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Enhancements in Alginate Microencapsulation Technology & Impacts on Cell Therapy Development

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ENHANCEMENTS IN ALGINATE MICROENCAPSULATION TECHNOLOGY & IMPACTS ON CELL THERAPY DEVELOPMENT

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DEDICATION

To the memory of my father, Omar Belhaj (1937-2014), who would have been proud of me reaching my goals.

To my mother, Aziza Abo-Janah, for her constant, unconditional love, and support toward success.

To my dear husband, Suliman Arebi, who has been a constant source of support and encouragement during the challenges of graduate program and in daily life. I am truly thankful for having you in my life, and for helping me figuring out my path for bright future.

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ABSTRACT

Biodegradable alginate microcapsules (MC) are recently becoming widely used in the biomedical field, and have shown effectiveness as a drug delivery system. Alginate has been used in microencapsulation due to its simple gelling process, biodegradability, excellent biocompatibility, and its stability under in-vivo conditions. Alginate polymer is a natural polysaccharide derived from brown seaweed and has the ability to polymerize rapidly in the presence of cations to form a porous matrix. In order to advance the previous system for efficient protein and peptide delivery, we further lyophilized the peptide-filled microcapsules and we were able to rehydrate, and test them for sustained release. Electrospray method, as a microencapsulation technique, has been previously used to encapsulate peptides and proteins successfully using alginate. A second coating of poly-l-ornithine (PLO) polymer can be used to increase the integrity of alginate microcapsules. Alpha-carboxy terminus 1 (αCT1) peptide, Human platelet lysate (HPL), and bovine serum albumin (BSA) were examples of peptides and proteins that were successfully encapsulated by our lab.

Cell-based therapies represent a revolutionary bio-technique that has been applied widely in medicine. Recently, they have been successfully applied in treatment of neurodegenerative diseases, eye diseases, cardiovascular disease, diabetes, liver disease and cancer. Suppression of the host immune system is considered the main challenge in such therapies. In order to avoid the side effects of immunosuppressive drugs,

encapsulating cells into polymeric matrices is considered a promising strategy. Encapsulation systems utilize permeable materials that allow diffusion of nutrients, waste and therapeutic factors into and out of cells, while masking the cells from the host immune response. In our model, we have been encapsulating ARPE-19, human immortal cell line, that was genetically designed to express and secret CR2-fH protein to test them for safe and effective long term inhibition in vivo. CR2-fH is a complement inhibitor molecule that has been recently found to express promising therapeutic effects in both in vivo and in vitro models of Age-related Macular degeneration (AMD). Furthermore, human skeletal muscle differentiating cells (skMDC) that were recently used in biomedical research to investigate skeletal muscle behavior, function, and ability to be implemented in tissue regeneration field for further applications of disease advanced therapies.

In this study we hypothesize that alginate encapsulation using high voltage method can be enhanced and advanced further to use it in different clinical approaches and applications. The present study shows the successful formation of alginate-poly-L-ornithine microcapsules using the above-mentioned method, according to specific parameters, to produce microcapsules about 200 μm in diameter. The microcapsules derived from this encapsulation technique according to our hypothesis can be freeze dried without harming the shape of the microcapsules in which they retain the original shape after hydration. The main purpose of lyophilization is to increase the shelf-life storage of encapsulated biomaterials as well as preserve its activity to be used for treatment. Moreover, the technique has the ability to preserve the metabolic activity of genetically engineered ARPE-19 cells and skMDC cells resulting in effective and safe long-term

drug delivery for treatment. In addition, hydrogel systems have been utilized in combination with the previous approaches, lyophilized microcapsules and encapsulated cells, for more improvements in regenerative medicine and targeted delivery therapy fields.

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CHAPTER I

INTRODUCTION

1.1 Microencapsulation and Drug Delivery Systems

Drug delivery systems (DDS) are engineered approaches or technologies for controlled release of therapeutic agents and/ or delivery of them to target organs in the body in order to eliminate or minimize systemic side effects. Recently, DDS have been improved steadily, and the interest in controlled release dosage forms has increased. DDS that include carriers, composed mainly from lipids and/or polymers, and their associated therapeutic agents on macro, micro, and nano scale are primarily designed to manipulate biodistribution and pharmacokinetics of the associated drugs. In addition, DDS are functioning as sustained release systems especially with advances in biotechnology related to macromolecules such as proteins, peptides, oligonucleotides, and plasmids [1,2].

Microencapsulation systems are one of DDS that are recently getting more attention in advancement for targeted delivery of therapeutic agents. Microcapsules yielded from this technique are characterized by their semipermeable membrane that allows diffusion of the encapsulated materials from the inner core. The permeability of the microcapsules can be adjusted by coating with one or more layers which provide a controlled release property or add protection of the encapsulated materials. There is a diversity of materials that can be encapsulated successfully for different clinical

applications such as peptides, proteins, enzymes, and antibiotics. Properties of microcapsules (size and shape) are controlled by different parameters such as applied voltage, spraying distance, flow rate, method of encapsulation, and polymer concentration[3]. The synthesis procedures can vary such as electrospray method, emulsification, jet flow, and solvent evaporation; which result in microcapsules with different morphologies and shapes including spheres, cubes, rods, and irregular patterns[3–5]. There are two main categories in the field of electro-hydrodynamics: electro-spraying and electro-spinning.

Microparticles, as drug delivery systems, have been developed due to the advancement of pharmaceutical materials technology that allowed creation of polymeric microcapsules using electrically charged fluids of different natural and synthetic polymers. The polymeric microcapsules have distinctive properties in terms of shape, particle size, drug loading, and profile release[6]. Currently, there are a wide range of polymers that are used for microencapsulation technology including sodium alginate, chitosan, poly-lactic-co-glycolic acid (PLGA), poly-ethylene glycol (PEG), poly-l-ornithine (PLO), and poly-l-lysine (PLL)[7–12]. Unique properties of each polymer such as biocompatibility, viscosity, reactivity with the encapsulated materials, and charge; make it unique for its desired application.

All the different encapsulation procedures share the same methodology concept. One of the polymers used for encapsulation is placed in a positively charged syringe attached to a high voltage generator, and the flow rate of the polymer is controlled by using a syringe driver or pump where the polymer is extruded to a grounded gelling bath. A cone jet is formed at the needle tip due to application of an electric polarization stress

(electrically conductive solution passes through electrical potential difference), flow and gravity. The cone jet is a formation of conical shape of liquid at the needle tip where the charged particles of the liquid accumulate at the apex of the cone. Once a minimal steady state has been reached of both flow and electrostatic voltage, the charged particles separate from the apex and break into reproducible droplets. The material's nature and properties plays main role in this stage of the procedure[13,14]. Without applying electrical forces to the procedure, the droplet will be controlled by the surface tension of the liquid polymer and effect of the gravity[15]. The formed droplet, as micro or nanoparticles, will finally be collected in a grounded solution (as in electrospray method) or device (as in electrospinning method). Both methods are becoming more popular as methods of drug delivery because they can be easily adjusted or altered to form particles possessing a desired release rate and pharmacokinetics. On the other hand, other methods of encapsulation may require additional steps such as heat, shear stress, and pressure which might negatively affects the encapsulated therapeutic agents by decreasing its functionality or degrading its structure[3]. Our method of interest in this work is the electrospray method to produce spherical microcapsules using sodium alginate as a primary polymer.

Alginate is a natural polymers and it is used widely in microcapsule formation because of its unique properties including low toxicity, biocompatibility, mechanical stability, bioadhesive properties, gelling properties and abundance[2,16,17]. It is a naturally occurring polysaccharide extracted from brown seaweed, and it's chemical structure is composed from a linear copolymer of (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid as it is described in Figure 1.1 [10,18–23]. Different applications of alginate

has been explored such as its usage as coating material, preparation of controlled release drug delivery systems including microspheres, gels, and membranes; as well as forming a hydrogel when crosslinked to cations such as calcium of barium[16,24,25].

1.2 Freeze drying—Lyophilization

Lyophilization, or freeze drying, is a technique often used mainly to enhance the stability and shelf life of pharmaceutical products. This technique is based on dehydrating a pre-frozen material at low temperature and low pressure, and resulting in formation of a porous structure. In addition to preserve color, scent, and shape of lyophilized materials, freeze drying technique keeps the biochemical characteristics and pharmacological properties to the greatest extent. Freezing and drying stresses applied by lyophilization could tend to destabilize or denature protein compounds, but different proteins endure freezing and drying stresses to various degrees. Although lyophilization of protein formulations is a challenging process, it is considered a common procedure in the pharmaceutical field to improve stability of therapeutic proteins especially to maintain their original characteristics upon reconstitution[26–29]. Freeze drying has been used previously to dehydrate proteins, plasma, vaccines, blood platelets, and sperm cells and it has been shown to increase their shelf-lives[29–34]. Also, seeking activity preservation of most biological materials containing live bacteria and viruses through freeze drying become widely used to ensure long term preservation.

The lyophilization process is typically performed initially by freezing the biological material to freezing temperature range of (-50°C)-(-80°C), followed by drying the biological material under vacuum to remove frozen water from the material at two

different stages of drying. Primary drying at temperatures below 0°C and the drying rate at this stage is limited to avoid foaming of the product due to the high temperature and the excess liquid (solvent) amount at this stage. Then the secondary drying stage begins at temperatures above 0°C to bring the residual moisture of the biological material to an optimum level. If the moisture content is above or below the optimum value, the effective life of biological material will be reduced[35–37].

Freeze drying of microencapsulated human retinal pigment epithelial cells (ARPE-19) has been shown to be successful in a recent study[38]. It has been demonstrated that the cells were able to retain their viability and structural integrity after freeze drying[39]. Cell were encapsulated in polycation coated alginate microcapsules and lyoprotective solutions such as trehalose that protects proteins, cellular membrane, and nucleic acids from the harmful effects of the dehydration[32,40–43]. Trehalose replaces the cellular water molecules and forms intracellular glass to protect the viability of the cells from the damage that can be caused by the dehydration. Also sugars and antioxidants can be added in different protocols for the same protection purposes[43–47].

1.3 Cell encapsulation

Cell encapsulation technology refers to entrapment of living cells as targeted delivery systems. Encapsulated cells can be used for two purposes: delivering living, functional cells to the target organs for tissue engineering purposes; delivering therapeutic molecules produced from genetically engineered cells to a targeted tissue or organ. More important, this technique provides the advantage of long term targeted treatment without the necessity for recurrence. As described in Figure 1.2, cells after

encapsulation will be entrapped within a polymeric semipermeable membrane that allow influx of nutrients and oxygen; and outflux of metabolic waste products and therapeutic molecules. The membrane also protects the cells from the host immune system[48].

Cell encapsulation technique is a promising approach in the development of cell based therapies to treat acute and chronic illnesses. Recently, research studies are focused on encapsulating different cell types such as ARPE-19 cells, peripheral blood mononuclear cells, non-human primate multipotent stromal cells, mesenchymal stem cells, and islet cells[39,49–52]. Alginate polymer is widely used in encapsulating different types of cells and the purification degree of alginate is playing a great role in the success of cell encapsulation process. It has been shown that poorly purified alginate resulted in failure of many microencapsulation processes; indeed, ultra-purified raw product (clinical grade) of alginate in combination with PLO showed to be highly proficient in rendering immunity stimulation of the host body where the mannuronate dimeric blocks of alginate covalently bound to PLO[49].

In a previous clinical study it has been shown that cells encapsulated within alginate/PLO fabricated microcapsules had enough durability and robustness over a long period of time lasts up to 5 years without any immunity sensitization[49]. The team of this study was able to retrieve physically intact microcapsules after 5 years post-transplant from one of the patients with type I diabetes included in the study. Although the study was successful in transplanting encapsulated islet cells in diabetic patients for a long period of time without any adverse effects, no therapeutic effect has been recorded including regeneration of native islet cells or downregulation of plasma glucose levels.

However, such a successful clinical study brings motivation and hope that this technique can be developed to play a vital role in cell targeted therapy in the near future[49].

1.4 Hydrogel

Hydrogels are composed of hydrophilic polymer chains that form a three-dimensional system with high water content. The characteristics and properties of each hydrogel depend on the type of polymer used to prepare it, and also can define the purpose of use or the results to be pursued of each application. These properties are varied such as: biocompatibility, elasticity, capability to mimic extracellular matrix (ECM), and adaptable chemical properties[53–55]. Polymers used in preparing hydrogels are classified in two categories: naturally derived from materials such as collagen, alginate, chitosan, and hyaluronic acid or synthetically derived from materials such as poly hydroxyethyl methacrylate (poly HEMA) and poly ethylene glycol (PEG)[56,57]. Hydrogels are gaining attention in biomedical research field and regenerative medicine due to their ability to serve as cell and drug delivery therapies, and due to the advancement accomplished in biomaterial systems which set the foundation for therapeutic strategies [54,58–62].

Stem cell therapy using hydrogel as a bioactive material is one of the important applications of hydrogel where it is known as regulation of stem cell fate in the field of cell delivery therapy. Hydrogels act as platform that allows better viability, proliferation, and retention of the stem cells[63–67]. The similarity in the structure between hydrogel scaffolds and extracellular matrix of many tissues is beneficial and increases the

successfulness rate of this approach in engineering tissue replacements; as well as in drug and growth factor delivery[68].

Usage of natural polymers in preparing hydrogel scaffolds is becoming more popular in regenerative medicine recently. Collagen is considered one of the most commonly used natural polymers in this field due to its biocompatibility, biodegradability, and high versatility[69–71]. It is one of the main components of extracellular matrix proteins existing in the mammalian tissue, and it is derived from skin and tissue of vertebrate species[67,72,73]. It has been shown previously that type I collagen based hydrogels can cooperate with polyacrylamide to create matrix models of scars which considered soft matrices that exhibit less cell to cell noise. Moreover, type I collagen is able to covalently cross-link to protein patterns to stimulate interactions between the hydrogel surface and the cells[74,75]. Collagen hydrogels have been used with great success as three-dimensional media for cell culture and showed promising results acting as scaffolds for engineered tissues and tumors. More important, adjustment of quantitative characteristics of collagen hydrogel properties is a critical phase to fulfill engineering requirements and match specific tissues in order to be considered as viable tissue mimics[76].

My studies aim to further investigate different approaches to test the hypothesis that microencapsulation technique is a unique tool or device which could be advanced to fulfill the necessity of different clinical applications and therapies. These studies are described in the following chapters:

- Chapter II: The role of lyophilization process in advancing microencapsulation technique.

- Chapter III: The effect of electrospray method of encapsulation on the viability of living cells within alginate based microcapsules.

- Chapter IV: Involvement of hydrogel systems in advancing alginate based microcapsules.

