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Alternate formulations for optimization of PLGA microsphere-comprised glucose biosensor coating against foreign body inflammation.

Klair Lubonja
University of Connecticut - Storrs, klair.lubonja@uconn.edu

University of Connecticut

Honors Scholar Thesis

"Alternate formulations for optimization of PLGA microsphere-comprised glucose biosensor coating against foreign body inflammation"

By: Klair Lubonja

Department of Molecular and Cell Biology

College of Liberal Arts and Sciences

University of Connecticut

Honors Thesis Advisor

Dr. Diane J. Burgess

Department of Pharmaceutical Sciences

School of Pharmacy

University of Connecticut

Honors Advisor:

Dr. Michael A. Lynes

Department of Molecular and Cell Biology

College of Liberal Arts and Sciences

University of Connecticut

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I would like to dedicate this work to my parents Klarita and Genci Lubonja. Their hard work, support, and dedication has been vital to my educational success. Also, I would like to thank my brother, Migen Lubonja, for always pushing me to do better and leading by example.

Abstract

In this study, dexamethasone loaded PLGA microsphere/PVA hydrogel composites were explored as an outer drug-eluting coating for implantable biosensors to provide defense against acute inflammation of the foreign body response. The microspheres, with a dexamethasone release target period of approximately two weeks, were manufactured under various conditions: different co-solvents and homogenization speeds. Particle size measurement, scanning electron microscopy and differential scanning calorimetry were performed for all the microsphere formulations prepared. The addition of acetone as a co-solvent for dexamethasone, at normal homogenization speeds, shows promise for the improvement of drug loading and the attainment of superior release profiles. PLGA microsphere/PVA hydrogel composites were prepared using the optimized microsphere formulation to coat microdialysis probes. The microdialysis probes were used as surrogates for biosensors to evaluate inflammation inhibition *in vivo* via histological staining. The results of this study indicate that the aforementioned PLGA microsphere/PVA hydrogel composite shows promise as being an exceptional coating material for the application of acute inflammation prevention using semi-implanted microdialysis probes.

Table of Contents

Section 1:

Introduction

Diabetes Mellitus is a metabolic disorder that affects the pancreas, causing an imbalance in blood glucose levels. There are two types of diabetes: those with type one diabetes have a problem with producing adequate amounts of insulin and those with type two diabetes have insensitivity to insulin. For optimal diabetes care, it is important to monitor blood glucose tightly. The conventional method of monitoring blood glucose involves pricking the finger and drawing blood onto a test strip, as seen with technologies like OneTouch; this method of tracking glucose levels is inconvenient. A glucose sensor that is totally implanted in the body (and capable of communicating with a personal electronic device) could alleviate the painful effects of intermittent finger pricking for diabetics. [4]

Significant progress has been made toward the development of wireless, fully implantable biosensors for real-time monitoring of various metabolic analytes to aid in the treatment and management of diseases such as diabetes. Continuous monitoring of glucose, as an example, provides information on not only the glucose level at a specific time point, but also the pattern of change over time. [4] By having the ability to take real time readings, those that are diabetics have the capability to fully comprehend the disease's adverse effects and adapt their lifestyle to fit their specific needs. Furthermore, continuous monitoring would give the patients and the physicians alike the ability to allow for early detection of complications such as hyperglycemia/hypoglycemia and their associated risks. Finally, continuous monitoring of glucose levels in the body allows for the realization of what is considered an "artificial pancreas", a concept that ensures tight control of glucose through the most favorable insulin dosing. [5,6] As of now, only semi-implantable biosensors are currently available on the market.

The reason for this limitation is as a result of the poor *in vivo* stability seen with fully implantable biosensors. This poor stability is due to the fact that once implanted, foreign objects

become subject to the host tissue's response, otherwise known as the "Foreign Body Reaction" (FBR). The response consist of a cascade of events that includes inflammatory cell recruitment, collagen deposition and a fibrous encapsulation which isolates the biosensor from the tissue it was implanted on and renders it useless, as it can no longer record analyte readings. In order to suppress this unwanted tissue response, biocompatible coatings in combination with tissue modifier delivery systems have been investigated. [5] These biocompatible coatings consist of dexamethasone-loaded poly (lactic-co-glycolic acid) (PLGA) microspheres dispersed in polyvinyl alcohol (PVA) hydrogel. Dexamethasone is a synthetic corticosteroid with potent anti-inflammatory properties. PVA was selected, as it possesses similar strength characteristics as those of human soft tissue, when cross-linked. In this study, it was used as both a stabilizing and emulsifying agent. Concurrently, PLGA is programmable for controlled drug delivery and biodegradable.

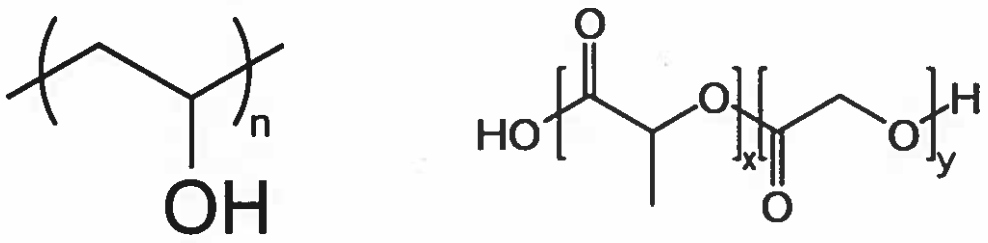

Figure 1: Molecular structures of PVA (left) and PLGA (right)

When localized, the effects of this coating have been shown to reduce the negative FBR seen in cases like the one aforementioned. With that being said, not long after, the dexamethasone would be close to, if not completely, dispersed and a delayed tissue reaction would be observed. This rapid release of drug is termed "burst release". As previous research

had determined, new methods for creating these dexamethasone loaded microspheres needed to be looked into so as to increase drug loading and reduce burst release.

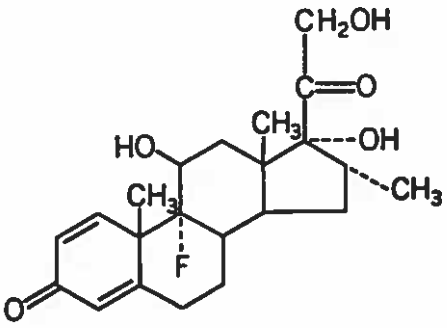

Figure 2: Molecular structure of dexamethasone

The research presented here deals with optimizing the dexamethasone loaded PLGA microspheres so as to have maximal drug loading, low burst release and stable continuous release thereafter. Using the standard method of preparing the microspheres as a control group, four other formulations were prepared and tested accordingly so as to determine which formulation of preparation gave us the characteristics we desired. The formulations differed slightly from one another by either having higher homogenization speeds, using co-solvents, or both. The two homogenization speeds used during testing were the standard 10,000-rpm and a higher speed of 15,000-rpm. Homogenization speed has been shown to have an inverse relationship with particle size. The solvents used were methylene chloride (DCM), acetone/ methylene chloride mix, and methanol/methylene chloride mix. Co-solvents were used to increase the solubility of the dexamethasone. Acetone and methanol, although in different classes of low toxicity, share the same characteristic of aiding in solubilizing dexamethasone.

