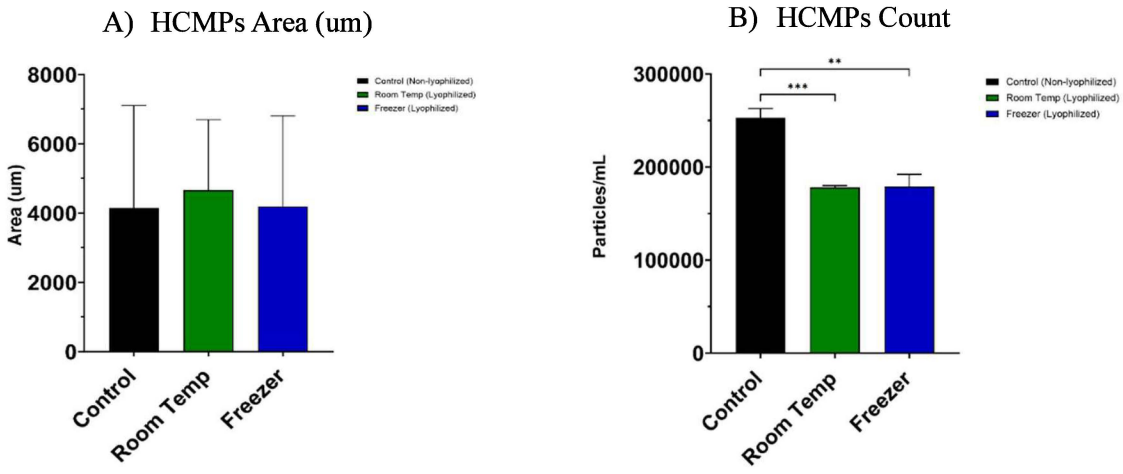

Figure 5. Comparison of particle area and counts between non-lyophilized and lyophilized 10 kPa HCMPs. Bar graph displaying the average area and particles/mL of non-lyophilized and lyophilized samples with their respective standard deviations as error bars. Non-lyophilized samples are considered the control group and are displayed in black. Lyophilized samples include the samples stored at room temperature in green, and samples stored in a -20 ° C freezer in blue. No differences observed in area between groups (A). Significantly lower counts of particles were found in both groups of lyophilized samples compared to the non-lyophilized control group (B).

Drug Dissolution

At 24 hours, the curcumin-encapsulated PLGA microparticles had dissolved ~100% of loaded drug into the sink, but double curcumin-encapsulated microparticles did not dissolve completely throughout the entire duration of the experiment regardless of lyophilization status (Fig. 6). Non-lyophilized samples were observed and measured for 192 hours, whereas lyophilized samples were measured for up to 96 hours. This difference was due to both time constraints and procedural differences: non-lyophilized samples were introduced to the H2O sink immediately, and thus samples could be taken immediately, while lyophilized samples required an additional four days for processing and storage before being introduced to the H2O sink. Among the double-encapsulated HCMPs, both groups had significantly greater percent dissolution than their non-lyophilized counterparts at all time points, with the exception of the 48-hour mark (one-way ANOVA: $F(3, 16) = 228$, Dunnett's post hoc test: $P < 0.05$; Fig. 6).