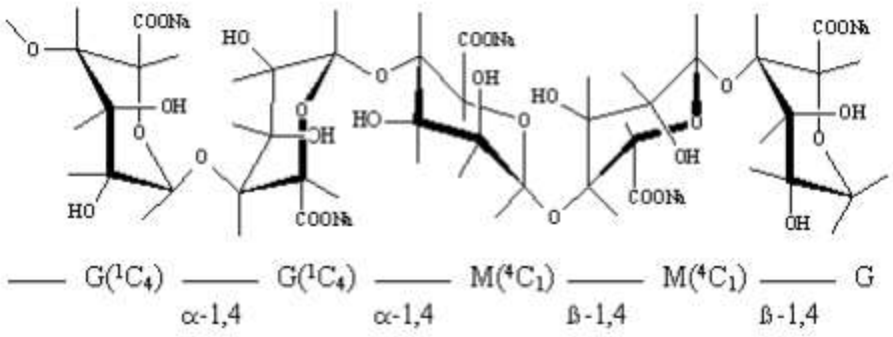

Figure 1.1 The chemical structure of sodium alginate polysaccharide building units. (G) Glucuronic acid, and (M) Mannuronic acid. Image produced by the Food and Agriculture Administration of the United Nations.

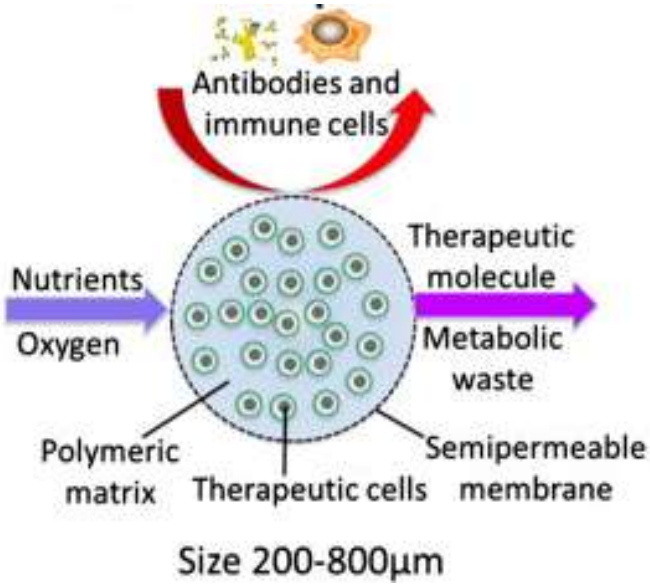

Figure 1.2 Schematic illustration of cell encapsulation technology.
(Reproduced with permission from Bhujbal SV, de Vos P, Niclou SP. Drug and cell encapsulation: Alternative delivery options for the treatment of malignant brain tumors. Advanced Drug Delivery Reviews. 2014;67-68:142-153. doi:10.1016/j.addr.2014.01.010)

CHAPTER II

THE ROLE OF LYOPHILIZATION PROCESS IN ADVANCING MICROENCAPSULATION TECHNIQUE[1]

2.1 Background

Targeted drug delivery and controlled release delivery are considered key areas in drug delivery systems. Advancements in technology are becoming more focused on precisely targeting therapeutic agents to the exact cell, tissue, or organ; and in controlling the fate of a therapeutic agent entering the body[77]. The encapsulation method is a major factor in encapsulation and release of therapeutic agents. Other factors also control the characteristics of microcapsules; the type of polymer icluding its molecular weight, viscosity, and surface charges contribute in defining its characteristics as well as the characteristics of formed microcapsules[78]. Using electrospray method as a method of fabrication of polymers is the method of choice in this study. Also, alginate is the core polymer used to form microcapsules in adjacent to poly-l-ornithine which is used as a double coat to increase the integrity and enhance the selective porosity of the microcapsules[79,80]. In this work, we hypothesized that lyophilization of A-PLO microcapsules might advance this technology and enhance its usage for a variety of applications.

[1] Parts of this chapter have been excerpted from the following research article:
Belhaj M, Menon V, Rohrer B, Potts J. Alginate Microcapsule Technology and Impacts on Cell Therapy Development. Microsc Microanal. 2017;23(S1):1214-1215.

Lyophilization is a process that operates at different conditions and temperatures which may result in products with different characteristics[26,81,82]. However, lyophilization has been used widely to preserve different biological materials used for a variety of clinical purposes such as antibiotics, vaccines, proteins, and cells[31,35,36,45,83]. Moreover, lyophilization is a promising procedure in maintaining different therapeutics, including pharmaceuticals and biological materials, in a dry form for long-term storage, transport, and rehydration at the time of clinical administration[84].

2.2 Materials and Methods

Microencapsulation:

Electrospray Method was used to prepare the microcapsules[7,85,86]. Sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) was mixed under sterile conditions at a concentration of 2% w/v. The resulting mixture was purified by filtration at 0.2 mm. Human platelet lysate (HPL) (Cook Biotech) was added to the alginate and small aliquots (280 mL) of filtered alginate and HPL mixture were loaded into a 3cc syringe and attached to a syringe pump. Alginate-poly-Ornithine (A-PLO) microcapsules were produced by a one-step method. Polymer solution of poly-l-ornithine (Alfa Aesar L-ornithine hydrochloride 99%) was mixed at a 0.5% w/v in 0.15 M calcium chloride (CaCl2) (Sigma-Aldrich). The pH was buffered accordingly by 0.1 M HEPES.

A volume of 30 mL of the gelling solution was placed in a beaker below the syringe pump as a gelling bath, with a needle-to-bath working distance of 7 mm (spray distance). A high voltage generator was attached to the tip of 30cc blunt tip needle (purchased by Amazon Supply, Seattle, WA, USA) with a ground placed in the CaCl2

solution. Next, a constant voltage was set on the high voltage generator to pass a field of current through the needle tip attached to the syringe. The syringe pump was started to push the filtered alginate solution through the needle tip and into the $CaCl_2$ gelling bath with a flow rate of (60 mm/h). As the extruded alginate was pushed by the syringe pump, it passed through the positively charged needle into the negatively charged $CaCl_2$ gelling bath creating spherical microcapsules. The voltage remained constant throughout the microcapsulation process by setting the generator prior to starting the syringe pump. Synthesized microcapsules were allowed to gel 12 minutes then removed from the gelling bath post-synthesis and rinsed several times in sterile deionized water to remove any traces of $CaCl_2$.

Lyophilization of Microcapsules:

Microcapsules were prepared as mentioned above. The suspended microcapsules in deionized water were frozen using liquid nitrogen. Once the sample was frozen, the beaker was covered with parafilm or a piece of foil and pierced many times to allow escaping of water vapor. Then the sample was placed into a lyophilizer vessel (Labconco, Kansas City, MO, USA) overnight to get a dry powder of microcapsules. The lyophilized microcapsules were rehydrated using deionized water and tested morphologically to ensure complete recovery and shape retaining.

Scanning Electron Microscopy (SEM) of Microcapsules:

It was performed to characterize surface morphology of lyophilized and non-lyophilized microcapsules and examine the effect of lyophilization process on microcapsules morphology. Prior to SEM preparation, microcapsules from both groups

were exposed to UV power at 9,000 μJ x 100 on a plastic petri dish for 30 minutes. An incubation of 24 hours at 4°C was then allowed. Exposure to UV power has been done to increase microcapsule rigidity that insures maintaining the shape during harsh SEM processing. The method of processing was carried out based on a modified protocol using the osmium-tannic acid osmium method (Murphy, 1980). A buffer of 0.1M Na cacodylate, 3mM CaCl2 at pH 7.2 was used initially to wash microcapsules followed by a buffered fix of 2% paraformaldehyde, 2% glutaraldehyde, and 0.1% ruthenium red.

The microcapsules were then rinsed with (Na cacodylate/ CaCl2) buffer and placed in a secondary buffered fix consisting of 1% osmium tetroxide and 0.1% ruthenium red. The same buffer solution, (Na cacodylate/ CaCl2) buffer, was used to rinse fixed microcapsules, and then placed in a series of alternating buffered fixatives containing either 1% tannic acid in 1% glutaraldehyde or 1% osmium tetroxide. Rinses in these buffered fixatives were repeated eight times at 20 min each with rinsing with buffer in between steps. Finally, the microcapsules were rinsed with water to remove any buffer residues, followed by deydrating in a series of ethanol baths, critical point drying (Tousimis Samdri PVT 3B; Tousimis, Rockville, MD, USA), and gold sputter coating (Cressington 108 Auto/SE; Cressington Scientific Instruments Ltd., Watford, UK). SEM images were taken using a JEOL JSM-6300 SEM at 10 kV (JEOL Ltd., Tokyo, Japan).

Microencapsulation and Release Profile of BSA Protein:

Release profiles of BSA were studied over 10 days from alginate microcapsules formation or from lyophilized microcapsules rehydration at different temperature settings, 37°C and room temperature. In our studies we have elected to study release in

alginate microcapsules before lyophilization and rehydrated microcapsules after lyophilization.

For these experiments, encapsulation parameters were kept constant at 6.0kV initial voltage, flow rate of 60mm/hr, and 7 mm spray distance. Initial BSA protein concentration was kept constant at 1mg/ml. Alginate concentration was also kept constant at 2%, while the $CaCl_2$ gelling bath was synthesized at 0.15M $CaCl_2$ as previously listed. As it has been suggested in a previous study held in the lab, pH as well kept constant at 7.3. Microcapsules used for release profiles were not coated with poly-l-ornithine polymer. Post synthesis, microcapsules were rinsed in deionized water to remove excess gelling solution. The microcapsules were then re-covered in 0.5mL deionized water, with time points taken over 10 days. Early time points of 1, 2, 4, 6, and 8hrs were taken to account for early burst release of peptide from the microcapsules. Analysis of peptide concentration was performed using a Micro BCA protein assay kit (Thermo Scientific) with BSA standard solution for our standard curve. All tests were run on a BioTek Synergy 2 spectrophotometer.

2.3 Results

Microencapsulation technique in our lab has been performed using electrospray method under specific parameters to produce ~200 μm microcapsules in diameter. The voltage was 6.0 kV, the flow rate was 60 ml/hour, the spray distance was 7 mm, and 2% concentration of alginate solution. Also, the microcapsules were coated with poly-l-ornithine to increase the integrity of alginate microcapsules. Before processing the microcapsules for electron scanning microscopy, the microcapsules were crosslinked

using ultraviolet cross linker to increase their stiffness in order for the microcapsules to maintain their shape during the preparation protocol for microscopy. As shown in Figure 2.1 A, the microcapsules were spherical in shape at its hydrated state with an average diameter of 200 μm. Microcapsules after encapsulation process were washed thoroughly to remove any traces of $CaCl_2$; then the microcapsules were loaded into lyophilizer for dehydration. Dried microcapsules appeared as white powder, and its structure appeared to be shrunk when examined with light microscopy and SEM as it is shown in Figure 2.1 B.

Adding deionized water to the lyophilized microcapsules led to its rehydration and regaining almost the original spherical shape compared to control microcapsules (before lyophilization). In Figure 2.1 C, rehydrated microcapsules appeared to have rough surface compared to control microcapsules; however, most of the microcapsules retained back their spherical appearance where very few rehydrated microcapsules get burst after rehydration.

Loading efficiency experiments were conducted on four samples. Two identically synthesized sets of microcapsules, one used immediately after encapsulation process and one after rehydration following lyophilization, were both analyzed for release at 37°C and room temperature. Our preliminary data in Figure 2.2, indicated a rapid initial burst of BSA prior to the initial 1 hour time point in the examined samples. Release from non-lyophilized microcapsules at room temperature does not show the initial burst release, and it shows a delayed release until day 6 of incubation when it starts releasing. Previous reports have suggested that the insets of release are at the five hours of release for better appreciation of the rapid release of peptide[85]. The fluctuating release of BSA over all the

samples is not clear. The impact of lyophilization and incubation temperature on BSA release is unknown and must be further investigated in order to determine significance.

2.4 Discussion

A-PLO microcapsules previously showed a great potential as a controlled release delivery system of connexin 43 peptide to increase wound healing rates *in vivo*[80,85]. Parameters of encapsulation procedure are what control the characteristics of the microcapsules produced. Nature and features of polymers have played an integral role in advancements of delivery systems which defines the characteristics of microcapsules.

Accordingly, in this study we are using electrospray method to produce approximately 200 μm microcapsules by adjusting alginate concentration, flow rate, spraying distance, and voltage rate. Our main goal is to optimize this technology and improve its versatility for variety of clinical applications; therefore, lyophilization is one of the approaches that will be examined in the current study. Lyophilization, freeze drying, has been utilized since 1958 to enhance the pharmaceutical products in order to be utilized at different administration strategies[87].

As demonstrated through our results in Figure 2.1 that A-PLO microcapsules were successfully lyophilized; not only that but also they were able to retain their morphology back after rehydration. The microcapsules shown were empty, not loaded with any material. Proteins were one of the biological materials to be lyophilized successfully for stability and storage purposes. Although lyophilization was ideal to prepare solid protein pharmaceuticals achieving stability at acceptable storage shelf life; however, this process generates different stresses such as temperature changes, vacuum

forces, pH changes, and formation of ice crystals. Protein denaturation at various degrees might occur as a result of exposure to all of the stresses listed earlier[26,87]. Numerous research studies have been conducted to identify critical effects of protein lyophilization on protein stability and effectivity[37,82,88]. It has been shown that the need for stabilizers is very critical in maintaining encapsulated biological materials' effectivity during freeze drying and storage afterwards as well as preserve the microcapsules structure[40,42,89]. The results shown in our study indicates that the lyophilization process was capable of preserving the original structure of our alginate-PLO microcapsules. However, further investigations needed to verify the effects lyophilization on characterizations of microcapsules which serve as controlled release delivery system.

Release profiles of biomaterials have proven to be controlled by different factors including encapsulation parameters and incubation environment. As a result, it has been previously shown that release of αCT1, a low molecular weight peptide, can be controlled by altering extrusion parameters or by including an additional polymer coating to in order to interfere with diffusion pathway[85]. According to our preliminary data, the effect of lyophilization on release of BSA is not clearly understood. In addition, the change in incubation temperature presumed to have an effect that was not clearly correlated to the control of BSA release from alginate microcapsules. Further investigations are highly recommended to identify a clear vision on the effect of lyophilization on releasing of encapsulated biomaterials which will dramatically affect the feasibility of lyophilization as technique intended for long term storage of encapsulated therapeutics.