For the *in vitro* studies, particle size, glass transition analysis, high performance liquid chromatography (HPLC), and scanning electron microscopy (SEM) imaging were used as

methods of particle testing. Particle size was measured to determine the effects of using a higher homogenization speed and of adding co-solvents. In addition, the size of a particle is revealing of the amount of drug it is able to load, with smaller particles having lower drug loading capacities. Glass transition temperature analysis was used to determine the stability of the different microsphere formulations. The formulations were all subjected to HPLC so release profile data could be collected at pre-determined time points. The release profiles of the different formulations were the most important aspect of the *in vitro* studies as they provided the primary source of data used in the selection of the optimal formulation. The last technique used to assess the microsphere formulations *in vitro* was SEM imaging. The images helped in better understanding both the internal and external characteristics of the microspheres.

For the *in vivo* component of the research, semi- implanted microdialysis surrogates were used to test the chosen formulation in rat models. Coating-free surrogates were used as the control group. The *in vivo* study is telling of the efficacy of the coating when subjected to a foreign body response and other factors unable to be reproduced *in vitro*. Histological data was collected to identify the effects of the coating on the surrounding tissue.

Knowledge gained from the present work will be useful in understanding the importance of co-solvent use in PLGA microsphere preparation and will speak to the comparability of *in vitro* and *in vivo* data. It will also prove to be useful in the future development of implantable biosensor specific coatings.

Section 2:

Methods

Preparation of PLGA Microspheres

Standard preparation of dexamethasone loaded PLGA/PVA microspheres

To prepare the microspheres through an oil and water emulsion method, 500 mg of PLGA and 50 mg of crystalline dexamethasone were weighed and placed into 5 mL glass vials. 2 mL of methylene chloride (DCM) were added to the mixture to dissolve the polymers. Once capped, the vials were subjected to sonication using a Solid State/Ultrasonic F-28 sonicator (Fisher Scientific). The sonicated product was then homogenized for 30 seconds using a T 25 digital ULTRA-TURRAX homogenizer (IKA Works, Inc.) at 10,000 rpm to obtain a homogenous suspension. The mixture was then added to 10 mL of 1% PVA solution and subjected to homogenization at a speed of 10,000 rpm for an additional 2 minutes. Once the homogenization step was complete, the solid/oil/water emulsion was transferred to 125 mL of .1% PVA solution (112.5 mL DI water and 12.5 mL of 1% PVA) and subjected to vacuuming through a vacuum chamber for 3 hours to remove the DCM. After the time limit had passed, the hardened microspheres were collected into three 50 mL tubes and prepared for centrifugation to purify. After three centrifugation cycles at 1,500 rpm for 3 minutes each, the particles were subjected to liquid nitrogen freezing and then dried overnight using a LABCONCO freeze-drying system. This method of preparation served as the control microsphere formulation. [5, 7]

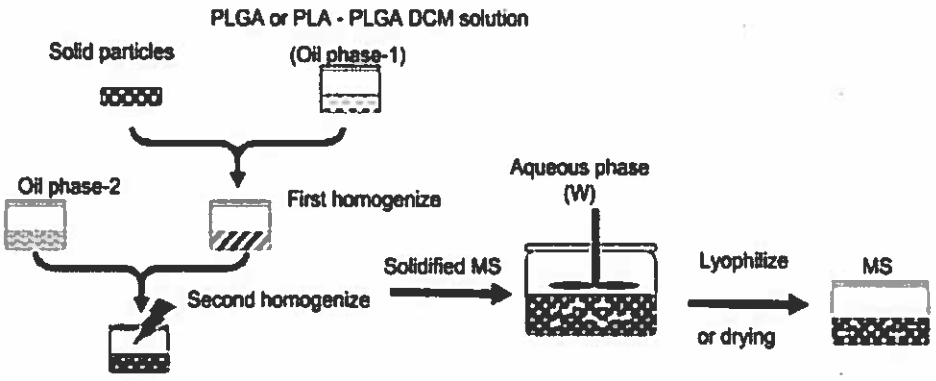

Figure 3: Standard method of preparation [11]

Alternate preparations of dexamethasone loaded PLGA/PVA microspheres

- Higher Homogenization Speed

  - Change standard method (above) slightly by increasing the first homogenization step speed to 15,000 rpm instead of the standard 10,000 rpm.

- Use of Co-Solvent (Acetone)

  - Change standard method (above) by adding 1 mL of acetone and 1 mL of DCM to dissolve the PLGA/dexamethasone composition instead of the standard 2 mL of DCM.

- Higher Homogenization Speed and use of Co-Solvent (Acetone)

  - Change standard method (above) by increasing the first homogenization step speed to 15,000 rpm instead of the standard 10,000 rpm and by adding 1 mL of acetone and 1 mL of DCM to dissolve the PLGA/dexamethasone composition instead of the standard 2 mL of DCM.

- Use of Co-Solvent (Methanol)

  - Change standard method (above) by adding 1 mL of methanol and 1 mL of DCM to dissolve the PLGA/dexamethasone composition instead of the standard 2 mL of DCM.

Table 1: Different formulation methods created and tested.

Formulation	1 (Control)	2	3	4	5
Speed (rpm)	10,000	15,000	10,000	15,000	10,000
Solvent Composition	2 mL DCM	2 mL DCM	1 mL Acetone 1 mL DCM	1 mL Acetone 1 mL DCM	1 mL Methanol 1 mL DCM

Characterization of PLGA Microspheres

Drug Release and Drug Loading

For the release study, microspheres were dispersed in poly-vinyl alcohol hydrogels, as is the case when used as biosensor coatings. This was achieved *via* the freeze-thaw cycling method described in the *in vivo* experimental procedure outline below. The microsphere/hydrogel composites were used for the *in vitro* release testing. Approximately 5 mg samples of the composites (n=2) were incubated at 37°C in 1.8 mL PBS (10 mM, pH 7.4) for the initial samples. Later samples were incubated in 0.9 mL of PBS. At designated time points (1 hr., 3 hrs., 24 hrs., and 48hrs x 6) over a two-week period, the release medium was totally removed, placed in HPLC vials, and replenished with the same amount of new medium. The samples were then placed in the Perkin-Elmer Series 200 HPLC system for analysis. [1] The mobile phase used for HPLC testing was composed of 1400 mL DI water, 700 mL acetonitrile and 10 mL of 10%

phosphoric acid. The flow rate used for HPLC is 1 mL/min and 20 μL of volume was injected for each sample. The column used for the HPLC was an Agilent Zorbax RX C18 column. The system allowed us to obtain the dexamethasone concentration in each sample when calculated using a calibration curve. The release profile was calculated and plotted as cumulative percent release at each time point.

