The Controlled Release of Rat Adipose-Derived Stem Cells from Alginate Microbeads for Bone Regeneration

A Thesis
Presented to
The Academic Faculty

By

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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in the
School of Biomedical Engineering

Georgia Institute of Technology
August 2013

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The Controlled Release of Rat Adipose-Derived Stem Cells from Alginate Microbeads for Bone Regeneration

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ACKNOWLEDGEMENTS

Firstly I would like to thank God for giving me the grace and strength to complete the degree. In addition, I thank my mentors, Dr. Barbara Boyan and Dr. Zvi Schwartz as well as my committee members, Dr. Todd McDevitt and Dr. Vlamidir Tsukruk for their advice and support. I am especially grateful towards my mentors for their guidance in the studies completed for my Master's degree. I am thankful to the past and present members of the Boyan/Schwartz Laboratory for their support over the years. Most importantly, I would like thank my immediate family who has been a strong tower of support through my educational endeavors. Lastly, but surely not least I would like to thank Ms. Sharon Knight for her support and help in correcting my written work. This research was funded by a grant from the Department of Defense.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	analysis of variance
BMP-2	bone morphogenetic protein 2
Col 1	collagen type 1
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
FGF	fibroblast growth factor
GFP	green fluorescent protein
HBSS	Hanks' balanced salt solution
IGF	insulin-like growth factor
MG-63	human osteosarcoma cell line
OCN	osteocalcin
OPG	osteoprotegerin
Osx	osterix
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLGA	poly (lactic-*co*-glycolic) acid
rASCs	rat adipose-derived stem cells
RPS18	40S ribosomal protein S18
RUNX2	Runt-related transcription factor 2
TUNEL	dUTP nick end labeling
VEGF	vascular endothelial growth factor

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Summary

Approximately 6.3 million fractures occur each year in the U.S. [1] and 5 to 20% of these fractures result in nonunions [2]. A fracture results in nonunion when the bone fails to heal completely. Despite the high prevalence of fractures, there are no methods that currently exist to prevent nonunions. Presently, both surgical and nonsurgical treatments exist, however most nonunions require surgery[3]. For patients who initially needed post fracture surgery, and resulted in a nonunion, a second surgery is not only painful but also very costly financially and time wise. Cell-based therapies would be especially advantageous for these patients since it's less invasive and would aid also in bone regeneration.

Cell-based therapies using rat adipose-derived stem cells (rASCs) provide one possible form of treatment for fractures. rASCs are known to be multipotent [4], easily isolated, and , and can be induced to express an osteoblastic phenotype [5]. Recent reports indicate that ASCs are not as osteogenic as bone marrow stem cells, but their ready availability supports their use as a potential cell source [6]. One disadvantage of cell-based therapies is cells tend to disperse to other tissues, away from the initial delivery site [7]. It is important that a form of delivery is developed to allow the controlled release of cells, maintain the cell's viability and their ability to differentiate along the osteoblast lineage. One solution is to use alginate, a well-known biocompatible hydrogel, which is derived from seaweed. This biopolymer is known to maintain the viability of cells after injection and could be used as a platform for stem cells as they are positioned in the body and.[8, 9] However, alginate does not readily degrade *in vivo* for six months [10]. Before alginate can be used to deliver stem cells, a method of controlled degradation must be established to achieve controlled cell release. To date, there are no methods that control the degradation of alginate hydrogels *in vivo*. Several attempts have been made by using gamma irradiation, oxidization and a bifunctional crosslinker, however they have not been successful. [10, 11]

The overall goal of this project is to develop a system of injectable hydrogels to deliver stem cells for the purposes of bone regeneration. First, a method was developed to incorporate

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alginate lyase in alginate microbeads to achieve controlled degradation. Two methods were employed; combining alginate with PLGA particles loaded with alginate lyase and combining the alginate lyase directly with the alginate. The latter of the two methods resulted in controlled degradation of alginate microbeads. Using this method, controlled release parameters were established for both wound healing alginate and medical grade alginate. Consequently, we determined if the by-products of alginate lyase - mediated alginate degradation had an effect on the cell's viability and osteogenic potential. This was determined through viability tests, real time PCR and biochemical assays. The development of this therapy could be used for any other tissue regeneration with stem cells once the controlled release parameters are optimized.

Methods: The rASCs were isolated from the inguinal fat pads of male Sprague Dawley rats. MG63s and GFP-rASCs were bought from Harlan Labs and the University of Missouri respectively. All cell types were microencapsulated at 25 x 10^6 cells/ml alginate.

Equal volumes of alginate lyase solution and 4% UP LVM alginate were combined. Microbeads were formed using a micoencapsulator with an electrostatic potential of 6kV and a calcium crosslinking solution (75mM $CaCl_2$, pH 7.4). The degradation of the microbeads was followed by the absorbance of the uronate products and the diameter of the microbeads over the period of twelve days every 48 hours. Once the cells were microencapsulated the degradation was followed by the number of cells released and in the case of GFP-rASCs, the fluorescence was also recorded. Additionally, the alkaline phosphatase specific activity and DNA content of these cells were measured. The viability of the released cells was analyzed by using live/dead staining and TUNEL assay. In addition, we ensured that the released cells maintained their osteogenic potential by demonstrating that they were able to produce osteogenic and angiogenic factors after treating the cells with osteogenic media for a week. We measured the mRNA levels

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of the following genes; Osx, Runx2, Col1, Vegfa, Fgf2 and Bmp2, Noggin, Gremlin, Ocn. The following soluble factors were also measured; OCN, OPG, VEGF and FGF2.

Results: Incorporating PLGA particles loaded with alginate lyase lead to no visible degradation of alginate microbeads. The controlled degradation of alginate microbeads was achieved by directly incorporating the alginate lyase with alginate. The wound healing alginate required more alginate lyase compared to medical grade alginate to be degraded within the same period. Using the controlled release parameters for the medical grade alginate the controlled release of cells was obtained. The viability of the cells released ranged from 87% on day 2 to 71% on day 12, and these cells were not in a process of apoptosis. The released cells produced factors associated with osteogenesis and angiogenesis.

Discussion: PLGA particles loaded with alginate lyase were not able to achieve degradation which was partly due to the low encapsulation efficiency of the double emulsion method. In addition, the enzyme released from the PLGA particles was not active as a result of the harsh environment inherent to the double emulsion method. Directly incorporating the alginate lyase with the alginate resulted in controlled degradation; however the amount of alginate lyase required for the wound healing alginate compared to the medical grade alginate is greater. This could be directly related to the composition of the two types of the alginate. The wound healing alginate is free of impurities such as the polyphenols and endotoxins. In the presence of polyphenols, the activity of the alginate lyase is reduced. The exact mechanism by which endotoxins affect alginate lyase mediated alginate degradation remains unknown. Using the degradation parameters for medical grade alginate, the controlled release of cells was also achieved. The higher ratios of alginate lyase to alginate lead to faster cell release. The viability of the cells with the highest concentration of alginate lyase was 87% on day 2 and with the lowest

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concentration 71% on day 12 indicating that the cells remained viable. The mRNA levels of Runx2, Osx, and Col1 were similar to the control while Bmp2, Grem1, Nog, Vegfa, Fgf were significantly higher than the control, indicating that genes involved in osteogenesis and angiogenesis were expressed. The levels of OCN, OPG, VEGF, and FGF2 produced, also demonstrated that the phenotype of these released cells was maintained. In fact, the released cells produced a significantly higher level of osteocalcin compared to the control, which is a late marker of osteoblasts differentiation.

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CHAPTER 1

Introduction

The Use of Hydrogels in Stem Cell Delivery

Hydrogels have a gained special focus because of their ability to maintain a temporary support for cells as they facilitate the bidirectional transport of nutrients, gases and metabolic wastes. Furthermore, hydrogels are tunable to allow the incorporation of specific biochemical cues. In typical cell-based therapies, stem cells are used either solely by themselves or in conjunction with a biomaterial which acts as a scaffold at the site of interest. In order for the tissue regeneration to be complete, it is important that the cells implanted are released from the scaffold to aid in tissue regeneration.

The controlled release of cells is critical to the success of cell-based therapies. In this project, controlled release of cells will be achieved through the controlled degradation of alginate microbeads encapsulating cells. Microencapsulation is a technique which entraps cells in a semi-permeable membrane, thereby immobilizing the cells. This semi-permeable membrane allows the inflow of molecules essential for cell survival and the out flow of metabolic products [12].

Alginate and Alginate Degradation

Alginate, a natural polymer is a polysaccharide which consists of (1-4)-linked-β-D mannuronic acid (M units) and α-L-guluronic acid (G units) monomers. It is a block copolymer where each block may be repeating units of mannuronic acid (poly (M)) or guluronic acid (poly(G)) or a random organization of mannuronic and guluronic units (poly(MG)) [13]. Alginate can be found in the cell wall of brown algae and can also be synthesized by bacteria.