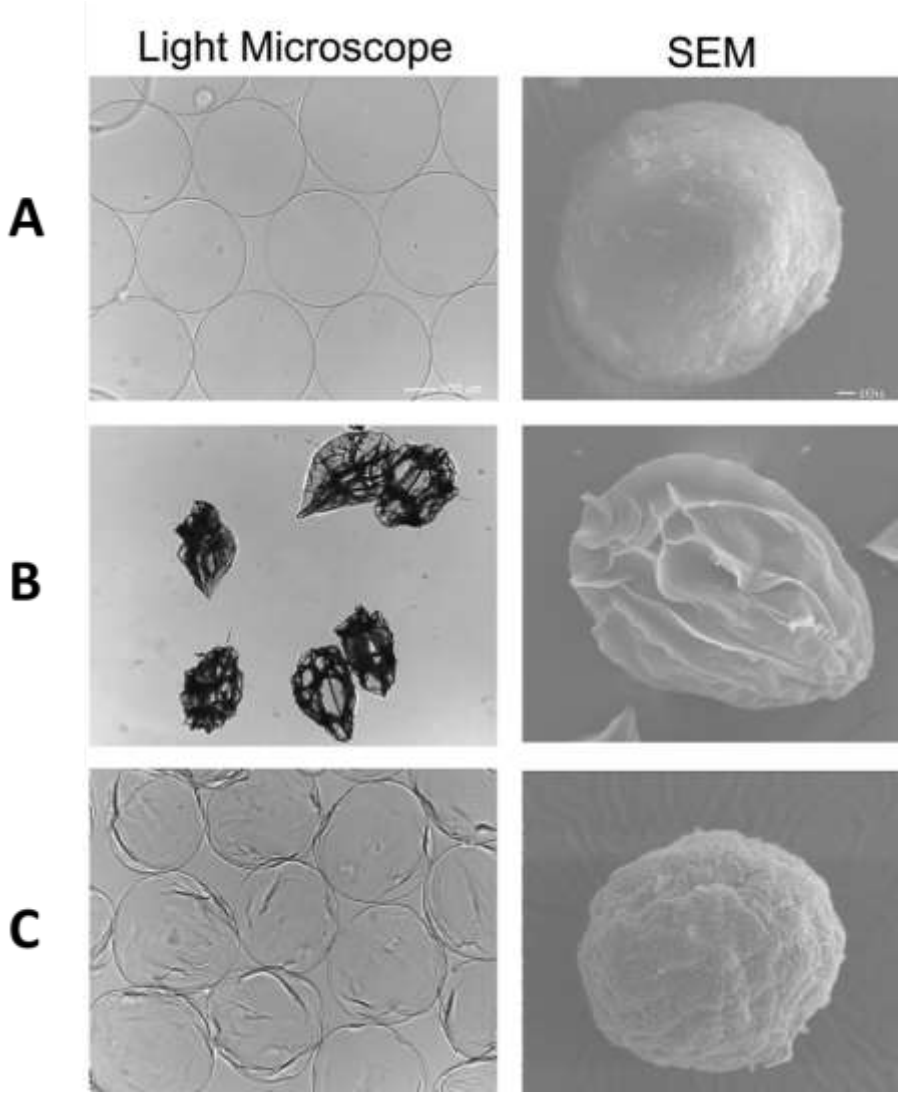

Figure 2.1 Light microscope and SEM images of empty alginate microcapsules.
(A) Microcapsules before lyophilization in a hydrated form and spherical shape with a diameter of ~200 μm. (B) Lyophilized microcapsules that appear shrunk in a dehydrated form. (C) Lyophilized microcapsules were rehydrated by adding DI water where the microcapsules retain their spherical shape. Scale bars are 100 μm (LM) and 10 μm (SEM).

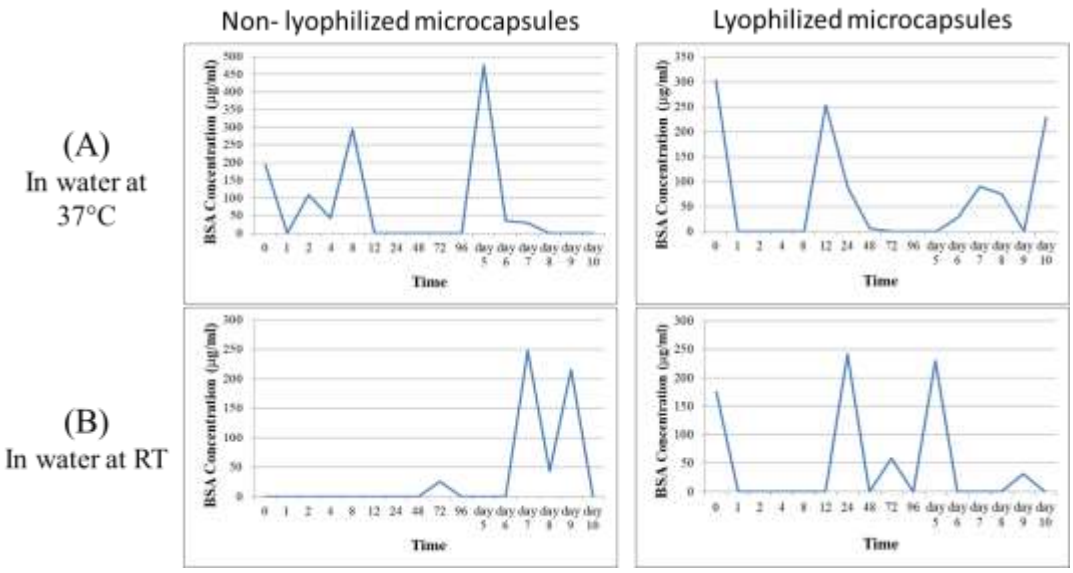

Figure 2.2 Release profiles comparison of loaded BSA within non-lyophilized and lyophilized alginate microcapsules at different incubation temperatures.
All 2% alginate microcapsules were loaded with BSA at concentration of 1 mg/ml. The microcapsules were synthesized and gelled in a 0.15M $CaCl_2$ bath at a pH of 7, a flow rate of 60 mm/hr, and a voltage of 6.0 kV. (A) Comparing release profiles of BSA in water at 37°C between non-lyophilized and lyophilized microcapsules. (B) Comparing release profiles of BSA in water at room temperature between non-lyophilized and lyophilized microcapsules. Measurements were examined using Micro BCA protein assay at 562nm.

CHAPTER III

THE EFFECT OF ELECTROSPRAY METHOD OF ENCAPSULATION ON THE VIABILITY OF LIVING CELLS WITHIN ALGINATE BASED MICROCAPSULES[2]

3.1 Background

Currently available drug delivery systems cannot fulfill the demand of several diseases for tight regulation of therapeutic factors. Accordingly, cell therapy shows a potential to serve as a new approach of delivery systems for multiple diseases including neurodegenerative diseases, diabetes, and muscle diseases[48,90]. Genetically engineered cells, stem cells, and primary cells are the main focus of recent research studies with the aim of avoiding the implant rejection by host immune response and maintaining cell viability. Therefore, cell encapsulation emerges as a promising therapeutic strategy for targeted management of numerous health problems. The goal of cell encapsulation is to serve as a drug delivery tool, sustained release of therapeutics from encapsulated cells, and regenerative medicine tool, delivering cells to particular tissues[49,90].Utilizing encapsulated cells as a therapeutic application was pioneered by Lim and Sun early in 1980 when they demonstrated the ability of inducing normal glycemic levels in diabetic rats by implanting microencapsulated islets[91].

[2] Parts of this chapter have been excerpted from the following research article:
Belhaj M, Menon V, Rohrer B, Potts J. Alginate Microcapsule Technology and Impacts on Cell Therapy Development. Microsc Microanal. 2017;23(S1):1214-1215.

Our aim in this work is utilizing A-PLO microcapsules as a carrier for different cell types genetically modified ARPE-19 cells, expressing inhibitory factor CR2 and CR2-fH, and skMDC cells that might be beneficial for future clinical approaches as delivery systems.

3.2 Materials and Methods

Cells Culture:

Human retinal pigment epithelial cells (ARPE-19), cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The medium was replaced every 2-3 days, and the cells were passaged after 70%-80% confluence was reached. Cells were used between 24-27 passages.

ARPE-19 cells were transfected using AAV- mediated delivery with plasmid constructs of CR2 and CR2-fH to express CR2 and CR2-fH proteins. These transfected cells were provided by our collaborators, Dr. Baerbel Rohrer's lab, at The Medical University of South Carolina, Department of Ophthalmology. The cells were cultured in High glucose/without L-glutamine- Dulbecco's Modified Eagle Medium (Sigma Aldrich®) supplemented with10% dialyzed FBS (Sigma Aldrich®), 2% Glutamine synthetase expression medium (GSEM- Sigma Aldrich®), and 0.28μl/ml L-methionine sulfoximine (MSX- Sigma Aldrich®). The medium was replaced every 2-3 days, and the cells were passaged after 70%-80% confluence was reached. Cells were used between third and fifth passage.

Primary human skeletal muscle derived cells (skMDC) were obtained from Cook Myosite®(Pittsburgh, PA). skMDC were cultured in MyoTonic™ basal medium supplemented with MyoTonic™ growth supplement. Cells were cultured according to the supplier recommendations at 37 °C and 5% CO2 and maintained below 80% confluence. For differentiating the cells, the growth media will be replaced with MyoTonic™ differentiation media (DMD- Cook Myosite) after cells reach confluence, and exchanged after 2 days. The differentiation will be apparent within 3-4 days following replacing the media.

Cell Encapsulation:

Electrospray method of microencapsulation has been used to encapsulate the targeted cells. In this method, sodium alginate (Sigma-Aldrich) was mixed with DI water under sterile conditions at concentration of 2% w/v and purified by filtration at 0.2 mm. Alginate solution (~300 μl aliquots) were loaded into a 3cc syringe and attached to a syringe pump, and pumped through a needle (30G blunt tip; Small Parts, Inc., Logansport, IN, USA) to a gelling bath placed in a beaker below the syringe at 7 mm of a needle to bath spraying distance. The gelling bath contains a volume of 40 mL of 10 mM HEPES buffered saline containing 100 mM calcium chloride (CaCl2) (Sigma-Aldrich), and 0.5% w/v poly-L-ornithine (PLO) (Alfa Aesar) that forms a second coating to the microcapsules as one step method for adjusting the porosity. The needle tip connected to a high voltage generator while the gelling bath grounded. By applying high voltage between positive and grounded bath, alginate is extruded from the needle tip to the gelling bath where calcium ions (Ca2+) replace sodium ions (Na+) forming alginate microcapsules coated with PLO and cells mixed with alginate solution will be trapped

inside the microcapsules. Cells were trypsinized, centrifuged, and washed with 10 mM HEPES buffered saline solution (pH 7.4), and a final cell concentration of $1x10^6$ in alginate solution were adjusted. Constant parameters of 60mm/hr flow rate and 6.0kV initial voltage were adjusted to produce microcapsules size of ~200 μm. The size of the microcapsules can be monitored by adjusting flow rate and voltage applied in which ~150 μm diameter microcapsules can be retrieved by applying parameters of 30mm/hr and initial voltage 8kV. Formed microcapsules containing cells were washed with washing solution (10 mM HEPES buffered saline containing 1.5 mM CaCl2, pH 7.4) twice, and then incubated with suitable media in a humidified incubator at 37°C and 5% CO2.

Cell recovery:

The cells were released from alginate microcapsules by adding 55 mM Sodium citrate in 10 mM HEPES buffered saline (pH 7.4) to the encapsulated cells with gentle shaking for 5 mins at room temperature. In this step, calcium ions will replace sodium ions in the microcapsule structure resulting in diluted sodium alginate and releasing the cells from the microcapsules. Furthermore, the cells were centrifuged at 1200 rpm for 5 mins and incubated with media at 37°C and 5% CO2 for 24 hrs.

Cell viability (Live/Dead) assay:

Encapsulated cells—after incubating the encapsulated cells for 24 hrs, approximately 30 microcapsules were washed twice with washing solution (10 mM HEPES buffered saline containing 1.5 mM CaCl2) and stained for live-dead viability. Live/Dead assay kit (Molecular Probes) was used to evaluate the viability of cells after

encapsulation. According to the manufacturer's instructions, CalceinAM indicated viable cells by green fluorescence (excitation/emission ~495/515 nm), whereas Ethidium homodimer-1 indicated dead cells by red fluorescence (excitation/emission ~495/635 nm). Encapsulated cells in washing solution were mixed with Calcein AM and Ethidium homodimer-1 at final concentrations of 2 μM and 4 μM respectively, and incubated for 30-45 mins in dark at room temperature. After staining, microcapsules were washed twice with washing solution and imaged using Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA).

Encapsulated ARPE-19 cells in hydrogel— hydrogel was washed with PBS to remove any media traces and stained for encapsulated cells viability. Live/Dead assay kit (Molecular Probes) was used to evaluate the viability of cells after encapsulation and mixing with collagen hydrogel. According to the manufacturer's instructions, CalceinAM indicated viable cells by green fluorescence (excitation/emission ~495/515 nm), whereas Ethidium homodimer-1 indicated dead cells by red fluorescence (excitation/emission ~495/635 nm). A cocktail of Calcein AM and Ethidium homodimer-1 at final concentrations of 2 μM and 4 μM respectively was added to the hydrogel and incubated for 30-45 mins in dark at room temperature. After staining, excess amount of staining solution was washed with PBS and we imaged the encapsulated cells in the hydrogel using Invitrogen EVOS FL Auto Cell Imaging System.

Immunofluorescence analysis of the skMDC:

skMDCs were trypsinized from confluent plates and then grown overnight on 24 well plate with initial seeding density of 10,000 cells per well. Cells then were fixed with

2% formaldehyde for 5 minutes and permeabilized with PBS- 0.1%Triton X-100 for 10 minutes. Fixed cells were washed with PBS and incubated at room temperature with Glycine then with 5% BSA/PBS for 30 minutes each in order to stop the fixation reaction using formaldehyde, and block non-specific protein binding accordingly. Cells after blocking were washed and incubated overnight at 4C° with Desmine, MyoD, and Pax-7 (Santa Cruz Technologies, Inc), 1:50 each. After washes, cells were incubated with 1:100 Texas Red (TX)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) together with DAPI for nuclear staining (Invitrogen/ Life Technologies). Samples were analyzed on Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA) in the Instrumentation Resource Facility (IRF) at the USC School of Medicine. Cells were observed with a 10X objective lens, using a DAPI filter to visualize nuclei and a TX filter for Desmin, MyoD, and Pax7. Results were observed in three separate experiments.

3.3 Results

Encapsulation of ARPE-19 cells:

Electrospray method at voltage rate of 6.0 kV enabled us to encapsulate ARPE-19 cells within 200 μm A-PLO microcapsules as it is shown in Figure 3.1(B). Encapsulated cells were then incubated in 10% FBS DMEM media at 37°C/ 5% CO_2 for a period of time, microcapsules were imaged at different time points 3days, 6 days, and 20 days. As it is shown in Figure 3.1 the encapsulated cells were proliferating inside the microcapsules and appeared as clumps of cells. After 20 days of incubation, the microcapsules start to burst and cells find their way outside the microcapsules as shown

in Figure 3.1 (E). We revealed that FBS supplement in media has to be decreased from 10% to 2% to slow down their proliferation to prevent losing the microcapsules.