Drug loading was determined by measuring 10 mg of microspheres of each formulation type and adding them to separate volumetric flasks. Dissolving those same microspheres in tetrahydrofuran (THF) and then taking 0.1 mL of the solution, mixing it with 0.9 mL of dexamethasone mobile phase and running the samples through HPLC using the method described above.

Particle Size

The average particle size measurements of each microsphere type were determined using an AccuSizer 700A dilution particle sizing system. 10 mg of microspheres were dispersed in 1 mL of 0.1% PVA solution. Approximately 0.2 mL of the dispersion was then added to the sizing system for analysis. [5, 6]

Glass Transition Temperature Analysis

Approximately 5 mg of microspheres were placed in a DSC Q1000 specific standard pan and then analyzed using a DSC Q1000 system. The samples were heated at a rate of 2 °C/min from 4 °C to 200 °C at a modulating oscillatory frequency of 0.83 °C/80 seconds in the differential scanning calorimeter. The thermograms were recorded and the glass transition temperatures (Tg) were analyzed using Universal Analysis software (TA Instruments).

Scanning Electron Microscopy

Microspheres from each formulation were collected, crushed and imaged using an SEM system. 2,500x and 5,000x images were collected. The microspheres were crushed to see both external and internal characteristics. Samples were mounted on carbon taped aluminum stubs and sputter coated with gold for 1 min at 6 mA. Images were taken with an accelerating voltage of 2.0 kV and a working distance of 4 mm.

In Vivo Experimental Procedure

Microdialysis probes were coated with PVA hydrogel to form PVA hydrogel/PLGA microsphere composite coatings *via* freeze-thaw cycling. PLGA microspheres were dispersed in 5 %(w/v) PVA solution and filled into an 1-mL syringe to experience the first freeze thaw cycle including 2-hours in a freezer at -20 C and 1-hour at room temperature. After the first cycle, the dispersion with enhanced viscosity was injected into sterile plastic tubings (4 mm in diameter and 25 mm in length) and the microdialysis probes were then inserted into the tubings. The coated probes experienced two additional freeze thaw cycles to form composite coatings with the desired mechanical property. For animal experiments, all procedures were performed under approved protocols by the University of Connecticut IACUC. Two microdialysis probes (one coated and one uncoated) were subcutaneously implanted into the dorsal side of each rat. The rats were sacrificed at predetermined dates and the tissue surrounding the microdialysis probes was collected. Histological sections were prepared after tissue fixation in 10% formaldehyde solution. H&E staining was performed to the sections in order to evaluate the inflammation reaction to the coated and uncoated probes.

Section 3:

Results and Discussion

In Vitro Release Study

The PLGA polymer used to make the microspheres had a molecular weight of approximately 10 kD. This type of polymer is used for 2-week drug release studies. According to the data shown in Figure 4, formulation 3, with an initial (day-zero) 16% cumulative drug release, showed promise of having low burst release as compared to the other formulations, which all centered at about 23-28%. After 24 hours, formulations 2, 4 and 5 reached 82-87% cumulative release, while formulation 3 remained at 62% and formulation 1 (control) reached 73% (Figure 4). Formulations 2, 4 and 5 had higher burst release levels compared to formulation 3 and by the third day of testing, more than 90% of the drug encapsulated within those microsphere formulations was completely released. As a continuous release profile over seven days was desired these three formulations were not considered for further testing. When looking at the formulation 3 data on the third day, it stood at 75% cumulative release. By comparison, the control formulation had reached 80% cumulative release. All the other formulations had exhausted the drug days prior.

Although the initial burst release level of formulation 3 was not as high as that of the other formulations, it was more than adequate to suppress the initial inflammatory response *in vivo*. It is considered that the more porous structures of the other formulations made them susceptible to a higher burst release and thereby reducing the amount of drug available for release on days 2-9. The release profile is related to the structure of the microspheres, which is demonstrated in the section below.

Particle Size

As mentioned in the introduction, homogenization speed is known to have an effect on particle size; the higher the speed the smaller the particle size. Furthermore, solvents other than DCM can also have an effect on particle size since solvents determine much about porosity and composition. As shown in Figure 5, the average diameter of the formulation 3 prepared microspheres was 5.76 μm. As compared to formulations 1, 2, 4 and 5, that had larger average diameters of 7.88, 5.68, 6.55, and 6.85 μm, respectively (Table 2). The particle diameters of the formulations are all significantly different ($p>0.05$), except when comparing formulations 4 and 5 to one another. Interestingly, formulation 3, prepared without an increase in homogenization speed, had smaller particles than formulation 4, which was prepared with acetone as the co-solvent and had a higher homogenization speed. Although larger particle size may result in more drug encapsulation (Table 2) and slower release profiles, it should be noted that the formulations 1, 4 and 5, which had larger particles than formulation 3, a had a greater burst release and a faster overall release profile, which as previously stated is attributed to the greater extent of porosity of these formulations. The data showed that formulation 2 had the smallest particle size (5.68 μm) and the least desirable release profile with low drug loading. Accordingly this formulation was not considered for further study *in vivo*.

Overall, the release characteristics of formulation 3 are closest to the desired release profile. It is possible that the release profile of formulation 3 may be further improved in the future by increasing the particle size through a reduction in homogenization speed.

Glass Transition Temperature

The temperature at which an amorphous compound undergoes transformation from a glassy state to a rubbery state is called the glass transition temperature. As the temperature of the human body is 37 °C, it is important that any controlled release formulation to be administered *in vivo* has a glass transition phase greater than 37 °C, so that the polymer being tested is not rapidly degraded, leading to rapid release of the drug. According to Figure 7, formulation 3 had a glass transition temperature ($T_{g)}$ of 41.55 °C. Since this T_g is higher than body temperature, it will help in maintaining controlled release of the drug. According to Figure 8, the T_g's formulations 1, 2, 4, were 43.25, 41.60, 42.71 and 40.70 °C, respectively.

SEM Images

SEM imaging of the microsphere particles is important as the images provide an insight into the internal and external characteristics of the microspheres. The crushed microspheres shown in Figure 9 reveal that formulation 3 microspheres have an eggshell structure. This type of structure is likely to be responsible for the release profile of formulation 3. In addition, upon further inspection, a fibrous structure is seen enclosed within the microsphere walls. This is thought to be the dexamethasone that precipitated as a result of the acetone/methylene chloride co-solvent mixture. This fibrous, matrix-like structure, may also explain the release profile of formulation 3. It is possible that precipitation makes encapsulation easier, contributing to enhanced encapsulation efficiency.

