A Thesis

entitled

Evaluation of Calcium Alginate Microparticles Prepared Using a Novel Nebulized Aerosol Mediated Interfacial Crosslinking Method

by

Junkyu Shin

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Pharmaceutical Science with Industrial Pharmacy Option

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August 2016
An Abstract of
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Alginate is a very popular biopolymer used in microencapsulation. Applications of alginate based microparticles can be found in pharmaceuticals, food, cosmetics, and tissue engineering industries. In this work a novel nebulized aerosol mediated interfacial crosslinking method was developed and evaluated as a new potential particle formulation method. The apparatus design for microparticle production, and the recovery process was established. The evaluation studies such as particle sizing, zeta potential, morphology observations by optical microscopy, scanning electron microscopy, and atomic force microscopy, thermal behavior by dynamic scanning calorimetry, structural composition by Fourier transform infrared spectroscopy, and encapsulation efficiency, were performed to characterize the microparticle. A granular shape with embossed bubble surface texture was observed in optical micrographs and SEM. The particle size range was between 9.9 µm and 60.5 µm. The drug loading was 15.1 ± 3.1 µg of methylene blue per 1 mg of microparticle.
Acknowledgements

I would like to express my special gratitude to my advisor, Dr. Jerry Nesamony for providing me an opportunity to start researching in his group. I deeply appreciate all his support and assistance for completing the research. Dr. Jerry Nesamony offered me advice and always listened to my opinions as a leader and a research partner, shared his valuable life experiences as a father, and spent memorable moments as a friend. I will remember my great experience in his lab all the time.

I would like to thank Dr. Sai Boddu for being my committee member, providing me with his valuable lectures and advices. I thank Dr. Gabriella Baki for being my committee member and allowing me to work as a TA in her lab class. I specially thank Dr. Kenneth Alexander for his advanced knowledge and motivation, during my undergraduate and graduate program. I also thank Dr. Caren Steinmiller for serving as my graduate faculty representative and her great teaching. I thank Dr. Youssef Sari for his help with optical microscopy, Dr. Pannee Burckel for helping me with SEM, Dr. Elisha Injeti and Dr. Steven Gollmer at Cedarville University for their generous help with AFM study, Dr. Joseph Lawrence and Dr. Sam Imanieh for their assistance with DSC, and Dr. James Slama for allowing me to use his FTIR.

I also would like to thank all my fellow classmates for beautiful memories and helpful guidance. I finally thank my family for limitless support and love. I would like to offer my deepest appreciation for their encouragement to help me finish my studies.
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**List of Abbreviations**

AFM ......................... Atomic Force Microscopy
DI water .................. DeIonized water
DLS ......................... Dynamic Light Scattering
DSC ......................... Differential Scanning Calorimetry
ELS ......................... Electrophoretic Light Scattering
FTIR ......................... Fourier Transform Infrared Spectroscopy
G ............................. α-L-guluronic acid residue
M ............................. β-D-mannuronic acid residue
MAN ........................ Micro-Airflow-Nozzle
PVC ........................ PolyVinyl Chloride
SAL ........................ Sodium Alginate
SEM ........................ Scanning Electron Microscopy
Chapter 1

Introduction

1.1 Introduction

Hydrogels are a well-known and very useful technology in drug delivery. They have been extensively studied and many applications were developed over many years in pharmaceutical\textsuperscript{1}, cosmetic\textsuperscript{2}, tissue engineering\textsuperscript{3}, and food industries\textsuperscript{4}. One of the most commonly used biopolymers for producing a hydrogel is alginate. Alginate turns into a gel when it comes in contact with divalent cations such as calcium ions.\textsuperscript{5} The alginate based gel was first introduced in 1980.\textsuperscript{6} It was developed primarily for encapsulation purposes and currently is one of the most commonly used gelling materials because of its various features such as biodegradable nature, biocompatibility, non-toxic nature, high safety profile, reproducible behavior and low cost of production.\textsuperscript{7} It also serves as a controlled release polymer, and its properties can be modified by formulators, depending on the requirement.\textsuperscript{3,8} Due to its several advantages, many studies on production methods were performed and developed for optimization of alginate particle preparation.\textsuperscript{1}

This chapter describes properties of alginate, its gelling characteristics, and various gelation methods. Also, various types of equipment set-up and materials used in the preparation of alginate hydrogel are described in detail.
1.2 Alginate (Source and Structure)

Alginate is a naturally occurring anionic polysaccharide obtained from brown seaweed algae, from three different species: *Ascophyllum nodosum*, *Laminaria hyperboreana*, and *Macrocystis pyrifera*; and occurs as a mixture along with various types of cations: \( \text{Na}^+ \), \( \text{Mg}^{2+} \), \( \text{Sr}^{2+} \) and \( \text{Ba}^{2+} \), in nature. Alginate is commercially available in a powder form of sodium alginate, which is also called alginic acid sodium salt. Sodium alginate is a water soluble biopolymer compound that has linear polysaccharide fibers. The linear fibers consists of (1-4) linkages of \( \alpha\)-L-guluronic acid (G) and \( \beta\)-D-mannuronic acid (M) residues, meaning that \( \alpha\)-L-guluronic acid and \( \beta\)-D-mannuronic acid residues bind each other and form alginate fibers.
Figure 1-1. (Top) Chemical structures of α-L-guluronic and β-D-mannuronic acid residue monomer, (Bottom) (1-4) linkages of M blocks and G blocks: MM, MG, GG, and GM

