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Synthesis and Characterization of Hyaluronic Acid Based Biomaterials for Drug Delivery and Virus Modification

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SYNTHESIS AND CHARACTERIZATION OF HYALURONIC ACID BASED BIOMATERIALS FOR DRUG DELIVERY AND VIRUS MODIFICATION

by:

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DEDICATION

For you, Glenda. My person, my angel, my protector, my ride or die, my best friend, who just also happened to be my mom. You will never be forgotten. “Love you forever.”

ACKNOWLEDGEMENTS

I would like to first thank my two beautiful Maine Coons, Grendel and Dren (my little miracle baby and fighter). Thank you for showing me unconditional love and for helping me get through each day. You both are my absolute world and I don't think I would still be here if it wasn't for you two.

Second, I would like to thank my sister, brother, and dad for being supportive of my crazy endeavors and putting up with me. Thank you for being stuck with me.

I would also like to thank Mal, Selina, Kayla, Courtney, Poonam, Katya, and Steven for being there for me through this chaos. In addition, thank you to those not specifically mentioned who I also consider my friend or help along the way.

Lastly, to you, "May the bridges I burn light the way."

ABSTRACT

In this thesis, hyaluronic acid based biomaterials are studied. The properties and potential applications of these polymers are characterized and discussed.

In Chapter 1, biomaterials are introduced as well as hydrogels and nanogels. Hyaluronic acid is highlighted as a remarkable bio-polymer. The overall objectives of my research are also discussed.

Chapter 2 is focused around trials for utilizing graphene oxide as a nanomaterial for controlling the drug release of an aromatic compound. Hyaluronic acid based hydrogels were synthesized as a delivery vessel. The results of the drug release profiles are examined and discussed.

A different direction is taken in Chapter 3. A nanogel is formed using hyaluronic acid and a thermoresponsive polymer. The synthesis of the grafted polymer itself as well as the nanogel are outlined and the characterization of each are discussed.

In Chapter 4, two methods for surface modification of virus particles are discussed. In one approach, a boronic acid grafted hyaluronic acid polymer is synthesized and characterized. In another approach, "click" chemistry is utilized as an alkyne-terminated polymer is synthesized and characterized for conjugation with an azide-modified virus.

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CHAPTER 1: GENERAL INTRODUCTION

1.1.Biomaterials

Biomaterials can be defined as any matter or construct that is able to interact with biological systems.[1] These materials can be comprised of natural or synthetic components and are generally used in medical applications to aid in healing after disease or injury.[1] Polymers, metals, glass, and even living cells or tissue all can be used as components when making a biomaterial. These components are often combined and reengineered into coatings, films, gels, foams, or fabrics for biomedical use.[1] Biomaterials are not only biocompatible, but they are also often biodegradable. Some applications where biomaterials are currently being used include drug delivery systems, tissue engineering, biosensoring, imaging, and medical implants.[1]

1.2. Hyaluronic Acid

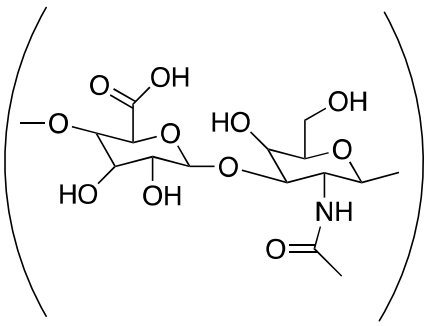


Figure 1.1. Structure of hyaluronic acid

A biopolymer which is being utilized in the field of biomaterials is hyaluronic acid (HA). Hyaluronic acid is a naturally occurring glycosoaminoglycan consisting of alternating N-acetyl-D-glucosamine and D-glucuronic acid units.[2] HA is a major component of the extracellular matrix and is also found in bodily fluids and connective tissues. HA is responsible for playing a part in several vital roles in the body such as cell differentiation, tissue hydration and lubrication, and nutrient diffusion.[2,3] Due to HA's biocompatibility, biodegradability, and unique viscoelastic properties, HA has often been used in drug delivery, tissue engineering, and cell encapsulation.[2,3] Cross-linking HA with copolymers provides the ability for the formation of hydrogel or nanogel networks.

1.3. Hydrogels and Nanogels

Hydrogels are three-dimensional networks comprised of cross-linked, water-soluble polymers. A variety of crosslinking methods are currently being employed to synthesize hydrogels such as Michael-addition reactions, Schiff-base formations, and photopolymerization.[4] These hydrogel networks can be classified into two categories based on the nature of the cross-linking: physical networks and chemical networks. Physical networks are based upon non-covalent interactions such as hydrogen bonding, ionic interactions, or hydrophobic interactions. On the other hand, chemical networks are held together by covalent bonds formed from the polymer chains.[4]

Hydrogels are currently being used in a wide range of applications. Some of these applications include pharmaceuticals, food additives, tissue engineering, drug delivery, cellular encapsulation, biomedical implants, and biosensors[5]. Hydrogels have also been recently used in the making of artificial muscles, contact lenses, wound dressings, and super absorbent materials.[5]

Nanogels are comprised of small, nanoscale sized hydrogel like materials on the nanoscale range.[6] A major advantage of nanoscale hydrogels is the ability to form emulsions which can be systemically delivered to organs, tissues, and cells that are difficult to reach. Another advantage of nanogels over bulk hydrogels include the ability for rapid pharmacokinetics.[6] Nanogels are globular particles, however, advances in new synthesis strategies allows for different shapes.[6] They are also capable of being designed to have a core-shell or a core-shell-corona structure.

Due to the fact that these gels are highly swollen and can incorporate 30% wt. or more of drugs or biological materials, nanogels have high loading capacities which is advantageous in applications where cargo is needed to be entrapped and stabilized.[6] The cargo is held in place within the nanogel through either hydrophobic, electrostatic, or van der Waals interactions or through chemical means such as covalent bonding. Recent attention has been gained for nanogels in the field of drug delivery, diagnostics, imaging, glucose sensing, and tissue engineering.[6]

1.4. Research Objectives

The first goal of this work was to create a hyaluronic acid based hydrogel using "click" chemistry with controlled drug releasing abilities. Graphene oxide was employed to serve as a nanomaterial to control drug release of an aromatic based drug.

The second goal of this work was to synthesize a thermoresponsive hyaluronic acid based nanogel. This study first involved grafting poly-n-isopropylacrylamide (pNIPAM) onto the backbone of a hyaluronic acid polymer, followed by the synthesis of the corresponding nanogel.

The third goal of this work was to develop grafted biopolymers for the application of encapsulation and delivery of the vaccinia virus (VACV). There were two methods: (1) to synthesize HA-g-$B(OH)_2$ for the linking to VACV via formation of a boronate ester on the surface of VACV and (2) DBCO-pNIPAM to be used as a "smart" coating of the VACV via "click" chemistry.