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The advantage of alginate compared to synthetic polymers is its biocompatibility, availability in nature, economical value, forms hydrogel under relatively mild pH and temperature, and its ability to be sterilized [13]. Alginate is used to form a hydrogel by cross-linking it with divalent cations such as calcium or barium. The crosslinking occurs between the guluronic acids of neighboring chains.

When ASCs are encapsulated using an electrostatic potential in a calcium crosslinking solution containing glucose as an osmolyte, the resulting microbeads contain a tunable number of cells in a bead diameter of 200μm [14]. These microbeads were used successfully as a delivery vehicle for subcutaneous injection of ASCs in mice and the cells remained viable for two months [8, 9]. Mass transfer issues are obviated due their small size and high surface to volume ratio. These studies have also shown that encapsulated ASCs can be induced to express a chondrocytic phenotype and to produce chondrogenic factors, suggesting that similar approaches will be successful for bone regeneration [15, 16].

Despite the many advantages of alginate, a central challenge remains – alginate does not degrade enzymatically within the human body and non-enzymic breakdown occurs very slowly. In vitro, release of cells is achieved by chelating Ca^{++} thereby breaking the crosslinks between chains. There have been attempts to control the rate of degradation of alginate constructs *in vivo* by decreasing the molecular weight through oxidation and γ-irradiation, thus inducing hydrolytically labile groups [10]. After six months, these constructs maintained their structure but elastic modulus was decreased and cell migration was increased. Another approach, involved the encapsulation of PLGA particles loaded with alginate-lyase [17], an enzyme that cleaves the β-1,4-glycosidic linkage between the monosaccharide units of the alginate through a β-elimination reaction [18]. Finally another strategy used covalent crosslinking with bifunctional

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groups to degrade alginate constructs [19]. These efforts have been partially successful in larger alginate constructs (4mm), but there are no methods to control the degradation of smaller alginate microbeads (200µm – 500µm), which are important for injectable applications.

Evaluating Alginate Degradation

Currently, there are no methods to quantify alginate degradation therefore we have used multiple methods to follow the degradation. Measuring the absorbance of uronate products or the byproducts from a ketal reduction is possible, but for the purposes of quantification, a standard curve cannot be constructed. No single method is sufficient in characterizing the degradation, but together they complement each other thus aiding in the understanding of the degradation process. First degradation is followed by recording the absorbance of the uronate products at 235 nm. However, after the first 4 days our results show not much uronate products are produced but the morphology of the microbeads continues to change. Hence, the other method involves observing the bead's morphology. This method is coupled with measuring the diameter of the alginate microbeads over the duration of the experiment. Therefore for most of the studies the absorbance readings are accompanied by pictures of the alginate microbeads.

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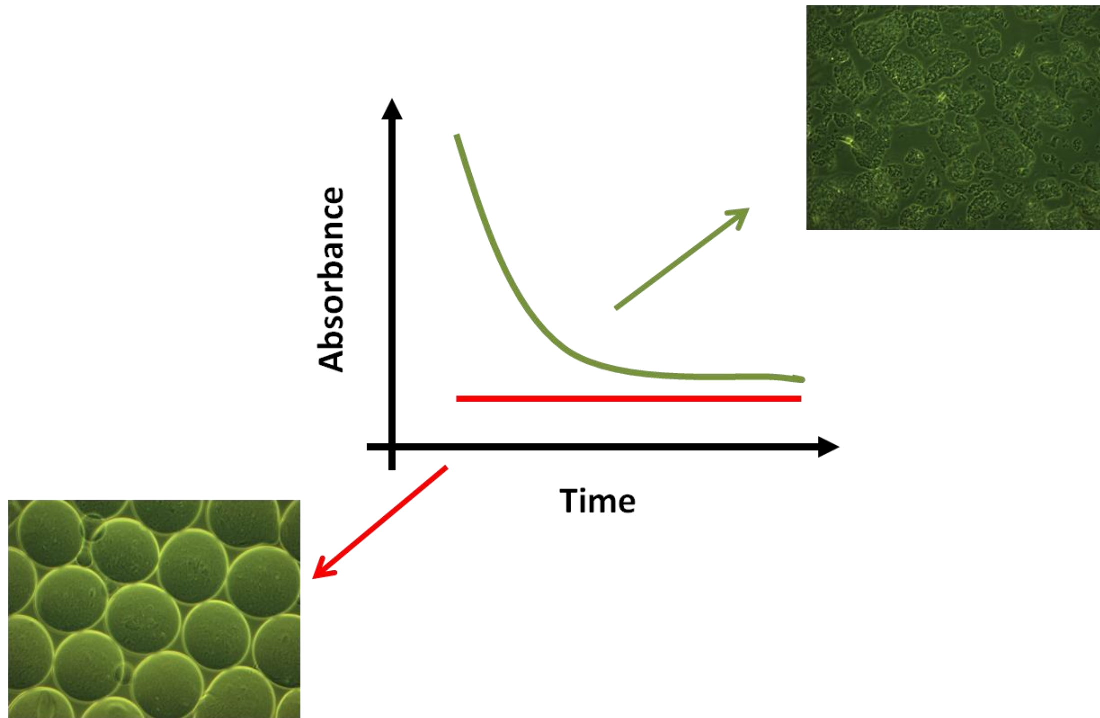

Figure 1: Schematic of interpreting alginate degradation - degradation and no degradation of alginate microbeads through the morphology of the microbeads and absorbance of uronate products.

The third method employed to follow the alginate microbead degradation, is through the release of the microencapsulated cells. As the integrity of the beads is compromised through degradation, the cells are released and migrate out of the microbeads. In the cell release studies, the number of cells released was counted at each time point to follow the degradation of the alginate microbeads.

SPECIFIC AIMS AND EXPERIMENTAL DESIGN

The **overall objective** of this thesis is to develop a system of injectable microbeads to deliver stem cells for the purposes of bone regeneration, thereby allowing the cells to remain at

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the area of injury, to proliferate and secrete soluble factors that will facilitate tissue regeneration. The **overall hypothesis** states that controlled degradation of alginate microbeads can be used to control the release kinetics of viable cells.

Specific Aim 1: To determine if alginate-lyase incorporated into alginate hydrogels can effectively catalyze alginate degradation in a time and dose – dependent manner. There are two main approaches in achieving the controlled degradation of alginate hydrogels (a) PLGA particles loaded with alginate-lyase which could then be encapsulated in the alginate microbeads and (b) mixing alginate-lyase directly with alginate at temperature outside the enzyme's active temperature range. The enzyme alginate-lyase is known to cleave the glycosidic bonds in the alginate through a β-elimination reaction [10]. The **objectives** of this specific aim were to (i) develop and optimize a method of incorporating alginate-lyase in alginate microbeads and (ii) establish controlled release parameters using wound healing alginate and medical grade alginate. Alginate microbeads will be made by using a microencapsulator and a calcium crosslinking solution. The degradation of the beads will be followed by the absorbance of uronate products, the diameter of the beads, and the number of cells released for the cell release studies. The **working hypothesis** is incorporating alginate-lyase into alginate microbeads can control the degradation kinetics of alginate microbeads.

Specific Aim 2: To determine if alginate lyase - mediated alginate degradation has an effect on cell viability and osteogenic potential. Once the stem cells are released from alginate microbeads they must remain viable and multipotent to play an active role in bone regeneration**.** The **objectives** of this specific aim were to use Live/Dead staining, and the TUNEL assay to determine viability of the cells released from alginate microbeads and secondly, to use real time PCR and ELISAs to determine if the osteogenic potential of the released cells is maintained. The

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mRNA levels of Col1, Runx2, Osx, Ocn, Noggin, Gremlin, Bmp2, Vegfa, Fgf2 will be measured and the protein levels of BMP2, VEGF, FGF2, and OPG using ELISAs, and osteocalcin using a radioimmunoassay. The **working hypothesis** was that the viability and phenotype of cells will be maintained as they are released from degrading alginate microbeads.

METHODS

Alginate Degradation Studies

To test our hypothesis that time controlled degradation can be achieved by the incorporation of alginate-lyase into alginate microbeads, various ratios of alginate-lyase (units [U] of enzyme activity) to alginate [g] were combined to form microbeads. Two types of alginates were used: "wound healing" grade alginate (55% to 60% mannuronate units) (Protonal® LF10/60LS, FMC BioPolymer, Sandvika, Norway) and medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginates, FMC BioPolymer). Low viscosity alginate was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX) at a concentration of 40 mg/ml. Microbeads were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.175 mm, an electrostatic potential of 6 kV and a calcium crosslinking solution [19, 20]. The crosslinking solution contained 75 mM $CaCl_2$, 90 mM glucose with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.4 (Sigma Aldrich) (HEPES-buffered). Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca^{++}, and suspended in saline supplemented with calcium chloride (1.8 mM Ca^{2+}).