1.3 Alginate Gel Formation

The unique conformations of residues: α-L-guluronic acid (G) and β-D-mannuronic acid (M), provide homogenous blocks of MM and GG, and it can also be alternating blocks of MG and GM as shown in [Figure 1-1. bottom]. As described and shown in [Figure 1-1], G block is aligned vertically, while M block is arranged horizontally. Hence, the configuration of two GG blocks that face against each other provides a little space for a divalent cation to be entrapped inside of the cavity of two GG blocks. This special structural result is called an egg box model [Figure 1-2. bottom], which will further form alginate gels. The flat configuration of MM or alternating sequence of MG or GM, is not suitable for confining divalent cations. After forming egg box structures, each egg box structure is stacked with other structures to produce a gel network. This process undergoes aqueous sol-gel transformation under mild conditions which provides an ideal environment for drug encapsulation because no external forces are applied to damage the carrier.¹ secrecy
In order to cover divalent cations (e.g., Ca$^{2+}$), a sodium-calcium exchange reaction has to occur. The affinity and ability for ionically cross-linking alginate gel is dependent on the type of cation used. Calcium ions are frequently used for the formation of alginate gel. However, calcium ions do not result in the strongest gel bonding when compared to other
cations. It is reported that Sr$^{2+}$ and Ba$^{2+}$ ions form stronger gel matrices than Ca$^{2+}$.\textsuperscript{12,14,15} Mg$^{2+}$ and monovalent cations are not good options for producing alginate gel, since they do not form strong alginate gel bonding.\textsuperscript{9} Additionally, the high concentration of guluronic acid (G) residues produces the increased affinity between alginate and divalent cations. This further suggests that calcium ions possess variable and different strength of affinity for the guluronic acid and mannuronic acid residues, and show a tendency to react with the guluronic acid first.\textsuperscript{12,16}

The degree of cross linking (gel strength) depends on three factors: molecular size, composition, and sequential structure.\textsuperscript{17-19} Alginates with more G residues form stiffer gels when compared to alginates with more M residues. The residue block of GG as demonstrated previously [Figure 1-2. bottom] for forming the egg box model, is known to possess the lowest flexibility, while the residue block of MG has the highest flexibility of various alginate polymers. The mannuronic acid residues gather together and produce $\beta$ (1-4) linkages. This structure adds more linear arrangements and behaves as a flexible gel, while the guluronic acid residues have $\alpha$ (1-4) linkages which gives a more rigid gel due to the orientation of egg box model and the steric hindrance around carboxyl groups.\textsuperscript{12,20,21}

It is reported that the order of inflexibility of alginate polymer is as follows: GG > MM > MG.\textsuperscript{18,22} Also, the presence of Na$^+$ in alginate, significantly influences the shear stiffness of alginate gel.\textsuperscript{23} The concentration of the divalent cation solution used significantly affects the gel formed and has a role in determining the stability of the gel bonds. When highly concentrated calcium chloride solution is used, the guluronic acid in alginate have more chance to react with calcium ions leading to permanent chain associations within
the gel network. Lower amounts of calcium chloride solution results in temporary chain associations.\textsuperscript{9} The formed alginate gel holds more than 95\% of water by weight, and this high content of water supports the shape and resisting stress of gel.\textsuperscript{1,9}

1.4 Drug Encapsulation and Release

Alginate is used in the pharmaceutical industry as an excipient for gel forming, stabilizing, and thickening formulations. Since it can be easily used for encapsulation, the delivery of small molecules, tissue localized drugs, and oral dosage forms are possible applications. Alginate gels are known to deliver low molecular weight drugs.\textsuperscript{10} This is because the surface of alginate gels is porous and the size of the smallest pores is around 5 nm.\textsuperscript{24} Thus, there is a rapid release of incorporated small molecule drugs from alginate gels and the degree and extent of drug release can be delayed with ionic and covalent cross linking. Partial oxidization of alginate gels and the chemical structure of polymer chains can also influence the controlled release of drugs from alginate gels.\textsuperscript{25,26} Hydrophobic drugs can also be incorporated into modified amphiphilic alginate gels and the drug release can be controlled.\textsuperscript{27}

1.5 Gelation Mechanisms

Typically, alginate hydrogels are formed by an external or internal gelation method. Other methods that are not categorized into either of the two methods mentioned in the previous sentence are gelation by cooling \textsuperscript{14}, inverse, interfacial, and multi-step interrupted gelation\textsuperscript{28}. Each mechanism imparts different properties of gel homogeneity, morphology, density, diffusion, and polymer matrix.
1.5.1 External Gelation

The most known and commonly used method is external gelation. This mechanism is also referred to as diffusion method. This gelation method is done by introducing the alginate solution as discrete droplets either in air or liquid, into a cation containing solution (e.g., calcium chloride solution). Cations like Ca$^{2+}$, in the continuous phase permeate into the inner parts of the alginate droplet. The cross linking between cations and alginate polymer starts from the external surface of the droplet. This process is considered to occur first and proceeds rapidly. Gelation quickly occurs on the surface of alginate droplet, and proceeds into the core of the droplet producing an inhomogeneous gel density. As the polymer droplets are immersed in the ionic continuous phase longer, gelation advances toward the center of droplet and the core of the droplet becomes more solid with increased cross linking.

1.5.2 Internal Gelation

Unlike external gelation, internal gelation starts its gelation from the core of the droplet. Since it forms from the interior of the alginate droplet, it is also referred to as in situ gelation. This method is considered as an emulsification gelation because an oil phase is used for alginate solution dispersion. The alginate solution is first mixed with a water insoluble calcium salt (e.g., calcium carbonate). Then an acidic solution is introduced into the calcium salt loaded alginate solution dispersed in oil. The water insoluble calcium salt in the alginate droplets is gradually dissolved and is released throughout the alginate droplet. The released calcium ions react with alginate and start to
form a gel from the core of the droplet.\textsuperscript{31,32} The internal method results in more homogenous gelation when compared to external gelation, because of the presence of uniformly dispersed water insoluble calcium salt. The gelation method can be controlled by modulating the concentration of water insoluble calcium salt and the pH level.\textsuperscript{33}

### 1.5.3 Other Gelations: Inverse Gelation, Interfacial Gelation, Multi-step Interrupted Gelation, and Gelation by Cooling