When compared to the other formulations, formulation 3 has a unique morphology (Figure 10). Formulation 1 has a thick inner lining and also appears to have surface associated drug on its outer lining. This is as a result of un-encapsulated drug sticking to the surface of the

microspheres. SEM images of formulation 2 show that it has a hollow structure, almost like that of formulation 3, but it too has surface-associated drug that is unseen in formulation 3. Formulation 4 microspheres have weak eggshell structures as all of the SEM images show the microspheres in a crushed form, more so than the other formulation microspheres (Figure 4-C). This may explain their poor release profile. Lastly, although unable to see the internal structure of the microspheres prepared with the 5th formulation, poor encapsulation is possibly due to the layered structures observed in Figure 4-D. These structures may be caused by precipitated dexamethasone due to addition of methanol as a co-solvent. Addition of methanol can lead to surface distribution of dexamethasone due to phase separation of the polymer and drug. This indicates that careful selection of an appropriate co-solvent is critical to obtain optimal release profiles.

Overall, formulation 3 microspheres had a lower burst release and a smaller lag phase, stable post-burst release, particle size in the desired size range, acceptable glass transition phase temperatures, and optimal morphologies. The other formulations failed to satisfy all of the desired criteria.

Histological Images

Through H & E staining of histological samples obtained after *in vivo* testing, it was found that the microdialysis tubes coated with the formulation 3 dexamethasone loaded microspheres were effective at suppressing the foreign body response. Figure 11-A shows a lack of immune cells surrounding the probe after 5 days of testing. Figure 11-B, which shows the probe without coating, expresses the inflammatory cells engulfing the probe. When comparing the two figures, the difference in terms of immune suppression is noteworthy.

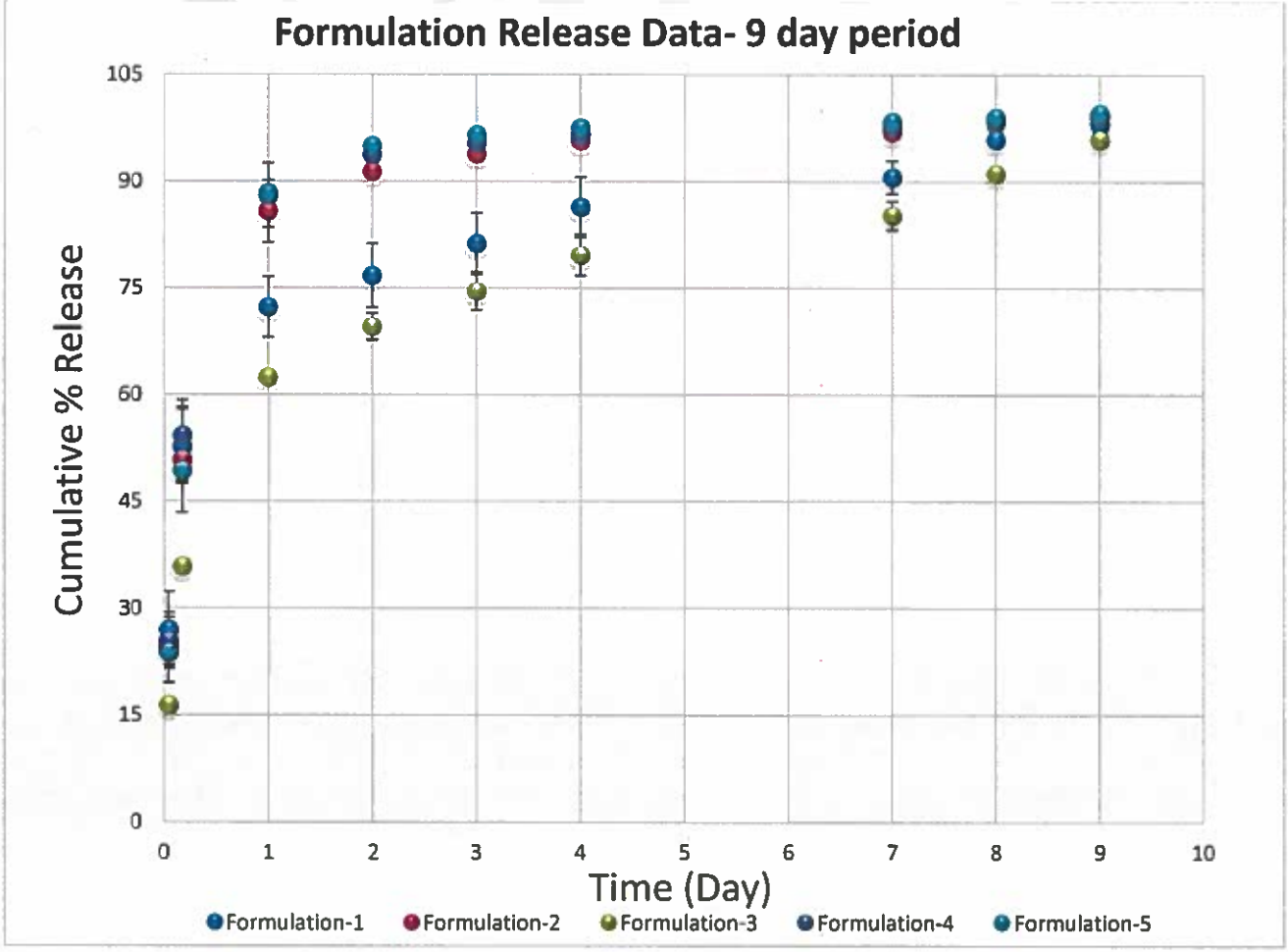

Figure 4. Nine-day composite release profiles for all formulations. Data points for Formulation 3 are shown in green. The release testing was conducted using PBS (10 mM, pH 7.4) at 37 °C.