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PLGA Particle Formation

1. Solid in Oil in Water Double Emulsion

PLGA particles were formed using a previously published solid in oil in water double emulsion method. [14] PLGA particles contained 1:10 ratio of alginate-lyase to PLGA (w/w) and the control PLGA particles had no alginate-lyase incorporated. Briefly, PLGA (50:50) [Sigma Aldrich] was dissolved in dichloromethane (DCM) [Sigma Aldrich] to obtain a final concentration of 20% (w/v). In addition, 2% alginate-lyase [Sigma Aldrich], trehalose [Sigma Aldrich] and 2% magnesium hydroxide [Sigma Aldrich] were added to the dissolved PLGA to create the first emulsion. A sonicator was used to combine components for one minute. This emulsion was added drop wise to an 8% PVA (w/v) solution [Sigma Aldrich] with continuous stirring to form a second emulsion. This double emulsion was combined with 4% (w/v) PVA to ensure the protein of interest was in the PLGA particles. Following this step, the DCM was removed using a rotor evaporator; the particles were washed twice with deionized water, collected and lyophilized.

2. Solid in Oil in Oil Double Emulsion

Prior to the production of PLGA particles, an alginate-lyase and trehalose mixture was made (1:4 ratio of alginate-lyase to trehalose) and lyophilized. PLGA particles contained 1:10 ratio of alginate-lyase to PLGA (w/w) and the control PLGA particles had no alginate-lyase incorporated. Briefly, PLGA (50:50) [Sigma Aldrich] was dissolved DCM to obtain a final concentration of 20% (w/v). In addition, the lyophilized mixture of alginate-lyase and trehalose to produce a final concentration of 2% (w/v)) and 2% magnesium hydroxide [Sigma Aldrich] were added to the dissolved PLGA to create the first emulsion. A sonicator was used to combine components for one minute. Poly(dimethylsiloxane) (silicone oil) [DC 200, Sigma Aldrich] was

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added to the mixture to create a homogenous emulsion. This emulsion was added to heptane and constantly mixed for two hours to allow for the hardening of the PLGA particles. The PLGA particles were collected through filtration, washed twice with heptane [Sigma Aldrich] and allowed to dry in a dessicator. This method proved to be very difficult because of the silicone oil's viscosity. As a result, another sample of PLGA particles were made following the protocol above but using a 1:1 ratio of silicone oil to peanut oil (from Arachis hypogaea) [Fluka, Sigma Aldrich].

Protein Release Studies

To confirm that the PLGA particles were loaded with alginate-lyase, a protein release study was conducted. This was done by suspending the various PLGA particles in saline, and at each time point the samples were centrifuged, supernatant collected, and the particles were re-suspended in saline. The protein released was quantified by the Micro Protein Assay [Pierce Biotechnology, Rockford, IL] and normalized by the weight of PLGA particles used. It is also important that the protein released is active, therefore the ability for the enzyme to produce uronate products was measured. This was done by using an aliquot of the samples collected each day for the protein release study and combining it with 0.5% (w/v) alginate solution, which was allowed to stay overnight and the absorbance of the uronate products were measured at 235 nm.

Micro Protein Assay

The total protein content was measured using the Pierce Macro BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). Reagents A and B were mixed in a 50:1 ratio to make the working reagent. 25 μl of each sample was aliquoted to 96-well plates, and 200 μl of

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the working reagent was added to the sample plates. They were incubated at 37 ^{0}C for 30 minutes and read on a plate reader at 570 nm.

Incorporation of PLGA particles loaded with alginate-lyase into alginate microbeads to achieve controlled degradation

PLGA particles were incorporated into alginate microbeads by encapsulating PLGA particles in a 1:70 (1 mg PLGA particles: 70 mg alginate) ratio. Once the beads were made they were suspended in saline with physiological levels of calcium (1.8 mM $CaCl_2$) at 37 ^{0}C.

Adipose Derived Stem Cells

ASCs were isolated from 100-125g male Sprague-Dawley rats ($n = 6$) [4, 5]. Briefly, the inguinal fat pad was removed and transferred to a container with Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank's balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco, Carlsbad, CA) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of MSCGM. The cells were separated, plated in T-175 flasks at 5,000 cells/cm^2 and cultured in MSCGM at 37 °C and 5% CO_2. For the next two days the monolayers were washed with Dulbecco's phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media

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were changed every 48 hours and cells were cultured to confluence. First passaged cells were used for studies.

ASCs were also isolated from green fluorescent protein (GFP) positive rats and cultured up to passage 6 for the cell release studies.

Cell Release Studies

To test our hypothesis that controlled release of cells can be achieved through the controlled degradation of alginate microbeads, cells were encapsulated in various ratios of alginate-lyase to UV-sterilized medical grade alginate. ASCs were released from the culture plate by trypsinization, and collected by centrifugation after rinsing the cell pellet with MSCGM, the cells were suspended in the alginate lyase-alginate mixture at a concentration of 25 x 10^6 cells/ml. Alginate microbeads were then formed as previously described. Microencapsulated ASCs and GFP-ASCs were suspended in 40μm cell strainers (BD Falcon, Franklin Lakes, NJ) and cultured in MSCGM in non-tissue culture treated 6-well plates to limit proliferation of released cells in the well. Media were changed every 48 hours and each cell strainer was washed twice with 0.9% (w/v) saline to ensure that all the cells released were collected.

1. Cell Number

The number of cells released was counted using a Z1 particle counter (Beckman Coulter, Brea, CA). For the groups where the alginate microbeads did not fully degrade by the end of the 12 day period, 82.5 mM sodium citrate was used to release the remaining cells. All cells were collected, suspended in 0.05% Triton-X100 (Sigma Aldrich) and used to determine the DNA content [Quant-iT ™ Picogreen® ds DNA Reagent, Invitrogen, CA] and the alkaline phosphatase specific activity. The percent cell release was calculated as a function of the total

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number cells in the 0 U/g group (control). The total number of cells microencapsulated in the control group was determined by the summation of all the cells released during the 12 days and all the cells released on the 12th day using 82.5 mM sodium citrate.

To determine if our observations were applicable to other cell types, microbeads were prepared containing human osteoblast-like MG63 cells (American Type Culture Collection, Rockville, MD). Microbeads were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 1% penicillin-streptomycin.

2. Alkaline Phosphatase Specific Activity

To measure alkaline phosphatase specific activity, released cells were suspended in 0.05 % Triton-X100 and lysed by sonication. Enzyme activity was measured as the ability to convert *p*-nitrophenylphosphate to *p*-nitrophenol pH at 10.25. This was normalized by the total protein content (Pierce Macro BCA Protein Assay kit, Pierce Biotechnology, Rockford, IL) in the cell lysate.

3. Cell Viability

a. LIVE/DEAD Assay

Viability of cells within the microbeads and released cells was measured using a LIVE/DEAD Viability kit (Invitrogen). Samples (200µl) were collected at each time point (days 0, 2, 4, 6, 8, 10, and 12) for microbeads without alginate-lyase and on the days where the greatest cell release occurred for the microbeads incorporating alginate-lyase. The 200µl aliquots were transferred to a chamber slide and incubated for 30 minutes at 37°C in 0.9% saline containing 4µM ethidium homodimer-1 and 2µM calcein-AM. Images were obtained using a Zeiss LSM 700-405 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Three images

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were obtained for each well and then red and green cells were counted using Image-Pro Plus software (Media Cybernetics) to determine the percent viability.

b. TUNEL Assay

To determine if cell death was due to apoptosis, the integrity of the cell's DNA was measured using the TiterTACS™ Apoptosis Detection Kit following the manufacturer's protocol (Trevigen, Gaithersburg, MD). At each time point, 300,000 cells from each well were used for the assay.

Osteogenic Potential of Released rASCs

For these experiments ASCs that were not microencapsulated were compared to ASCs released from microbeads containing either 0 U/g or 0.13 U/g alginate-lyase. To investigate the osteogenic potential of the cells released, the cells were collected at day 8 from the 0 U/g and 0.13 U/g groups and treated with OM for 7 days. In addition ASCs that were never microencapsulated were also treated with OM for 7 days. First passage ASCs and released ASCs were plated 5000 cells/cm^2 and cultured in MSCGM. Confluent cultures were treated with osteogenic medium (OM, Lonza) for 7 days followed by MSCGM for 24 hours.

1. Alkaline Phosphatase Specific Activity

Alkaline phosphatase specific activity was measured as previously described in 2.3.2.

2 Soluble Factor Quantification

Enzyme-linked immunosorbent assays (ELISA) were used to measure the levels of fibroblast growth factor-2 (FGF2), osteoprotegerin (OPG), and vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) in the conditioned medium. The levels of osteocalcin were measured with a radioimmunoassay kit (Human Osteocalcin RIA Kit,

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Biomedical Technologies, Stoughton, MA). All ELISAs and the radioimmunoassay were normalized by the DNA content.

