Synthesis and Evaluation of Polysialic Acid-Polycaprolactone Based Micelles for Drug Delivery

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Synthesis and Evaluation of Polysialic
Acid-Polycaprolactone Based Micelles for Drug Delivery

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Abstract

The growing use and investigation of pharmaceuticals to treat disease has offered the field of drug delivery an opportunity to further improve upon the effects provided, as well as address therapeutic complications. An effective long-term treatment for rheumatoid arthritis (RA) has yet be developed, while many current treatments pose disadvantageous side effects due to undesirable reactions outside of the diseased tissue. Therefore, there is a need for an improved strategy for RA treatment. A previously developed drug carrier system based on polysialic acid (PSA) and polycaprolactone (PCL) micelles was shown to be effective for *in vitro* therapeutic use. The evaluated PSA-PCL micelles were capable of passively accumulating in joint tissue. To actively target diseased tissue, the possibility of improving site specificity through the addition of hyaluronic acid (HA) was explored further here. These micelles were synthesized from separate PSA and PCL polymer conjugates, incorporating HA to the micelle backbone. However, initial characterization revealed large, inconsistent, and unstable micelle structures poorly suited for effective drug delivery. Therefore, additional research is warranted to identify and develop targeted, polysaccharide-based micelles.
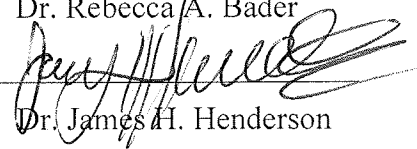
Synthesis and Evaluation of Polysialic Acid-Polycaprolactone Based Micelles for Drug Delivery

Executive Summary

  The pharmaceutical industry is a vast and capitalizing field, with research institutions and universities across the world funneling large amounts of resources into pharmaceutical research. Almost $800 billion is spent on pharmaceutical research and sales within the U.S. annually. The drug development procedure usually spans 10-15 years from the start of initial research through clinical trials, and can require $1-2 billion of initial investment in that time. Therefore, any potential to improve a drug's effectiveness and reduce unfavorable side effects is of medical and economic interest. Controlled pharmaceutical transportation within the body by drug delivery systems can be used to accomplish such objectives. This project aims to evaluate a method to increase drug efficacy while reducing side effects by exploring the viability of adding a tissue-specific targeting element on an existing drug delivery platform.

  Rheumatoid arthritis (RA) is the primary focus of this project, with possible applications to other inflammatory diseases. Although a number of FDA drugs exist for RA management, there lacks a long-term treatment that consistently and effectively alleviates inflammatory symptoms from patient to patient and has minimal side effects. Tofacitinib (commonly known as Xeljanz) is of key interest in this project due to its past clinical success, although many users still experience adverse events. The circulation of the drug throughout the body post-injection has adverse effects as the drug reacts in unintended areas.

Altering inflammatory and immune responses outside of the diseased site can put the patient at risk of chronic infection. Integrating a site-specific carrier system with current RA treatments in order to preferentially localize small molecule therapeutics to diseased tissue will increase drug efficacy while avoiding the complications that arise with non-site specific drug delivery.

Throughout the body, different cells and tissues possess differing attributes that can allow their individual distinction. Specifically, RA synovial fibroblasts (RASFs) are found locally around joint tissue and have a specific receptor for hyaluronic acid (HA). HA is a glycosaminoglycan (GAG), which is a long unbranched polysaccharide consisting of repeating disaccharide monomers. The external receptors on the RASFs allow for potential drug localization through active targeting of an encapsulated drug to these sites by preferentially binding attached HA molecules.

The active targeting of a small molecule drug like tofacitinib involves the use of a carrier system for drug delivery. In this work, the proposed carrier system is a polymer micelle, or self-assembling particle, that can be loaded with a drug and linked with HA. This project focused on the addition of an active targeting element onto an existing micelle system. The new system proposes a mechanism that results in greater site specificity, as well as increased circulation time and decreased degradation by shielding the encapsulated drug. Polysialic acid (PSA) is a hydrophilic saccharide polymer that has ben previously shown to work well as part of a carrier system. Micelles form spherical particles as a result of having a hydrophilic (water-loving) region, and hydrophobic (water-resistant)

iv

region. When in an aqueous environment, the hydrophobic constituents gather as far away from the aqueous surroundings, while the hydrophilic segments assemble to face the outside, thus forming an enclosed sphere. The PSA functions as the hydrophilic element, while polycaprolactone (PCL), a polyester with long carbon chains, acts as the hydrophobic component. Linking a PSA-PCL copolymer with HA is anticipated to result in the ability to preferentially deliver the micelle to RASF receptors.

To synthesize the micelles, PSA and PCL were linked through functional groups along the polymer backbone before HA was similarly added to the polymer chain. The conjugation was performed at a specified percentage of PCL and HA allowing only a small number of sialic acid monomers to be reacted, leaving room for micelle assembly and further additions. The constrained equivalents also facilitated control over the micelle size and physical characteristics. The critical micelle concentration (CMC) is a measure of the density at which the polymers arrange into micelles. This was determined by fluorescence testing, using pyrene as a marker for when micelles formed. As micelles form, they encapsulate the small pyrene molecule, which alters its environment into the hydrophobic inside of the micelle, and shifts the pyrene fluorescent output. When a range of concentrations are used, the shift can be seen, yielding the critical micelle concentration as the inflection point of the curve. Additional metrics used to characterize micelles are size and zeta potential. The size of the micelle effects how the body perceives it. Renal clearance and immunologic systems constantly act to remove the micelle and

encapsulated drug from the body. An optimal size is around 20-200nm, being large enough to avoid small ion filtration, yet small enough to not provoke an immune response. The zeta potential is a measure of the charged attraction/repulsion forces within the micelle solution. In order for stable micelle formation, micelles should possess a zeta potential between an absolute value of 30 and 40 mV.