CHAPTER 2: GRAPHENE OXIDE FOR CONTROLLED RELEASE OF AROMATIC BASED DRUGS

2.1 Abstract

In this study, the goal was to confirm graphene oxide as a nanomaterial for controlled drug delivery. A hyaluronic acid based hydrogel was synthesized and used as the delivery vessel for the hydrophobic drug, resveratrol. Graphene oxide (GO) was incorporated into the drug delivery system via π-π stacking. Drug release profiles were then studied.

2.2. Introduction

Due to its unique physical properties, biocompatibility, and biodegradability, hyaluronic acid (HA) has recently been studied for the use in localized and controlled drug delivery applications.[2-3] HA hydrogels allow the loading of drugs due to porosity and swelling, and the release of drugs depending on a diffusion coefficient of the small molecule. These hydrogels still have limitations such as non-homogeneous loading of the drug and burst drug release due to the high water content and large pore sizes of the network. [3] These limitations drastically reduce the practical use of HA hydrogel-based drug deliveries in the clinic setting.

In order to overcome the pharmacological limitations of HA hydrogel-based drug deliveries, modifying the microstructure of the hydrogel,[5] or incorporating nanoparticles can be performed. [7] Recently, the introduction of nanoparticles for the purpose of controlling drug release has gained a lot of attraction.[7] Graphene oxide is a nanomaterial

whose structure and delocalized surface π electrons can be used for drug loading via π–π stacking, electrostatic, or hydrophobic interactions.[8]

The delivery of hydrophilic drugs has been most studied because of their compatibility with the hydrophilic hydrogel and the aqueous solvents.[6] Thus, the loading capacity and subsequent release are easy to be studied. Hydrophobic drugs, present a greater challenge. Non-homogeneous drug loading and burst, or rapid, release are often observed.[6] A previous study utilizing an aromatic drug, Senexin A, showed successful incorporation of GO into the HA system with controlled release of Senexin A.[9] The interaction with SNX and GO was via π-π stacking and H-bonding.[9]

Resveratrol (RSV) is another aromatic drug and is commonly cited as the main active polyphenol in red wine.[10] RSV is an antioxidant stimulates cell proliferation through protection against protease enzymes, increase of fibroblast function, and increase of collagen formation.[10] However, resveratrol exhibits several drawbacks including poor stability, low aqueous solubility, degradation to pH, light, and temperature. RSV also has poor oral bioavailability and is extensively metabolized in the body.[10]

The goal of this study was to create a synergistic hydrogel system consisting of hyaluronic acid, resveratrol, and graphene oxide for controlled drug release. HA serves as the degradable biocompatible matrix to house the GO and drug conjugate through H bonding. First, the nanocarrier was synthesized by adhering RSV to GO via π–π and electrostatic interactions. Next, the *in situ* encapsulation of GO-RSV was performed with HA-based hydrogel. The drug release test was then determined.

2.3. Experimental

2.3.1. Materials and Instrumentation

All chemicals were purchased from VWR, Sigma Aldrich, and Click Chemistry Tools, LLC. ^{1}H NMR spectra were recorded on a Brucker ARX300 spectrometer. UV−vis spectrophotometry was done on a Nanodrop 2000.

2.3.2. Experimental Procedures

2.3.2.1.Preparation of Drug-Loaded Graphene Oxide

Resveratrol (RSV) (2 mg) and GO (2, 4, 6, 8 mg) were added to 2, 4, 6, and 8 mL Kphos buffer respectively (pH 7.4) (sonicated together for 2 h) and stirred for 24 h at 4 °C in darkness. The product (RSV-GO) was collected by centrifugation at 3000 rpm, 4 °C, for 20 minutes and washing with PBS until the supernatant became color free. The resulting RSV-GO pellet was freeze-dried. UV-Vis was used to measure the amount of unbound RSV at an absorbance of 306 nm from the supernatant. The measurements were performed relative to a calibration curve recorded under identical conditions, which then allowed for the drug loading efficiency to be estimated.

The RSV-loading efficiency was calculated as follows, where $W_{feedRSV}$ is the loaded amount of RSV and $W_{freeRSV}$ is the amount of unbound RSV in the supernatant:

$$\text{RSV-loading efficiency \%} = 100\ (W_{feedRSV} - W_{freeRSV})/\ W_{feedRSV}$$

2.3.2.2.Characterization of Graphene Oxide and Drug-Loaded Graphene Oxide

GO and RSV-GO were characterized by UV−vis spectrophotometry (Nanodrop 2000), and Scanning Electron Microscope.

2.3.2.3. Formation of Drug-Loaded Graphene HA hydrogels

Methacrylated modified HA polymer was first synthesized at approximately 50% molar modification based on monomer unit (50DM MeHA) following previous reports.[11] The RSV-GO was dispersed in 1x PBS at 0.1% w/v by sonication for 2 h. Next, the 50DM MeHA was added into the GO-RSV suspension at concentration of 3%. Once the polymer completely dissolved in GO-RSV mixture, 0.5 M dithiothreitol (DTT) was added into the mixture to form the hybrid GO-HA-RSV hydrogel.

2.3.2.4. Drug Release

Hydrogels were formed as described in Section 2.3.2.3 and placed in 96-well plates with 100 μL of release PBS, covering the hydrogel. The amount of released RSV in the withdrawn PBS was determined by UV- vis spectra at 306 nm. The PBS was then removed and replaced every 24 h at an equal volume. The drug release profiles were performed until RSV no longer released. A standard curve of RSV concentration versus absorption peak intensity (at 306 nm) was established before performing the RSV release.

2.4. Results and Discussion

2.4.1. Preparation of Drug-Loaded Graphene Oxide and Drug-Loaded Graphene HA Hydrogels

The sub-microsized GO was created by further sonicating GO, providing a size range around 500 nm to 10 μm, as verified by SEM imaging (Figure 2.1).

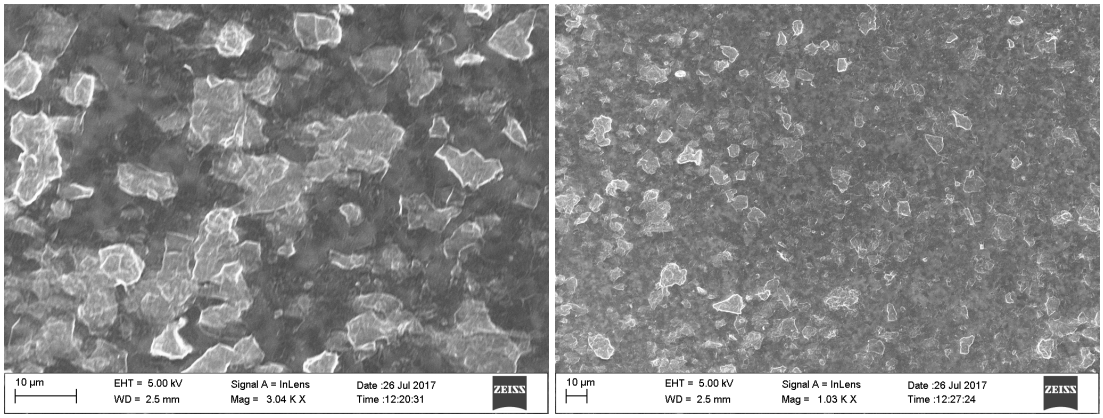


Figure 2.1. Scanning electron micrograph (SEM) of the graphene oxide after sonication for 2 h.