3. RNA Extraction and Real Time PCR

RNA was extracted 8 hours after the last media change using TRIzol ® [Invitrogen] and quantified with the Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were then converted into cDNA by the reverse transcription of 1 µg RNA with random primers (Applied Biosystems, Warrington, UK) and Multiscribe Reverse Transcriptase (Applied Biosystems). The cDNA was analyzed for mRNA levels by real-time PCR using the StepOne Plus PCR System (Applied Biosystems). mRNA levels for the following proteins were measured; BMP2 (Bmp2), type I collagen (Col1), FGF2 (Fgf2), Osterix (Osx), Runx2 (Runx2), osteocalcin (Ocn), and VEGF-A (Vegfa). In addition the mRNA levels for BMP antagonists, Noggin (Nog) and Gremlin 1 (Grem1) were measured. All primers (Table 1) were designed using the Beacon Designer 7.0 program and then synthesized by the Eurofins MWG Operon (Huntsville, AL). The mRNA levels were quantified relative to a standard curve of known concentration, and results were normalized to the transcript levels of the housekeeping gene, 40S ribosomal protein S18 (Rps18).

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Table 1: Primer sequences used for the analysis of mRNA levels

Gene	Type	Sequence
Bmp2	R	CTT CCG CTG TTT GTG TTT GG
	F	TGT GAG GAT TAG CAG GTC TTT G
Col 1	R	AGT GAT AGG TGA TGT TCT GG
	F	CGA GTA TGG AAG CGA AGG
Fgf2	R	global gene sequence unknown
	F	global gene sequence unknown
Grem1	R	ATC AGC GAC AGA CGG GGC ATC T
	F	AAG CAC ATC ACC GGC CAA CGG
Nog	R	CAG CAG CGT CTC GTT CAG
	F	GCC AGC ACT ATC TAC ACA TCC
Ocn	R	Quiagen QuantiTect Primer_013414
	F	Quiagen QuantiTect Primer_01084573
Osx	R	GLOBAL GENE
	F	GLOBAL GENE
Rps18	R	TGT ATT GTC GTG GGT TCT GC
	F	TCG CTA TCA CTG CCA TTA AGG
Runx2	R	AGA GGC AGA AGT CAG AGG
	F	TCC CCA TCC ATC CAT TCC
Vegfa	R	TCC AGG GCT TCA TCA TTG C
	F	GGA CAT CTT CCA GGA GTA CC

Statistics

The results of the qualitative and morphometric analyses were calculated as the means ± SEM of six independent cultures per variable. Statistically significant differences between groups were determined by one-way ANOVA followed by Bonferroni's modification of Student's t-test. P values ≤ 0.05 were considered significant. All experiments were repeated to ensure validity of results.

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CHAPTER 2

Alginate-Lyase Incorporated into Alginate Microbeads can Effectively Catalyze Alginate Degradation in a Time and Dose Dependent Manner

INTRODUCTION

Hydrogels used to deliver stem cells must be degradable *in vivo* to allow for the release of cells thus facilitating tissue regeneration. Alginate does not readily degrade *in vivo*. It has been shown to be present six months after implantation [10]. In this thesis alginate degradation is achieved by using alginate-lyase which is an enzyme that catalyses the depolymerization of alginate. It cleaves the glycosidic bonds between the monosaccharide units of the alginate through a β-elimination reaction. This reaction is specific to the alginate-lyase; it may only cleave the glycosidic bond between the mannuronic acids or guluronic acids or mannuronic acid and guluronic acid. In this study the alginate-lyase cleaves the glycosidic bonds between the mannuronic acids. This enzyme is found in organisms of the marine ecosystem such as algae, mollusks and microorganisms, and both marine and soil bacteria [21]. By using this enzyme we are able to control the time for complete degradation of alginate microbeads. This enzyme was incorporated in two ways (i) PLGA particles loaded with alginate-lyase which could then be encapsulated in the alginate microbeads and (ii) the enzyme can be mixed directly with alginate. The first method included the use of solid in oil in water, double emulsion to create PLGA particles loaded with alginate-lyase which were then incorporated into alginate microbeads. Only the latter method resulted in controlled degradation of alginate microbeads. Cell release studies were also conducted to follow the degradation of the microbeads.

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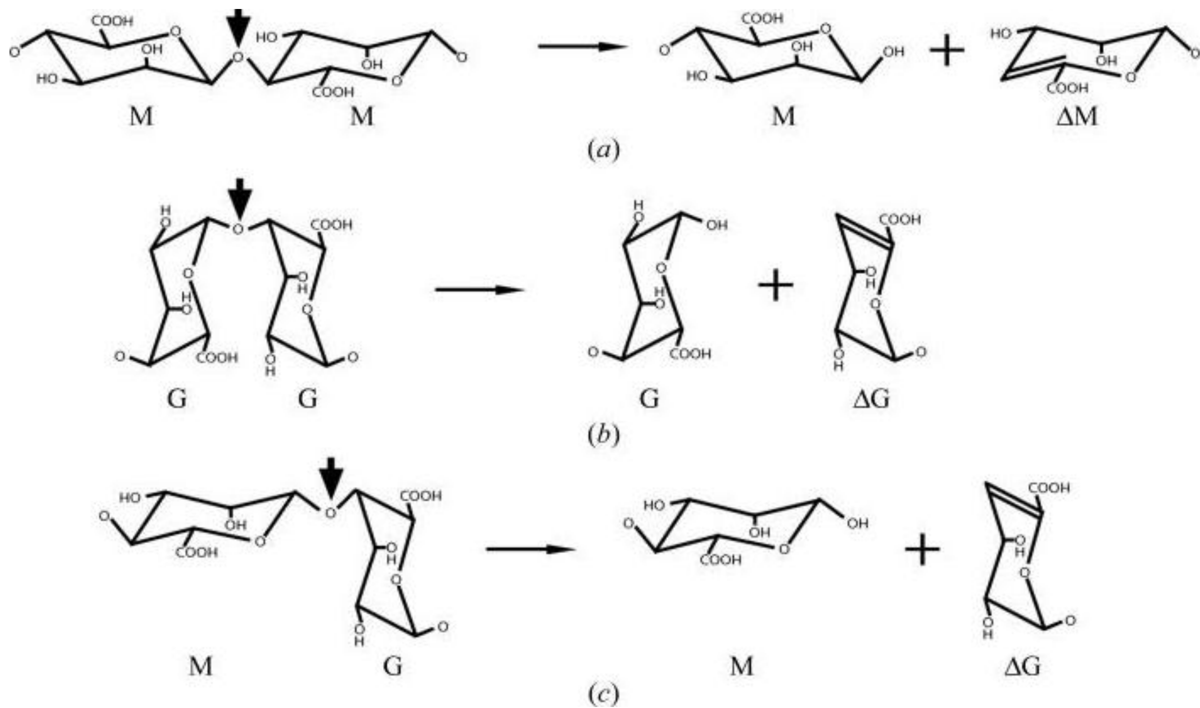

Figure 2: β-elimination reaction of alginate-lyase with alginate. The vertical arrows indicate the cleavage site between (a) MM blocks (b) GG blocks and (c) MG blocks. Taken from M. Yamasaki et al. 2004 [18].

RESULTS

PLGA particles were made using four different formulations. The quantity and activity of the enzyme released from these particles were measured. Group C had the highest protein released however only group A had the ability to produce uronate products (Fig.3A). In any case, these PLGA particles were too big to be microencapsulated. As a result, we went ahead and incorporated group D's PLGA particles in the alginate microbeads but there was no degradation (Fig. 4A). This was further confirmed when the PLGA particles were suspended in the surrounding media which also resulted in no degradation.