After completing polymer synthesis, we found the PSA-PCL conjugate polymer showed a low CMC (37 µg/mL) as well as relatively small micelle size (≤50nm), consistent with previous tests on PSA-PCL. The PSA-PCL-HA was similarly characterized, having a critical micelle concentration of around 169 µg/mL and a dispersed size distribution ≥100nm. This increase in CMC and size can be attributed to the HA addition, sterically hindering micelle formation through the size of the molecule. The variable size associated with PSA-PCL-HA suggests inconsistent micelle formation and a lack of reproducibility, traits not conducive to drug delivery. The greatest concern with a larger CMC values is the possibility of dissociation in the body. It is known that when administered to a patient, the micelle-encapsulated therapeutic is readily diluted throughout the body's fluid volume, potentially causing micelle collapse if the CMC is not reached. To address this issue, the PSA-PCL-HA was remade with only half of the initial HA amount. The resulting compound was characterized as before, with very little change in CMC and size measurements. It was therefore concluded that using a single polymer system to incorporate HA did not show potential as a targeted drug delivery system.

In an attempt to address the resulting problems with PSA-PCL-HA conjugation, a novel approach was explored in which separate blocked polymers were made with PSA-PCL and PCL-HA, co-assembled to micelles. The separated HA and PSA components were anticipated to form an aggregate micelle with randomly dispersed PSA-PCL and HA containing regions. The reduced block polymer size would hopefully correlate to a reduced micelle size. In a similar reaction to conjugating PCL to PSA, the backbone carbonyl groups of the HA polymer were used to attach PCL, resulting in an amphipathic (containing both hydrophilic and hydrophobic regions) molecule. A 5wt% combination of PSA-PCL and PCL-HA was made in order to maintain the previous ratio of HA within the micelle structure, and subjected to CMC and size testing. Even greater size distributions were seen with this approach, with possible segregation between the PSA and HA containing polymers.

Each explored method of HA incorporation within the established PSA-PCL micelles revealed material properties that are not well suited for a functional drug delivery system. Micelle formation was characterized by increased CMC values and highly dispersed size. The inconsistent nature of the synthesized polymer micelles posed problems with reproducible results and viability as a therapeutic tool. Although the developed micelle systems were not ideal for drug delivery, the success of previous PSA-PCL micelles warrants further research to identify possible solutions to create targeted polysaccharide-based drug carrier systems.

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Acknowledgements

I'd like to extend a special thanks to Dr. Rebecca Bader and the rest of the Bader lab for the opportunity to pursue this project, and the great amount of help while doing so. It has been a tremendously educational, eye opening, and has given me great respect for all those working in the medical research field. I have greatly enjoyed being a part of the lab and the opportunity to constantly learn.

Advice to Future Honors Students

   To my fellow honors students: As an engineer, I have had the opportunity to be a part of multiple amazing research opportunities. Each has been unique and, dare I say, exceptionally educational. In a scientific or engineering major, early experience is very valuable and motivational as you can apply what you have learned in the classroom. Many struggle to initially get research experience on campus, despite its importance. The advice I would give you is to be proactive in your search for experience. Go to office hours of professors the first week of class your first semester at school. Get to know them, and for them to know you. Show interest in what they do. Now, when you ask to work in their lab, they know you and your character. Getting that first project cannot come soon enough, as that leads to another project, and another, and then with your capstone, you have a full, competitive résumé; this leads to internships (money!) and job offers (lots of money!) or graduate school admissions. Whatever your aspirations, be proactive and go get it.

Chapter 1

Introduction to Drug Delivery & Rheumatoid Arthritis

When addressing ailments of the human body, there has been an increased utilization of directed small molecule pharmaceuticals to target biological pathways and elements.[1,2] The drug development procedure usually spans 10-15 years from the start of initial research through clinical trials, and can require $1-2 billion of primary capital investment over that time.[3] Therefore, any potential to improve a drug's effectiveness and reduce unfavorable side effects is of medical and economic interest. The focus of modern drug design is primarily to identify and target specific abnormality related to the cause of a disease. These irregularities can take many shapes, and require large time commitments to fully understand due to the highly complex mechanisms and signaling pathways they can affect. For example, multiple types of nonmelanoma skin cancers like squamous cell carcinoma overexpress regulatory proteins involved in cell cycle progression. Therefore, a logical treatment path would be to discern the involved proteins and the specific affects of their overexpression. Then, an exclusive small molecule inhibitor could be designed to down regulate that protein, hopefully restoring homeostatic conditions.

With the *in vivo* delivery of small molecule drugs, side effects commonly arise attributed to the non-site specific nature of the treatment. An intravenously delivered drug circulating in the bloodstream will interact with healthy tissue, even with a preferential passive pathway such as increased vasculature leakage or cell division in particular tissues related to certain diseases. A well-known example is the severe nausea and hair loss associated with chemotherapy. Toxic chemotherapeutic drugs have an increased effect on cells with high proliferation rates, most characteristic of the invasive tumorous tissue, however, the fast dividing stomach lining and hair follicle cells are equally effected.[4] Alternate approaches are being explored to potentially alleviate adverse side effects by using a drug delivery carrier system to 1) increase circulation time in the body for passive targeting, 2) prevent therapeutic degradation, and 3) improve treatment site specificity, reducing any related unfavorable effects.