ARPE-19 cells viability test after encapsulation and their recovery from microcapsule structure:

After encapsulation and incubation in 10% FBS media for 3 days, cells were stained for live/ dead assay to evaluate their viability. Green color indicates viable cells whereas dead cells were stained red. In Figure 3.2 (A), Cells in green (viable) were more than dead cells (Red).

We examined the ability of the cells to be recovered back from microcapsules and their ability to be incubated and proliferate after exposure to high voltage rate during encapsulation. Encapsulated cells were incubated in 55 mM sodium citrate solution HEPES buffered saline. Sodium ions from the solution will replace calcium ions attached to alginate which will convert it into a soluble form and allow us to collect cells after centrifugation. Recovered cells were cultured back in media and incubated at 37°C and 5% CO_2. Cell showed viability after being cultured back in their desired media, and we were able to passage it up to 3 times after recovery.

Encapsulation of genetically modified ARPE-19 cells, viability, and recovery:

Genetically modified ARPE-19 cells were provided by our collaborators from MUSC which expresses CR2 and CR2-fH proteins. Cells were encapsulated successfully as indicated in Figure 3.3 B and examined for viability where the viable cells were highly found in our sample; dead cells were barely found in our sample of live/dead assay as

shown in Figure 3.3 (A). Cells were then recovered from microcapsules and incubated in media as shown in Figure 3.3 (C) where the cells showed viability after recovery and they were passaged for up to 3 times.

Encapsulation of skMDC cells, viability, recovery, and differentiation:

In Figure 3.4, skMDC cells encapsulated using the same method of encapsulation, and the encapsulated cells examined for viability where in Figure 3.4 (C) most of the cells were green indicating its viability.

Cells were recovered and were recovered back in media as shown in Figure 3.4 (D). In addition, a group of recovered cells were cultured in differentiating media Figure 3.4 (E) where the cells showed viability in the new media and incubated to give them a chance to differentiate into skeletal muscles.

Staining skMDC cells for myogenesis markers Desmin, MyoD, and Pax-7:

The main purpose of staining the cells for myogenesis markers is to examine the effect of encapsulation process has on the cell viability and metabolic activity. Staining skMDC cells against the myogenic markers showed that the cells from the entire 3 group: Control (before encapsulation), recovered, cells in differentiation media (differentiated), were able to express MyoD, desmin, and Pax7. Nuclei were stained blue (Dapi) while the targeted protein stained in red as it shown in Figure 3.5.

Freezing the encapsulated cells was another aspect that we utilized with ARPE-19 cells. After encapsulation and incubation in media for 3 days, we changed culture media to a freezing media were the encapsulated cells mixed with and prepared for freezing.

Vials of encapsulated cells were stored in Mr. Frosty and kept at -80°C for up to one week. Finally, the encapsulated cells were thawed and cultured back in supplemented media, and then they were tested for viability using live/dead assay. As shown in Figure 3.8, the cells were stained in green indicating that their viability still preserved; red signal indicating dead cells was barely expressed.

3.4 Discussion

Since enhancement of A-PLO microcapsules is the main goal of this work, cell encapsulation is another approach in which we utilize our microcapsules to encapsulate different type of cells. The polymer membrane serves as immunoisolation barrier for the cells intended for applications as therapeutic delivery system and regenerative medicine. Indeed, there are some preliminary clinical studies of prolonged survival of cells; however, cell encapsulation technology has not yet accomplished the expected clinical advances[49,92]. Recently, a wide range of research studies focused on investigating the effects of different fabrication methods and variety of polymers on viability and effectivity of variety of encapsulated cells[47–50, 91–102]. In this work, we investigated the effect of electrospray method of fabrication on cells encapsulated in A-PLO. Cells used for this study were intended for therapeutic agent delivery system, ARPE-19/ CR2-fH, and for regenerative medicine, skMDC.

Encapsulated ARPE-19 cells through our fabrication method showed that the viability and effectivity of cells was preserved after encapsulation in Figure 3.1 and Figure 3.2. Consequently, genetically modified ARPE-19 cells, steadily express CR2-fH protein, proved to be viable and effective after encapsulation in Figure 3.3. ARPE-

19/CR2-fH cells were generated in order to develop innovative therapeutic tool targeting treatment for age-related macular degeneration (AMD). Factor H serve as an inhibitory agent of the alternative pathway (AP) of complement activation, whereas polymorphisms in FH are associated with AMD risk. CR2-fH, the novel recombinant form of fH, has found to be effective inhibitor in different AP-dependent disease models such as collagen-induced arthritis, and acute lung reperfusion injury. Importantly, CR2-fH has shown that it is able to reduce the choroidal neovascularization (CNV) *in vitro* when injected intravenously[105–109]. Encapsulated ARPE-19 cells expressing CR2-fH showed to be effective *in vivo* as therapeutic agent delivery system. It has been presented that CR2-fH was secreted in the eye from the encapsulated cells after intravitreal injection, and was effective in reducing compliment activation and can decrease CNV in mouse[93].

Primary human skeletal muscle cells (skMDC) are considered and targeted for cell therapy[110]. Encapsulation of skMDC cells might be useful as a potential therapeutic tool for treatment of severe muscle diseases and conditions. The results of this study demonstrated that cells viability and morphology characteristics were preserved after encapsulation in Figure 3.4. It was been showed that cells were recovered from microcapsules in which it continue to proliferate indicating that encapsulation process did not affect its biological characteristics. In addition, cell staining for the expression of myogenic markers such as desmin, MyoD, and Pax-7 showed that the cells in all the samples were able to express the targeted proteins Figure 3.5, 3.6, and 3.7 to proof that encapsulation technique does not affect the metabolic activity of encapsulated cells.

Moreover, we showed in this work that cryopreservation of A-PLO encapsulated cells is a feasible for future therapeutics delivery and regenerative medicine projects.

Previous research studies have been demonstrated that cryopreservation is a promising approach for preserving encapsulated cells for future optimization[101]. Encapsulated ARPE-19 cells have been shown to preserve the viability and biological activity after cryopreservation Figure 3.8. The goal of this approach was to generate the foundation for cryo-banking for future utilization of encapsulated cells in regenerative medicine and therapeutics delivery projects.

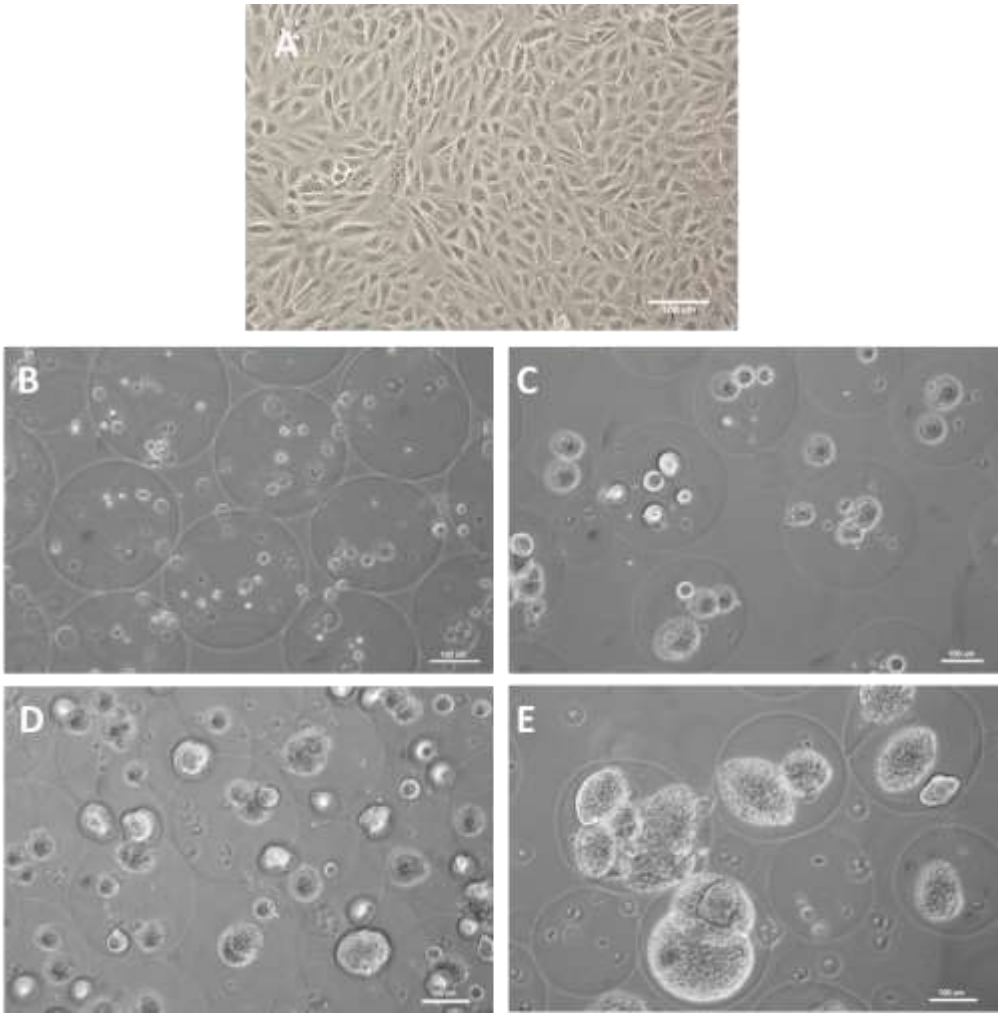

Figure 3.1 Effect of electrospray method on encapsulation of ARPE-19 cells.
(A) ARPE-19 cells cultured in DMEM media. (B) Encapsulated cells at initial concentration of 1x106 cells/ ml after 1 hour of encapsulation. (C) Cells after 3 days of encapsulation. (D) Cells continue to grow inside the microencapsulation after 6 days of incubation in media. (E) Microcapsules became full of growing cells after 20 days of incubation in 10% FBS media. Scale bars = 100 microns.

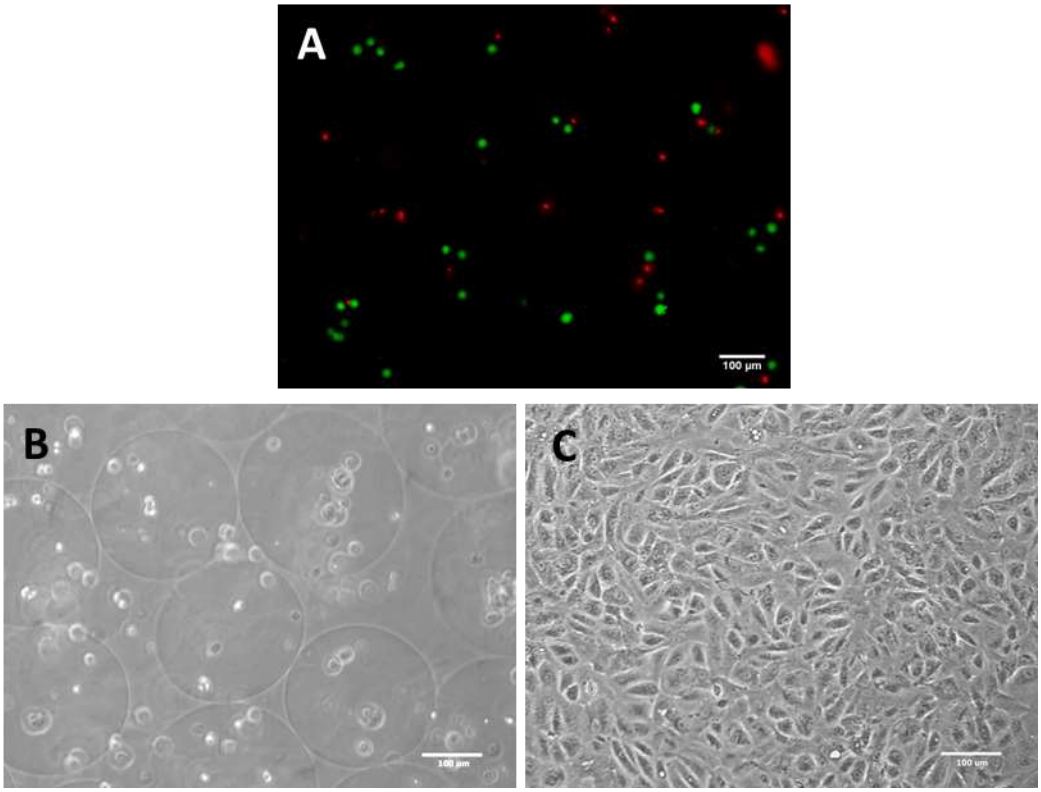

Figure 3.2 Viability of ARPE-19 cells after encapsulation and Recovery.
(A) Live/ dead staining performed on encapsulated cells after 3 days of encapsulation. Viable cells stained in green (CalceinAM), whereas cells in red (Ethidium homodimer) indicates cells with massive membrane damages. (B) ARPE-19 cells after encapsulation and before recovery. Cells were recovered from microcapsules using sodium citrate solution. The recovered cells have been passaged up to 3. Scale bars = 100 microns.

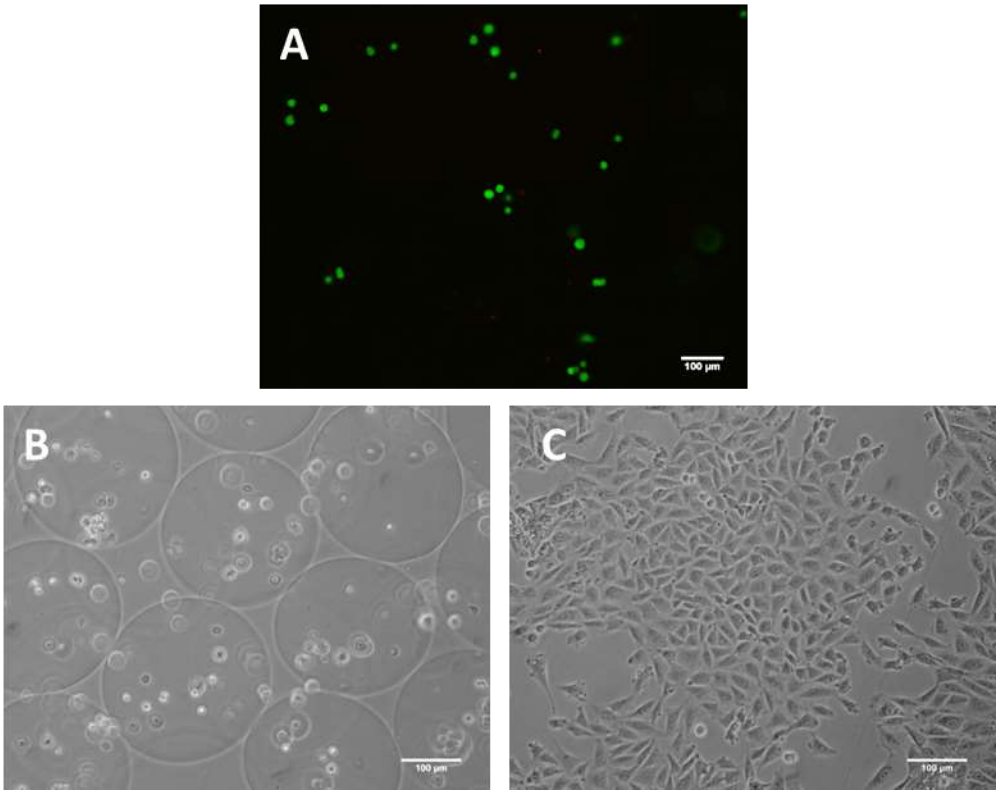

Figure 3.3 Viability of ARPE-19/CR2-fH cells after encapsulation and Recovery.
(A) Live/ dead staining performed on encapsulated cells after 3 days of encapsulation. Viable cells stained in green (CalceinAM), whereas membrane damaged (dead) cells in red (Ethidium homodimer). (B) ARPE-19 cells after encapsulation and before recovery. Cells were recovered from microcapsules using a sodium citrate dissolving solution. The recovered cells have been passaged up to 3 times. Scale bars = 100 microns.