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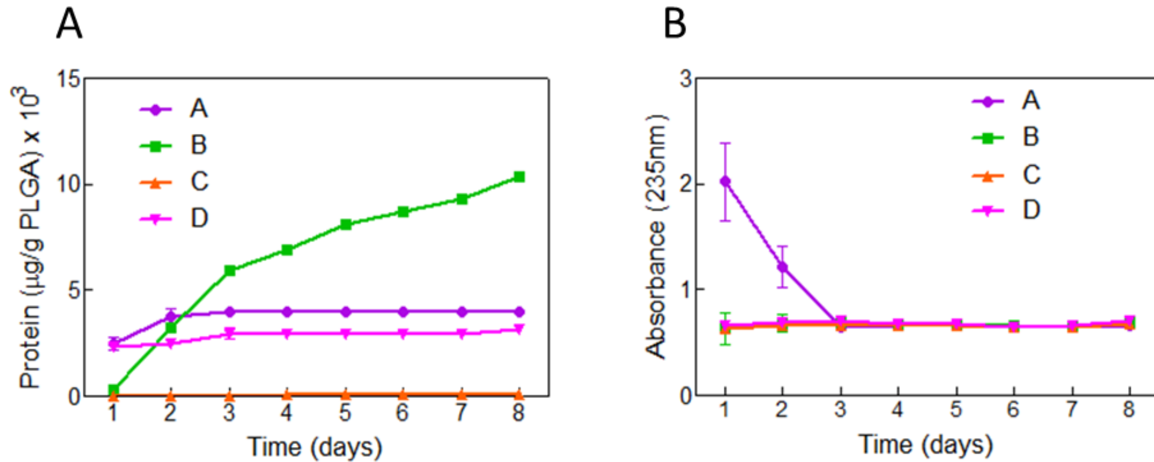

Figure 3: Characterization of PLGA particles formed (A) Cumulative protein release from PLGA particles loaded with alginate-lyase and (B) the ability of the enzyme released to produce uronate products. A- Solid in oil in oil method using silicone oil, B-Solid in oil in oil using a ratio of 1:1 peanut oil: silicone oil, C- Solid in oil in oil using a ratio of 1:1 peanut oil: silicone oil (no alginate-lyase incorporated), D- Solid in oil in water method with trehalose and magnesium hydroxide

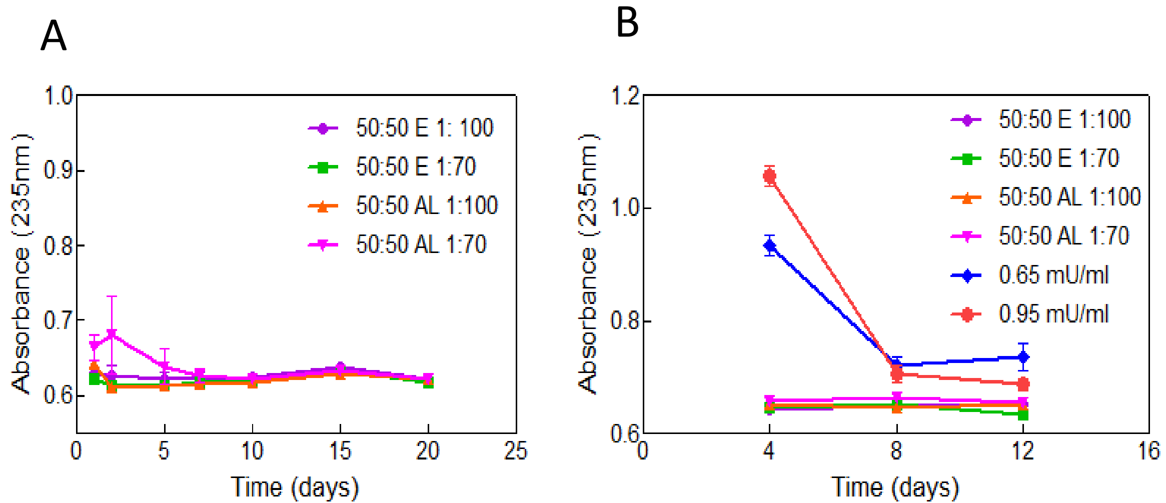

Figure 4: Degradation of alginate (wound healing) microbeads with PLGA particles (A) Degradation of alginate microbeads encapsulating PLGA particles and (B) Degradation of alginate microbeads with PLGA particles in the surrounding media 50:50 PLGA Empty 1:100 (1 mg PLGA particles: 100 mg alginate), 50:50 PLGA Empty 1:70 (1 mg PLGA particles: 70 mg alginate), 50:50 PLGA Alginate-lyase 1:100 (1mg PLGA particles loaded with alginate-lyase: 100 mg alginate), 50:50 PLGA alginate-lyase 1:70 (1 mg PLGA particles loaded with alginate-lyase: 70 mg alginate), 0.00065 U/ml and 0.00095 U/ml (controls)

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Controlled degradation of alginate microbeads made from both wound healing (Figs. 5, 6) and medical grade (Fig. 7) alginate was a function of alginate-lyase concentration. For both alginate types, as the ratio of alginate-lyase to alginate increased, the time required for total degradation of the alginate microbeads decreased (Figs. 6A and 7A). Complete degradation was not observed in microbeads produced using 0.7 U/g in either alginate formulation over the 12 day time course. Degradation of the wound healing grade alginate occurred within 4 days (Fig. 6B) whereas degradation of the medical grade alginate required 6 days (Fig. 7B).

Microbead diameter changed overtime in an enzyme-dependent manner that was formulation specific. Newly generated microbeads had an average diameter of 400 µm when placed in the degradation solution (saline with 1.8mM Ca^{2+}). Incorporation of 28 U/g alginate-lyase caused wound healing grade bead diameters to decrease by >100µm within 2 days. In contrast microbeads containing 0.7 U/g did not decrease significantly until day 12. There were no changes in diameter in the absence of enzyme (Fig. 6C). Similarly, medical grade alginate beads did not change size in the absence of enzyme (Fig. 7C). However, diameters increased by 100µm in the presence of alginate-lyase and this effect occurred more rapidly at higher enzyme concentrations.

Degradation of the medical grade alginate microbeads with no cells in MSCGM (data not shown) occurred faster compared to the same microbeads suspended in saline supplemented with physiological levels of calcium (1.8 mM Ca^{2+}) (Fig 8).

Cell release kinetics varied as a function of alginate-lyase content (Fig. 9A). Medical grade alginate microbeads with the highest concentration of alginate-lyase (1.4 U/g) released the cells in the shortest period of time (2 days) (Fig. 9B). A similar result was seen in the percent

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release where the highest percentage of cells (63%) was released on day 2 from the 1.4 U/g microbeads (Fig. 9C). DNA content of the released cells confirmed these observations (Fig. 9D). Released cells had measureable alkaline phosphatase specific activity (Fig. 9F). The highest levels of enzyme activity were seen in cells that were encapsulated in beads with alginate-lyase content varying from 0.09 to 0.24 U/g. Similar cell release kinetics were observed using GFP-ASCs (Figs. 10A and 10B), and confirmed by measuring fluorescence (Fig. 10C). Release of MG63 cells exhibited comparable kinetics (Figs. 10D, 10E). Unlike the ASCs, however, alkaline phosphatase specific activity of the released MG63 cells was low and did not vary with alginate-lyase content of the microbeads (Fig. 10F).

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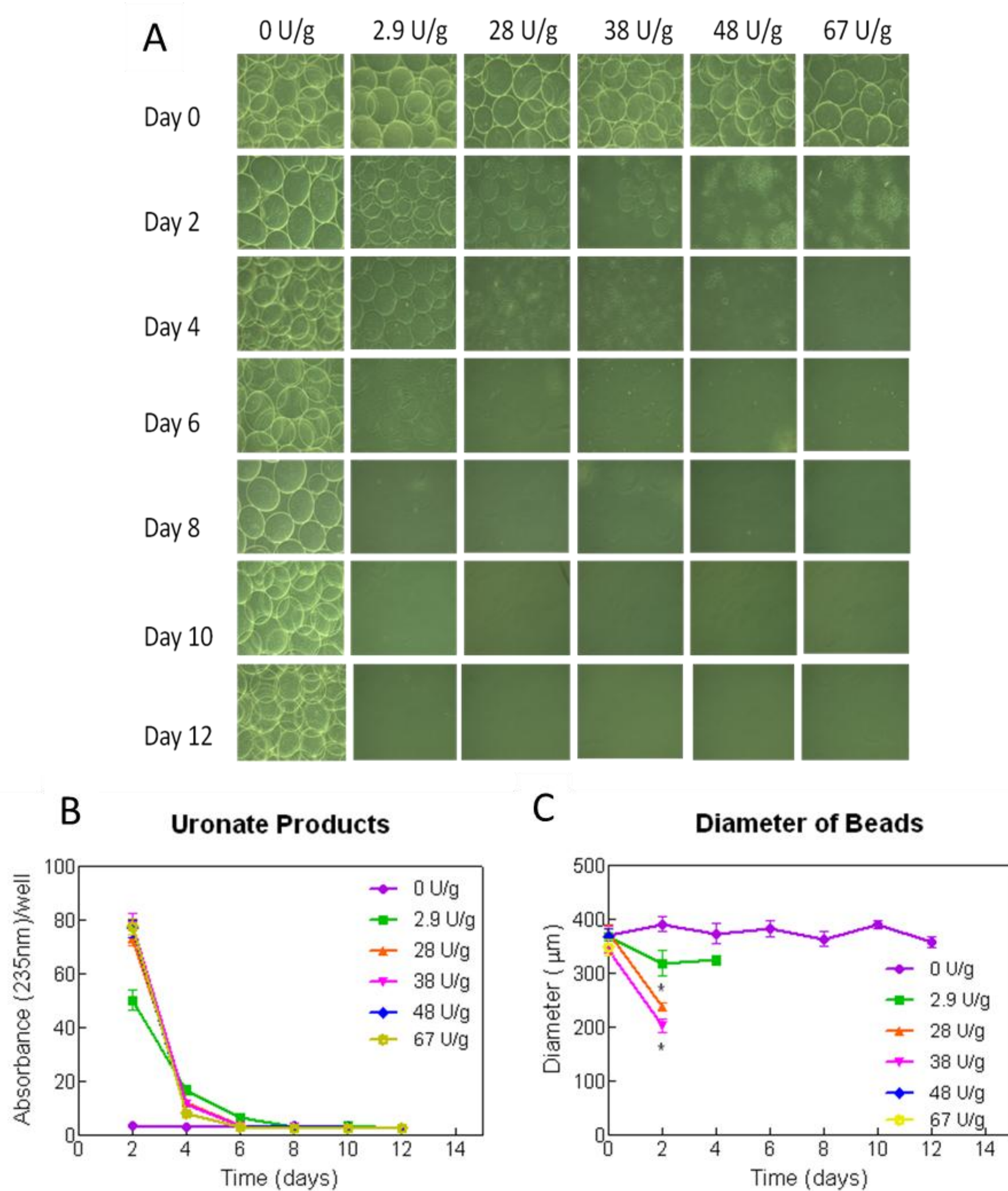

Figure 5: Degradation of alginate (wound healing) microbeads with different formulations of alginate-lyase (U): alginate (g) (a wide range). (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) absorbance of uronate products, and (C) diameter of beads as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 0.