Rheumatoid arthritis (RA) is a chronic autoimmune disease associated with progressive debility and additional medical complications. RA is characterized by chronic inflammation and swelling, with articular joint localization, ultimately leading to joint pain and destruction. Consequently, cartilage, bone, and RA synovial fibroblasts (RASFs) are predominately affected and involved in the mechanisms behind RA origination and development, with chemical linkages to immune cells like B and T cells. Disparities in signaling anti-inflammatory and pro-inflammatory cytokines released by articular cells like RASFs have been particularly well linked to the inflammation and tissue degradation associated with RA.[5-6] They play a central role in the signaling from

local macrophage cells, osteoclasts (and other degradative cells), and immune system elements. Most commonly, signaling molecules such as tissue necrosis factor α (TNF-α) and the interleukin (IL) family (most notably IL-6, IL-8 and IL-1β) are involved as pro-inflammatory immune system promoters, directing leukocytes/ lymphocytes to the joint.[5,7,8]

Additional pathways have also been explored as possible therapeutic targets, involving janus kinase/ signal transducer and activator of transcription (JAK/STAT) signaling. These kinases, JAK1, JAK2, JAK3, act through specific tyrosine residue phosphorylation. Upon phospho-activation, JAK molecules conjugate with and phosphorylate localized STATs, initiating their dimerization and nuclear relocation, where they act as pro-inflammatory transcription factors.[9,10] Consequently, the development of JAK inhibitor drugs has provided a possible treatment path that utilizes a differing approach to traditional therapeutics. One such example is tofacitinib, a JAK inhibitor (JAK1 and JAK3 preferentially) that has shown clinical success and gained FDA approval, although still exhibits adverse side effects in some patients. The positive anti-inflammatory properties of this drug suggests that through localization and controlled administration, the side effects could be greatly reduced while improving drug efficacy.

A modified polysialic acid (PSA)-polycaprolactone (PCL) based drug carrier system is hypothesized to achieve these enhancements. PSA-PCL micelle systems have previously shown success with drug delivery, allowing prolonged circulation times and thus passive accumulation in diseased tissue. The proposed

addition of a targeting element aims to increase site specificity, while benefiting

from advantages of the micelle system. Incorporating a targeting element to the

micelle requires a distinguishing element. In this case, the RASFs contain CD44

receptors that bind hyaluronic acid (HA). By synthesizing a micelle with

conjugated HA at the surface, the micelle could be preferentially targeted to these

cells in the tissue of interest. Therefore, both active and passive targeting could

be used to specify drug accumulation at the site of interest.

Chapter 2

PSA-PCL-HA Polymers: Synthesis Methods

Polysialic acid (PSA) has shown to be an effective hydrophilic foundation for carrier systems, extending circulation times and preventing therapeutic degradation, ultimately improving drug efficacy.[11-14] The amphipathic characteristics for micelle formation are satisfied when linked with the hydrophobic polyester polycaprolactone (PCL). Previously in the Bader lab, this PSA-PCL conjugate has been demonstrated to effectively load and deliver small molecule drugs *in vitro*.[14] Here, additional conjugation methods of a chondrocyte-targeting hyaluronic acid (HA) moiety were performed and similarly evaluated for carrier system potential. Incorporation of HA into the PSA-PCL micelle allows for potential targeting of the drug delivery system to joint tissue, localizing the drug's effect to predominantly diseased tissue.

Materials

Colominic acid sodium salt (PSA, isolated from E. coli, MW 30 kDa) was obtained from Nacalai USA (San Diego, CA). N-(3- Dimethylaminopropyl)-N0-ethylcarbodiimide (EDCI), N,N0-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), ethylenediamine, m-chloro-peroxybenzonic acid,

benzyl alcohol, 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD), e-caprolactone (e-CL), and Boc-gly-OH were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. (Dimethylamino)pyridine-p-toluensulfonic acid (DPTS) was formed from hydrated p-toluenesulfonic acid and 4-(dimethylamino) pyridinium following a known protocol.[14] Trifluoroacetic acid (TFA) (peptide synthesis grade), HPLC grade acetonitrile, dichloromethane, and anhydrous DMSO were also obtained from Sigma-Aldrich. Hyaluronic Acid (50K MW) was obtained from Creative PEGWorks (Chapel Hill, NC).

PCL-Amine Synthesis

As shown in Figure 1, PCL-OH was transformed into an amine-terminated variant in order to improve amide bond connectivity to PSA, done through a Boc-glycine intermediate structure. 5.2 mg of DCC, 7.3 mg DPTS, and 4.4 mg Boc-glycine-OH were dissolved in 12mL of distilled dichloromethane (CH_2Cl_2), stirring under a nitrogen atmosphere. 200 mg of PCL-OH (.05mmol) were added and allowed to stir for 48 hours at room temperature. DCC activates the Boc-glycine to be conjugated to the PCL through the terminal hydroxyl group acting as the nucleophile with the carbonyl group. The PCL-Boc complex was added drop-wise to 300 mL of cold methanol, precipitating out the product. After filtration, the product was dried at 75°C under vacuum for 24 hours.

The amine product was obtained by deprotection of the Boc group. The PCL-Boc was re-dissolved in 12mL CH_2Cl_2, 12 mL TFA was added via syringe, and the solution was stirred under nitrogen for 2 hours. The final product was obtained by rotary evaporation to remove excess CH_2Cl_2, followed by drying in