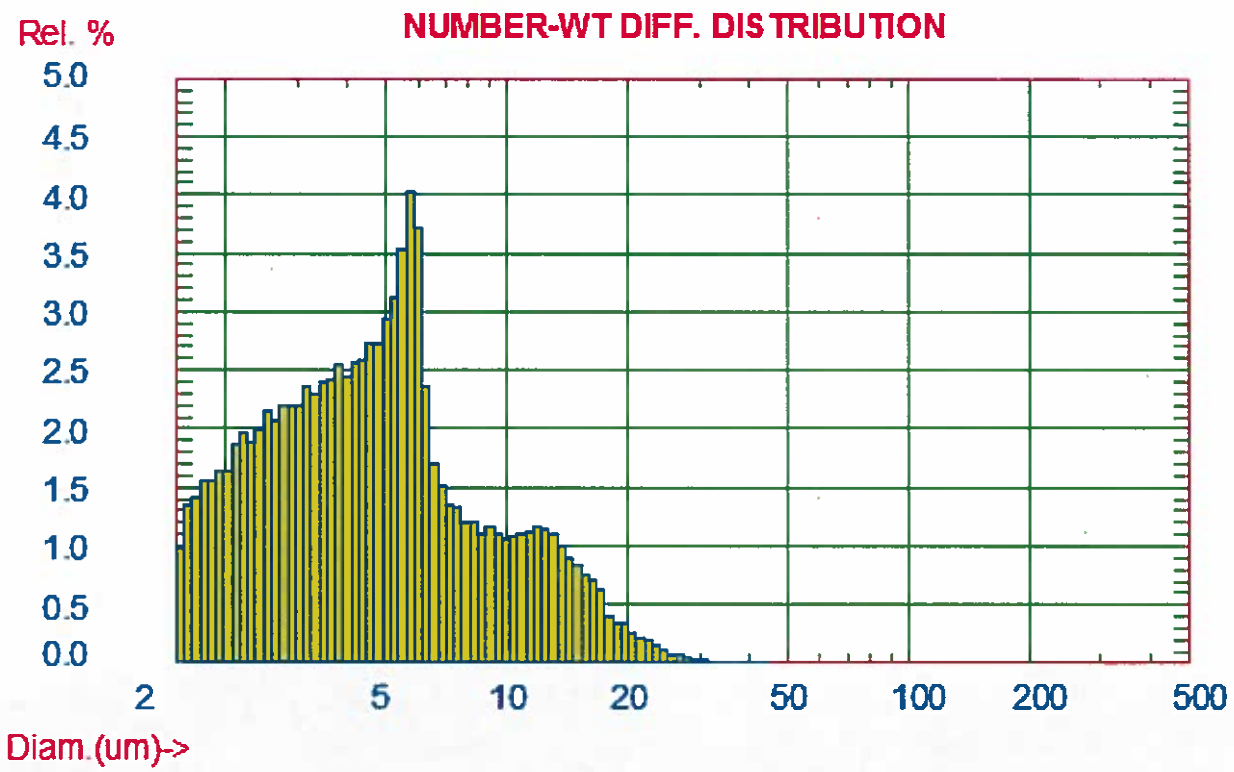

Figure 5. Particle size data for Formulation 3. The mean, medium and mode values attained were 5.76 μm, 4.56 μm and 5.99 μm, respectively.

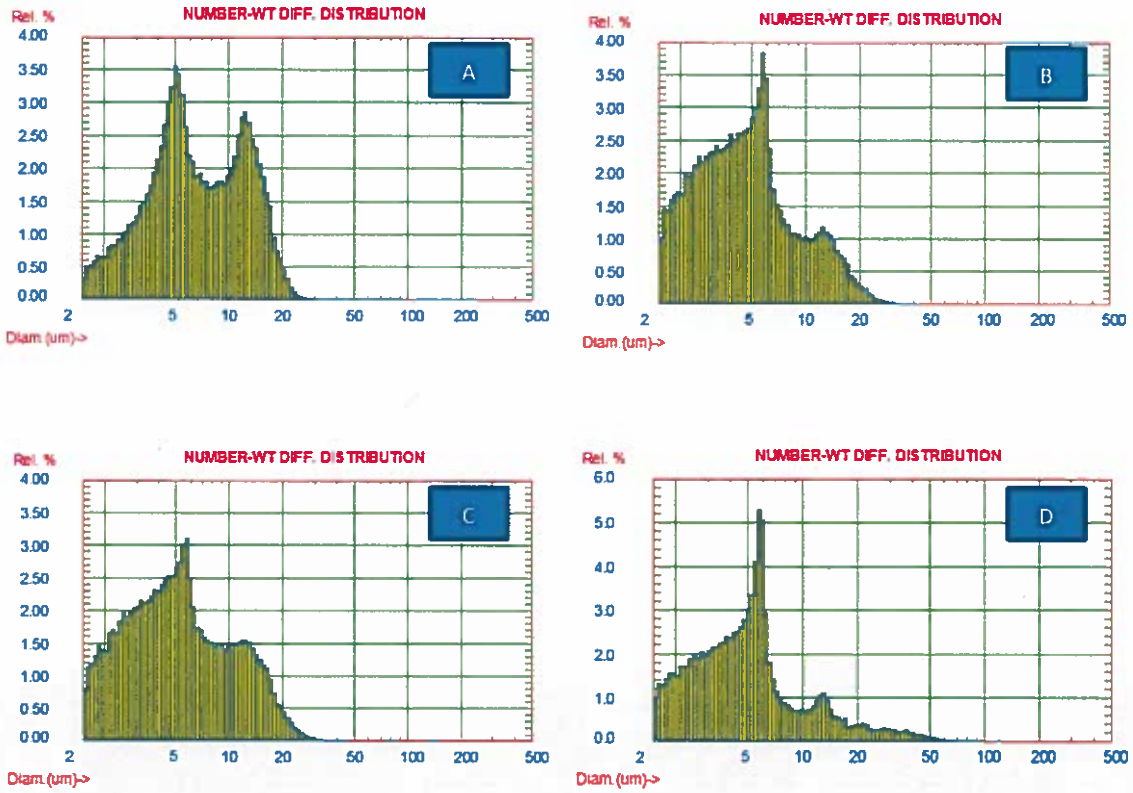

Figure 6: Number-Weight Difference Distribution graphs for Formulations 1 (A), 2 (B), 4 (C), and 5 (D).

Table 2: Number-Weight Difference Distribution data for Formulations 1, 2, 4, and 5.

Formulation	1	2	3	4	5
Mean	7.88μm	5.68μm	5.76μm	6.55μm	6.85μm
Median	6.27μm	4.41μm	4.56μm	4.99μm	4.77μm
Mode	4.99μm	5.72μm	5.99μm	5.72μm	5.99μm
Volume Distribution	154.80um	15.69μm	15.98μm	18.01μm	40.69μm
Standard Deviation	98.42μm	6.54μm	6.85μm	14.14μm	18.43μm
Of Distribution	63.6%	41.7%	42.9%	78.5%	45.3%