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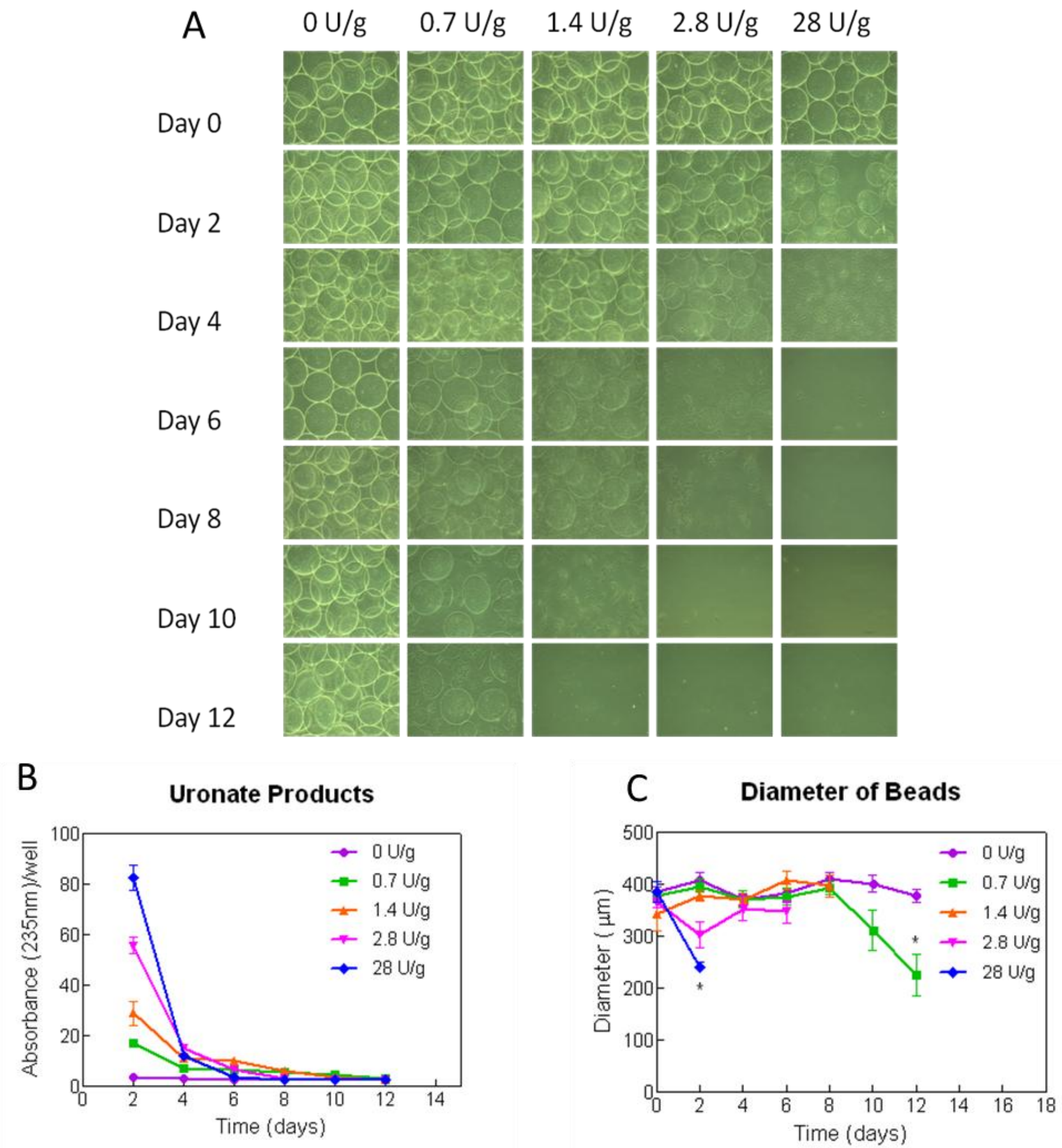

Figure 6: Degradation of alginate (wound healing) microbeads with different formulations of alginate-lyase (U): alginate (g) (a small range (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) absorbance of uronate products, and (C) diameter of beads as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 0.

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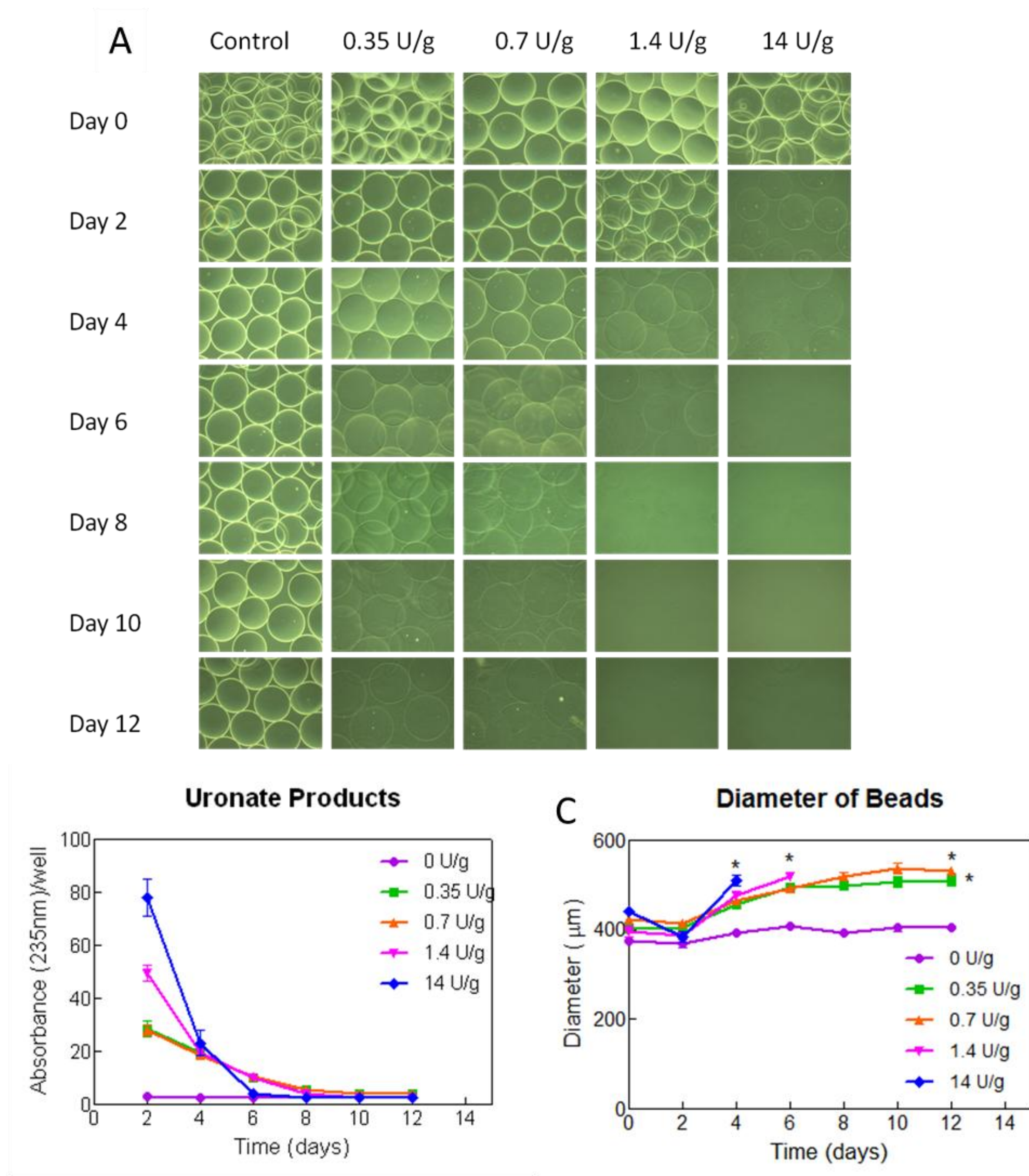

Figure 7: Degradation of alginate (medical grade) microbeads with different formulations of alginate-lyase (U): alginate (g). (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) absorbance of uronate products, and (C) diameter of beads as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 0.