vacuum oven at 75°C for 48 hours, resulting in a slightly red transparent solid. The process had a yield of 79.4% and successful Boc addition and deprotection was confirmed by ^{1}HNMR.
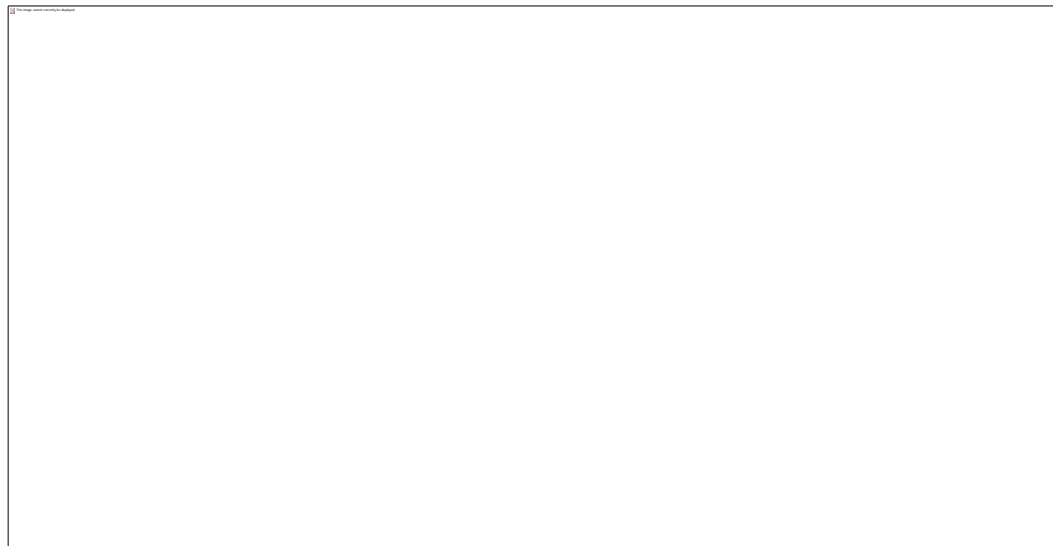

Figure 1. The synthesis outline of amine-terminated PCL

PSA Ion Exchange

With the primary colominic acid sodium salt, the sodium ion was exchanged with tetrabutylammonium using ion exchange resin. 600 mg of Dowex 50 WXZ resin was mixed with 750 mg tetrabutylammonium bromide (TBAB) (2.26 mmol) in 15 mL DI water for 3 hours. The resin particles were vacuum filtered and washed with DI water to remove excess TBAB. The resin was added to 10 mL of 2% aqueous PSA (weight/weight) (200mg PSA, 0.65 mmol sialic acid monomer). This mixture was stirred overnight at room temperature. After stirring, the mixture was filtered to remove the resin and the flow-through was lyophilized to obtain the final product, as seen in Figure 2.
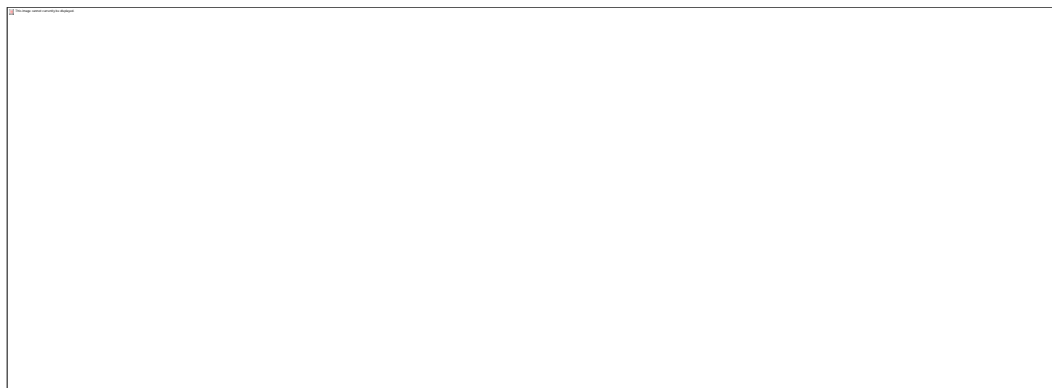
Figure 2. Synthesis outline for PSA t-butylammonium salt

PSA-PCL Conjugation

Amine terminated PCL was conjugated to the PSA chain to complete the micelle complex as shown in Figure 3. Initially, 109 mg of PSA (.1965 mmol monomer) was dissolved in 10 mL anhydrous DMSO with 4.5 mg DCC and 6.4 mg DPTS (.0197 mmol, .1 eq each) and stirred under nitrogen for 1 hour to activate the carbonyl groups for addition. 159 mg PCL (.0197 mmol, .1 eq) was dissolved in 10 mL CH_2Cl_2 simultaneously and stirred, for a 10% PCL conjugation. After 1 hour, the PCL solution was added to the PSA via canula and stirred for 48 hours.

The reaction was stopped, filtering the solution through ~2cm diatomaceous earth to remove urea side product. The off-white opaque flow through was transferred to a 6-8,000 dalton molecular weight cut-off dialysis membrane, and dialyzed against 50 mM NaCl for 24 hours, then dialyzed against DI water for another 24 hours. The product was next lyophilized, yielding a white solid. Upon ^{1}HNMR analysis, excess PCL was found in the product, which was subsequently dissolved in excess DI water (25 mL) and filtered through a medium