The GO was then mixed with RSV at different weight ratios (Figure 2.2A) in PBS for 24 h at 4 °C to generate RSV-loaded GO (GO-RSV). RSV could attach to GO at different loading ratios with almost 100% loading efficiency (Figure 2.2B).

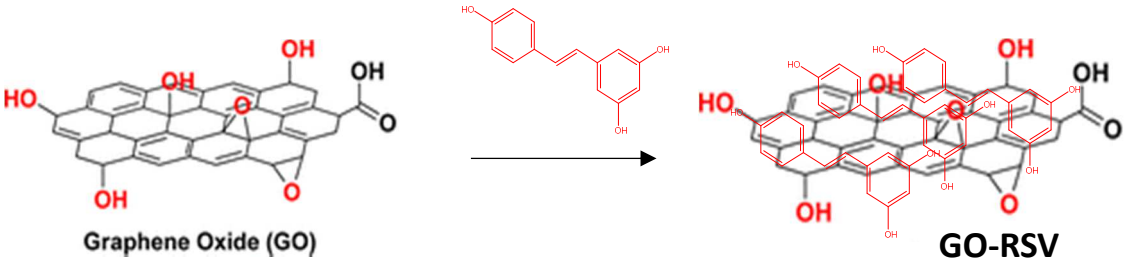
The UV-Vis spectrum of GO-RSV showed the absorbance at both 230 and 306 nm, which corresponded to the absorbance peaks of GO and RSV respectively (Figure 2.2C).

Lastly, the GO-RSV HA hydrogel was formed by mixing 3% of MeHA polymer with 0.1% of GO-RSV in PBS and adding DTT (Figure 2.3).

2.4.3. Drug Release of Resveratrol-Loaded Graphene HA Hydrogels

Next, an investigation took place to determine if RSV could be delivered and released in a controlled fashion from the GO-HA hydrogels. It has been reported that drug release from a GO nanocarrier depends multiple factors such as pH, GO sizes, and interactions between drug and GO carriers.[8]

A



B

GO:RSV (mg:mg)	306nm (% eff)
4:1	100
3:1	99.99
2:1	100
1:1	99.97
1:2	99.99
1:3	99.99
1:4	99.99

C

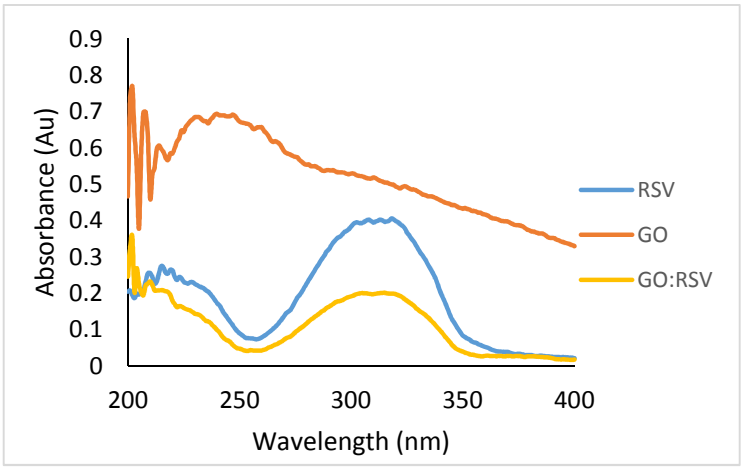


Figure 2.2. Preparation and characterization of Resveratrol-loaded graphene oxide. (A) Schematic illustrating the preparation of Resveratrol-loaded graphene oxide (GO-RSV). (B) . Loading efficiency of RSV on to GO at different weight ratios (n = 3) (C) UV-Vis of GO, RSV, and GO-RSV.

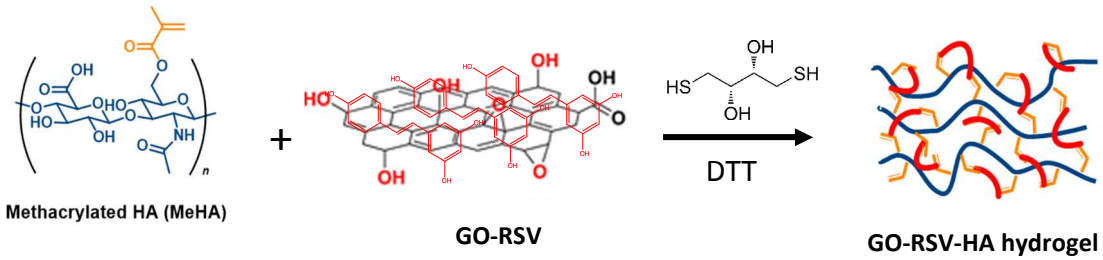


Figure 2.3. Schematic illustrating the synthesis of Resveratrol-loaded graphene HA hydrogels via thiol–ene "click" reaction.

In this research, the release behavior of RSV from GO-RSV HA hydrogels was done in PBS with pH 7.4, which represents normal physiological pH. The release of RSV without GO from RSV-HA hydrogels was also studied. Figure 2.2A shows that the RSV released from the RSV-HA hydrogels reached equilibrium within 6 h at concentration of 220 μM in PBS, while a concentration of 160 μM was achieved in GO-RSV HA hydrogels. The release of RSV from both the RSV-HA and GO-RSV HA systems showed a burst release followed by a maintained release (Figure 2.5B). The cumulative release of RSV from RSV loaded HA-based hydrogels was also the same as the GO-RSV HA hydrogels.

Further, to optimize the release of RSV, increasing polymer weight percentage and polymer molecular weight was performed. It was found that increasing the polymer weight percentage and molecular weight could slightly slow down the drug release (shown in Figure 2.5C). This is due to the fact that higher polymer weight percentage and higher molecular weight can lead to a greater degree of entanglement within the hydrogel matrix, further trapping GO-RSV to lead to an even slower release.