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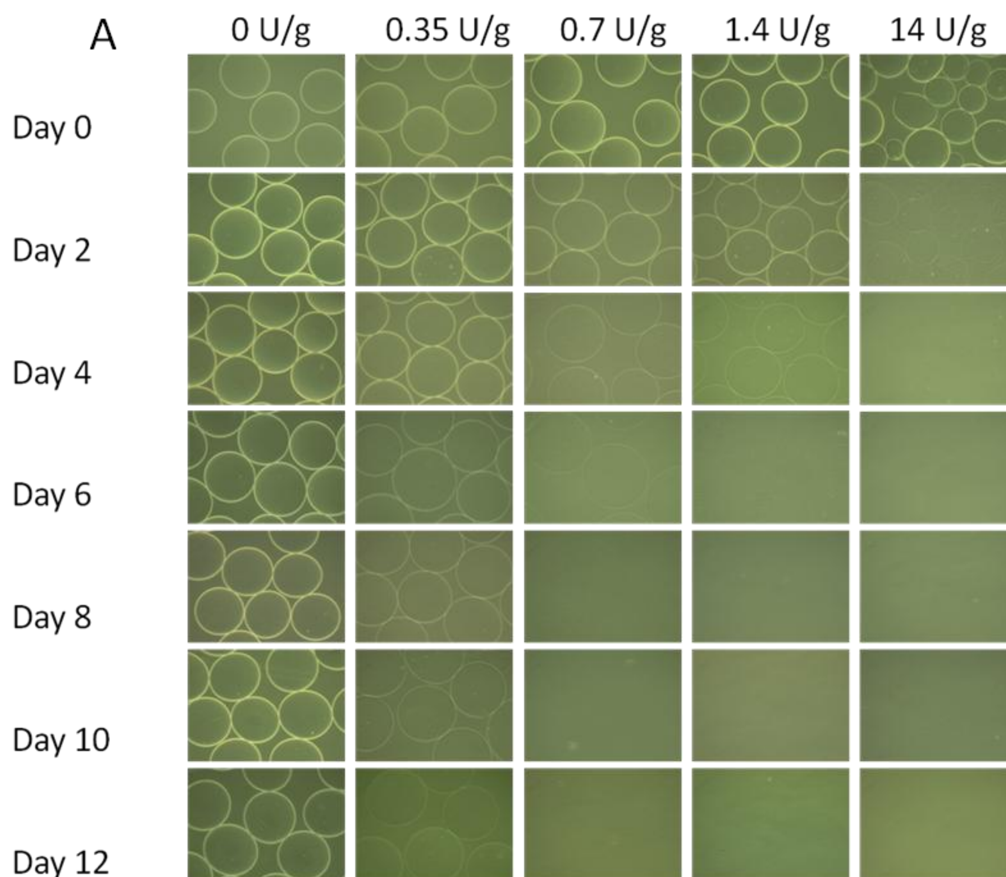

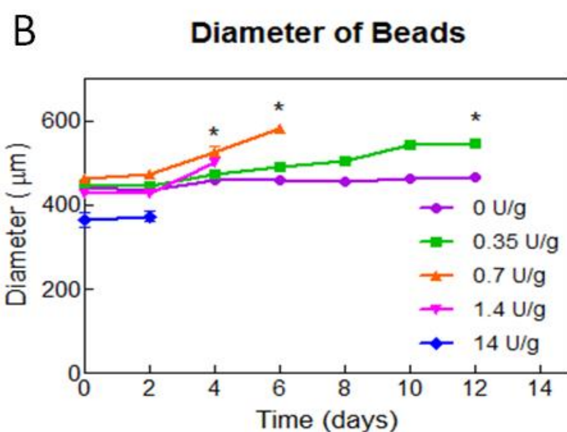

Figure 8: Degradation of alginate (medical grade) microbeads with different formulations of alginate-lyase (U): alginate (g) in MSCGM. (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) diameter of beads as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 0.

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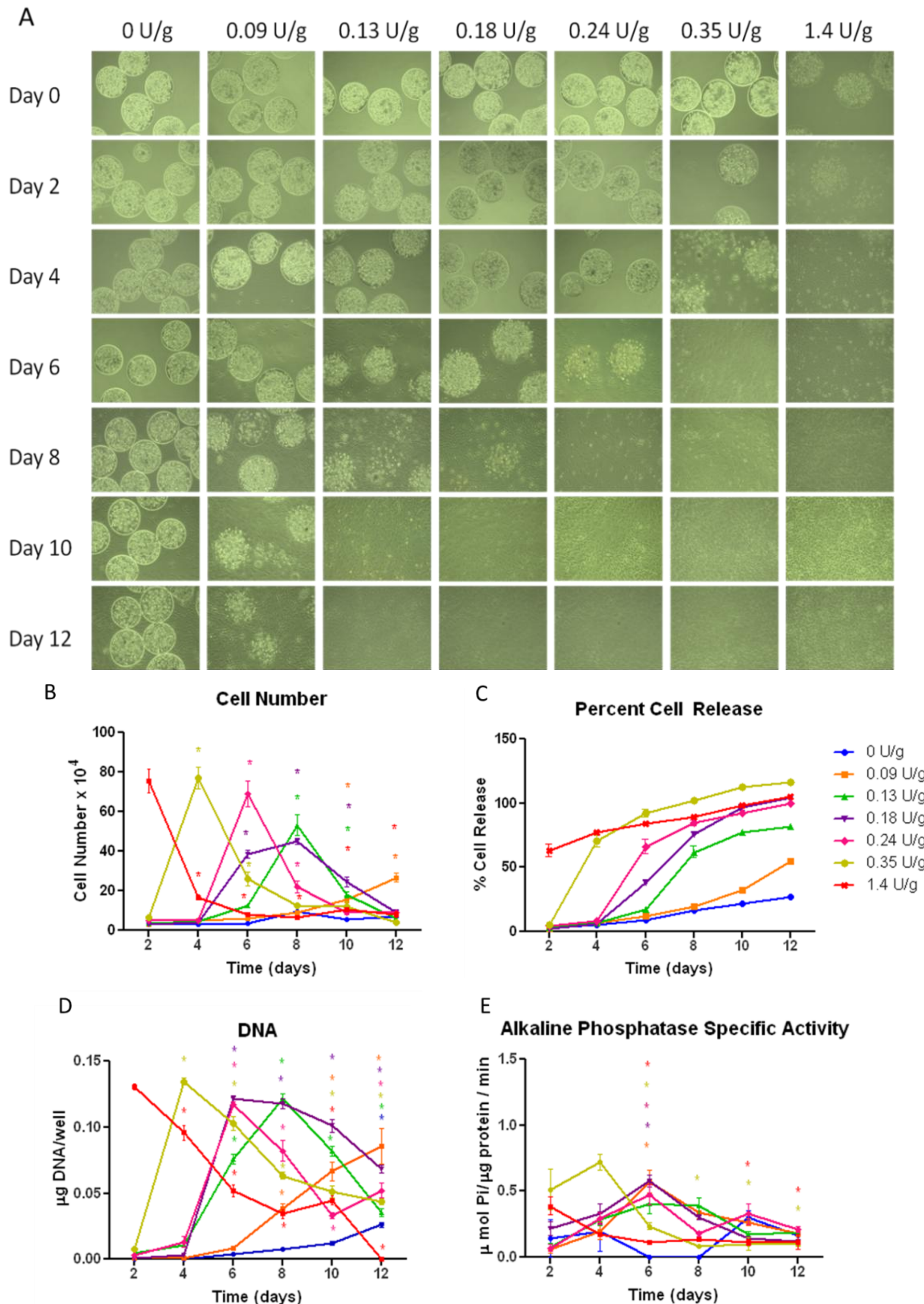

Figure 9: The release of rASCs from alginate (medical grade) microbeads with different formulations of alginate-lyase (U): alginate (g) over the 12 day period. (A) Visualization of the released rASCs from alginate microbeads under an inverted light microscope (mag. = 10x), (B) the number of released rASCs, (C) percent cell release, (D) total DNA content, and (E) total alkaline phosphatase specific activity in the cell lysate. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 2.