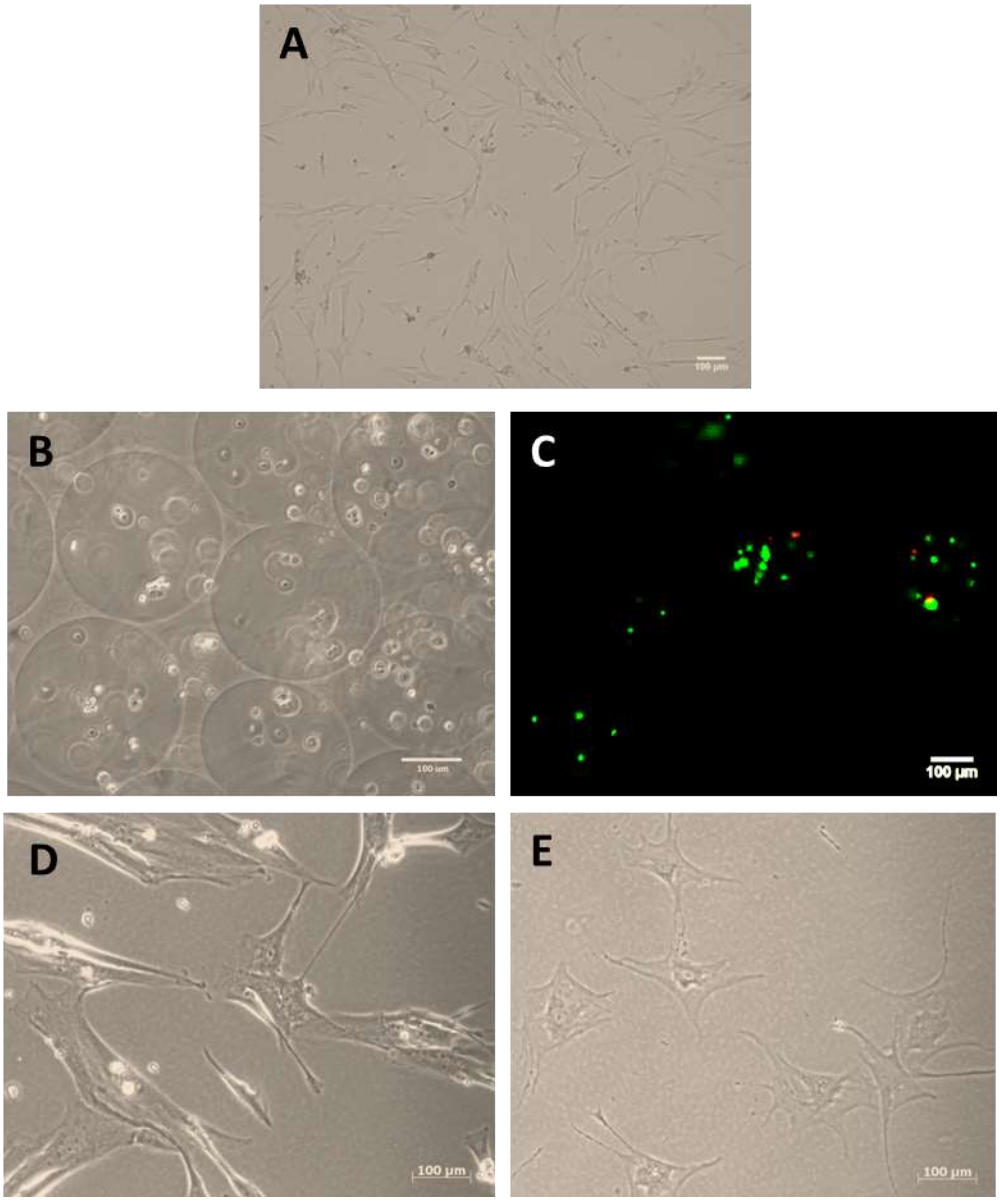

Figure 3.4 Viability of skMDC cells after encapsulation and Recovery.
(A) skMDC cells cultured in supplemented MyoTonic media. (B) Encapsulated skMDC cells at initial encapsulation concentration of 1x106 cells/ml. (C) Encapsulated cells after 3 days of encapsulation were stained with CalceinAM (2 µM/ Green) and Ethidium homodimer (4 µM/Red). Viable cells stained in green whereas dead cells in red. (D) skMDC were recovered from microcapsule structure using citrate sodium solution and cultured back into supplemented MyoTonic media. (E) A Group of recovered cells was cultured in differentiation media to allow them to differentiate and change phenotype. Scale bars = 100 microns.

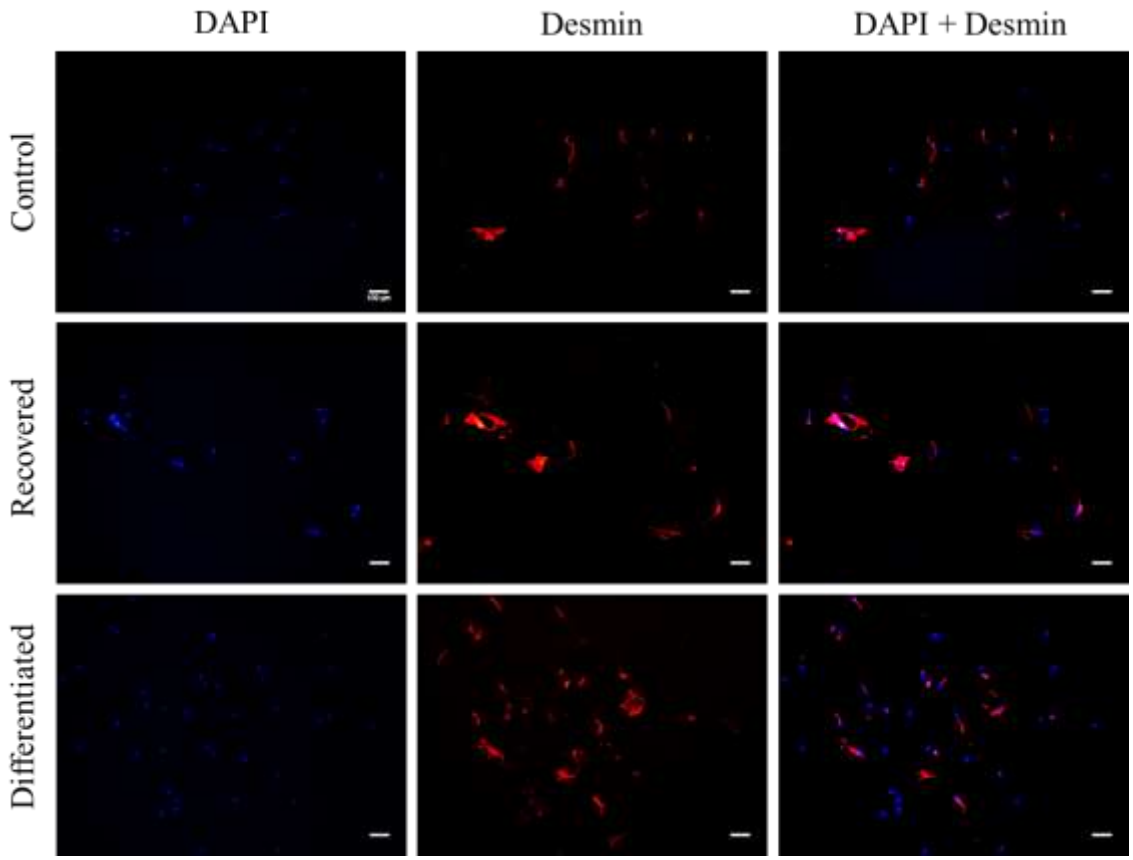

Figure 3.5 The effect electrospray method of encapsulation on Desmin expression in skMDC cells.
Control cells (before encapsulation), recovered, and differentiated cells were probed for DAPI (Blue) and desmin (Red). Scale bars = 100 microns.

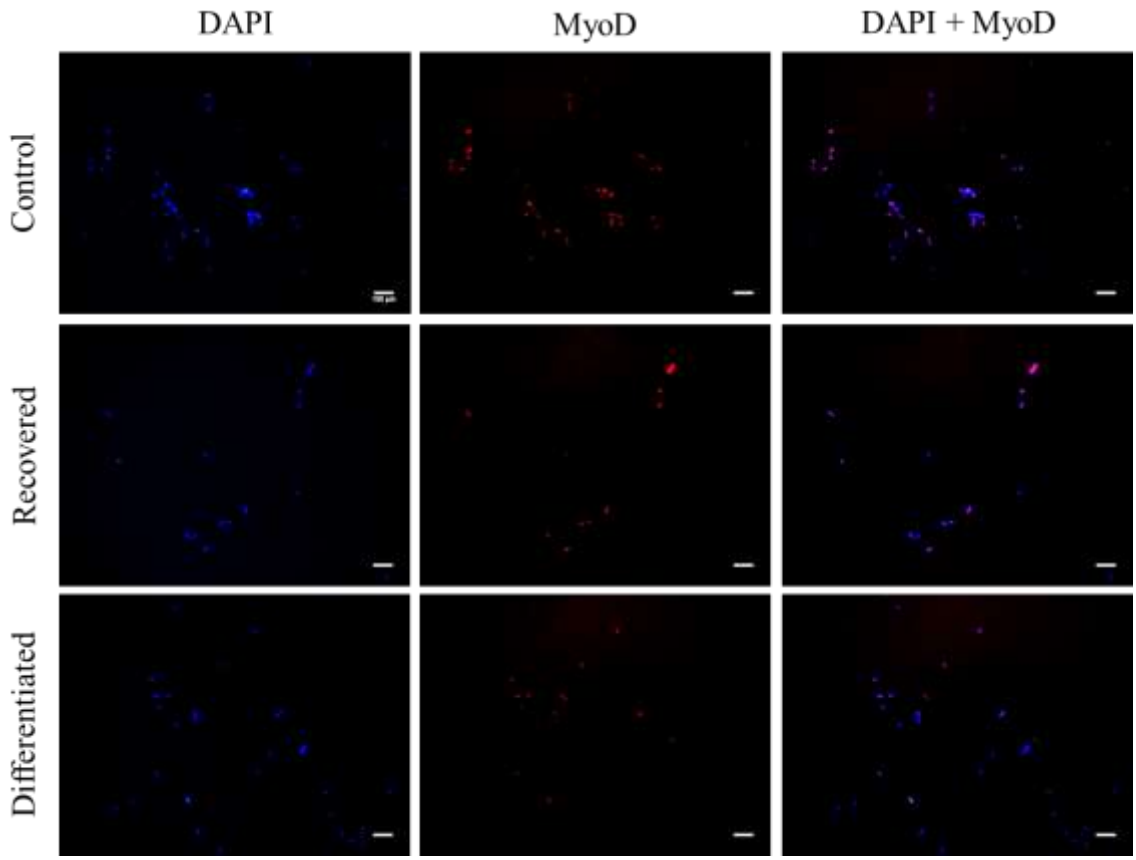

Figure 3.6 The effect electrospray method of encapsulation on MyoD expression in skMDC cells.
Control cells (before encapsulation), recovered, and differentiated cells were probed for DAPI (Blue) and MyoD (Red). Scale bars= 100 microns.

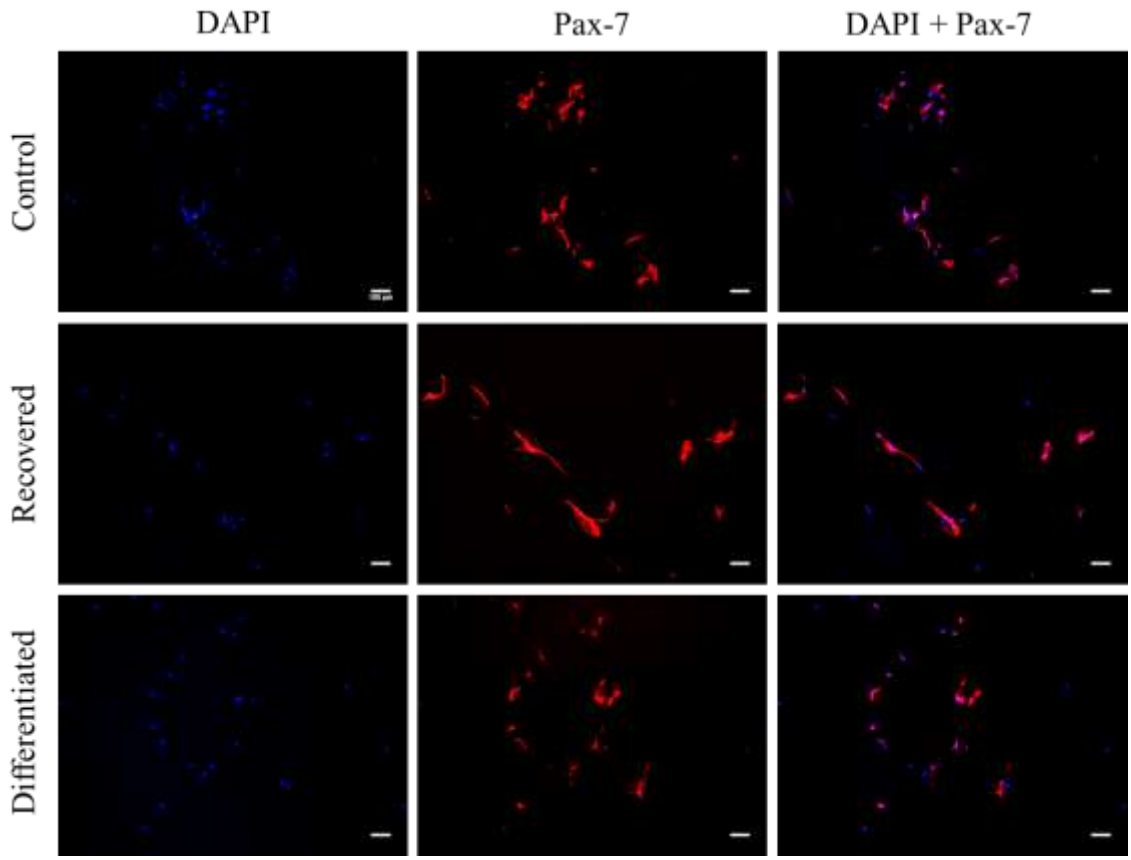

Figure 3.7 The effect electrospray method of encapsulation on Pax-7 expression in skMDC cells.
Control cells (before encapsulation), recovered, and differentiated cells were probed for DAPI (Blue) and Pax-7 (Red). Scale bars = 100 microns.