filter, removing an off-white solid. The cloudy flow-through was re-lyophilized, yielding a purer PSA-PCL product.
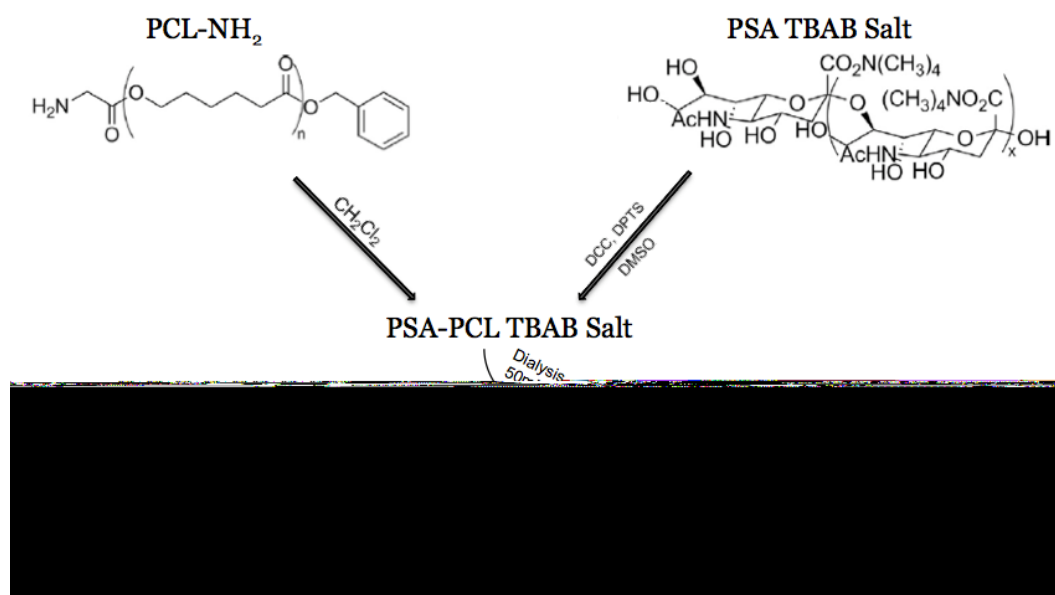

Figure 3. PSA-PCL synthesis scheme

HA Addition

Following PSA-PCL synthesis, similar carbonyl addition chemistry was used to attach the HA ligand through a dihydrazide linker as in Figure 4. 125 mg of PSA-PCL (.321 mmol) was dissolved in 20 mL DI water, and 10 mg EDC (.0643 mmol, .2eq), 7.4 mg NHS (.0643 mmol, .2 eq), and 5.6 mg ADH (.0321 mmol, .1 eq) were added and allowed to stir, targeting a 10% ADH conjugation. EDC/ NHS chemistry was used as an alternative to the DCC/DPTS used in PSA-PCL conjugation, in order to capitalize on the amine nucleophile from the ADH linker, as opposed to the hydroxyl of the PCL-OH. 1M HCl was added drop-wise to adjust pH to 4.7, and the solution was allowed to stir at room temperature for 2 hours. The pH was normalized to 7.0 with NaOH. To utilize the other end of the amide linker, 10 mg EDC (.0643 mmol, .2eq), 7.4 mg NHS (.0643 mmol, .2

eq), and 96.3 mg HA (.0321 mmol, .1 eq) were stirred in 10 mL DI water for 30 minutes at room temperature to activate the HA carbonyl groups. The solution was added to the PSA-PCL-ADH solutions and stirred overnight, resulting in a cloudy white solution, but with no precipitates. The solution was added to a 6-8,000 dalton molecular weight cut-off dialysis membrane to aid in product isolation and solvent exchange, and then dialyzed against DI water for 24 hours before lyophilization to obtain the final product.

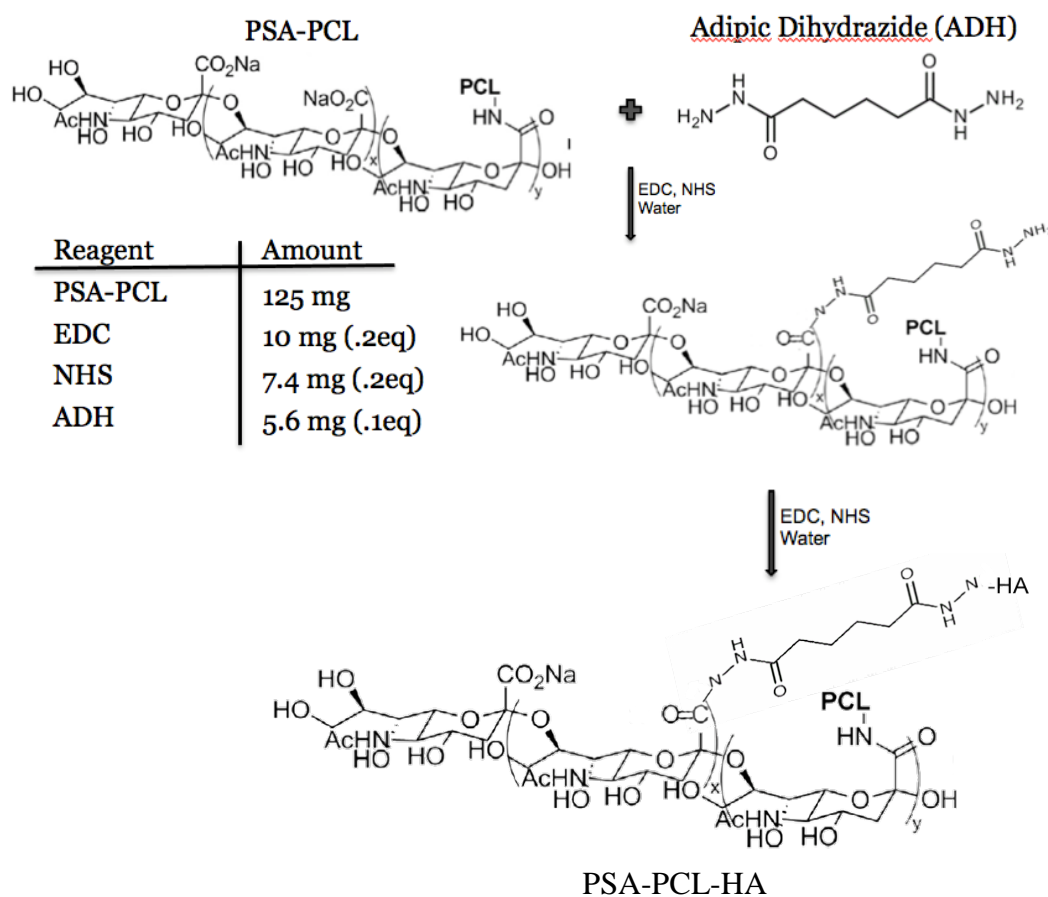| Reagent	Amount
PSA-PCL	125 mg
EDC	10 mg (.2eq)
NHS	7.4 mg (.2eq)
ADH	5.6 mg (.1eq)

PSA-PCL-HA

Figure 4. Synthesis scheme for ADH & HA addition to make PSA-PCL-HA

Additionally, PSA-PCL-HA was synthesized with only 5% HA conjugation in order to evaluate the effects of altered HA content on micelle properties. Smaller individual particles were hypothesized to yield smaller

micelles and facilitate micelle assembly. This was achieved by scaling the ADH

and HA reagents in the previously described reaction by .5 (along with EDC and

NHS components).