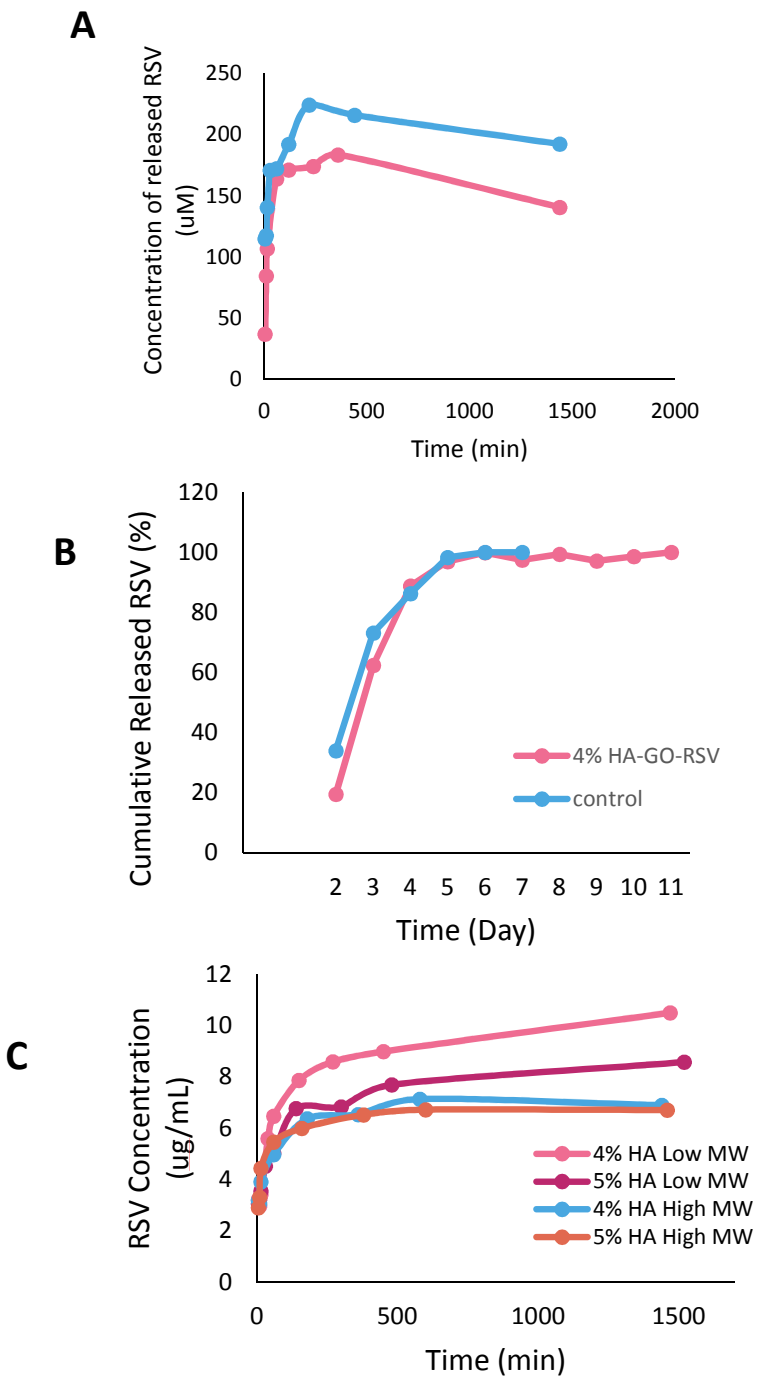


Figure 2.4. RSV release profiles of Resveratrol-loaded graphene (GO-RSV) HA hydrogels. (A) RSV release profiles of GO-RSV HA hydrogels compared to RSV loaded HA hydrogels in first 24 hours in PBS. The values expressed are means (n = 3) ± SD. (B) RSV cumulative release profiles of GO-RSV HA hydrogels and RSV loaded HA hydrogels for 11 days in PBS. The values expressed are means (n = 3) ± SD. (C) Influence of the HA molecular weight and percentage on RSV release profiles of GO-RSV HA hydrogels in PBS. The values expressed are means (n = 3) ± SD.

2.5. Conclusion

A GO hybridized HA hydrogel for drug delivery was created. The hydrogel could control the release of RSV and could be tunable to provide different drug release rates by varying the polymer molecular weight and density. This study revealed a novel interaction of the of resveratrol with graphene oxide in HA based hydrogels, which could be used for prolonged delivery of aromatic small molecule drugs.

A hybridized GO-HA hydrogel was created to study the controlled release of RSV. The drug release profiles showed that the release of RSV could be slightly controlled using GO. Varying the polymer molecular weight as well as the polymer density were also investigated. Increasing the molecular weight and density of the polymer further retarded the release of RSV.

In future work, varying the ratio of loaded GO:RSV could be studied in order to further slow and tune the release of RSV. Biocompatibility studies of the GO-HA hydrogel could also be performed as well as *in vitro* drug release profiles. These studies would help confirm the novel interaction between GO and RSV and the controlled release of RSV. The GO-HA hydrogels could then be used for controlled delivery of small aromatic molecules for various applications.

CHAPTER 3: THERMORESPONSIVE HYALURONIC ACID BASED NANOGEL

3.1 Abstract

The purpose of this study was to design and synthesize "smart" nanogels that could be utilized as biomaterials. A thermoresponsive polymer, poly-n-isopropylacrylamide (pNIPAM) was grafted onto the backbone of the biopolymer, hyaluronic acid using EDC/NHS coupling. Nanogels were then created using a sonication method. Both the polymer and nanogels were successfully synthesized and characterized and a thermoresponsive effect was observed.

3.2 Introduction

Modern nanocarrier systems include liposomes, nanogels, lipid or polymeric nanoparticles, and microemulsions.[6] These carrier systems can facilitate the movement of cells, drugs, or other cargo. Recently, hydrophobic drugs have gained interest as cargo due to improved pharmacokinetics due to improved solubility and stability.[6]

Nanogels, like nanoparticles, are able to protect drugs and can be easily administered. Over the past few years, "smart" or stimuli-responsive (e.g. pH, specific ions, or temperature) gel systems have gained interest due to the advantages such as tunable size, porous structure, and large surface areas for bioconjugation. The "smart" nature of these polymers have facilitated applications in fields such as medicine, technology, and biomedicinal engineering.[12]

Among the most studied thermoresponsive polymers is poly(N-isopropyl)acrylamide (pNIPAM). The lower critical solution temperature (LCST) of pNIPAM, or the temperature at which the polymer will undergo a phase transition from a flexible solvated to a more rigid unsolvated state, is 32 °C.[13] Poly(N-isopropyl)acrylamide has a reversible soluble-insoluble behavior because of the LCST. The phase change is a result from the heat driven release of water molecules from the isopropyl side chains of pNIPAM. Above the LCST temperature, the hydrophobic interactions increase, resulting in desolvation from the aqueous phase.[12]

Grafting pNIPAM onto the backbone of hyaluronic acid not only creates a natural biopolymer with thermoresponsive abilities, but it also presents an opportunity to tune the LCST temperature of pNIPAM and thus, the gelling properties of the network.

3.3 Experimental

3.3.1 Materials and Instrumentation

All chemicals were purchased from VWR, Sigma Aldrich, and Click Chemistry Tools, LLC. ^{1}H NMR spectra were recorded on a Brucker ARX300 spectrometer. Nanogels were prepared using a Fisher Scientific FS110D Ultrasonic Cleaner. The hydrodynamic radius of the nanogel particles was determined using dynamic light scattering on a Zetasizer. Nanogel particle size was also examined with Hitachi H-8000 scanning TEM operated at an accelerating voltage of 200 kV. The particles were collected in 300 mesh copper grids for imaging.