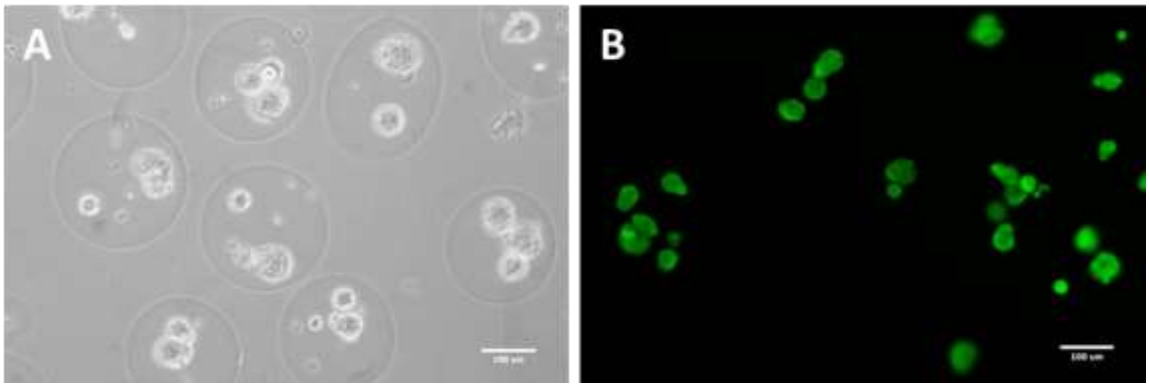

Figure 3.8 Viability of encapsulated ARPE-19 cells after cryopreservation.
(A) Encapsulated cells thawed after freezing at -80C° for 1 week. (B) Encapsulated cells were tested for their viability after freezing. Viable cells were stained in Green (CalceinAM), and dead cells were stained in red (Ethidium homodimer). Scale bars = 100 microns.

CHAPTER IV[3]

ENCAPSULATED CELL TECHNOLOGY-BASED DELIVERY OF A COMPLIMENT INHIBITOR REDUCES CHOROIDAL NEOVASCULARIZATION IN A MOUSE MODEL

4.1 Background

Age-related macular degeneration (AMD) is a slowly progressing condition that is classified according to the pathological mechanism into two categories, wet and dry AMD. In wet AMD, fluid accumulates in the subretinal space due to choroidal blood vessels invasion through damaged blood barrier (BRB), and the fluid accumulation could cause retinal detachment that consequently lead to photoreceptor deterioration as complications of untreated condition. On the other hand, the pathological mechanisms of dry AMD are still not clearly understood. The main characteristics of dry AMD are degeneration of retinal pigment epithelium (RPE) and choriocapillaries that correlates to the loss of photoreceptor cells. Involvement of genetic and environmental risk factors are responsible on the complexity of AMD[111]. Wet AMD occurrence has been showed to be associated with single nucleotide polymorphisms (SNPs) in vascular endothelial growth factor (VEGFa), VEGFR2 receptor, and genes in the proliferation subpathway[112–115]; however, there is no clear pharmacogenetic association in anti-VEGF therapy between patient's response to the treatment and patient's VEGFa or VEGFR2 SNPs[116,117].

[3] This chapter have been excerpted from the following research article:
Annamalai B, Parsons N, Belhaj M, Brandon C, Potts J, Rohrer B. Encapsulated Cell Technology-Based Delivery of a Complement Inhibitor Reduces Choroidal Neovascularization in a Mouse Model. Transl Vis Sci Technol. 2018;7(2):3-3.

In addition to the multiple SNPs in genes of the complement pathway including polymorphisms in Y402H variant in the complement inhibitor factor H (CFH) which are linked with an increased risk of developing AMD[118]. According to association between AMD and Y402H, this study focused on investigating complement activation mechanisms in AMD models as well as examining improvements in complement therapeutics for treatment of AMD pathologies.

The complement system is a part of innate immune system that is activated in response to stress and/or injury stimulators. The activation of the complement systems followed by multiple biological molecules activation cascade including C3a and C5a (anaphylatoxins), opsonins C3d and C3dg (opsonization of damaged cells), membrane attach complex (MAC- lysis of cells)[119]. A complement inhibitor molecule (CR2-fH) has been developed and effectively inhibits complement activation at RPE levels in vitro in AMD models[120–122]. In addition, CR2-fH has been shown via in vitro models that it was effective in reducing choroidal neovascularization (CNV) development by suppressing complement activation and VEGF production[123–125]. Moreover, CR2-fH has shown effectivity as an inhibitor indifferent AP dependent disease conditions such as collagen induced arthritis, and acute lung and ischemia perfusion injury[126,127]. The inhibitory activity of CR2-fH is prompted due to presence of AP-inhibitory domain that linked to a complement receptor (CR2) and targets the inhibitor fH to bind complement activation products[128]. Importantly, the efficacy and safety of subretinal administration of AAV vector encoding the CR2-fH inhibitor has been confirmed in mouse model of CNV[129]. In the current study, efficacy of delivering CR2-FH using encapsulation technique to encapsulate genetically modified ARPE-19 cells has been examined in mouse CNV

model. The small size of produced alginate microcapsules is required for intravitreal injections into the eye of an experimental mouse.

4.2 Materials and Methods

Cells Culture:

ARPE-19 cells, a human RPE cell line that express the differentiated phenotype of RPE cells. The plasmid constructs of CR2 and CR2-fH were transfected into ARPE-19 cells with FuGene HD transfection reagent according to the manufacturer's instructions (Roche Applied Science, Indianapolis,IN). Stable expression in a mixed population was produced by L-methionine sulfoximine antibiotic selection. Secreted protein into apical and basal compartments was monitored in polarized RPE grown on transwell plates[121]. The molecular weight of CR2-fH was examined using sodium dodecyl sulfate acrylamide gel electrophoresis (SDS-PAGE; data not shown). The cells were cultured in High glucose/without L-glutamine- Dulbecco's Modified Eagle Medium (Sigma Aldrich®) supplemented with10% dialyzed FBS (Sigma Aldrich®), 2% Glutamine synthetase expression medium (GSEM- Sigma Aldrich®), and 0.28µl/ml L-methionine sulfoximine (MSX- Sigma Aldrich®). The medium was replaced every 2-3 days, and the cells were passaged after 70%-80% confluence was reached. Cells were used between third and fifth passage.

Cells Encapsulation:

Electrospray method of microencapsulation has been used to encapsulate the targeted cells. In this method, sodium alginate (Sigma-Aldrich) was mixed with DI water

under sterile conditions at concentration of 2% w/v and purified by filtration at 0.2 mm. Alginate solution (~300 μl aliquots) were loaded into a 3cc syringe and attached to a syringe pump, and pumped through a needle (30G blunt tip; Small Parts, Inc., Logansport, IN, USA) to a gelling bath placed in a beaker below the syringe at 7 mm of a needle to bath spraying distance. The gelling bath contains a volume of 40 mL of 10 mM HEPES buffered saline containing 100 mM calcium chloride (CaCl2) (Sigma-Aldrich), and 0.5% w/v poly-L-ornithine (PLO) (Alfa Aesar) that forms a second coating to the microcapsules as one step method for adjusting the porosity. The needle tip connected to a high voltage generator while the gelling bath grounded. By applying high voltage between positive and grounded bath, alginate is extruded from the needle tip to the gelling bath where calcium ions (Ca2+) replace sodium ions (Na+) forming alginate microcapsules coated with PLO and cells mixed with alginate solution will be trapped inside the microcapsules. Cells were trypsinized, centrifuged, and washed with 10 mM HEPES buffered saline solution (pH 7.4), and a final cell concentration of $1x10^6$ in alginate solution were adjusted. Constant parameters of 60mm/h flow rate and 8.0 kV voltage were adjusted to produce microcapsules size of ~150 μm. Formed microcapsules containing cells were washed with washing solution (10 mM HEPES buffered saline containing 1.5 mM CaCl2, pH 7.4) twice, and then incubated with suitable media in a humidified incubator at 37°C and 5% CO2.

Cells Recovery:

The cells were released from alginate microcapsules by adding 55 mM Sodium citrate in 10 mM HEPES buffered saline (pH 7.4) to the encapsulated cells with gentle shaking for 5 mins at room temperature. In this step, calcium ions will replace sodium

ions in the microcapsule structure resulting in diluted sodium alginate and releasing the cells from the microcapsules. Furthermore, the cells were centrifuged at 1200 rpm for 5 mins and incubated with media at 37°C and 5% CO2 for 24 hrs.

Cells Viability (Live/Dead) Assay:

After incubating the encapsulated cells for 24 hours, approximately 30 microcapsules were washed twice with washing solution (10 mM HEPES buffered saline containing 1.5 mM CaCl2) and stained for live-dead viability. Live/Dead assay kit (Molecular Probes) was used to evaluate the viability of cells after encapsulation. According to the manufacturer's instructions, CalceinAM indicated viable cells by green fluorescence (excitation/emission ~495/515 nm), whereas Ethidium homodimer-1 indicated dead cells by red fluorescence (excitation/emission ~495/635 nm). Encapsulated cells in washing solution were mixed with Calcein AM and Ethidium homodimer-1 at final concentrations of 2 μM and 4 μM respectively, and incubated for 30-45 mins in dark at room temperature. After staining, microcapsules were washed twice with washing solution and imaged using Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA).

Microcapsule injections:

C57BL/6J mice 8-10 weeks of age of both sexes were used in this study (Jackson Laboratory, Bar Harbor, ME) that were bred in the facility animal house. All the experiments were carried out in compliance with the Guide for the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee at the Medical University of South Carolina. Prior to

intravitreal injections, mice were placed under using an anesthesia mixture of (xylazine and ketamine, 20 and 80 mg/kg, respectively) applied by intraperitoneal injection. Phenylephrine HCL (2.5%) and atropine sulfate (1%) were used to dilate the mouse's pupils. Under dissecting microscope, an amount of 1 μL of DMEM containing microcapsules with CR2-fH- or CR2-expressing ARPE-19 cells, native ARPE-19 cells, or no content was injected intravitreally with a 27G needle attached to a Hamilton syringe angled at 45-degree during the injection to avoid the lens. Before injecting the microcapsules, the sclera was punctured at the limbus with a 25G needle. Post injection, antibiotic ointment was applied to the animals' eyes.

Laser CNV:

Argon laser photocoagulation (532 nm, 100-lm spot size, 0.1- second duration, 100 mW) was used one month post injection to create four laser spots around the optic nerve of each eye[124]. Bruch's membrane (BrM) rupture was confirmed by bubble formation at the laser burn site where those lesions only were used for lesion growth assessment[130]. Five days post CNV induction, mouse eyes were imaged using optical coherence tomography (OCT) before scarifying the animals on day 6 for tissue collection.

Dot blot and Western Analysis:

Examining CR2-fH production from transfected cells—Cell lysates were collected after centrifugation (20,000g for 30 minutes at 48C). 25 μl of total protein was loaded into 96- well plate and samples were transferred on a nitrocellulose membrane using the Bio-Dott Microfiltration Apparatus (Bio-Rad Laboratories, Inc. Hercules, CA).

The dotted membrane blocked in 5% nonfat milk in TBST buffer for 2 hours at room temperature after being washed with Tris-buffered saline and 0.1% Tween 20 (TBST) wash buffer. After blocking, the membranes were incubated overnight with primary antibody against CR2 at 1:1000 dilutions in 5% nonfat milk/ TBST. Horseradish peroxidase- conjugated secondary antibody (anti-rat; Santa Cruz Biotechnology, Inc., Dallas, TX) was used for visualization followed by incubation with Claritye Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc.).

Examining antibody generation in mice against secreted CR2-fH following intravitreal administration of ARPE-19 cells—Supernatants from CR2-fH-expressing cells were added to Laemmli sample buffer and boiled. Two different concentrations of the samples were separated electrophoretically using 4%–20% Criterione TGXe Gels (Bio-Rad Laboratories, Inc.). Proteins were then electro-transferred to PVDF membranes, and incubated with primary antibody against CR2, or serum (1:50) from mice treated with intravitreal CR2- or CR2-fH microcapsules. Horseradish peroxidase-conjugated secondary antibody (antimouse IgG and IgM; Santa Cruz Biotechnology) was used for visualization followed by incubation with Claritye Western ECL Blotting Substrate and chemiluminescent detection (Bio-Rad Laboratories, Inc.).

Immunohistochemistry:

Tissue samples of eye lens and anterior chamber were removed from eyecups which was collected as showed in previous study[124], and the samples were then fixed overnight with 4% paraformaldehyde (PFA). After thoroughly washing in PBS, samples were embedded in Neg-50 cutting medium (Richard-Allen Scientific; Thermo Fisher

Scientific) and sectioned into 14-μm sections using a cryostat. Blocking solution consisting of (10% normal goat serum, 3% bovine serum albumin factor V, and 0.4% Triton-X in PBS) was used to block the sections before staining overnight with (1:200) CR2 (7G6) antibody at 4°C. after washing the stained sections with blocking solution, secondary antibody was added (Alexa Fluor 488 goat anti-mouse IgG; 1:500; Invitrogen) and mounted using Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL). Images were obtained by fluorescence microscope (Zeiss, Thornwood, NY).

Optical Coherence and Tomography:

SD-OCT Bioptigent Spectral Domain Ophthalmic Imaging System (Bioptigen, Inc., Durham, NC) was used to visualize the injected microcapsules in the eye and to analyze CNV lesion size after 5 days of laser treatment as has been described in previous studies[131,132]. For the analysis of CNV lesion, rectangular volume scan images set at 1.63 1.6 mm were acquired which consists of 100 B-scans (1000 A-scans per B scan)as well as measurements of cross-sectional area of the lesion using the en-face fundus reconstruction tool. The center of the lesion was determined passing through the RPE-BrM rupture with the axial interval positioned at the level of the RPE/choroid complex as described in previous work[133]. Image J software for date analysis (Wayne Rasband, National Institutes of Health, Bethesda, MD; available online) was used to measure the area around the hyporeflective spot produced on the fundus image, with vertical calipers set at 0.100 mm at the site of each lesion.