An alternative novel approach was also explored in regards to HA addition

into the PSA-PCL micelle system. Separate amphipathic copolymers, PSA-PCL

and PCL-HA, were synthesized and then combined to create a random aggregate

micelle with HA containing regions, PSA-PCL[PCL-HA]. The significantly

reduced polymer size and steric mobility was hypothesized to lower size and

CMC values. As seen in Figure 4., a similar reaction to PSA-PCL conjugation

was performed to link PCL and HA.   37.9 mg 50k MW HA (.1mmol, 1eq) was

stirred in ~8mL DMSO under nitrogen for 1 hour with 2.1 mg DCC (.01mmol,

.1eq) and 3 mg DPTS (.01mmol, .1eq) to activate the HA carbonyl groups. 80 mg

PCL-NH$_2$(.01mmol, .1eq) was simultaneously dissolved in equal volume CH$_2$Cl$_2$.

The mixtures were combined to form a hazy solution, but with no distinguishable

particles, and stirred under nitrogen for 48 hours. The reaction solution was

filtered through diatomaceous earth over a fine fretted filter, and rinsed

thoroughly with 1:1 DMSO and CH$_2$Cl$_2$, yielding a relatively clear flow through.

The flow through was rotary evaporated to reduce the solvent volume and then

dialyzed against DI water for 24 hours to trade solvents, exchanging bath after 2,

4, and 8 hours. As the dialysis progressed, an opaque white solution formed, but

with no precipitates. The product was lyophilized to obtain a white clumpy

powder. H^{1}NMR analysis revealed excess unbound PCL-NH$_2$. Following

suspension of the product in DI water, repeated washing in a separatory funnel

with CH₂Cl₂ followed by lyophilization of the aqueous layer purified the final

product. A yield of 38% was achieved after the subsequent purification steps.

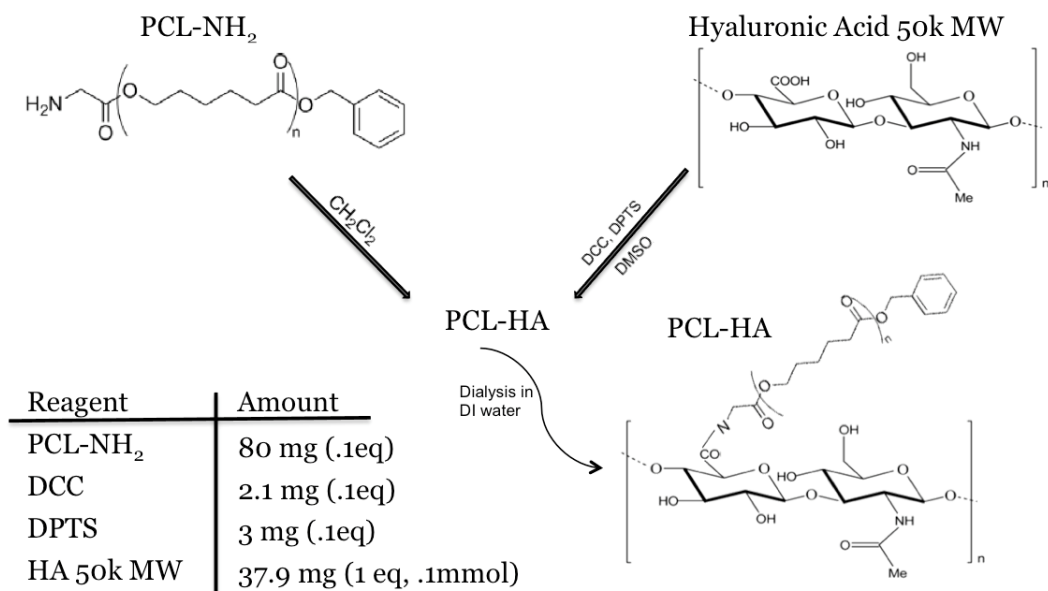| Reagent	Amount
PCL-NH$_2$	80 mg (.1eq)
DCC	2.1 mg (.1eq)
DPTS	3 mg (.1eq)
HA 50k MW	37.9 mg (1 eq, .1mmol)

Figure 5. Synthesis scheme for PCL & HA conjugation to yield PCL-HA. This could be used in a controlled combination with PSA-PCL.

Chapter 3

PSA-PCL-HA Polymers: Characterization Methods & Results

Following synthesis of PSA-PCL-HA, characterization methods allow

elucidation of pharmaceutical properties.