3.3.2 Experimental Procedures

3.3.2.1 Synthesis of HA-g-pNIPAM Polymer

Hyaluronic acid (MW = 47kDa, 0.25 g based on monomer unit) was dissolved in deionized water (50 mL). Next, EDC·HCl (0.506 g, 2.64 mmol) and N-hydroxysuccinimide (0.303 g, 2.64 mmol) were added to the solution. The pH was then adjusted to 5.5 using 5 M HCl and the solution was stirred for 1 hour at room temperature. Next, amine terminated poly(N-isopropyl)acrylamide (MW = 5.5 kDa, 0.181 g,3.30 x 10^{-5} mol) was added to the solution. The pH was adjusted to 7.5 using 5 M NaOH and the reaction mixture was stirred for 48 hours at 4 °C. Dialysis was then performed against 1000 mL deionized water for 72 hours using dialysis tubing with MWCO = 10,000 g/mol. The water was changed every 2 hours. Next, lyophilization was performed for 72 hours to obtain the purified polymer.

3.3.2.2 Preparation of HA-g-pNIPAM Nanogels

The HA-pNIPAM nanogel was prepared using a sonication method in aqueous conditions. A 0.5% w/v HA-pNIPAM solution in 1x PBS was then sonicated for 15 minutes on ice. The nanogels were allowed to settle overnight at 4 °C followed by 5 min centrifugation at 3,000 g.

3.4. Results and Discussion

HA-g-pNIPAM was synthesized from HA polymer using EDC and NHS peptide coupling with amine terminated pNIPAM. The amine-terminated pNIPAM was grafted onto hyaluronic acid at 25% based on molar ratio corresponding to the disaccharide units. Successful grafting of pNIPAM onto HA backbone was demonstrated through ^{1}H-NMR and TGA analysis.

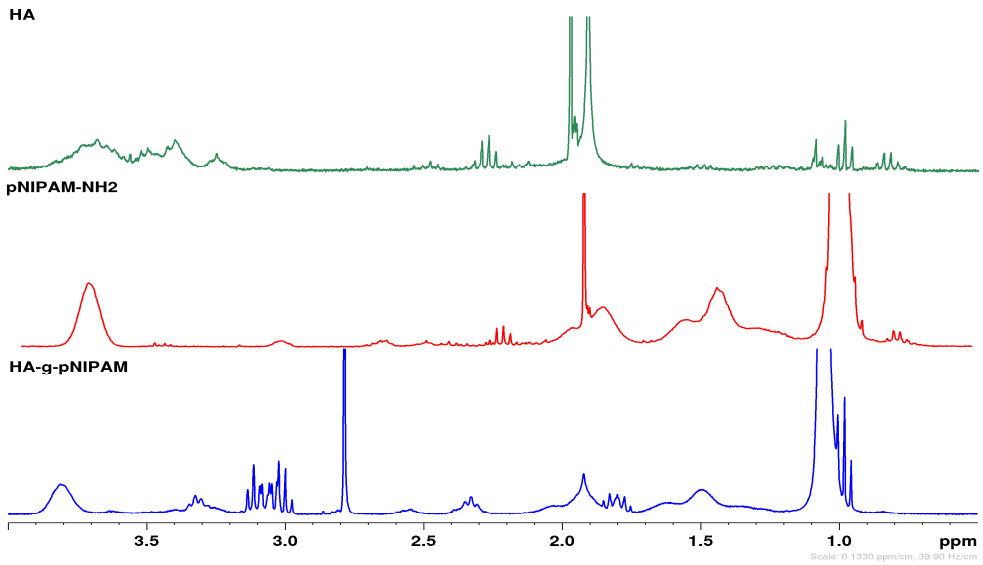


Figure 3.1. ^{1}H NMR spectra of (top) hyaluronic acid, (middle) amine terminated poly(N-isopropyl)acrylamide, and (bottom) hyaluronic acid grafted poly(N-isopropyl)acrylamide.

Thermogravimetric analysis (TGA) was performed in order to establish the composition of the HA-g-pNIPAM product. It was found that the degradation profile of the grafted hyaluronic acid was different than that of the unmodified hyaluronic acid. The initial degradation temperature of unmodified HA was around 233 °C, while the grafted hyaluronic acid displayed two different degradation temperatures of 221 °C corresponding to HA and 377 °C which corresponded with pNIPAM. The appearance of two degradation temperatures for the grafted hyaluronic acid indicates the presence of two chemical components within the polymer. Thus, the TGA could also demonstrate the successful grafting of pNIPAM onto the backbone of HA.