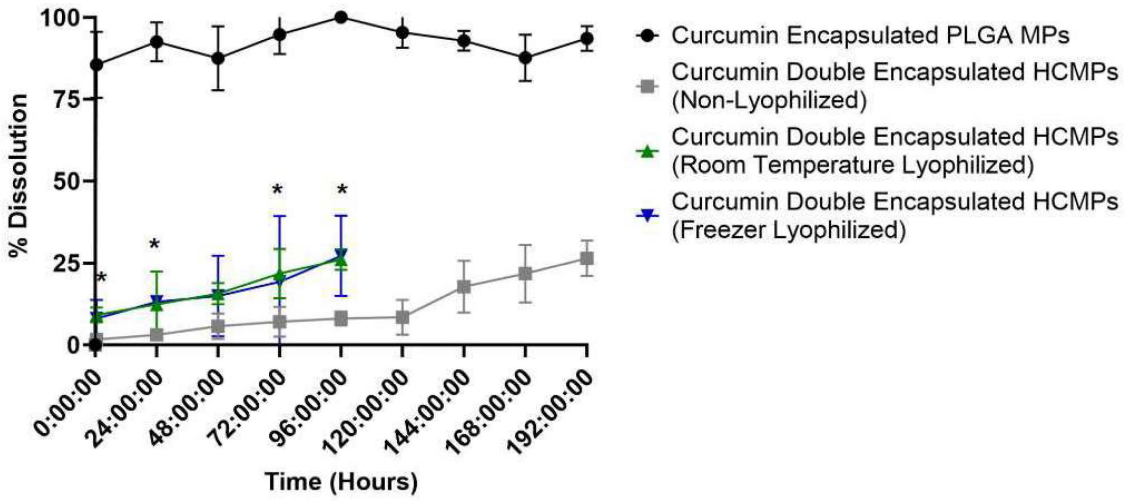

Figure 6. Dissolution profile of double curcumin-encapsulated HCMPs, non-lyophilized vs. lyophilized over 96-192 hours. PLGA microparticles served as the control and exhibited ~100% dissolution in the first 24 hours. At the end of the experiment, both non-lyophilized and lyophilized sample groups did not reach 50% dissolution.

The release kinetics of the control PLGA microparticles were modeled using the Korsmeyer–Peppas equation, $M_t / M_\infty = kt^n$. The model showed an excellent fit to the experimental data, with an R^2 value of 0.99, a diffusional exponent of $n = 0.02$, and a kinetic constant of 0.87. All the double encapsulated HCMPs sample groups were plotted instead to fit the Peppas-Sahlin model, $M_t / M_\infty = k_1 t^m + k_2 t^{2m}$.

The release profiles for the groups fit the model with a range for the R^2 values from 0.89 – 0.95, indicating good fit with the model. For the non-lyophilized double-encapsulated HCMPs, the release exponent was $m = 1.0$, with a diffusion constant of $k_1 = 0.00045$, and a swelling rate constant of $k_2 = 0.00000191$. The double encapsulated lyophilized sample stored at room temperature, the release exponent was $m = 0.23$, with a diffusion constant of $k_1 = 0.05$ and a swelling constant of $k_2 = 0.01$. Finally, for the double encapsulated lyophilized sample stored in the freezer, the release exponent was $m = 0.25$, with a diffusion constant of $k_1 = 0.015$ and a swelling constant of $k_2 = 0.001$.

Discussion

The goal of this study was two-fold: to evaluate how different lyophilization techniques affect certain properties of hyper-compliant microparticles, and double encapsulating curcumin into HCMPs and comparing dissolution rates for lyophilized and non-lyophilized samples. Results demonstrated that regardless of freezing method, whether flash freezing via liquid nitrogen or slowly freezing overnight utilizing a Mr. Frosty freezing container, lyophilization significantly contorts and destroys the HCMPs. This is consistent with literature that outlines how lyophilization causes significant physical stress on microparticles, affecting their size and shape.[19] Due to HCMPs being softer than other microparticles, it stands to reason that they would be more affected by lyophilization. Additionally, HCMPs are mostly made of water, making them vulnerable to developing ice crystals during the freezing process of lyophilization. These ice crystals deform the shape of the HCMPs, as well as tear them apart either during lyophilization or during the rehydration process. This can be seen in the data as lyophilized HCMPs without any additives were consistently smaller than the control, non-lyophilized particles. Furthermore, because HCMPs are so soft, they are more susceptible to breakage from these ice crystals, contributing to the significant low counts of HCMPs present after lyophilization regardless of any additions or modifications.