Other gelation methods that do not fall into the two categories described above are inverse gelation, interfacial gelation, multi-step interrupted gelation\textsuperscript{28} and gelation by cooling.\textsuperscript{1}

Inverse gelation was first introduced in 1988.\textsuperscript{34} In contrast to the external gelation method in which the alginate solution is dripped into the calcium chloride solution, inverse gelation drips the calcium chloride solution into the alginate solution.\textsuperscript{35} Interfacial gelation occurs in an emulsion by formation of the gel at the interface of emulsified droplets. This method produces relatively small particles and a narrow size distribution.\textsuperscript{36}

In 2008, Ladet’s group introduced a multi-step interrupted gelation technique and described it as an onion-like multi-membrane hydrogel structure. It is prepared by repeating external gelation multiple times until the desired number of layers are obtained.\textsuperscript{37} This method was recently used by several research groups for developing controlled release formulations using alginate hydrogels.\textsuperscript{38-40}

Gelation by cooling was described by Papageorgiou in 1994.\textsuperscript{14} This method uses the principle that the gelation does not occur at high temperature, the polymer forms its
gelling network when the temperature decreases. At an elevated temperature the alginate alignment is loosely spread. The cross-linking interaction occurs after the temperature of solution cools.

1.6 Production Methods

Leong categorized the alginate dispersion methods into three groups: liquid-air, liquid-liquid, and self-assembly; depending on the type of phase the alginate solution exists before forming the alginate droplet and after extruding the alginate droplet. Production methods in this article are also arranged based on the type of phase in which the alginate solution is present. The most popular type is liquid-air method which is a simple dripping which means that the alginate solution is extruded through a needle or a nozzle, and formed into droplets in the air phase. Currently existing methods mostly follow by this type and sometimes may be modified from the simple dripping method and includes co-axial laminar air flow, co-extrusion dripping, electrostatic, vibration, jet cutting, spinning disk, rotating nozzle, rotating micronozzle, air-atomization, and impinging aerosol method.

A typical liquid-liquid method is known as an emulsification method. This method uses the alginate solution in a continuous immiscible liquid phase, (e.g., oils are commonly used to form w/o emulsion). Since the apparatus setups that are used in the self-assembly method, are adapted from the techniques used in liquid-air and liquid-liquid methods, this chapter does not cover the details of self-assembly method. The following are explanations regarding various apparatus setups used in the different alginate particle preparation methods.
1.6.1 Simple Dripping

Simple dripping method is the most commonly known technique to produce hydrogels because it is simple, easy and no special equipment is required. Some authors call this method the extrusion dripping method as this apparatus extrudes the alginate solution drop wise into a cationic solution (e.g., calcium chloride solution). This method usually utilizes a syringe to extrude the alginate solution. The syringe containing the gelling solution (sodium alginate solution) and the gelling bath which contains the cationic solution, are aligned vertically. A magnetic bar is sometimes placed in the gelling bath to stir the bath solution gently [Figure 1-3].

A drop of gelling solution flows out through the opening of the needle that is attached to a syringe, as the pressure is applied to the syringe plunger. Once the drop is released from the needle, the drop travels via air and is introduced into the liquid phase of the gelling bath. Since the drop of gelling solution which is in the form of liquid, travels to the air phase, this method is considered as liquid-air mechanism.
1.6.2 Co-axial Laminar Air Flow

Co-axial laminar air flow (airjet) uses a nozzle that is equipped with two tubes: an inner tube and an outer tube. The inner tube is connected to an alginate solution reservoir and extrudes the alginate solution into droplets. The outer tube surrounds the orifice of inner tube and is connected to an inert gas (e.g., nitrogen gas). Thus, it can produce relatively small diameters of droplets by blowing the inert gas with the alginate solution. A slight modification of co-axial laminar air flow method was developed by Sugiura. Sugiura applied this co-axial laminar air flow concept to his device and named it as a micro-airflow nozzle. Alginate channel nozzles with airflow channels are placed on a micro-airflow-nozzle (MAN) and the alginate solution is delivered directly into calcium chloride solution which results in more efficient size distribution of microparticles.
1.6.3 Co-extrusion Dripping

This technique was developed to encapsulate the liquid polymer core because the liquid core hydrogel was found to have difficulty controlling the morphology of beads, the thickness of the outer membrane, and caused agglomeration.\textsuperscript{50} This technique uses a nozzle similar to that is used in the co-axial laminar gas/air flow method. Instead of blowing inert gas through the outer tube, this technique extrudes the polymer solution (e.g., the alginate solution) in the outer tube. Therefore, the liquid core that flows in the inner tube is entrapped by the alginate solution.
1.6.4 Electrostatic

An electrostatic potential is utilized in this method. An electrostatic generator is connected to the simple dripping apparatus setup. The electrostatic potential flows between the needle tip and the gelling solution producing smaller beads, when compared to the simple dripping method.\textsuperscript{51}
1.6.5 Vibration

Vibration method uses mechanical vibrations or waves on either the syringe or the nozzle that extrudes the alginate solution. Some authors call this method as a jet-break up method.\textsuperscript{58-62} This method follows the basic set up of simple dripping. The only difference is that a vibration generator is connected to the syringe or nozzle to produce more discrete drops of gelling solution. As a variation, instead of employing the vibration generator, a loudspeaker that can produce a sound wave was introduced by Zhou.\textsuperscript{63} A multinozzle system was also developed by Brandenberger.\textsuperscript{64} This method combined the vibration method with multiple nozzles on the nozzle plate. This method was developed specially to produce highly reproducible sterile gel particles.