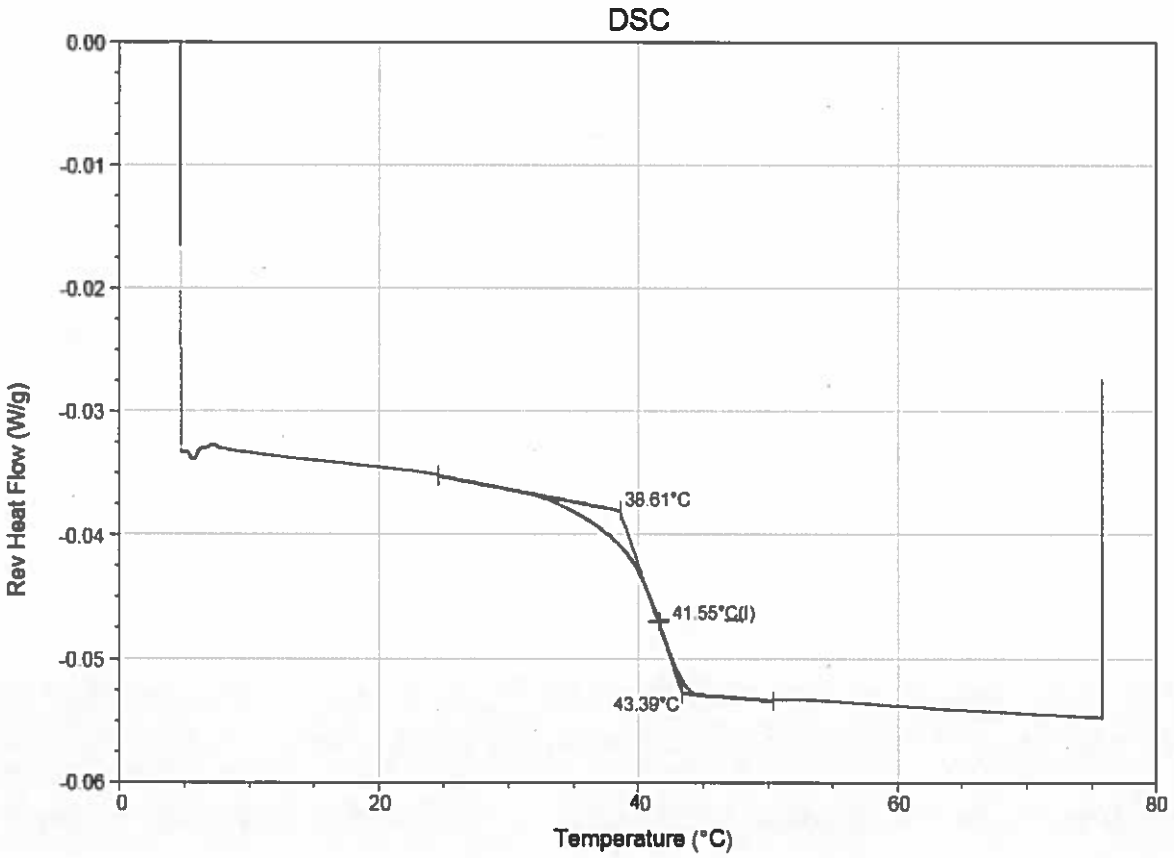

Figure 7: Glass transition temperature graph for Formulation 3. The temperature attained was 41.55°C.

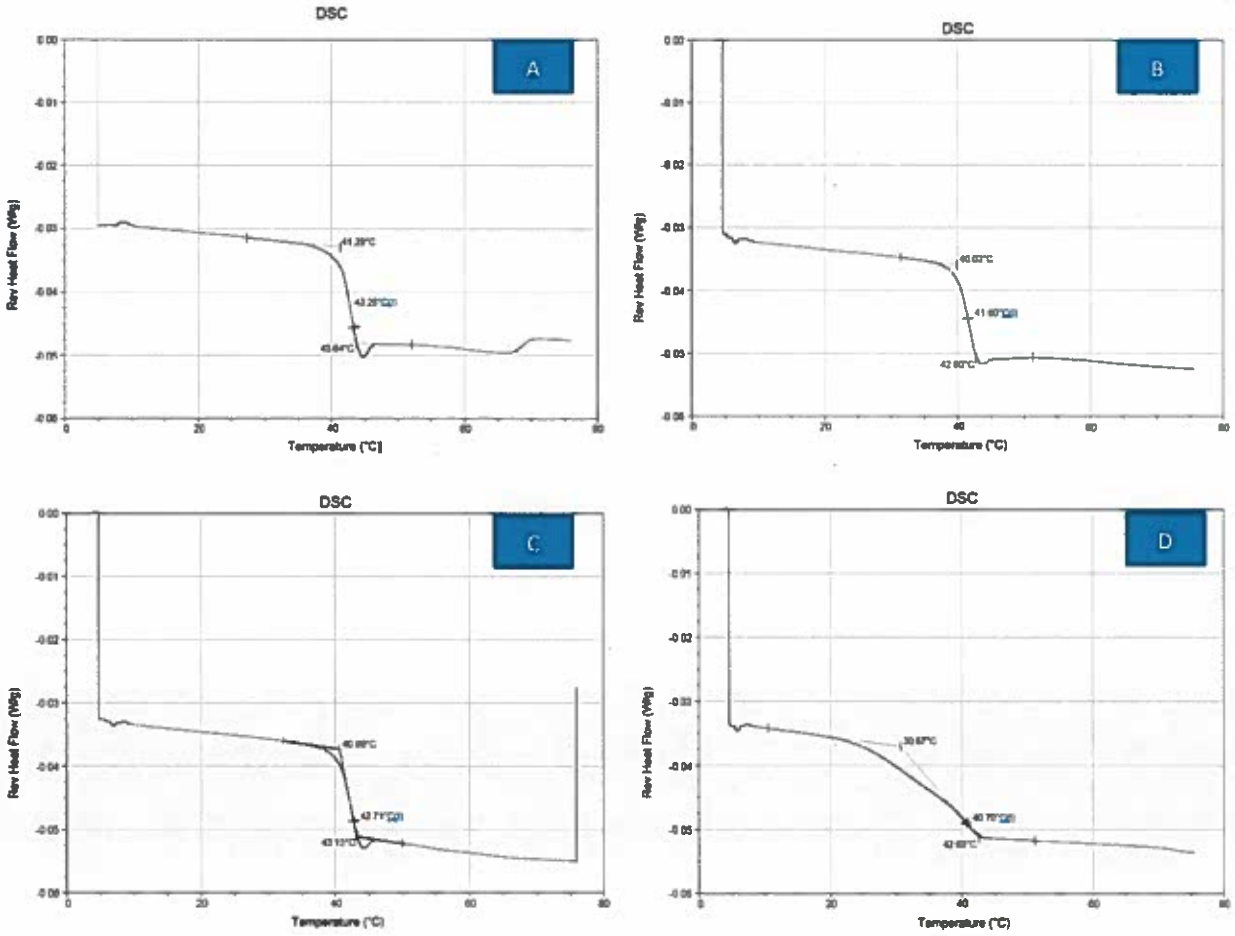

Figure 8: Glass transition temperature graphs for Formulations 1 (A), 2 (B), 4 (C), and 5 (D). The temperatures were 43.25, 41.60, 42.71 and 40.70°C, respectively.