However, it was also seen that with the addition of a surfactant (triton X-100), a sugar (sucrose), or formulating stiffer HCMPs, the area between lyophilized and non-lyophilized sample groups were the same. Surfactants reduce surface and interfacial tension, stabilizing the interface between solids and liquids.[20] Triton X-100 is a nonionic

surfactant, making it useful in this case where it is compatible with both the HCMPs and water. This binding has been found to inhibit the formation of crystals, including ice crystals which are theorized to be the main culprits as to why HCMPs collapse and deform following lyophilization.[21] Sugars such as sucrose get dissolved in water as the polar water molecules attract the sucrose molecules and form multiple hydrogen bonds with each sucrose molecule. The addition of sucrose to our HCMPs and water solution creates space between particles, preventing the HCMPs from sticking to one another during lyophilization, and averting possible breakage once rehydrated. This is important since previous studies have shown that particle size may increase after lyophilization and rehydration.[22]

While the addition of triton or sucrose did not significantly aid in the preservation of HCMPs following lyophilization, it did help HCMPs maintain their size, keeping it comparable to that of the control. This suggests that even a minuscule amount of surfactant or sugar can prevent HCMPs from collapsing. Moreover, there was an observed improvement in concentration with the addition of triton or sucrose, yielding higher values compared to experiments without any additives. This effect is likely due to triton's ability to lower surface tension as a surfactant, which minimizes stress and prevents particle collapse during lyophilization. Similarly, the addition of sucrose appears to provide sufficient spacing between microparticles, preventing clustering during lyophilization and rehydration, and thereby reducing breakage.

HCMPs have an elastic property range of 0.25 to 10 kPa.[12] The HCMPs that were created for this study were 4 kPa and 10 kPa. Hyper-compliant microparticles that were 4 kPa and did not have triton or sucrose added displayed a significant decrease in size compared to the control. In contrast, the 10 kPa HCMPs maintained a size comparable to the control non-lyophilized HCMPs, demonstrating that higher stiffness aids in HCMPs maintaining their size post-lyophilization. Higher stiffness enhances the HCMPs structural integrity by increasing their resistance to deformation under applied forces. This observation aligns with previous findings using hybrid hydrogel microspheres, where stiffer microspheres had high resistance to deformation, allowing the microspheres to keep their original shape and size after being exposed to external forces.[23]

In the drug dissolution experiment, it showed that PLGA microparticles released approximately ~100% of the drug within 24 hours. In contrast, the double-encapsulated microparticles did not release the entire drug load even by the end of the experiment. This seems to suggest that double encapsulation aids in the sustained, long-term release of curcumin. One possible explanation for this is that the drug must pass through two different barriers, the PLGA and HCMP shells, instead of just one. Additionally, the method of forming the double-encapsulated HCMPs may have caused the PLGA and HCMP shells to stick to one another, further restricting drug diffusion and creating a bottleneck within the system. This dual-shell structure likely contributed to the sustained release observed but may also have impeded curcumin from diffusing effectively from the polymers.

Furthermore, due to curcumin's poor solubility in water, it tends to aggregate and form clumps, especially when not uniformly dispersed. As a result, even after prolonged immersion in water, complete dissolution of curcumin may not occur.[16] This clumping can skew concentration measurements, as undissolved curcumin may not be evenly distributed within the sink, leading to an underestimation of the true concentration.

The difference in % drug dissolution between lyophilized and non-lyophilized samples may also be explained by structural changes produced by lyophilization. As previous results demonstrated, lyophilization can lead to significant collapse and deformation of HCMPs due to ice crystals during the freezing phase. These ice crystals can distort the microparticle matrix, resulting in increased porosity. Upon rehydration, these structural openings create additional pathways for the drug to diffuse out of the microparticles, accelerating its release compared to the non-lyophilized microparticles.