Figure 1-6. Schematic representation of electrostatic

Reference: \textsuperscript{44,49,51-57}
1.6.6 Jet Cutting

A mechanical cutting technique is utilized along with the simple dripping method. A motor with cutting wires or blades is typically placed next to the liquid jet stream of the alginate solution. By rotating the cutting wires, the jet stream is cut into cylindrical segments.\textsuperscript{65}
1.6.7 Spinning Disk

A characteristic point of this system is a spinning/rotating disk. A feeder of alginate solution is placed above the spinning disk. Once the alginate solution is fed to the top surface of the spinning disk, discrete alginate droplets are sprayed from the edge of the spinning disk by the centrifugal force. The alginate droplets are eventually introduced into the gelling bath (e.g., calcium chloride solution) and form alginate gel beads/particles.\textsuperscript{66} Senuma added a zigzag shaped rim on the edge of spinning disk for efficient spreading of the alginate solution.\textsuperscript{67} Also, Champagne introduced a bowl-shaped disk to modify the basic concept of spinning disk method. This method was initially developed to scale up the biomass production of lactic acid bacteria.\textsuperscript{68}
1.6.8 Rotating Nozzle

Similar to the spinning disk method, a rotating nozzle also uses the centrifugal force. However, instead of feeding the alginate solution on the top surface of a spinning disk, the alginate solution in this method flows through a vertical shaft which is directly connected to the rotating nozzle. The orifices of the rotating nozzle are placed perpendicular to the angle of the vertical shaft and extrude the alginate droplets by the centrifugal force. The alginate droplets from the rotating nozzle finish forming gel beads/particles in the gelling bath (e.g., calcium chloride solution).
1.6.9 Rotating Micronozzle

Haeberle modified the centrifugal force method and developed a rotating micronozzle setup for encapsulating living cells. Unlike the spinning disk method, the alginate solution reservoir is attached and placed on the top surface of the rotating disk and one end of a micronozzle tube is connected to the alginate solution reservoir. The receiving tubes are hung on the edge of the rotating disk. The receiving tubes are hung at an angle of 90 degrees to the rotating disk when the rotating disk is not in motion. Once the rotating disk spins, the receiving tubes are spread out in parallel by the centrifugal force. The centrifugal force also allows the alginate solution to flow through the tube in which its tip end faces the receiving tube. The alginate droplets are eventually generated by the centrifugal force, and dropped into the gelling solution in the receiving tube.\textsuperscript{71}

![Schematic representation of rotating micronozzle](image)

Figure 1-11. Schematic representation of rotating micronozzle

Reference: \textsuperscript{71}
1.6.10 Air-atomization

When compared to the simple dripping method, an atomizer reduces the particle size even more. By applying an external aerodynamic force, viscosity and surface tension of the alginate solution are influenced resulting in smaller particles. This method takes advantage of producing small particles by adopting the atomizer, instead of using a needle in the simple dripping method. The gelling bath is typically placed under the atomizer to collect and gel the alginate droplets.72

Figure 1-12. Schematic representation of air-atomization

Reference: 72-79

1.6.11 Impinging Aerosol

Two solutions: the alginate solution and the calcium chloride solution (cationic gelling solution), are sprayed separately by atomizers or spray nozzles. According to the schematic diagram reported in papers80-83, two atomizers that spray both solutions are
aligned and placed vertically in a cylinder shaped chamber. The alginate solution is sprayed by the atomizer from the top of the chamber and the calcium chloride solution is sprayed from the bottom of the chamber. The solutions are introduced in the form of mist and form the hydrogel particles in the chamber. The formed particles are collected from the base of the chamber.$^{80}$

![Figure 1-13. Schematic representation of impinging aerosol](image)

Reference: $^{80-83}$

1.6.12 Emulsification Method

Emulsification method does not require any particular apparatus setup. Typically, a stirrer for simple agitation is used to dissolve the ingredients: the sodium alginate and the calcium chloride.
There are two major emulsification methods for preparing alginate particles: complexation and alginate in oil emulsification.\textsuperscript{1} Poncelet proposed emulsification-internal ionotropic gelation method to overcome the main issue associated with classic internal gelation which is that it takes longer to complete gelation due to gradual hardening of the polymer matrix. Poncelet first developed gel beads by the internal emulsification method, then shortened the gelation time by using the external gelation method. The alginate solution that contains an insoluble calcium salt is prepared first. Oil is added to this to make the w/o emulsion with continuous agitation. An acidic solution (e.g., acetic acid) is added to the emulsion to dissolve the calcium salt. Then, calcium chloride solution is added to the emulsion to lead the external gelation and also for collecting the formed beads efficiently by inducing phase inversion of the emulsion (from w/o emulsion to o/w emulsion).\textsuperscript{84} No special apparatus design is needed for this method. This emulsification-internal ionotropic gelation method has utilized by many researchers to prepare alginate particles.\textsuperscript{31,85,86}

![Figure 1-14. Schematic representation of emulsification](image)
1.6.13 Microfluidic

Microfluidic method is done by utilizing a small platform that has a micro scale channel. Two immiscible fluids: hydrophilic and hydrophobic fluids; are injected into the microfluidic device to prepare an emulsion. For example, the hydrophilic fluids are the alginate solution and the calcium chloride solution, and the hydrophobic phase can be oils or organic solvents used as the continuous phase in the microfluidic device. The alginate solution comes as emulsion globules within the continuous phase of oil or organic solvent and flows through the microchannel. Once the alginate emulsion globule is introduced into the calcium chloride solution, the alginate emulsion globule forms the hydrogel.\textsuperscript{87}

Reference: \textsuperscript{31,33,41,84}

Figure 1-15. Schematic representation of microfluidic

Reference: \textsuperscript{87-93}
1.6.14 Jet Homogenizer (Leeds)

Recently, the use of a high pressure jet homogenizer, which is called Leeds jet homogenizer, was developed by Pravinata group to produce alginate microgels. The jet homogenizer is comprised of two cylinders that contain the alginate and calcium chloride solutions separately, pistons to push cylinders, a hydraulic ram to move pistons, and a pinhole to extrude the microgels. The author reported that the effects of high turbulence, cavitation, shear and impact resulted smaller gel particles.