C3a ELISA:

Mouse complement C3a ELISA (LifeSpan Biosciences, Inc., Seattle, WA) was used to perform C3a ELISA according to the manufacturer's protocol. The RPE/ choroid tissues were ultrasonicated with 500 μL ice cold PBS (lysing process) after being thoroughly rinsed with ice cold PBS to remove excess blood. The homogenized sample was centrifuged at 5000g for 5 minutes, and then the assay performed according to the manufacturer's instructions. Standards of C3a were used along with samples, and the absorbance was read at 450 nm using microplate reader.

Statistics:

Unpaired t-tests was used for single comparison, and ANOVA (Fisher PLSD) was used for in-between group analysis with P value = 0.05 for significance (StatView, Cary, NY). Data are reported as mean ± SEM.

4.3 Results

Encapsulation of genetically modified ARPE-19 cells—secretion of CR2 and CR2-fH was confirmed by dot analysis using collected supernatants from cells grown as stable monolayers on transwell plates as shown in Figure 4.1 (D). Encapsulation of genetically modified ARPE-19 cells within 2% alginate microcapsules was successfully accomplished and it has been confirmed by brightfield microscopy Figure 4.1 (A). Viability of encapsulated cells post encapsulation was assessed using live dead assay that showed a viability of 90% of encapsulated cells Figure 4.1 (C). After dissolving the microcapsule structure using sodium citrate solution, ARPE-19 cells viability has been

assessed by incubating the cells back into their desired media where they have been grown for 90% confluency Figure 4.1 (B).

Delivery of encapsulated ARPE-19 cells intravitreally—the microcapsules size was adjusted through the encapsulation method to produce microcapsules at an approximate size of 150 μl as an optimum size. The optimum size was ideal to be injected through 27G needle which its inner diameter 210μm, and to minimize the microcapsules during intravitreal injection. OCT imaging was performed after injecting the microcapsules in the vitreous of mouse's eye to examine the presence of intact microcapsules in that area as indicated in Figure 4.2 (A). In addition, presence of CR2-FH in the microcapsules as well as its secretion into the vitreous and retinal tissue has been confirmed using immunohistochemistry Figure 4.2 (B). Moreover, it has been confirmed that CR2-fH specifically retained in CNV injury site compared to sites without CNV lesions by dot blot analysis of RPE/choroid fractions as shown in Figure 4.2 (C) and as previously reported in previous study[134]. Examining antibody production against secreted CR2-fH which might access the blood stream has been performed. Mouse serum was collected after 1 month of microcapsules injection and tested for anti-CR2-fH antibody production. It has been shown that neither IgG nor IgM antibodies recognizing CR2-fH could be identified in both CR2-fH microcapsules injected group or control group Figure 4.3. Similar results were previously reported when mice were systemically injected with CR2-fH protein[135].

Effects of secreted CR2-fH on reducing complement activation and attenuating CNV development—the effect of secreted CR2-fH on CNV lesion size has been evaluated after 5 days of laser-induced photocoagulation. Experimental mice were

injected intravitreally with alginate microcapsules loaded with ARPE-19 expressing CR2, ARPE-19 expressing CR2-fH, native ARPE-19, or empty microcapsules. It has been reported in this study that lesion size in animals received microcapsules with cells expressing CR2-fh reduced significantly (P0.02) compared to other experimental animal groups as demonstrated in Figure 4.4 (A,B). Moreover, ELISA measurements showed significant reduction in C3a levels in mice injected with microcapsules containing cells expressing CR2-fH Figure 4.4 (C). This reduction was expected because it has been known previously that the presence of factor H prevents C3-convertase formation. On the other hand, animals injected with microcapsules loaded with cells expressing CR2, native cells, or empty microcapsules showed an increase in C3a in RPE/choroid fractions.

4.4 Discussion

The focus of this study was to investigate the effectivity of encapsulation technique in encapsulating cells as cell therapy approach to deliver CR2-fH to the eye as a therapeutic strategy for complement dependent diseases. It has been demonstrated through this study that encapsulation technique can preserve the viability of encapsulated ARPE-19 cell within alginate based microcapsules. The encapsulated cells showed secretion activity of CR2-fH that is able to diffuse into the vitreous of the mouse while anti-CR2-fH antibody was not detected. Importantly, secretion of CR2-fH by encapsulated ARPE-19 cells in the eye showed a great effect on reducing CNV development as well as complement activation. Indeed, the obtained results propose the effectivity of encapsulated cell therapy as a therapeutic strategy in treating wet AMD.

It has been recently discussed that the essential aim of encapsulated cells as a therapy approach is to provide a sustain release delivery of therapeutics secreted by encapsulated cells without triggering the immune response against it[136]. According to the present study, the viability of encapsulated cells was preserved and the cells were able to be recovered from the alginate microcapsules to continue proliferating after plating and incubating them back in media. Also, it was confirmed by dot blot analysis that cells secreted CR2-fH for 1 to 2 months after cells being encapsulated and injected; however, antibodies against CR2-fH were not generated by testing CR2-fH antibodies (IgG and IgM). On the other hand, antibodies formation against ARPE-19 epitopes have never been tested in this work because according to a number of clinical trials it has been shown that immunosuppressants are not required while utilizing devices loaded with ARPE-19 cells[137,138].

In biotechnology industry world, Neurotech is a biotechnology company focused on the development of encapsulated cell therapy as a therapeutic approach for chronic eye diseases. Neurotech has loaded ARPE-19 cells into a device (NT-501) that has been tested in the eye in which it is delivering CNTF and NT-503, a soluble anti-VEGF-R protein. NT-501 had a good safety results and ongoing trials in glaucoma and Macular Telangiectasia type 2 are taking place to deliver CNTF. However, delivering NT-503 protein using this device has been discontinued in phase II trial because rescue medications were highly required in the treatment arm by a large number of patients according to Neurotech Company. Also, according to Neurotech, it is very important to consider the fact that ARPE-19 cells secrete not only therapeutic proteins but also other proteins consistently that might interfere with the efficacy of the targeted therapeutic

proteins. It has been demonstrated that ARPE-19 cells secret a diversity of proteins that are involved in maintenance and regulation of the complement pathway, extracellular matrix, inflammatory responses, and angiogenesis[139]. The quantity and the composition of proteins secreted can vary according to the eye under specific disease or treatment application technique; however, the effect of delivering encapsulated ARPE-19 cells on protein secretion and composition has not been determined in this study. Indeed in this work, data show the presence of ARPE-19 in the mouse eye do not interfere with the growth of CNV lesion, but also active CR2-fH showed a protective effect comparing to the inactive CR2, the native cells, or the empty microcapsules Figure 4.4 (B). Moreover, complement activation has not been affected by the presence of ARPE-19 cells in the mouse eye according to the measurements of complement C3a, and this has been determined through the effect of encapsulated native ARPE-19, encapsulated ARPE-19 expressing CR2, and empty microcapsules Figure 4.4 (C). The concentration of microcapsules loaded with ARPE-19 expressing CR2-fH loaded reduced complement activation effectively to the base line levels. The preliminary data of this study showed that approximately 30 microcapsules were responsible on reducing the complement activation and interfere with CNV development. Accordingly, delivering the therapeutic dose of CR2-fH via cell encapsulation technique will promote normal functions of the complement system that is envision as a mediator as a global mediator in immune surveillance, cell homeostasis, and tissue development and repair[140].

ARPE-19 cells have a potential of initiating complications on disease outcomes when they being used a therapeutic approach. The need of delivering anti-complement materials is a necessity, and AAV gene therapy is considered an attractive tool that has

been utilized for disease models[141]. The expression of the inhibitor in dry or wet AMD should be inducible when required when new CNV lesion being developed, and it can be repressed once the inhibitory effect is no longer needed to avoid the toxic effects of excessive treatment. It has been demonstrated previously that using expression system that can be controlled exogenously, rapamycin or RU486 regulation, or endogenously, hypoxia response element (HRE), could be a feasible approach in controlling CR2-FH overtreatment, especially in case of HRE since hypoxia is highly correlated to wet AMD[142].

This study showed that encapsulated cells therapy has been used to deliver the AP-complement inhibitor factor H using genetically modified ARPE-19 cells to the eye at complement activation sites. The inhibition of AP complement activation reduces complement activation, slows CNV development, and allows retention of physiological levels of complement required for cellular homeostasis and immune surveillance[140].

Future investigations are recommended to examine the feasibility of cell encapsulation-mediated therapeutic delivery of a complement inhibitor as a potential long term inhibition approach in the smoke-induced ocular model[125], as well as in dry AMD.

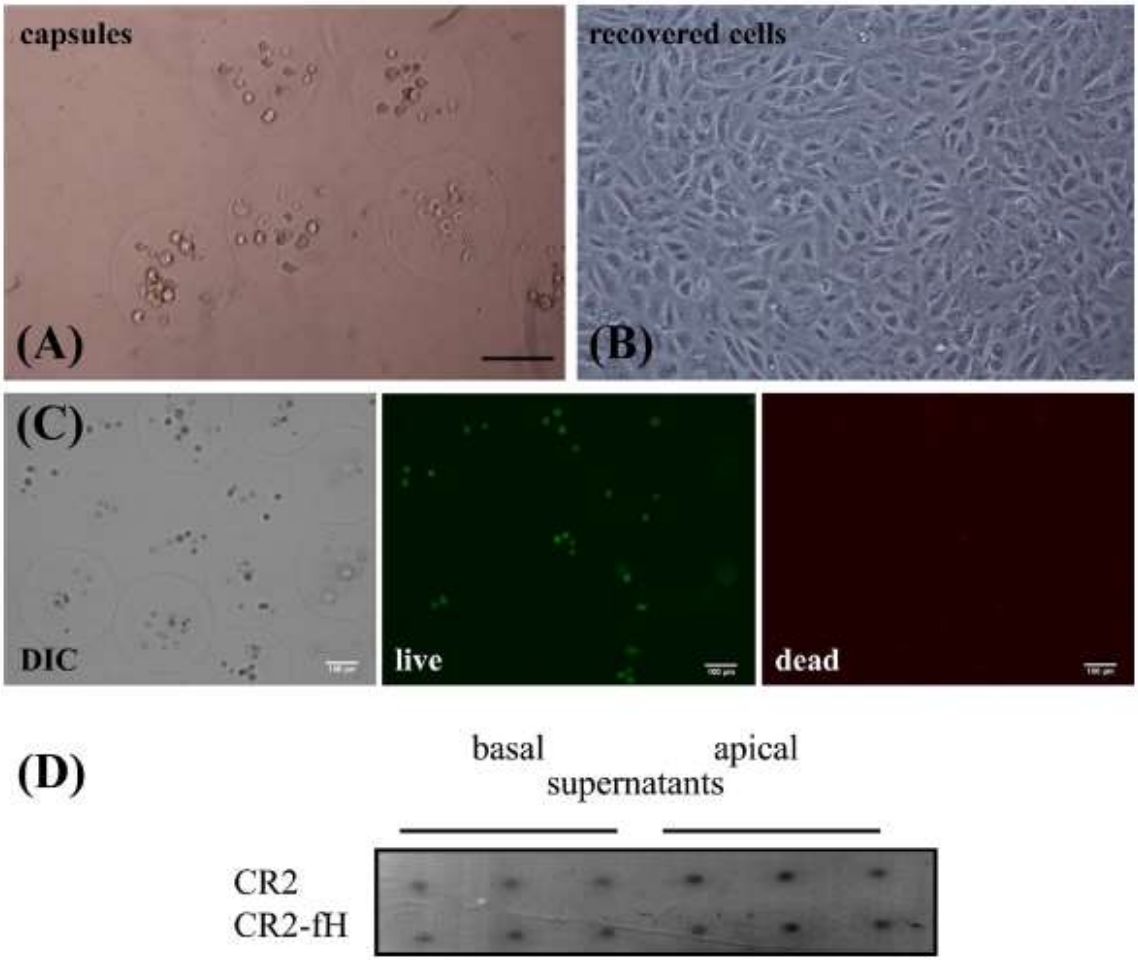

Figure 4.1 Encapsulation technique development to encapsulate genetically modified ARPE-19 cells to deliver CR2-fH.
(A) Encapsulated ARPE-19 cells in culture. (B) Recovered cells after dissolving the microcapsule alginate structure and incubated in culture to examine viability. (C) Cell viability within the microcapsules was assessed using live/ dead assay where Calcein AM indicating live cells (Green) and Ethidium homodimer-1 indicating dead cells (Red). Secreted CR2 and CR2-fH towards both the apical and basal side from the stably transfected ARPE-19 cells that were grown as monolayers on transwell plates (secretion assessed from three different cultures).

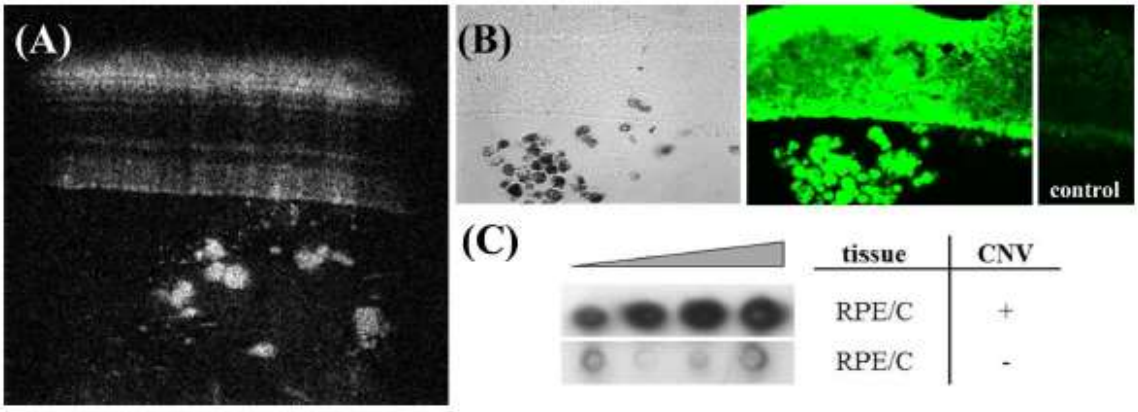

Figure 4.2 Assessment of delivering CR2-fH using encapsulation technique in the eye. (A) The microcapsules were detected in the eye using optical coherence tomography (OCT). (B) Immunohistochemistry was used to confirm production and diffusion of CR2-fH protein from the microcapsules throughout the retina layers via antibody staining against CR2. Results were compared between uninjected control eyes and injected eyes where the staining was negative in the control. (C) A dot blot of RPE/choroid samples were used to detect CR2-FH after intravitreal injection of the microcapsules in the RPE/choroid region of the eye with CNV lesions or without. CR2-fH was detectable in the samples with CNV lesions comparing to the other group. Results are shown from more than three independent experiments.