Drug release is influenced by various factors including but not limited to polymer erosion and diffusion through the polymer shell or matrix.[24] A model used to describe drug release is the Korsmeyer-Peppas model. The additional model used in the analysis of the lyophilized sample is known as the Peppas-Sahlin models. The Peppas-Sahlin model, extrapolates on the aforementioned Korsmeyer-Peppas model, to fit more complicated delivery systems, specifically systems that are prone to swelling or contain multiple polymers.[23] The Korsmeyer-Peppas model, $M_t / M_\infty = kt^n$ specifies that the value of n, the diffusional exponent, indicates the type of diffusion. If $n = < 0.43$, the drug release mechanism is Fickian diffusion, if $0.43 < n < 0.85$, it is anomalous transport and if $n = >$

0.85, it is zero order transport.[25] The *n* value for the curcumin encapsulated PLGA microparticles was observed to be significantly lower than 0.43 ($n = 0.02$), indicating Fickian diffusion through the matrix of the PLGA microparticles.[25] Additionally, because the *n*-value is so low, this is also evidence of the burst release that is observed since ~100% of the curcumin was released within 24 hours.

The Peppas-Sahlin model is different in that it considers the swelling and relaxation of the polymer that may occur during drug release. This is relevant as HCMPs are considered to be hydrogels, a swelling-controlled device.[25] The Peppas-Sahlin model is also used in cases where different forces may be at play due to the complexity and number of polymers. In this model, $M_t / M_\infty = k_1 t^m + k_2 t^{2m}$, on the right-hand side, the first term accounts for diffusion, similar to the original Korsmeyer-Peppas model, and the second term accounts for the relaxation contributions. *M* is the diffusional exponent, similar to the *n* value in the Korsmeyer-Peppas model.[25]

For the lyophilized samples stored at room temperature, the diffusion constant (k_1) was 0.04 and the swelling constant (k_2) was 0.01, indicating that diffusion is the dominant release mechanism, although there is some contribution from polymer relaxation mechanisms. For the lyophilized samples stored in the freezer, the diffusion constant (k_1) was 0.015 and the swelling constant (k_2) was 0.001, suggesting similarly to the room temperature samples, that diffusion is the dominant release mechanism. For both lyophilized sample groups, the release exponents are below 0.43, which indicates Fickian diffusion through the matrix of both the PLGA and HCMP matrices. While the diffusion

and swelling constants differ between the two storage groups, they have similar release exponents. This suggests that storage temperature has little effect on drug release profiles following lyophilization, only affecting release rates minimally.

For the double-encapsulated samples that were not lyophilized, the diffusion constant (k_1) was 5.74×10^{-6} and the swelling constant (k_2) was 3.12×10^{-10}, both considerably lower than the lyophilized sample groups, but still indicates that diffusion is the main force behind drug release. The m value for double-encapsulated non-lyophilized particles was 1.0, suggesting a zero-order drug release mechanism.[26] Zero-order drug release kinetics describes when drug releases at a constant rate from a device, independently from the concentration of the drug remaining in the system.[27] This is significant as its behavior contrasts with the Fickian diffusion observed in the lyophilized samples and strongly suggests that lyophilization alters the drug release mechanism in HCMPs, shifting it from a constant-rate release to diffusion through the polymer matrix.

The most likely explanation for the observed differences in drug release profiles lies in the structural changes caused by lyophilization, as seen in previous experiments. Lyophilization creates a more porous matrix, facilitating water penetration and diffusion-driven release. In contrast, non-lyophilized samples likely retain a denser, more intact structure that leads to a different release mechanism, more controlled by swelling or matrix erosion. Additionally, lyophilized samples exhibit much faster drug dissolution, likely due to microcracks and increased surface roughness formed during the freeze-drying process. These openings provide additional pathways from which curcumin may dissolve out from,

accelerating release. The porous structure resulting from lyophilization increases the surface area, which speeds up water uptake and rehydration, thus increasing drug dissolution. The shift from zero-order release in non-lyophilized microparticles to Fickian diffusion in lyophilized microparticles suggests that lyophilization disrupts matrix integrity, likely through deformation, and alters the release mechanism from a constant release to diffusion-drive, rapid release process.