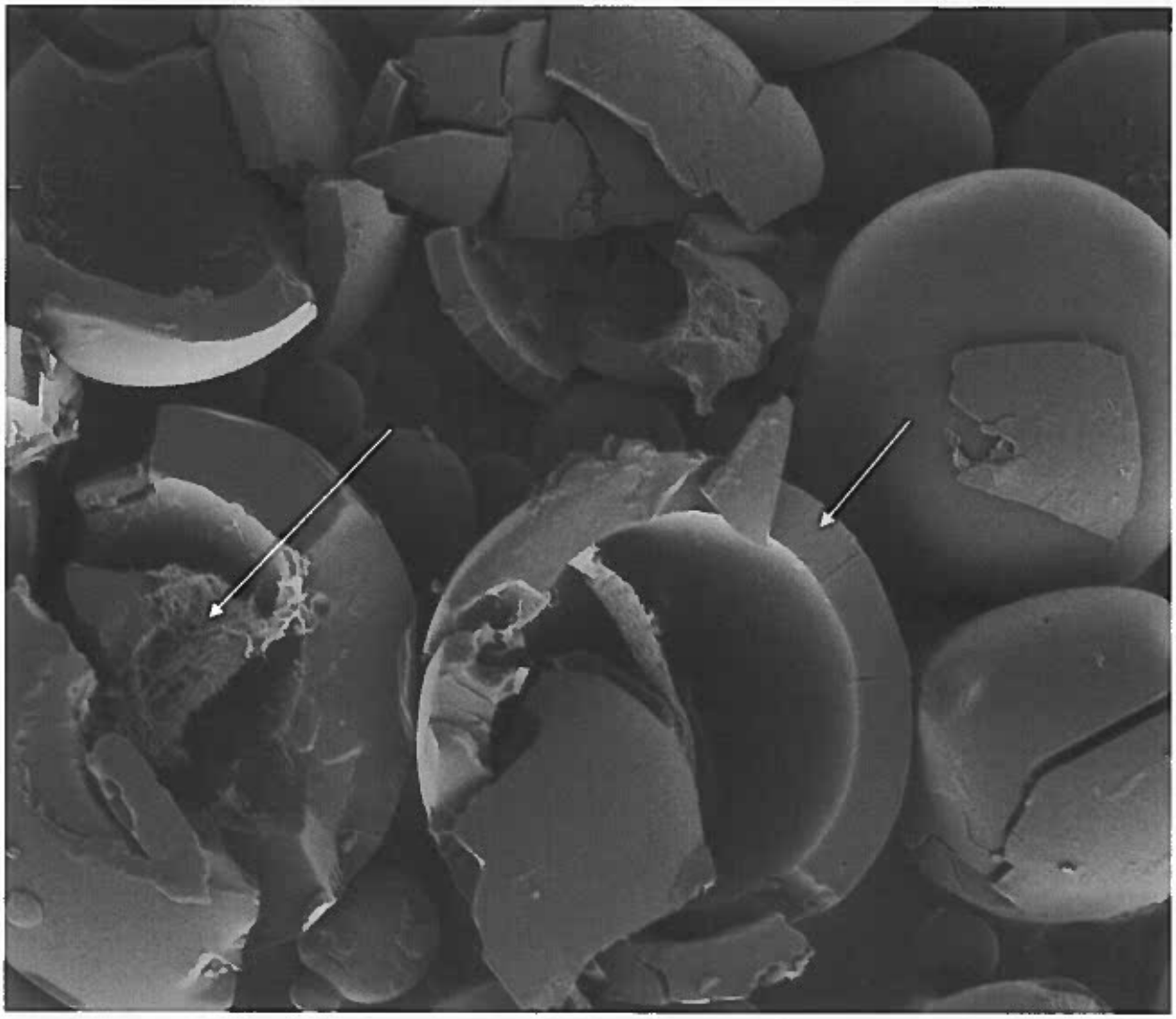

Figure 9. SEM images of Formulation 3 microspheres after crushing showing both external and internal structures. Yellow arrow indicates "eggshell" morphology. White arrow points at fibrous/matrix like structure believed to be dexamethasone.