Figure 1-16. Schematic representation of jet homogenizer (Leeds)

Reference: 94

1.7 Conclusion
Alginate gels have been widely utilized in different methods of encapsulation because of its biodegradable nature, biocompatibility, non-toxic, highly safe, reproducible behavior and low cost of production. Various production methods, apparatus setups and gelation methods are summarized in this chapter. Different devices or techniques were added to the simple gelation method to improve yield, obtain a consistent result (uniform size and shape), and process scale up. The external gelation method is used in a majority of the methods, in which the alginate solution is extruded in the air phase and introduced into the calcium chloride bath.
Chapter 1 References


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Chapter 2

Significance of the research

Alginate based microparticles possess very unique and versatile properties allowing them to be used in drug delivery as a controlled release drug carrier.

A novel nebulized aerosol mediated interfacial crosslinking method was developed and evaluated as a potential alginate formulation approach. This new method utilizing nebulizers provides ease of formulation. Alginate gels typically swell when they are in contact with water. Therefore its recovery from microparticle dispersion is challenging.

The goal of this research was to design an apparatus setup for formulating calcium alginate microparticles, which can employ the ionotropic-external gelation technique.

The microparticle recovery process was also established to separate the gelled particles from the microparticle dispersion to create a dried powder form of alginate microparticles without experiencing particle swelling.
Chapter 3

Evaluation of Calcium Alginate Microparticles Prepared Using a Novel Nebulized Aerosol Mediated Interfacial Crosslinking Method

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KEYWORDS: calcium alginate, microparticle, nebulization, ionotropic

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3.1 Abstract

Alginate is a very versatile and unique polymer that has numerous applications in pharmaceuticals, food, cosmetics, and tissue engineering industries. Several methods for microparticle production are available. In this study a novel nebulized aerosol mediated interfacial crosslinking method was developed and evaluated to be used as a new potential formulation method. The apparatus design for microparticle production and the recovery process were established. Characterization studies such as particle sizing, zeta potential, morphology observations by optical microscopy, scanning electron microscopy, and atomic force microscopy, thermal behavior by dynamic scanning calorimetry, structural composition by Fourier transform infrared spectroscopy, and encapsulation efficiency, were performed to evaluate the microparticles. A granular shape was appeared in general with the embossed bubble surface texture. The size range was between 9.9 µm and 60.5 µm. The encapsulation efficiency was resulted in 15.1 ± 3.1 µg of model drug per 1 mg of microparticle.
3.2. Introduction

Alginate based microparticles are a well-known drug delivery excipient in the pharmaceutical industry. Since its unique properties allow the drug delivery system to be used as a drug carrier and in controlled release, numerous studies on alginate hydrogels have been performed, and various apparatus setups developed for formulating alginate based particles.

Alginate microparticle production methods can be categorized based on where the alginate solution is present and subsequently travels through to form a hydrogel: liquid-air, liquid-liquid, and self-assembly method. A novel nebulized aerosol mediated interfacial crosslinking method, which was developed in our lab, employs the liquid-air, external gelation method. The unique feature of this method is that it allows the alginate to have crosslinking reactions with the calcium chloride vapor in air.

This article chapter focuses on various aspects involved in designing the apparatus setup and the microparticle recovery process. The produced microparticles were evaluated via particle size analysis, zeta potential analysis, morphology evaluation, thermal analysis, chemical composition, and drug loading efficiency. This method is expected to be a new approach for alginate based particle formulation.

3.3. Materials and Methods

3.3.1. Materials

Sodium alginate with medium viscosity was purchased from Sigma-Aldrich, St. Louis, MO. Calcium chloride (anhydrous, granular) was from Sigma-Aldrich, USA. Large volume nebulizers (400 mL) were purchased from Hudson RCI, USA. Methylene
blue chloride was from Fisher Scientific, IL, USA. All reagents in this study were analytical grade and used as received.

### 3.3.2. Methods

#### 3.3.2.1. Formulation of Microparticles

Calcium alginate microparticles were formed by ionotropic gelation method. The apparatus setup [Figure 3-1] was utilized for producing microparticles at ambient temperature.

Separate solutions of alginate (0.4 % w/v) and calcium chloride (5.0 % w/v) were prepared. The curing solution (120 mL) was placed in collecting chamber 1 and the curing solution (60 mL) was kept in collecting chamber 2 located separately as shown in [Figure 3-1]. Both curing solutions in collecting chambers 1 and 2 were 5.0 % (w/v) calcium chloride solution adjusted to pH 2 using HCl. The collecting chambers
(collecting chamber 1 and 2) were connected each other by PVC medical tubes, and one end of the PVC tube was placed to face the surface of the curing solution in collecting chamber 2 to expel the unreacted microparticles. Magnetic stirring bars were placed in the collecting chambers and stirred at 70 rpm. Both solutions, 0.4 % (w/v) alginate solution and 5.0 % (w/v) calcium chloride, that were prepared previously, were nebulized by large volume nebulizers and allowed to have crosslinking interactions in the air which is trapped inside of the collecting chamber 1 and 2. The formed microparticles in curing solutions were cured for 24 hours with gentle stirring.

3.3.2.2. Microparticle Recovery from Microparticle Dispersion

An aliquot of the aqueous microparticle dispersion was poured into a 100-kDa Amicon Ultra-15 centrifugal filter unit and centrifuged at 7,830 rpm for ten seconds. Then deionized water was added to the Amicon filter unit and centrifuged at 7,830 rpm for ten seconds. After adding sufficient amount of DI water to cover the filter membrane the Amicon filter unit was sonicated for one minute to prevent aggregation of microparticles. This rinsing step was repeated an additional two times more. The microparticles retained by the Amicon filter unit were transferred into a lyophilizer tube and frozen quickly at -80°C. The frozen sample was lyophilized for 72 hours in a lyophilizer (FreeZone 2.5-1 benchtop freeze dry system, Labconco, MO, USA) at -49°C and 0.120 mBar vacuum. The lyophilized microparticles were stored in a vacuum dessicator for further characterization.