Interestingly, non-lyophilized samples % dissolution increases at the 120-hour mark. In previous experiments, it was seen that HCMPs were fully rehydrated in 72 hours, however this was at room temperature and exposed to light. In contrast, the drug dissolution samples were stored in a refrigerator and shielded from light using aluminum foil. This colder, darker environment may have slowed the diffusion process. Curcumin is sensitive to both light and heat, drastically increasing its degradation when directly exposed to light and higher temperatures.[28] This same sensitivity to heat and light is also present for PLGA, with higher temperatures increasing the degradation rate.[29] Therefore, storing the samples in a cool, dark environment likely reduced the degradation rate of both curcumin and PLGA, and also slowed curcumin dissolution.

This study represents the first work to investigate and demonstrate the effects of lyophilization on hyper-compliant microparticles, as well as the drug release profiles of curcumin from double encapsulation in PLGA and HCMPs. The results offer new insights into the influence of lyophilization on not only hyper-compliant microparticle's properties, but also drug release mechanisms, revealing a shift from zero-order to Fickian diffusion.

It is important to remember however, that these HCMPs were made in bulk-emulsion, which lead to large polydisperse samples with a broad size distribution and variability. Future research should aim to fabricate monodispersed HCMPs with a droplet generator to ensure uniform particle size to get more consistent data. Additionally, this study only focused on the release of curcumin, a drug with low solubility in water, which limits the generalizability of the findings. Future experiments should investigate the release profiles of HCMPs as a microcarrier with other drugs, particularly those with higher solubility, to better understand how release mechanisms might change depending on the drug's properties.

One limitation of this study involves the method used to generate the standard curve for curcumin quantification. Due to curcumin's poor solubility in water, the standard curve was created using a 50:50 ethanol and water solution. While this solvent mixture was necessary to dissolve curcumin effectively and obtain reliable absorbance readings, it does differ from the pure water sink conditions used in the drug dissolution experiments. Nevertheless, the standard curve was linear across the concentration range ($R^2 > 0.99$), and absorbance measurements obeyed the Beer-Lambert Law. Additionally, absorbance values were measured against appropriate solvent blanks to maintain analytical validity.

Another important consideration is the type of stability assessed in this work. This study primarily demonstrated stability in vial, that is, how HCMPs hold up under storage conditions prior to use. However, stability in use or stability in syringes have not been tested. It is unclear whether HCMPs will maintain their structural integrity when exposed to

shear forces and pressure during syringe injection after lyophilization and rehydration. Given their soft composition, HCMPs may be especially vulnerable to rupture or deformation during administration, which could compromise their microcarrier functionality. Future research should investigate how the microparticles perform in simulated injection conditions and during active use.

Despite these limitations, this work demonstrates the potential that hyper-compliant microparticles have to be an innovative microcarrier. The ability of HCMPs to provide a sustained, long-term release rather than a burst release demonstrates their fit for applications where a steady low-dose administration of medicine is wanted. Furthermore, the unique properties of HCMPs, namely their size and elasticity, could potentially allow them to navigate through capillaries and softer tissues in the body. This offers the potential for targeted drug delivery to more specific sites within the body. These characteristics make hyper-compliant microparticles promising candidates as a microcarrier that may be used in a variety of therapeutic applications, especially in cases where controlled release is critical.