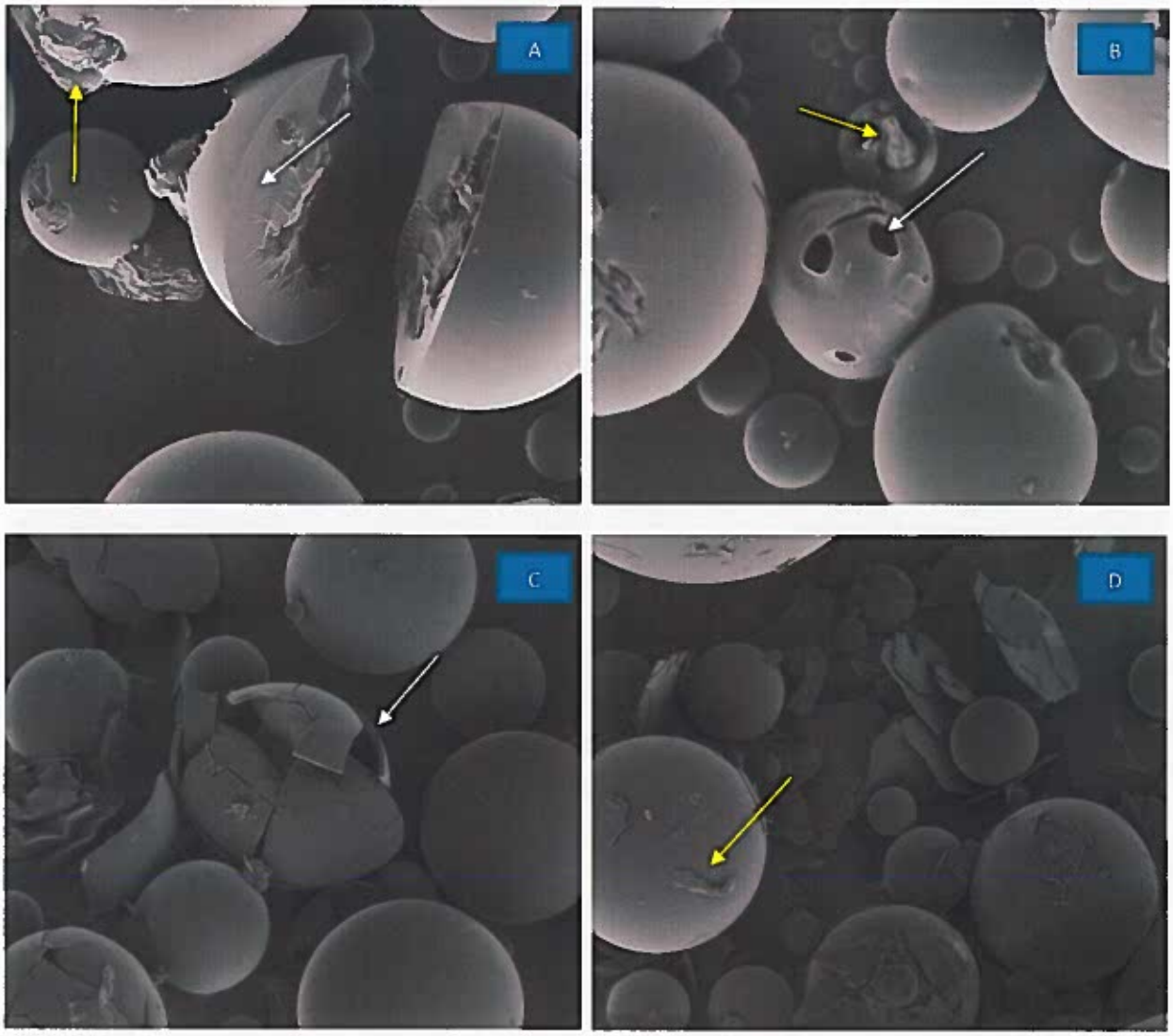

Figure 10. SEM images of Formulations 1 (A), 2 (B), 4 (C), and 5 (D) microspheres. Yellow arrows indicate surface associated drug (A, B & D). White arrows indicate the internal structure of the microspheres.

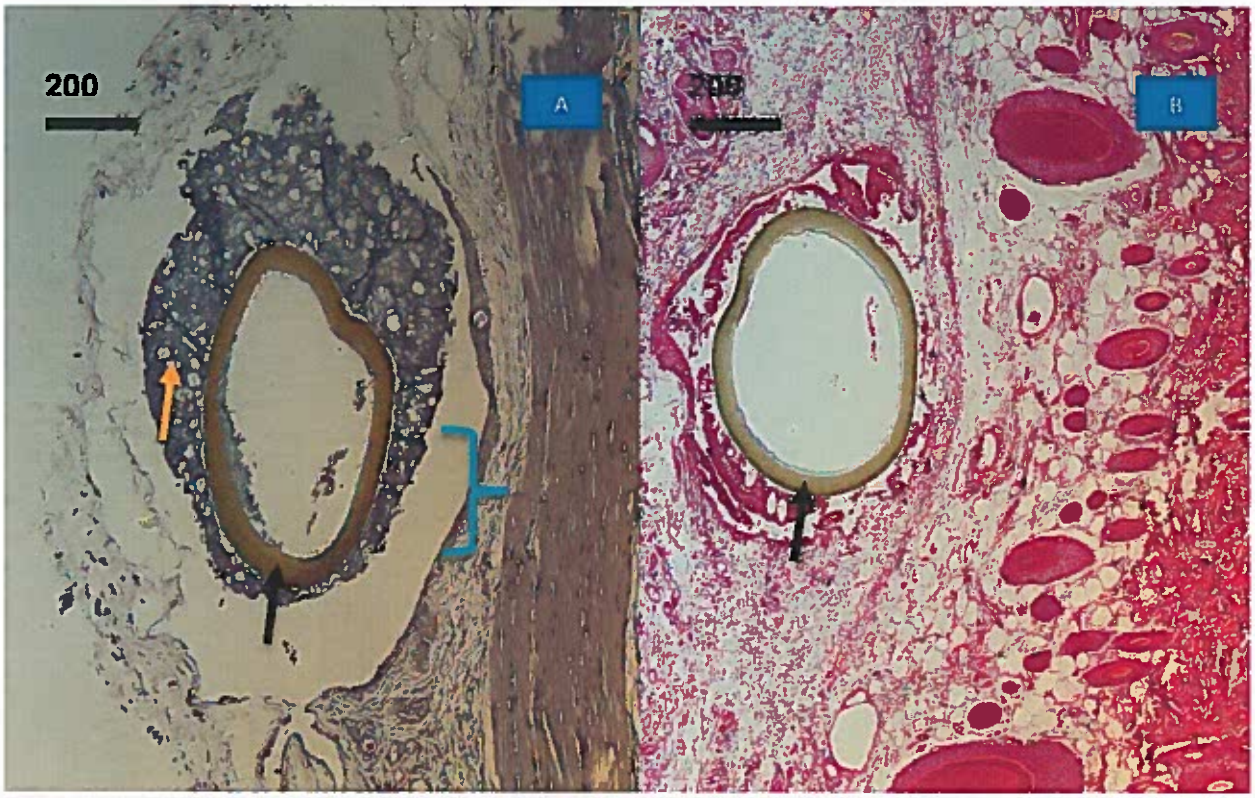

Figure 11. H & E stained samples showing the microdialysis probes, the Formulation 3 coating, and the respective inflammatory response. Figure A depicts the probe with the coating. The blue symbol represents the lack of an immune response. Figure B depicts the probe without the coating. These samples were taken after 5 days of *in vivo* testing. In both pictures, the black arrow points at the outline of the probe. In Figure 11-A the orange arrow points at the PLGA/PVA hydrogel coating.

Section 4:

Concluding Remarks

The aim of the research discussed in this work was to optimize the PLGA microsphere preparation method in order to have reduced drug burst release and lag phase, while concurrently increasing the overall life span of the dexamethasone loaded microspheres. After all of the formulations previously described were comprehensively tested, analysis showed that formulation 3 (1:1 acetone/methylene chloride solvent mixture) had a reduced burst release and resulted in the most continuous drug release profile over a period of approximately 9 days, as compared to the control group and the other formulations.

When looking at the histology images, one can infer that the coating is effective at suppressing the foreign body response. This proves that the *in vitro* data was plausible and that formulation 3 was a great choice to conduct further testing with. After a five day period, no immune agents had engulfed the microdialysis probes. Overall, the histological images presented in this work can now be used as a reference when the biosensor is coated rather than microdialysis tubes.

The data collected through this research will prove to be useful in future microsphere optimization studies. The formulations chosen for this research were selected based on the premise that they would have a positive influence on the overall characteristics of the PLGA microspheres. While some of the formulations portrayed a decrease in drug release lag phase, they failed on reducing drug burst release and maintaining stable release profiles thereafter. Although formulation 3 showed the most promise at meeting our criteria, it is possible that there are other adjustments that can be made to our chosen preparation method that would further improve its effectiveness both *in vitro* and *in vivo*.

Lastly, in terms of lengthening the time frame of the *in vivo* portion of this research, alternative methods of securing the microdialysis tubes to the rat species so as to prevent detachment due to regular movement would need to be looked in to, as this was an issue confronted with in this research. Though three rat models were used in the *in vivo* portion of this research, only one provided viable data as it was the only one that had the microdialysis probes still attached at the end of the one week *in vivo* study. It is possible that an adhesive or a jacket might be solutions to solving the issue.

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