Critical Micelle Concentration (CMC)

In order to assess the compound's ability to spontaneously assemble into

micelle particles, the CMC was found to quantify the minimum concentration at

which micelles form. A 1.24×10^{-2} M pyrene stock was made in acetone, and 8.1

µL (1nmol) was added to 9 glass vials. The vials were placed on a heating plate

at 65°C for 15 minutes to evaporate the solvent. Simultaneously, a 2x dilution

series of PSA-PCL and PSA-PCL-HA was made from 1mg/mL to 3.9µg/mL,

initially dissolving 2 mg of product in 2 mL DI water. The solutions were

transferred to the pyrene-containing vials and sonicated for 30 minutes, then

stirred at 65°C for 4 hours and cooled overnight in the dark to equilibrate the

solutions. A fluorescence excitation scan was performed with solutions in a

quartz cuvette, with an excitation scan from 300-380 nm and emission wavelength

of 390 nm. The hydrophobic and hydrophilic peaks were seen at around 320 and

335 nm. The log of the concentration was plotted against the ratio of the peaks at

337 and 334 nm (shifting in hydrophobic peak), and the CMC was determined to be the intersection of the tangent of the inflection point with the horizontal tangent of the low concentration points. PSA-PCL had a CMC of ~40 μg/mL, comparable to previously seen values.[14,16] PSA-PCL-HA had a CMC of 205 μg/mL, roughly 5 times the value without the HA ligand. Upon lowering the HA conjugation to 5%, a CMC of 169 μg/mL was seen.
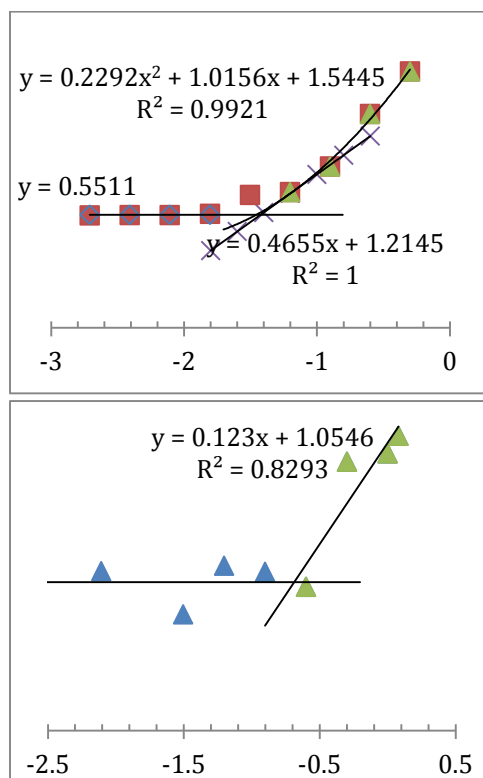

Figure 5. The CMC results for PSA-PCL (left) and PSA-PCL-10%HA (right), showing tangent lines for CMC calculations. X-axis is log(polymer concentration), y-axis is the

Size and Zeta Potential

Size and zeta potential of PSA–PCL and PSA-PCL-HA micelles were obtained as previously done[14] with a Zetasizer Nano ZS (Malvern Instruments). Aqueous samples were prepared by dissolving samples in DI water. For dynamic light scattering, the temperature for measurement was 25°C, and the angle of

scattered light was 173°. Micelle sizes are reported as the mean of the sizes derived from the number distributions, while size distributions are reported as the mean of the polydispersity indices (PDIs). PSA-PCL had a size of 35-50nm, low PDI near .08, and zeta potential of -33. This correlated to previously achieved values for PSA-PCL.[16] PSA-PCL-HA showed a highly distributed size around 100 nm, PDI of .4, and zeta potential of -20mV. Upon reduction of HA to 5% PSA conjugation, similar results were seen, with the observed size, PDI, and zeta potential around 100nm, .4, and -22mV, respectively. The large size distributions of PSA-PCL-HA required filtering through a .5µm filter to remove larger particles in the solution outside the range of detection of the instrument used.

PCL-HA was hypothesized to create an aggregate micelle with smaller individual assembled polymers. After initial synthesis, a 5 wt% PCL-HA in PSA-PCL solution was made for CMC analysis, yielding the PSA-PCL[PCL-HA] aggregate micelle solution. A 1mg/mL solution of the mixture was subjected to size and zeta potential testing after filtering through a 5µm cell culture filter. A double-peaked size distribution was seen with peaks near 85nm and 505nm. The sample had a zeta potential of -33mV.
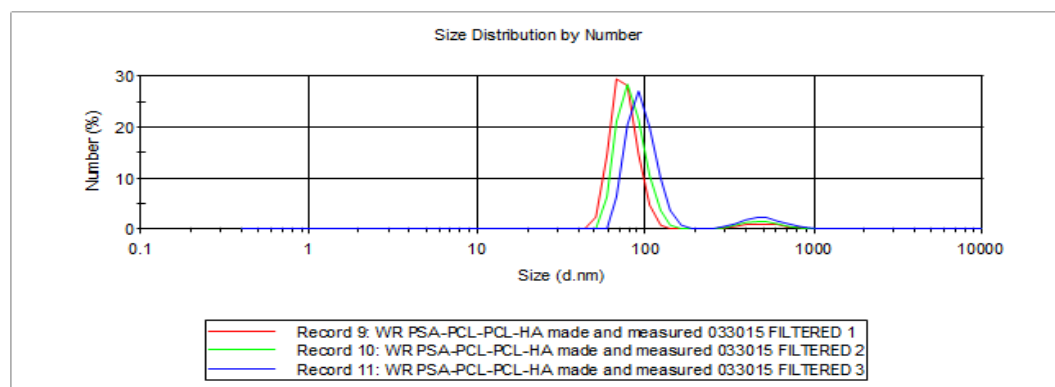
Figure 6. Size distribution for filtered solution of 5wt% PCL-HA and PSA-PCL micelles, showing bimodal peak distribution.

FILTERED	Peak 1	%	Peak 2	%	PDI	Z-Average
Run 1	498.1	0.052	75.49	0.948	0.371	537.5
Run 2	505.2	0.077	83.37	0.923	0.451	524.7
Run 3	512.8	0.115	96.37	0.885	0.428	531.5
Average	505.4	0.081	85.08	0.919	0.417	531.2

Table 1. The corresponding peak parameters, characterizing the PSA-PCL[PCL-HA] peaks. Note the 5-10% peak integral for the 500nm micelles.