References

1. Tan, M. L., Choong, P. F. M., & Dass, C. R. (2010). Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. *Peptides*, *31*(1), 184–193. https://doi.org/10.1016/j.peptides.2009.10.002
2. Penchala, S. C., Miller, M. R., Pal, A., Dong, J., Madadi, N. R., Xie, J., Joo, H., Tsai, J., Batoon, P., Samoshin, V., Franz, A., Cox, T., Miles, J., Chan, W. K., Park, M. S., & Alhamadsheh, M. M. (2015). A biomimetic approach for enhancing the in vivo half-life of peptides. *Nature Chemical Biology*, *11*(10), 793–798. https://doi.org/10.1038/nchembio.1907
3. Allen, T. M. (2004). Drug Delivery Systems: Entering the Mainstream. Science, 303(5665), 1818–1822. https://doi.org/10.1126/science.1095833
4. Gabizon, A., Shmeeda, H., & Barenholz, Y. (2003). Pharmacokinetics of Pegylated Liposomal Doxorubicin. Clinical Pharmacokinetics, 42(5), 419–436. https://doi.org/10.2165/00003088-200342050-00002
5. Amit Kumar Nayak, Md Saquib Hasnain, Laha, B., & Sabyasachi Maiti. (2023). Advanced and Modern Approaches for Drug Delivery. Elsevier.
6. *Microsphere - an overview | ScienceDirect Topics*. (n.d.). www.sciencedirect.com. https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/microsphere
7. Lengyel, M., Kállai-Szabó, N., Antal, V., Laki, A. J., & Antal, I. (2019). Microparticles, Microspheres, and Microcapsules for Advanced Drug

Delivery. *Scientia Pharmaceutica*, *87*(3), 20. https://doi.org/10.3390/scipharm87030020

8. Daly, A. C., Riley, L., Segura, T., & Burdick, J. A. (2019). Hydrogel microparticles for biomedical applications. *Nature Reviews Materials*, *5*(1), 20–43. https://doi.org/10.1038/s41578-019-0148-6
9. Siepmann, J., & Siepmann, F. (n.d.). Microparticles Used as Drug Delivery Systems. Smart Colloidal Materials, 15–21. https://doi.org/10.1007/3-540-32702-9_3
10. Cooley, M., Sarode, A., Hoore, M., Fedosov, D. A., Mitragotri, S., & Sen Gupta, A. (2018). Influence of particle size and shape on their margination and wall-adhesion: implications in drug delivery vehicle design across nano-to-micro scale. Nanoscale, 10(32), 15350–15364. https://doi.org/10.1039/c8nr04042g
11. Merkel, T. J., Jones, S. W., Herilhy, K. P., Kersey, F. R., Shields, A. R., Napier, M., Luft, J. C., Wu, H., Zamboni, W. C., Wang, A. Z., Bear, J. E., & DeSimone, J. M. (2011). Using mechanobiological mimicry of red blood cells to extend circulation times of hydrogel microparticles. PNAS, 108 (2) 586-591. https://www.pnas.org/doi/pdf/10.1073/pnas.1010013108
12. Labriola, N. R., Mathiowitz, E., & Darling, E. M. (2017). Fabricating polyacrylamide microbeads by inverse emulsification to mimic the size and elasticity of living cells. Biomaterials Science, 5(1), 41–45. https://doi.org/10.1039/c6bm00692b

13. Fonte, P., Reis, S., & Sarmento, B. (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *225*, 75–86. https://doi.org/10.1016/j.jconrel.2016.01.034
14. Trenkenschuh, E., & Friess, W. (2021). Freeze-drying of nanoparticles: How to overcome colloidal instability by formulation and process optimization. European Journal of Pharmaceutics and Biopharmaceutics, 165, 345–360. https://doi.org/10.1016/j.ejpb.2021.05.024
15. Sowers, Rebeka. (2023). *Ultrastructure and Release Characteristics of Hyper-Compliant Microparticles for Hydrophobic Drug Delivery*. [Master's Thesis, Brown University].
16. Carvalho, Deivis & Takeuchi, Katiuchia & Geraldine, Robson & Moura, Celso & Torres, Maria. (2015). Production, solubility and antioxidant activity of curcumin nanosuspension. Food Science and Technology (Campinas). 35. 115-119. 10.1590/1678-457X.6515.
17. Górnicka, J., Mika, M., Wróblewska, O., Siudem, P., & Paradowska, K. (2023). Methods to Improve the Solubility of Curcumin from Turmeric. *Life*, *13*(1), 207. https://doi.org/10.3390/life13010207
18. Jamnongkan, T., & Kaewpirom, S. (2010). Potassium Release Kinetics and Water Retention of Controlled-Release Fertilizers Based on Chitosan Hydrogels. *Journal of Polymers and the Environment*, *18*(3), 413–421. https://doi.org/10.1007/s10924-010-0228-6