3.3.2.3. Particle Size Determination
Dynamic light scattering (Nicomp 380 ZLS, CA, USA) was used for particle size analysis. The lyophilized microparticles were suitably diluted with pH 2 HCl solution. The microparticle dispersion was then transferred into disposable borosilicate glass culture tubes (Kimble Chase, Vineland, NJ, USA). The sample filled tubes were placed in DLS at ambient temperature, and the particle size data collected at a scattering angle of 90°. The data was obtained in triplicate and analyzed with Nicomp software to calculate the size and was expressed as a volume-weighted diameter.

3.3.2.4. Zeta Potential Measurement

Zeta potential measurement was performed in the DLS instrument (Nicomp 380 ZLS, CA, USA) in the Electrophoretic Light Scattering (ELS) mode. The lyophilized microparticle dispersion was placed in a standard glass cuvette. The zeta potential measurements were obtained at ambient temperature and a scattering angle of -14.06° using a helium neon laser of wavelength 658 nm. Triplicate measurements of 1 minute each were collected, and Nicomp software was used to calculate the mean value of zeta potential.

3.3.2.5. Morphology Observation

3.3.2.5.1. Optical Microscopy

An optical microscopy (Leica DM 4000B, Wetzlar, Germany) was used for viewing the morphology of microparticles. Droplets of the aqueous microparticle dispersion were placed on a standard microscopy glass slide (Microscope slides, plain, 3” x 1” x 1 mm, Fisher Scientific, USA). A cover glass slide (Cover glass, 24 x 50-1, Fisher
Scientific, USA) was gently placed on the sample. A magnification of 40x was chosen to observe the microparticles.

3.3.2.5.2. Scanning Electron Microscopy (SEM)

SEM images were obtained in SEM JEOL JSM-7500F (cold cathode analytical field emission SEM, Japan). Randomly selected lyophilized microparticles were gently sprayed on a double-sided carbon conductive tape which was adhered to an aluminum stub. The microparticles placed on the aluminum stub were sputter coated with gold by using a sputter coater (Denton Vacuum Desk II Sputter Coater, Moorestown, NJ, USA) under argon atmosphere to make the microparticles electrically conductive. The external morphology of the microparticles were then analyzed.

3.3.2.5.3. Atomic Force Microscopy (AFM)

AFM studies were carried out in Nanosurf EasyScan 2 Flex AFM system (Nanosurf AG, Switzerland) with EasyScan 2 software. A droplet of the aqueous microparticle dispersion was placed on circular mica disks (Tedpella Inc., Redding, CA, USA) and air dried for 24 hours prior to the AFM observation. AFM data was collected in the dynamic force tapping mode. The scanning range of 10 μm, and speed rate of 0.1 mm/s were used.

3.3.2.6. Differential Scanning Calorimetry (DSC)

Thermal behaviors of sodium alginate and calcium alginate microparticles were studied using a differential scanning calorimeter (PerkinElmer Diamond DSC, CT, USA)
equipped with an intercooler 1P. 20-µl aluminum pans with pin-hole lids were used for DSC measurements and as a reference pan. Lyophilized microparticles (5 to 7 mg) were weighed and crimped in the aluminum pan. Thermograms were recorded at a heating rate of 10 °C/min with the scanning range from 50 to 300 °C for sodium alginate and 10 to 300 °C for calcium alginate microparticles. The nitrogen purge gas was maintained at a flow rate of 20 ml/min. The collected thermal data were analyzed using the Pyris manager (v1.3) software.

3.3.2.7. *Fourier Transform Infrared Spectroscopy (FTIR)*

The IR spectra of calcium alginate microparticles were obtained by Fourier Transform Infrared spectroscopy (Thermo Scientific NICOLET iS5 Fourier Transform Infrared Spectrometer, iD3 ATR, USA) equipped with a Zinc-Selenium crystal. An aliquot of the lyophilized microparticles was evenly placed on the Zinc-Selenium crystal. The wavelength range for IR scan was from 4,000 to 500 cm$^{-1}$ at a resolution of 4 cm$^{-1}$.

3.3.2.8. *Model Drug Encapsulation Efficiency*

Remote drug encapsulation was performed by using methylene blue chloride as a model drug. Lyophilized microparticles (15 mg) were accurately weighed and dyed with 2.5 mM methylene blue chloride solution for 24 hours. The dyed microparticles were frozen at –80°C for 24 hours and then lyophilized for 24 hours in the lyophilizer (FreeZone 2.5-1 benchtop freeze dry system, Labconco, MO, USA) at –49°C and 0.120 mBar vacuum pressure. The lyophilized methylene blue chloride containing
microparticles were dissolved in pH 7.4 phosphate buffer solution for 24 hours to allow microparticles to release the methylene blue chloride sufficiently.

The concentration of methylene blue chloride in the pH 7.4 phosphate buffer medium was measured by UV-Vis spectrophotometry (Agilent 8453 UV-vis spectrophotometer, Shanghai, China). An aliquot of the dye dissolved phosphate buffer medium was placed in a standard glass cuvette. The scanning was done at an absorbance of 665 nm. The encapsulated concentration of methylene blue chloride from the dyed microparticles was obtained in triplicate.