Chapter 4

PSA-PCL-HA Discussion and Continuing Approach

The synthesis of PSA-PCL, as well as successful conjugation of HA to the PSA-PCL backbone, was verified by H^1NMR. PSA-PCL-HA synthesis demonstrated the ability to attach HA to an amphipathic micelle molecule in a controllable fashion through a symmetrical linking group. The subsequent characterization revealed that the HA moiety has an affect on both micelle assembly and physical properties. Compared to PSA-PCL, having a size of 35-50nm, HA containing micelles increased in size over two fold to near 100nm. A much wider range of sizes were observed as well, with a dispersed peak containing a small quantity of micelles over 1μm. The larger PSA-PCL-HA molecule was predicted to result in an increased micelle size due to the increase in polymer size, however the extent of the change in size was not anticipated. Even

with a decrease to 50% initial HA conjugation (.05 eq to PSA), significantly

larger particles were seen over a broad range. Similarly, CMC values increased

over 5 fold with the addition of HA to the PSA-PCL copolymer. The random

addition of PCL and HA to the PSA chain may sterically interfere with the

polarity separation during micelle formation, leading to less favorable micelle size

and polymer assembly. Although HA incorporation unfavorably affected micelle

size and CMC, the zeta potential was

Increases in size and CMC with HA incorporation pose problematic

characteristics for drug delivery. When administered *in vivo*, micelles must be an

adequate size within the vasculature so as to avoid renal clearance and

reticuloendothelial system recognition. Additionally, a low CMC is required to

maintain micelle stability post-administration, where a stock concentration is

effectively diluted to the *in vivo* aqueous volume. The novel approach to

construct an aggregate copolymer micelle was explored in order to possibly

facilitate micelle formation and reduce individual polymer and micelle size while

maintaining controllable incorporation of HA.

PCL-HA synthesis was successfully achieved by amide bond formation

between PCL and the activated carbonyl group of the glucaronic acid saccharide

in the HA monomer. A controlled ratio of PCL-HA and PSA-PCL could be

achieved through the mixture of known aqueous concentrations. After making a

5wt% PCL-HA in PSA-PCL solution, CMC testing revealed inconclusive results

with no definitive pattern in pyrene spectra shifting. Upon size and zeta potential

analysis, a bimodal size distribution was seen. The two peaks were near 80 and

500nm, suggesting somewhat similar micelle composition to PSA-PCL with an additional mechanism resulting in larger micelles. The lower PDI with two separate peaks than with PSA-PCL-HA alone suggested adequately formed micelles, further supported by an acceptable zeta potential of -33mV. However, micelle formation may not have occurred as predicted. The integration of the peaks revealed approximately 5-10% of the particles forming the larger micelles. This correlated well with the 5% PCL-HA in solution. A suggested mechanism involves the segregation of each copolymer (Figure 7), preferentially assembling as separate micelles. The size difference between PSA-PCL (~30k MW PSA) and PCL-HA (50k MW HA) could account for the behavior if the steric interactions were unfavorable to integration.
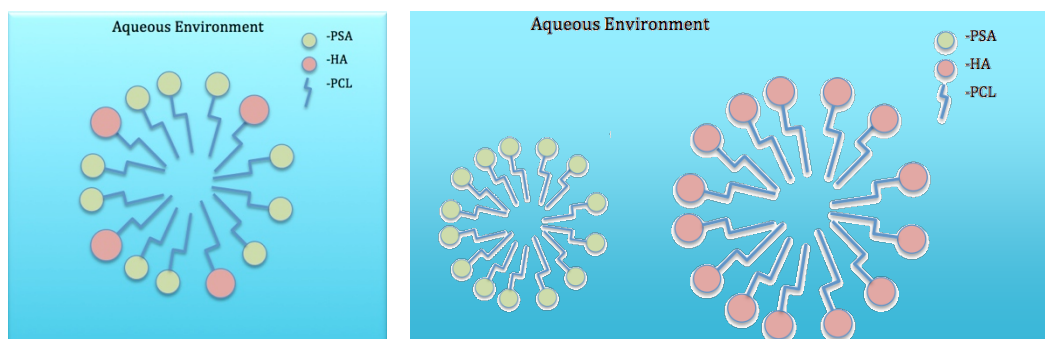

Figure 7. The anticipated micelle structure with the aggregate polymer approach (left) versus the proposed actual formation (right).

Continuing Approach

Additional characterization of HA containing PSA-based micelles will initially be necessary to optimize their assembly structure and synthesis. Both the single and aggregate copolymer systems show promise as a drug delivery modality. PSA-PCL-HA demonstrated micelle formation with slightly higher size

and CMC, while PSA-PCL[PCL-HA] has potential for more ideal properties, yet initially showed erratic micelle behavior. To address PSA-PCL[PCL-HA] aggregation, the compatibility of the copolymers to form a joint micelle with equal dispersion could be further investigated by altering the relative PSA and HA polymer sizes. The described possible mechanism of size separation could be evaluated by copolymer synthesis with varying PSA and HA polymer sizes in PSA-PCL and PCL-HA, respectively. Similar size, zeta potential, and CMC characterization could be compared to previously synthesized systems as described here, indicating optimal drug delivery performance.

Following the development of an HA-containing drug delivery system, drug loading and elution assays will reveal the performance and viability of the system after drug encapsulation. The ability to form a stable micelle with proper qualities would suggest similar outcomes with an encapsulated drug, however previous studies have revealed a trend of increased micelle size.[14,17,18] *In vitro* behavior can be evaluated barring successful drug loading characteristics. This includes cytotoxicity, cellular uptake, and ultimately protein expression levels being targeted by the drug.

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