CR2-fH (µL CHO cell sup)	20	40	20	40	20	40	20	40	20	40
primary antibody	CR2	CR2	S-E	S-E	S-E	S-E	S-C	S-C	S-C	S-C
secondary antibody	IgG	IgG	IgG	IgG	IgM	IgM	IgG	IgG	IgM	IgM

Figure 4.3 Immune response measurements against CR2-fH produced in the eye.
After one month of injecting encapsulated ARPE-19 cells expressing CR2 and CR2-fH, production of IgG or IgM were not detected. Two different concentrations of expressed CR2-fH were probed against anti-CR2 for the presence of CR2-fH in the supernatants in order to identify the size of the protein (positive control).Serum from both groups, experimental (S-E; injected with CR2-fH capsules) and control (S-E; age-matched animals without injections S-C), was used for probing at 1:50 followed by appropriate secondary antibodies.

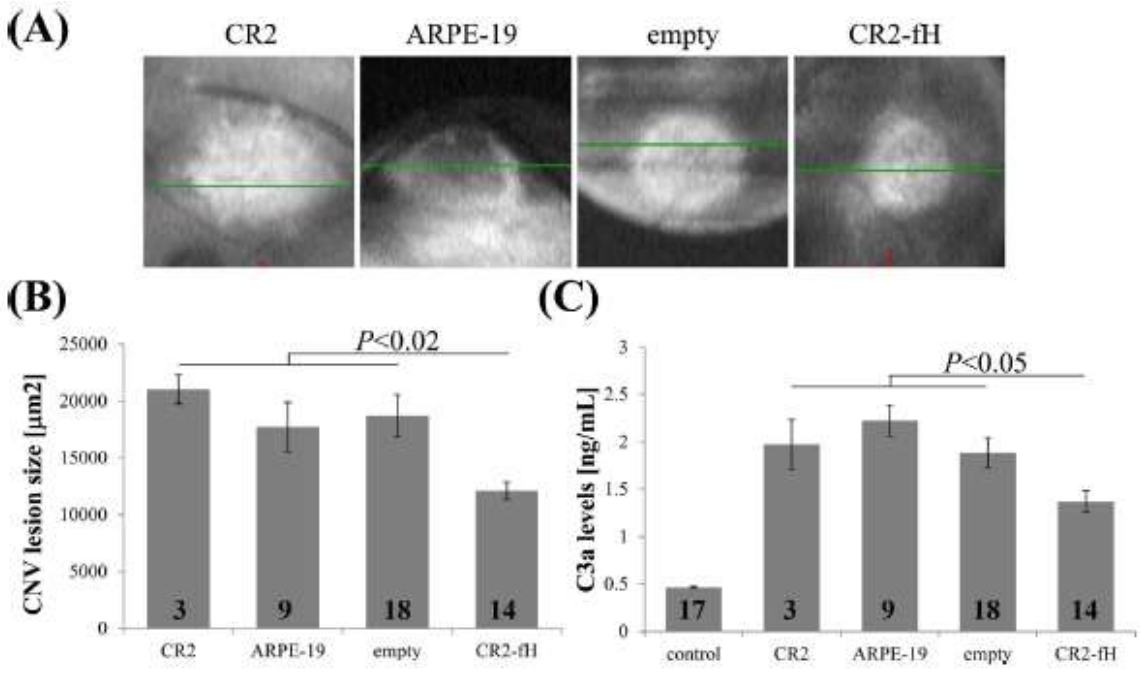

Figure 4.4 Delivered CR2-fH through encapsulation technique reduces CVV and complement activation.

Laser induced CNV was performed after one month of injecting encapsulated cells intravitreally. 5 days later, lesion spot sizes were analyzed using OCT and complement activation was assessed for anaphylatoxin C3a using ELISA. (A, B) CNV lesion sizes were reduced in the eyes of experimental group injected with encapsulated ARPE-19 expressing CR2-fH compared to other groups, encapsulated cells expressing CR2, native ARPE-19 without cargo, or empty alginate microcapsules. (C) C3a levels in RPE/choroid fractions were elevated in animals injected with encapsulated ARPE-19 expressing CR2, encapsulated native ARPE-19, and empty microcapsules. Levels of C3a were reduced significantly in animal groups injected with encapsulated CR2-fH expressing cells. Control group represents animals with no CNV lesions. Data shown are average values (6SEM) (n ¼ 3–18 animals per condition as indicated).

CHAPTER V

INVOLVEMENT OF HYDROGEL SYSTEMS IN ADVANCING ALGINATE BASED MICROCAPSULES

5.1 Background

Hydrogels have been utilized as scaffold polymers in different approaches including therapeutic agent delivery and regenerative medicine. There are a variety of applications in which hydrogels have been targeted such as delivery agents for bioactive materials, as three-dimensional structures that can organize cells and direct the formation of a desired tissue with presence of specific stimuli, as well as their application as space filling agents[68]. Properties including mechanical properties, swelling, diffusion, and degradation are important to the hydrogel crosslinked structure in which they are control the hydrogel utilization for each specific approach[143]. Type I collagen has been used widely as a viable scaffold for various applications; however, collagen scaffold properties can vary according to the fabrication protocol used by different researchers. Collagen source and gelation pH are significant fabrication parameters that contribute to identify hydrogel properties and applications[68,76,144–148].

The main goal of this study is developing new approach through embedding A-PLO microcapsules in collagen hydrogel as an enhanced delivery system. Through this

study we will show the successful implementation of lyophilized microcapsules as well as our encapsulated cells within collagen hydrogel.

5.2 Materials and Methods

Collagen hydrogels:

To prepare the hydrogels for incorporation of the lyophilized microcapsules, we used type I bovine collagen (Purecol®, Advanced Biomatrix, San Diego, CA), 0.2N HEPES buffer pH9.0, and 10X Minimal Essential Medium (MEM) at proportions of 8:1:1 accordingly. Lyophilized microcapsules were added immediately after mixing the hydrogel contents then aliquots of 300 μl of the mix poured into 24 well plates. The plate was then incubated at 37C° for 30 minutes to let it polymerize, then we added deionized water to keep hydrated.

To prepare the hydrogels for incorporation of the encapsulated ARPE-19 cells, we used type I bovine collagen (Purecol®, Advanced Biomatrix, San Diego, CA), 0.2N HEPES buffer pH9.0, and 10X Minimal Essential Medium (MEM) at proportions of 8:1:1 accordingly. Encapsulated ARPE-19 cells were mixed with freshly prepared hydrogel gel after aspirating the media from the microcapsules. Appropriate aliquots 300-500μl of the mix were poured into 24 well plate; then incubated at 37C°/ CO_2 for 30 minutes to let the hydrogel to polymerizing. Media was added after the hydrogel containing cells had been polymerized.

5.3 Results

Lyophilized microcapsules in hydrogel:

Lyophilized microcapsules were mixed with freshly prepared type I collagen hydrogel and incubated for 30 minutes at 37°C in order for the hydrogel to polymerize. Rehydration of lyophilized microcapsules were investigated under light microscope to verify that microcapsules were able to rehydrate within the hydrogel and retain their original shape (spherical) Figure 4.1 (B).

Encapsulated cells in hydrogel:

Encapsulated cells were mixed with freshly prepared type I collagen hydrogel and incubated at 37°C for 30 minutes. After polymerization of the hydrogel, we added media on the top of the hydrogel and we incubate it at 37°C and 5% CO_2 as it is shown in Figure 4.2 (B). Live/ dead assay was performed to test encapsulated cells viability within the hydrogel, and it was demonstrated that cells were still preserving their viability in Figure 4.2 (C) where live cells expressed in green.

5.4 Discussion

Hydrogels are natural and synthetic three-dimensional systems with hydrophilic polymer chains which provide ability to hold high content of water. They have broad uses in biomedical research including drug delivery, regenerative medicine, and tissue engineering.[58,61,149–151]. The diversity of hydrogel applications is dependent upon the special traits of hydrogel that spans from biocompatibility, adjustable chemical properties, ability to mimic extracellular matrix, and serve as growth medium[151] . As an

attempt to further improve A-PLO microcapsules for therapeutic applications, we utilized the lyophilized microcapsules and encapsulated cells to be mixed with type I collagen hydrogel. Our study shows that lyophilized microcapsules have the ability to rehydrate after being mixed with the hydrogel during its polymerization. The lyophilized microcapsules were empty; however we are demonstrating the effectiveness of the system as a tool for application in which the microcapsules can be filled with different therapeutic materials. Moreover, we also demonstrated the ability of the encapsulated ARPE-19 cells to be loaded into type I collagen hydrogels in which it can be used for regenerative medicine and as a delivery system.

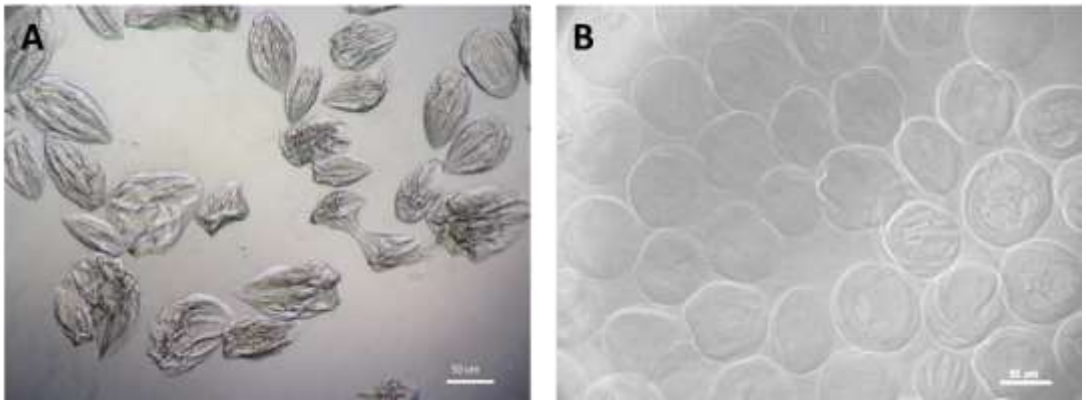

Figure 5.1 Lyophilized microcapsules and their rehydration after mix with collagen hydrogel.
(A) Microcapsules appear shrunk and dried after lyophilization. Lyophilized microcapsules rehydrated and returned their spherical shape again after mix with type I collagen hydrogel. Scale bars = 50 microns.

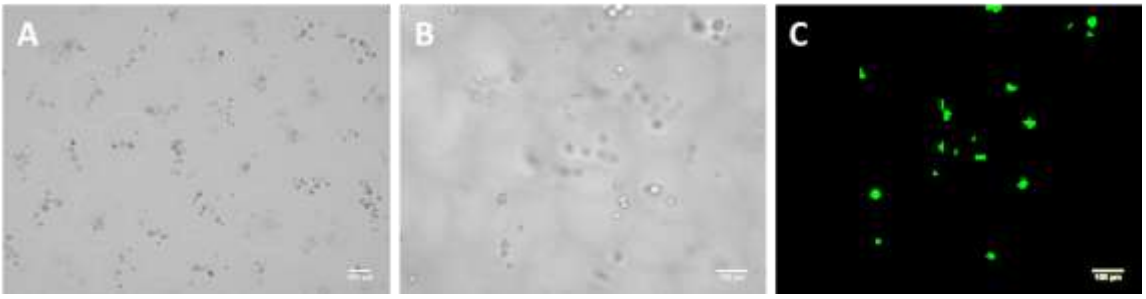

Figure 5.2 Viability of encapsulated ARPE-19 cells embedded within type I collagen hydrogel.
(A) Encapsulated ARPE-19 cells in DMEM media. (B) Encapsulated cells embedded in the hydrogel gel and incubated with media. (C) Encapsulated cells within the hydrogel were stained for their viability via live/dead assay. The Green was an indication of cell viability while the red indicated the damaged cells. Scale bars= 100 microns.

CHAPTER VI

SUMMARY AND FUTURE WORK

Summary

Electrospray microencapsulation has been used in our lab previously to show enhanced wound healing in a rodent model. The previous work concentrated on the encapsulation of a small peptide known as αCT1[85]; in addition, we have had success in encapsulating different constituents such as proteins, antimicrobial agents, and even cells. In this work, I was able to show several enhancements to the original encapsulation technique for both wound healing and cell therapy. I was able to lyophilize the alginate-PLO microcapsules without damaging their morphology following rehydration with the addition of DI water or Tyrodes buffer. Interestingly the microcapsules regain their spherical shape with some surface irregularity. I was also able to show that addition of lyophilized microcapsules to a gelling collagen matrix also showed the ability to hydrate back into their original spherical shape. To investigate the process for cell based therapies, I was able to encapsulate various cell types using the same method of encapsulation by tweaking the voltages and flow rates for optimum cell viability, and showed the ability of the cells to survive the voltage applied during microencapsulation technique. Encapsulated cells (ARPE-19, genetically engineered ARPE-19, and skMDC) were tested for viability after encapsulation using live/ dead assay, and the cells types

were shown to be viable within the microcapsules. We took this to an in vivo model of age related macular degeneration (AMD) and showed that addition of genetically modified ARPE-19 cells expressing the protein CR2-fh was able to ameliorate the symptoms of AMD in a mouse model. To determine whether the encapsulation process had interfered with the cells proliferative ability, we tested the cells when dissolved from the capsules and demonstrated their ability to continue to proliferate in culture and were passaged multiple times thereafter. Finally, we showed that the skMDCs retained several key markers, Pax7, desmin and MyoD following dissolving and further culturing. Indicating that the encapsulation process is not affecting the cells proliferative capabilities nor losing their inherent genetic profile.

We also demonstrated our ability to cryopreserve the microencapsulated cells for a period that resembles long term storage for future experimental use, and showed their viability after thawing and culturing in media. We froze the encapsulated cells and stored them at both -80C and also liquid nitrogen. Cells frozen in both conditions showed the ability to regain their growth potential. Finally, encapsulated cells were incorporated successfully within type I collagen hydrogels where the cells preserved their viability while cultured within this system which can be further utilized for tissue engineering and sustain release delivery of therapeutics.

Future Work

Presented here is a promising technique that can be tuned for its utilization according to each approach discussed in this study. Further investigations are needed for the effect of lyophilization on release profiles of materials such as αCT1, and BSA

loaded A-PLO microcapsules; a complete and comprehensive analysis of loaded lyophilized microcapsules for their sustained release and therapeutic effect on wound healing needs further investigation. In the future encapsulation of multiple cell types in the capsules is planned. It's believed that the cells interact in vivo could in fact be encapsulated to mimic their in vivo interactions. We would also begin examining the ability to further coat the microcapsules with antibodies to further target the addition of the microcapsules within the body to certain tissues or cells. Additionally, a single report exists of the ability to lyophilize microencapsulated cells. We plan to further our initial work to determine if this is another viable option of our system. Finally, we plan to generate an animal model for skeletal muscle atrophy and/or degeneration and use our encapsulated skMDCs to fully evaluate the therapeutic potentials of encapsulating skMDC cells in these types of muscle diseases. Thus providing us a true bench top to bedside application in the future.

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