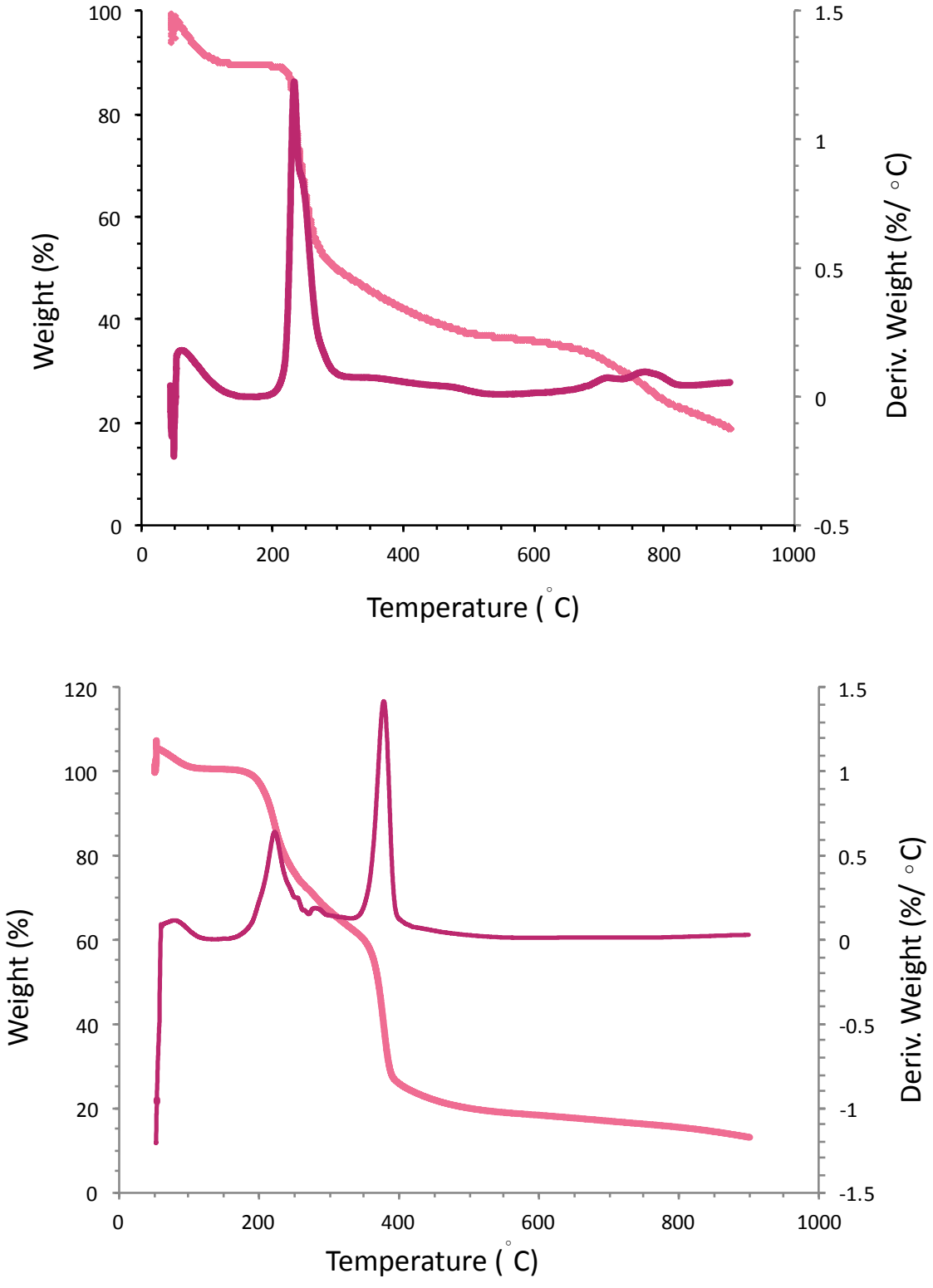


Figure 3.2. Thermal decomposition measured by thermogravimetric analysis (TGA) of (top) pure HA and (bottom) HA-g-pNIPAM showing the successful grafting of hyaluronic acid.

2.4. Preparation and Characterization of HA-g-pNIPAM Nanogels

Nanogels were prepared from HA-g-pNIPAM polymer through a sonication method under aqueous conditions. Confirmation of the formation of nanogels was carried out through differential light scattering (DLS) measurements and transmission electron microscope (TEM) images.

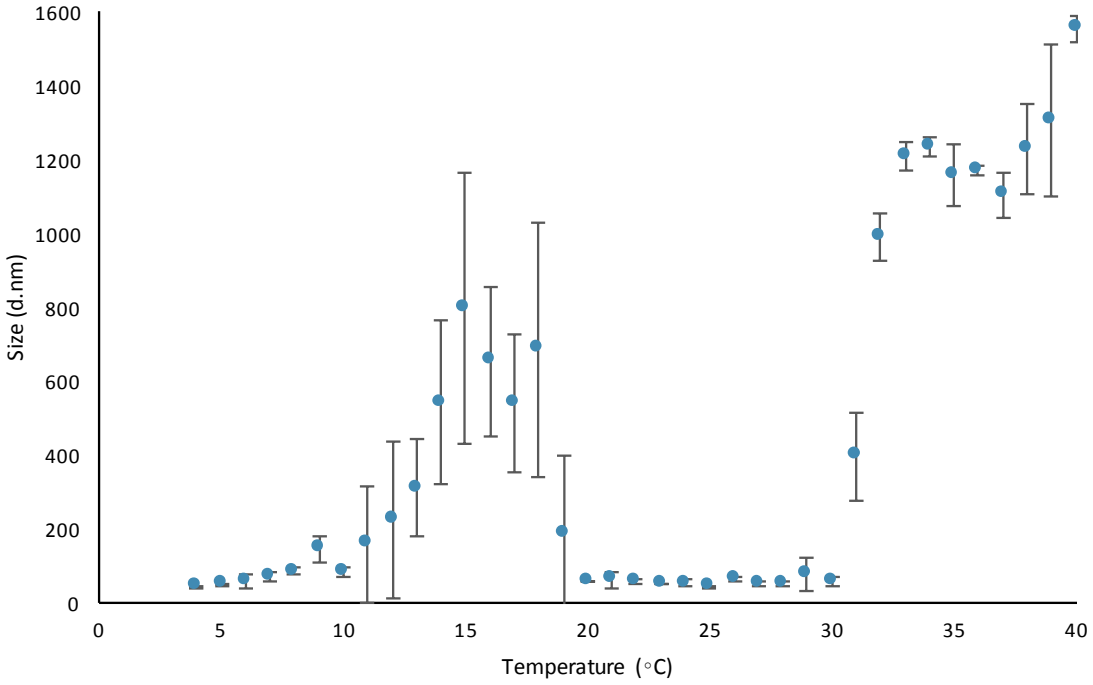


Figure 3.3. Particle size measured by dynamic light scattering (DLS) showing the thermo-responsiveness of the HA-pNIPAM nanogel. The significant change in size of naonogels indicated the changes in polymer assembly at the lowest critical solution temperatures (LCST).

Particle size as well as the lower critical solution temperature (LCST) was measured through DLS (Figure 3.3.). The thermoresponsive behavior of the polymer was also observed through DLS. Below 20 °C, it was observed that the particles size was unstable. The nanogel particles were in the hydrated state below 20 °C, which allowed for swelling

and a larger size. Once the temperature reached 20 °C, the size of the nanogel decreased and appeared to be stable, indicating the LCST had been reached. From 20 °C to 30 °C, the hydrophobic interactions were increased as water was removed from the particles. The nanogel particle size decreased as a result. Above 30 °C, the particle size increased indicating the dissociation of the hydrogen bonding responsible for the nanogel formation. TEM results confirmed the particle size of the nanogels as well as depicted a globular morphology (Figure 3.4.). The nanogel particles measured to be approximately 300 nm according to TEM.

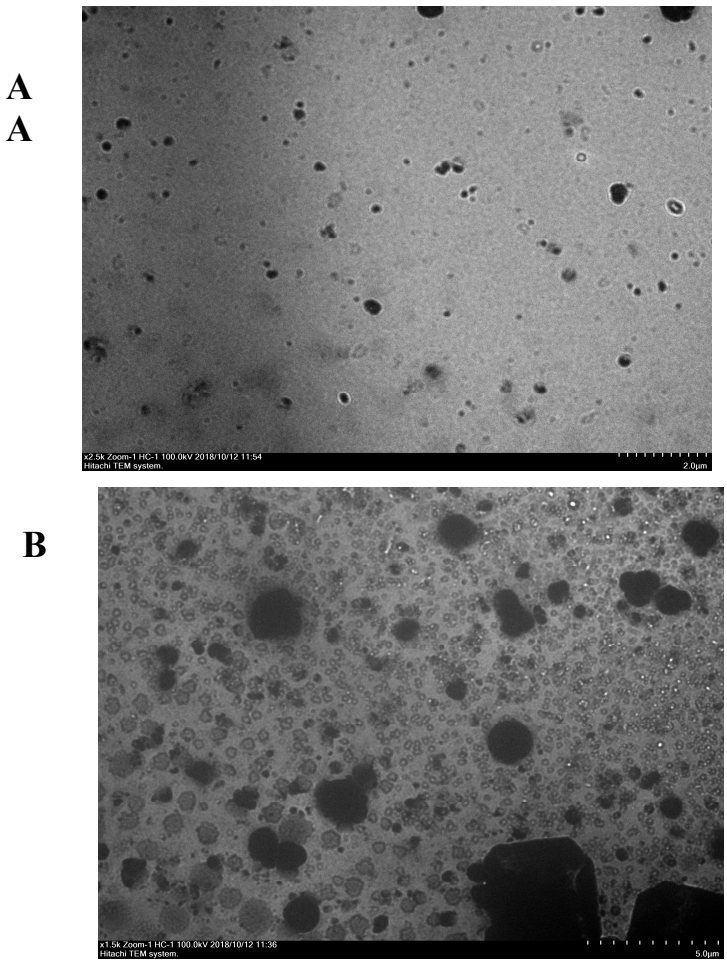


Figure 3.4. Transmission electron micrograph (TEM) showing the morphology of the nanogel at room temperature (25°C). Sizes measured by TEM were supported the measurements by DLS. (A) TEM performed in 1x PBS. (B) TEM performed in 10x PBS.