The obtained values were then subjected to the standard calibration curve which was developed to calculate the concentration of methylene blue chloride released from alginate microparticles. The equation obtained from the standard curve was:

\[ y = 25.044 x + 0.0641 \]

\[ R^2 = 0.9931 \]

3.4. Results and Discussion

3.4.1. Formulation of Microparticles

Few trials were performed in order to develop the formulation method. Different types of solvents: oils, organic solvents, and CaCl2 solution; were tried to collect microparticles. Oils such as castor oil and peanut oil were chosen first to collect microparticles. Since they are highly viscous when compared to other types of solvents its use however caused difficulty with isolation and rinsing/washing of microparticles. The use of organic solvents such as ethanol, methylene chloride, and t-butanol can enhance the freezing and drying process. However, the main issue with using organic
solvents was concerns related to toxicity associated with residual solvent and the observed particle swelling in organic solvents. The final formulation for collecting microparticle was done using pH2 CaCl2 solution because it was able to allow enough curing time for hardening the microparticles and prevented swelling of microparticle. The hydrogel typically swells when in contact with water, but not in the acidic solution (< pH 2).4 The typical simple dripping method extrudes the alginate solution in the air and allows it to form beads or particles in the liquid phase of a gelling bath. Unlike the simple dripping method, this method allows the alginate solution and the calcium chloride solution to be nebulized in the air and the interfacial crosslinking interaction occurs in the air.5 The formed microparticles settled down in the curing solutions located in collecting chambers 1 and 2 [Figure 3-1]. The unreacted vapors of sodium alginate and calcium chloride flows through the PVC tube that is connected between collecting chamber 1 and collecting chamber 2 and the interfacial crosslinking reaction concludes in collecting chamber 2. The initial trials of the apparatus setup design did not include the second collecting chamber. With the addition of the second collecting chamber the particle yield significantly increased. The production yield with two collecting chambers increased by about three times to 99.0 ± 3.7 mg when compared to the setup with one collecting chamber, where the yield was 31.7 ± 2.6 mg.

3.4.2. Microparticle recovery from microparticle dispersion

In order to have microparticles in the form of dry powder, a recovery process from the aqueous microparticle dispersion was required. The microparticles, which were
not obtained with the recovery process, aggregated and formed a gel film. The aggregated microparticles (the gel film) also showed crystalline particles on the gel film. The optical microscopy image below clearly showed the crystalized salts on the surface of the gel [Figure 3-2]. This is probably due to the unrinseed salt ions that remained from the curing solution on the gel particles.

![Image](image.png)

**Figure 3-2.** An optical microscopic image with magnification of 40x (Micromaster, FisherScientific, USA) of the aggregated gel particles with salt crystals.

Since pH 2 CaCl2 solution was chosen to collect microparticles, a rinsing process was necessary. This was because the microparticle does not completely dry in the presence of salt ions. In order to remove the salt ions and residual acid from the collecting solution (pH 2 CaCl2 solution), the following steps were tried during the recovery process: methylene chloride, t-butanol, separation funnel, centrifugation, chitosan coating, filter paper, sintered glass filter with/without vacuum, and 100-kDa Amicon Ultra-15
centrifugal filter units. As discussed previously in the formulation trials, the use of organic solvents: methylene chloride and t-butanol; had issues with toxicity and particle swelling. The separation funnel was used when oils were tried to collect microparticles. However, there was a limitation to move microparticles from the oil phase to aqueous phase due the surface tension difference between the two phases: the oil phase and the aqueous phase. Although the use of centrifugation can collect microparticles in the bottom of the centrifugation tube, it resulted in microparticle aggregation due to the gravitational force. Microparticles were also coated with chitosan to separate each microparticle individually. With this process severe microparticle aggregation was observed. The use of filter paper is easy and economic. It however contaminated the final microparticles with paper fibers. Therefore, a sintered glass filter was selected because it does not react with the microparticle dispersion and also does not contaminate microparticles. The only issue with using the sintered glass filter was a long filtration time which took over 12 hours to complete. During the filtration time, particle swelling was observed. To reduce the filtration time, vacuum was applied to the sintered glass filter. However, the microparticles continuously clogged the surface of sintered glass filter which further delayed the filtration time.

The collected particles were recovered by multiple washings of deionized water to eliminate all acidity and salt ions. The formed calcium alginate particles typically swell when in contact with water.\(^4\) 100-kDa Amicon Ultra-15 centrifugal filter units were chosen to rinse the microparticles because it filters the microparticle dispersion and DI water quickly, and reduces the particle contact time with DI water. The lyophilized microparticles did not show significant size increase after exposing the microparticles to
water. To see the particle size difference between the particles washed with the two different methods, the lyophilized microparticles were dispersed in pH 2 HCl acidic solution and regular DI water separately, and recovered.

The particle size of the prepared microparticles was measured to see if the microparticles swelled and resulted in any particle size growth. The size range of microparticles in pH 2 5.0% calcium chloride solution before recovery process was between 45.9 µm and 53.8 µm. The size of microparticles after the recovery process ranged from 27.3 µm to 51.8 µm. Based on two particle size data (the pH 2 HCl acidic solution and the regular DI water), it can be seen that there was no significant particle swelling. Therefore, it was concluded that the addition of a small amount of water for a short time (~10 seconds) during the microparticle recovery process did not affect the microparticle swelling. The recovered microparticles after lyophilization appeared as white solid powder. The appearance of final microparticles is shown in [Figure 3-3].

![Figure 3-3. The lyophilized calcium alginate microparticle powder by the nebulized aerosol mediated interfacial crosslinking method.](image)

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3.4.3. Particle Size Determination

The total observed particle size range varied from 9.9 µm to 60.5 µm. The size of particles that were formed immediately in the reacting chamber before curing process was between 9.9 µm and 24.8 µm. The majority of the size distribution after 24 hours of curing time in the curing solution was between 51 µm and 53 µm [Figure 3-4].

![Particle size analysis of calcium alginate microparticles.](image)

3.4.4. Zeta Potential Measurements

Zeta potential can be used to evaluate the electrical charge surrounding alginate particles. This measurement can provide indications about the stability of a colloid and
any interfacial interactions between the biomaterial and the drug. Surface charge studies also guide further surface modifications. In this study, the average zeta potential of lyophilized calcium alginate particles was \(-46.815 \pm 0.165\) mV.