19. Yu, Z., Rogers, T. L., Hu, J., Johnston, K. P., & Williams, R. O. (2002). Preparation and characterization of microparticles containing peptide produced by a novel process: spray freezing into liquid. European Journal of Pharmaceutics and Biopharmaceutics, 54(2), 221–228. https://doi.org/10.1016/s0939-6411(02)00050-4
20. Nakama, Y. (2017, January 1). *Chapter 15 - Surfactants* (K. Sakamoto, R. Y. Lochhead, H. I. Maibach, & Y. Yamashita, Eds.). ScienceDirect; Elsevier. https://www.sciencedirect.com/science/article/abs/pii/B978012802005000015X
21. Inaba, H., Inada, T., Akihiko Horibe, Suzuki, H., & Usui, H. (2004). Preventing agglomeration and growth of ice particles in water with suitable additives. *International Journal of Refrigeration*, *28*(1), 20–26. https://doi.org/10.1016/j.ijrefrig.2004.07.012
22. M. Glavas-Dodov, E. Fredro-Kumbaradzi, K. Goracinova, M. Simonoska, Calis, S., S. Trajkovic-Jolevska, & A.A. Hincal. (2005). The effects of lyophilization on the stability of liposomes containing 5-FU. *International Journal of Pharmaceutics*, *291*(1-2), 79–86. https://doi.org/10.1016/j.ijpharm.2004.07.045
23. Seong, Y.-J., Lin, G., Kim, B. J., Kim, H.-E., Kim, S., & Jeong, S.-H. (2019). Hyaluronic Acid-Based Hybrid Hydrogel Microspheres with Enhanced Structural Stability and High Injectability. *ACS Omega*, *4*(9), 13834–13844. https://doi.org/10.1021/acsomega.9b01475
24. Unagolla, J. M., & Jayasuriya, A. C. (2018). Drug transport mechanisms and in vitro release kinetics of vancomycin encapsulated chitosan-alginate polyelectrolyte

microparticles as a controlled drug delivery system. *European Journal of Pharmaceutical Sciences*, *114*, 199–209. https://doi.org/10.1016/j.ejps.2017.12.012

25. Talevi, A., & María Esperanza Ruiz. (2021). Korsmeyer-Peppas, Peppas-Sahlin, and Brazel-Peppas: Models of Drug Release. *Springer EBooks*, 1–9. https://doi.org/10.1007/978-3-030-51519-5_35-1
26. Tadej Ojsteršek, Franc Vrečer, & Grega Hudovornik. (2024). Comparative Fitting of Mathematical Models to Carvedilol Release Profiles Obtained from Hypromellose Matrix Tablets. *Pharmaceutics*, *16*(4), 498–498. https://doi.org/10.3390/pharmaceutics16040498
27. Talevi, A., & Ruiz, M. E. (2022). Zero-Order Drug Release. *Springer EBooks*, 1195–1200. https://doi.org/10.1007/978-3-030-84860-6_33
28. Castillo, M. L. R. del, López-Tobar, E., Sanchez-Cortes, S., Flores, G., & Blanch, G. P. (2015). Stabilization of curcumin against photodegradation by encapsulation in gamma-cyclodextrin: A study based on chromatographic and spectroscopic (Raman and UV–visible) data. *Vibrational Spectroscopy*, *81*, 106–111. https://doi.org/10.1016/j.vibspec.2015.10.008
29. *Factors Affecting PLGA Degradation: What You Need to Know*. (2023, September 1). https://polylactide.com/plga-degradation/