3.5. Conclusion

In this study, a thermoresponsive hyaluronic acid based nanogel was created. Poly-n-isopropylacrylamide was first grafted onto hyaluronic acid using EDC/NHS coupling and then the corresponding nanogels were formed. The thermoresponsive behavior of the nanogels was then tested and observed.

Future research could involve cargo loading such as hydrophobic drugs. By loading hydrophobic drugs into the HA-pNIPAM nanogel matrix, solubility issues could be improved as well as potential enhanced drug loading. The thermoresponsive nature of pNIPAM allows the nanogel to shrink in size when LCST is reached. The decrease in size has been previously reported as the mechanism by which the hydrophobic drug could then be released.[13]

CHAPTER 4: GRAFTED HYALURONIC ACID BASED POLYMERS FOR SURFACE MODIFICATION OF VIRUS PARTICLES

4.1 Abstract

In this study, the main goal was to synthesize biopolymers for the application of encapsulation and delivery of envelope viruses. A boronic acid grafted hyaluronic acid polymer was synthesized for the purpose of surface modification of the Vaccinia virus (VACV) through boronate ester formation. A DBCO modified thermoresponsive polymer was also synthesized so "click" chemistry could be performed to link the polymer to VACV. Characterization of these polymers were also performed.

4.2 Introduction

The Vaccinia virus, formerly used as a vaccine in the eradication of smallpox, has recently gained interest in a new light due to its oncolytic viral properties.[17] Oncolytic viruses are used in cancer therapy as they are designed to specifically target and replicate within tumor cells, while not harming normal cells.[17,18] The oncolytic viral agents are able to both kill the tumor cells and provoke an immune response against the tumor cells.[17,18] A major issue facing virus based vaccines as well as viral agents is the storage and delivery of these agents. Extreme temperature can damage and lead to viral death.[34] Thus, a "cold-chain" system is put into place from the site of manufacturing to the destination in order to ensure that proper conditions are met during the storage, handling, and transport of vaccines.[34] One possible solution to overcome this issue is to increase the thermostability

of the viral particles. Therefore, the goal of this study will be to modify the surface of VACV in order to elicit increased thermostability of VACV. Specifically, this study will aim to modify the surface of VACV by attaching "smart" coatings onto the virus composed of poly(N-isopropyl)acrylamide (pNIPAM), phenylboronic acid, and hyaluronic acid. The hypothesis is that by designing "smart" coatings consisting of biopolymers to encapsulate the enveloped viral particles, the stability and infectivity can be tuned, which can present solutions for a variety of applications.

An important feature of boronic acids are their ability to bond reversibly to diols to form boronate esters.[14] Five and six membered rings most likely form due to the prevalence of 1,2-diols and 1,3-diols. This notable reaction has encouraged the studies of boronic acid and polymers for the applications of self-regulated drug delivery systems, therapeutic agents, and sensors for biological species, including saccharides and glycoproteins.[14] Boronic acids appear to be non-toxic and have, therefore, been used in biomedical applications.[15]

Delivery of VACV is a multi-faceted issue for oncolytic viral therapy. On one hand, while systemic intravenous administration is easier than intratumoral injection, it presents issues such as sequestration in the liver, nonimmune human serum, or anti-oncolytic virus antibodies.[19] On the other hand, virus neutralization can occur as a result of attack from the immune system.[19] Protection from virus neutralization as well as extended vascular circulation has been achieved through coating the viruses in nanoparticles.[20]

Proteins, glycans, nucleic acids, and phospholipids are all sites of interest for chemical modification on enveloped viruses.[21-24] One method of linking agents such as fluorescent probes to the envelope proteins of enveloped viruses is through modification of amino

groups with various N-hydroxysuccinimide (NHS) esters.[25] The introduction of azide tags to viral proteins presents the use of the strain-promoted alkyne-azide cycloaddition (SPAAC) reaction for a chemospecific site modification.

Another site for chemical conjugation is a glycan residue on the glycoprotein structure. Specifically, vicinal diols are present in sugars that are able to bind to phenylboronic acid (PBA) analogs.[27] A recent study showed that the sialic acid present on the glycoprotein of the vesicular stomatitis virus was efficiently labeled with quantum dots conjugated with PBA.[28] In this chapter, a PBA modified HA polymer was synthesized to bind to the surface of VACV. Glycan residues can also be conjugated by adding azido-sugars through metabolic incorporation followed by a SPAAC reaction with alkyne-containing molecules.[29]

Lastly, phospholipids are a site of modification among envelope viruses. Modification on the lipid bilayer of the envelope have small effects on the biological properties of viruses versus modification of another site. A point of interest is that due to the fact that the viral envelope is composed of the cell membrane within host cells, the modification of viral phospholipids can be achieved by hijacking the inherent phospholipid biosynthesis.[30] Previous research has previously demonstrated that the surface of the measles virus could be modified with a number of functional groups through the metabolic incorporation of azide groups to either the phospholipid layer or surface proteins.[31,32] To then confirm that the azide-MV could undergo chemical modification, the strain-promoted alkyne-azide cycloaddition (SPAAC) reaction was exercised using dibenzylcyclooctyne (DBCO)-based fluorescent dyes or quantum dots. In another section of this chapter, a

DBCO-thermoresponsive polymer was synthesized to bind to an azide modified VACV via SPAAC.

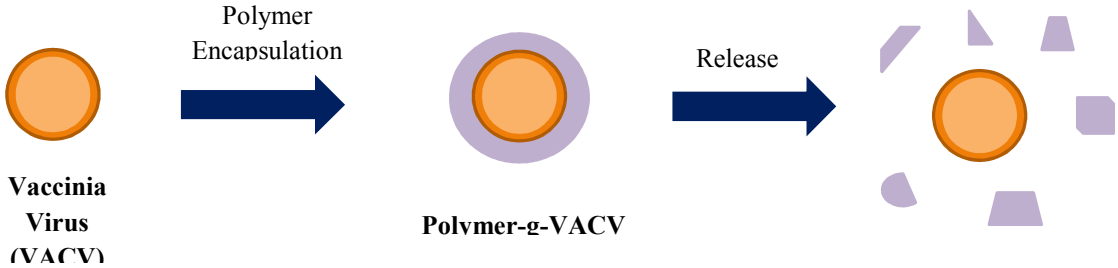


Figure 4.1. Schematic showing polymer coating onto VACV followed by release.