3.4.5. Morphology Observation

3.4.5.1. Optical Microscopy

The morphology of calcium alginate microparticles is presented in [Figure 3-5]. The shape of microparticles is generally granular shape, but they are not uniform overall. This irregular appearance is probably developed during the initial ionotropic reaction process and lyophilization. Since these microparticles are produced in the air phase and not formed in a completely surrounded cationic environment, this may provide insufficient gelling reactions to some microparticle population. Moreover, the process of freeze-drying may shrink the particles and produce irregular shapes.

Figure 3-5. Optical microscopy images of calcium alginate microparticles
3.4.5.2. **Scanning Electron Microscopy (SEM)**

SEM is a helpful analytical technique to observe microparticle appearance, surface morphology, and size. [Figure 3-6a] demonstrates the unique granular shape of microparticles. However, some microparticles in [Figure 3-6b] present sponge like spheres. This is probably due to the reason that the microparticles were formed in the air phase and resulted in partial gelling reactions as explained previously. The microparticles seen in the SEM micrographs are around 20 µm which concurs with the size range seen by DLS.
Figure 3-6. SEM images of calcium alginate microparticle of (a) granular shape and (b) irregular shape.

3.4.5.3. Atomic Force Microscopy (AFM)

AFM is specially designed for observing the topographical features of microparticles. Different locations of surface roughness and characteristic morphology of randomly selected microparticles are described in [Figure 3-7]. As shown in [Figure 3-7], microparticles depicted characteristic embossed bubble like surface texture.
3.4.6. Differential Scanning Calorimetry (DSC)

DSC thermograms of sodium alginate and the calcium alginate microparticles were obtained. The thermogram of sodium alginate exhibited an exothermic peak at 246.8 °C. Similar thermal behaviors were shown by other authors. Literature reviews suggest that this is probably due to the degradation of biopolymer since it also shows a noisy graph [Figure 3-8a]. The pure ingredient, sodium alginate, for formulating microparticle was characterized by an exotherm, while the thermogram of calcium alginate microparticle did not show any characteristic thermal behaviors [Figure 3-8b]. This is because of the formation of the characteristic egg box structure of sodium alginate with calcium ions.9

Figure 3-7. AFM images of calcium alginate microparticle.
Figure 3-8. DSC thermograms of (a) sodium alginate and (b) calcium alginate microparticle.
3.4.7. Fourier Transform Infrared Spectroscopy (FTIR)

The structural composition of calcium alginate microparticles was analyzed by FTIR study. A blank microparticle and bulk ingredients: sodium alginate and calcium chloride, were prepared separately, and the spectrum of each sample were obtained as described below [Figure 3-9].

Figure 3-9. FTIR spectrum of (a) calcium chloride, (b) sodium alginate, and (c) calcium alginate microparticle: (1) OH hydrogen bond, (2) carbonyl group (C=O), (3) asymmetric stretch of COO⁻, (4) symmetric stretch of COO⁻, and (5) single bond (C-O).
Peaks only above 1000 cm\(^{-1}\) were analyzed since they are the main fingerprint peaks to distinguish the chemical composition of samples.

IR spectrum of calcium chloride showed a broad peak at 3443 cm\(^{-1}\) and sharp peaks at 2334 cm\(^{-1}\) and 1622 cm\(^{-1}\) [Figure 3-9a]. Sodium alginate exhibited a broad peak at 3178 cm\(^{-1}\) and others at 1593 cm\(^{-1}\), 1401 cm\(^{-1}\), and 1018 cm\(^{-1}\) [Figure 3-9b]. The characteristic peaks of calcium alginate microparticles displayed a broad peak at 3388 cm\(^{-1}\) and other peaks at 2929 cm\(^{-1}\), 1724 cm\(^{-1}\), 1611 cm\(^{-1}\), 1415 cm\(^{-1}\), 1258 cm\(^{-1}\), and 1035 cm\(^{-1}\) [Figure 3-9c]. The broad peak around 3400 cm\(^{-1}\) represents OH hydrogen bond, and the shoulder peak in calcium alginate microparticles is not significant as it is shown in most organic compounds.\(^{13}\) The characteristic peaks that calcium alginate microparticles exhibit, at 1724 cm\(^{-1}\) and at 1258 cm\(^{-1}\) appears to be a carbonyl group (C=O) and a single bond (C-O), respectively.\(^{14}\) Other peaks, around 1600 cm\(^{-1}\) and 1400 cm\(^{-1}\), are corresponding to asymmetric stretch of COO\(^{-}\) and symmetric stretch of COO\(^{-}\).\(^{13}\)

3.4.8. Model Drug Encapsulation Efficiency

Encapsulation efficiency study was performed by the remote-controlled drug encapsulation method to see if the microparticles, which are formed by the nebulized aerosol mediated interfacial crosslinking method, have the potential to be used in drug delivery.\(^{15}\) Methylene blue chloride was selected to be as a model drug, since it is a water soluble model drug. In the present study, the drug loading was observed to be 15.1 ± 3.1 µg of methylene blue chloride per 1 mg of calcium alginate microparticle.

3.5. Conclusion

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Calcium alginate microparticles were successfully produced by the novel nebulized aerosol mediated interfacial crosslinking method. This apparatus setup formed microparticles by the ionotrophic external gelation method using sodium alginate and calcium chloride. The microparticle recovery process was able to separate the particles during the process without swelling and damaging the particles. The external morphology of microparticles was granular. The size range of the formulated microparticles was from 10 µm to 60 µm. A high positive zeta potential value was obtained. The microparticles prepared by this method also showed potential as a drug carrier. This apparatus design may provide another production approach for calcium alginate formulation.

3.6. Future Studies

The following studies are proposed to further develop and validate the microparticle preparation process.

- Scale-up
- Model API incorporation
- Drug loaded microparticle characterization
- \textit{In-vitro} release
Chapter 3 References


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