4.3. Experimental

4.2.1 Materials and Instrumentation

All chemicals were purchased from VWR, Sigma Aldrich, and Click Chemistry Tools, LLC. ^{1}H NMR spectra were recorded on a Brucker ARX300 spectrometer.

4.2.2. Experimental Procedures

4.3.2.1. Synthesis of HA-g-B(OH)$_2$ Polymer

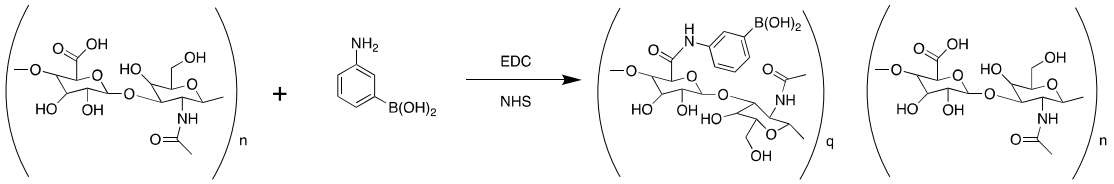


Figure 4.2. Scheme of HA-g-B(OH)$_2$ synthesis

Hyaluronic acid (MW = 47 kDa, 0.25 g based on monomer unit) was dissolved in deionized water (13 mL). Next, EDC·HCl (0.13 g, 6.59 x 10^{-4} mol) and 3-

aminophenylboronic acid (0.045 g, 3.30 x 10^{-4} mol) were added to the solution. The mixture was stirred at room temperature for 24 h and then lyophilized to obtain the crude polymer.

2.4.1. Synthesis of DBCO-pNIPAM Polymer

Amine terminated pNIPAM (MW = 5.5 kDa, 0.100 g, 1.82 x 10^{-5} mol) and DBCO-NHS-ester (0.007 g, 1.82 x 10^{-5} mol) were dissolved in 4 mL dichloromethane. The solution was stirred at room temperature for 12 hours. Next, the reaction mixture was frozen in -80 °C freezer overnight, followed by lyophilization for 84 hours.

4.4. Results and Discussion

HA-g-$B(OH)_2$ was synthesized by grafting 3-aminophenylboronic acid (APBA) onto HA using EDC/NHS peptide coupling. The grafted polymer was analyzed through ^{1}H-NMR. The phenyl peaks belonging to APBA appeared around 7 ppm, which confirmed the successful grafting of APBA onto HA backbone.

DBCO-pNIPAM polymer was synthesized from DBCO-NHS-ester and pNIPAM-NH_2 through the use of NHS conjugation and the formation of an amide bond. The product was characterized by ^{1}H-NMR. The introduction of the DBCO unit will allow for click chemistry through the SPAAC reaction on the modified surface of VACV.

4.5. Conclusion

Currently, HA-g-$B(OH)_2$, where the boronic acid portion was obtained from 3-aminophenylboronic acid (APBA), was grafted onto HA through EDC/NHS coupling via the carboxylic acid unit on HA and the amine on the APBA. DBCO-pNIPAM was also synthesized.

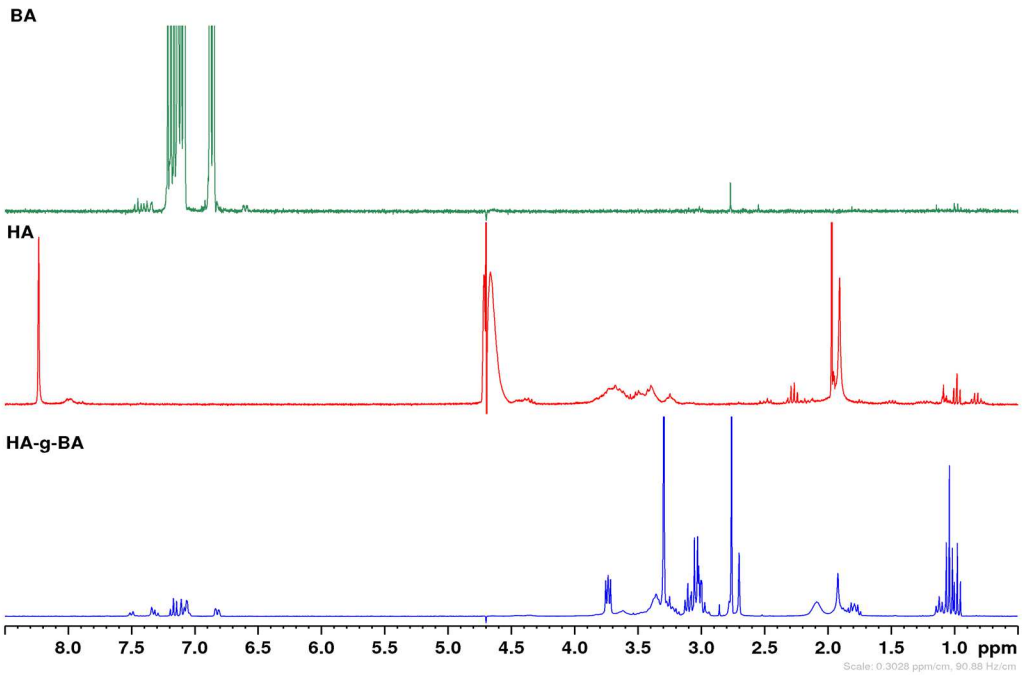


Figure 4.3. ^{1}H NMR spectra of (top) 3-aminophenylboronic acid, (middle) hyaluronic acid, and (bottom) phenylboronic acid modified hyaluronic acid.

In the future, research could involve attachment to the VACV and the investigation of the resulting thermostability, infectivity, and structural integrity of VACV. Self-assembly of biodegradable polymers will aid in the development of thermostable VACV particles by providing a shield around VACV. The hypothesis is that the biopolymer coating or "shield" will be able to protect the VACV particles and enhance its structural integrity and thermostability to prevent the deformation that may ultimately lead to viral death.

As a first approach, phenylboronic acid can be used as the method to attach HA to the surface of VACV. Specifically, HA-g-$B(OH)_2$ can be used in the assembly study. The sialic acid present on the envelope glycoprotein of VACV has been reported to bind to boronic acid through the formation of boronate esters, which will provide an anchor to the VACV.[25]

In order for release of VACV itself, the boronate ester bond is able to be cleaved by the addition of fructose or glucose, resulting in the controlled release of VACVs. Lastly, the use of HA allows for the enzyme, hyaluronidase, to aid in further degradation of the coating for the purpose of the release of VACV particles.

The thermostability of VACV can be enhanced through surface modification of VACV in order to develop "cold-chain" free delivery systems. Enhancing the thermostability will provide a more obtainable and sustainable approach to vaccine distribution. A focus could also be targeted and controlled delivery for oncolytic cancer therapy. Once the thermostable VACV coatings are established, cancer secreting peptides can be inserted as linkers into the polymer to be cleaved by enzymes, resulting in a targeted and controlled delivery of VACV.

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