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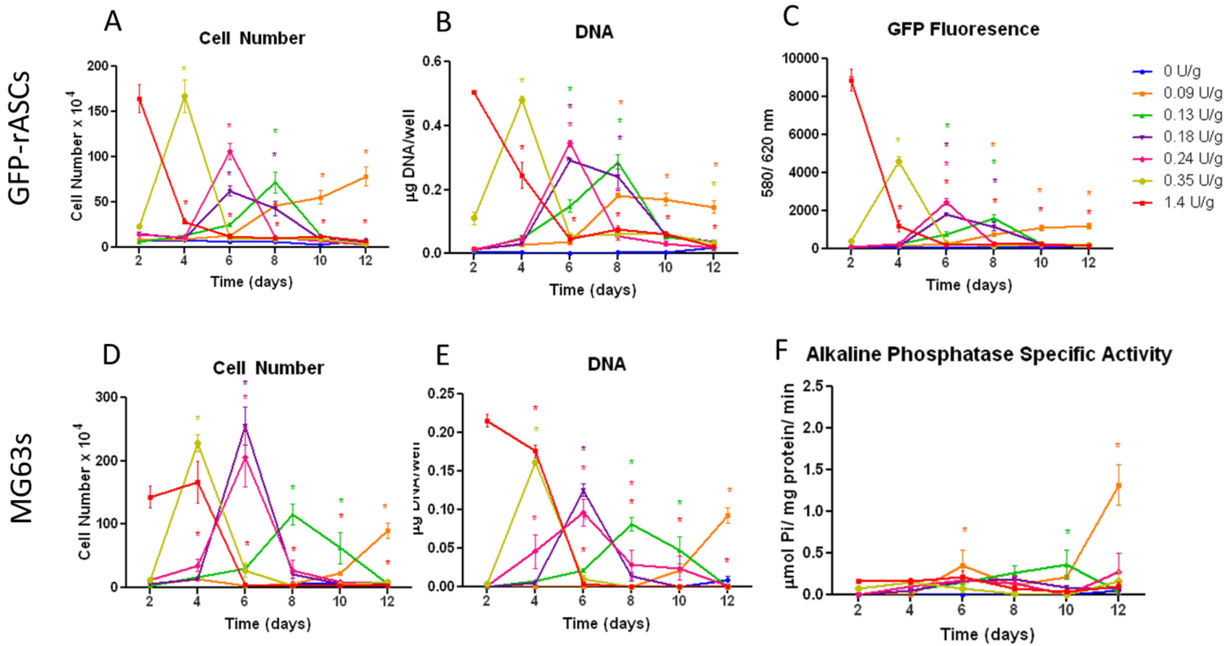

Figure 10: The release of GFP-conjugated ASCs and MG63 osteoblast-like cells from alginate (medical grade) microbeads with different formulations of alginate-lyase (U): alginate (g) over 12 days. Top row – GFP-rASCs: (A) the number of released cells, (B) total DNA content, and (C) fluorescence. Bottom row – MG63s: (D) the number of released cells, (E) total DNA content, and (F) alkaline phosphatase specific activity in the cell lysate. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 2.

DISCUSSION

The *in vivo* degradation of alginate microbeads is critical to cell based therapies using alginate as the carrier. Alginate-lyase was incorporated into alginate microbeads in two ways (i) encapsulating PLGA particles loaded with alginate-lyase in alginate microbeads and (ii) the enzyme can be mixed directly with alginate to form microbeads. However, the first approach did not result in degradation. Initially, these particles were made using the solid in oil in water method. This is a double emulsion method where the trehalose, magnesium dihydroxide, protein of interest is dispersed in a polymer solution then emulsified in an external aqueous phase after which the solvent is evaporated. [11] A trial was done with incorporating these PLGA particles within alginate microbeads which resulted in no degradation (Fig. 5A). We moved on to

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conducting another experiment where two controls were added representing the ideal case that all the alginate-lyase was released from the PLGA particles. Even with the alginate-lyase in the media it showed that there was not sufficient enzyme to degrade the alginate beads (Fig. 5B). Moreover, this double emulsion method is known to have a low encapsulation efficiency and due to its harsh environment inherent to the PLGA preparation, the protein may have been denatured.

As a result, we moved to the second approach which produced more favorable results. Here the alginate and alginate-lyase were combined at a low temperature and used to produce alginate beads. Controlled alginate degradation was achieved by incorporating alginate-lyase into alginate microbeads made from both types of alginate. The wound healing alginate was used to create a model of the alginate-lyase mediated degradation which could be applied to the more expensive medical grade alginate. However, the results showed that the wound healing alginate required more alginate-lyase to be degraded within the same time frame. Hydrogels used to deliver stem cells must be degradable *in vivo* to allow for the release of cells thus facilitating tissue regeneration. Our results demonstrate that this can be achieved by the incorporation of alginate-lyase during microbead production using an electrostatic potential. The effects of the alginate-lyase are concentration dependent. As the amount of incorporated enzyme is increased, the rate of microbead degradation is increased. This correlation between enzyme content and alginate degradation was found with two different alginate formulations used in medical applications but there were some differences. Wound healing grade alginate degraded more quickly based on release of uronate products and a decrease in bead diameter. In contrast, medical grade alginate degraded over a longer period of time and the diameter of the microbeads increased. Despite both types of alginate having similar ratios of M units to G units, differences in their purity including alginate chain length may have played a role [19]. The presence of

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polyphenols in the wound healing alginate does not appear to be responsible, however, since polyphenols have been shown to reduce the activity of alginate-lyase in aerobic degradation [22, 23] and degradation of the wound healing alginate microbeads occurred more rapidly.

Degradation of alginate microbeads via alginate-lyase occurs by two mechanisms; enzymatic cleavage and ion exchange. In the first few days, degradation is primarily due to enzymic cleavage of the glycosidic bonds between the M units. In our system, this was evidenced by the production of uronate products in the first four days of incubation. Once the glycosidic bonds are broken, the Ca^{++} ion crosslinks become more susceptible to ion exchange. The alginate structure disintegrates as a result of the out flux of Ca^{++} ions, which are replaced by monovalent ions resulting in decreased cross-linking density and, thus, a weakened hydrogel structure associated with an increase in pore size [24]. This two part mechanism likely contributed to the changes observed in microbead diameter over the 12 day period. The reduced crosslinking density coupled with the inward diffusion of other molecules resulted in the swelling observed in medical grade alginate microbeads. It is possible that a similar process takes place with microbeads produced using wound healing grade alginate, but it is more likely that the rapid cleavage of glycosidic bonds caused erosion of the surface of the microbead resulting in a decreased diameter.

The controlled release of cells was illustrated with various cell types demonstrating the versatility of the delivery system. For all three cell types, 1.4 U/g alginate-lyase released most of the cells on day 2, while 0.09 U/g caused a gradual release of cells up to day 12. Alginate degradation in the absence of cells occurred more slowly than when cells were present. Mammalian cells do not possess alginate-lyase activity, ruling out the possibility that the cells contributed to microbead degradation via an active process. The difference in degradation

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kinetics reflects the reduced alginate content of the microbeads due to the physical presence of the cells. In addition, the cells may begin to leave the microbeads before total degradation occurs as observed with 0.35 U/g group where the majority of the cells were released on day 4, but without cells the total degradation did not occur in 12 days. Also in the presence of the cells and as time increases, alginate has been shown to decrease in gel strength [24].

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CHAPTER 3

Alginate Lyase - Mediated Alginate Degradation does not have an Effect on Cell Viability and Osteogenic Potential

INTRODUCTION

In order for tissue regeneration to be complete, it is important that the cells implanted are not only able to secrete local factors but are also able to be released from the alginate microbeads. In addition alginate-lyase and the byproducts of alginate lyase - mediated degradation should have no diverse effects on the released cells so that their ability to aid in regeneration of the tissue is not decreased. Therefore the viability and osteogenic potential of the cells released must be maintained to ensure that the bone regeneration is complete. In the present study, we focused on developing a system of injectable hydrogels to deliver stem cells for bone regeneration. This injectable, cell delivery system would allow the cells to remain at the area of injury, to proliferate and secrete soluble factors that facilitate bone regeneration.

RESULTS

Cells microencapsulated in alginate without any alginate-lyase had a viability of 79% on day 2 to 64% on day 12 (Fig. 11B). During the same time period, the viability of the cells released from the microbeads in all the groups ranged from 87% to 71% (Fig. 12B). There was no difference in DNA integrity amongst all groups (Fig. 12C). As the cells were released from the alginate microbeads in all groups, they maintained their ability to produce alkaline phosphatase. The cells released from the 0.13 U/g group maintained their ability to differentiate along the osteoblast lineage by producing significantly higher levels of osteocalcin and similar

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levels of OPG, VEGF and FGF to monolayer cells that were neither previously microencapsulated nor exposed to alginate-lyase (Fig. 13A - F). The mRNA levels of RUNX2, Osx, OCN, and Col1 were similar to the control groups while the mRNA levels of VEGFa, FGF2, BMP2, Noggin, and Gremlin 1 showed a significant increase (Fig. 14A - I).

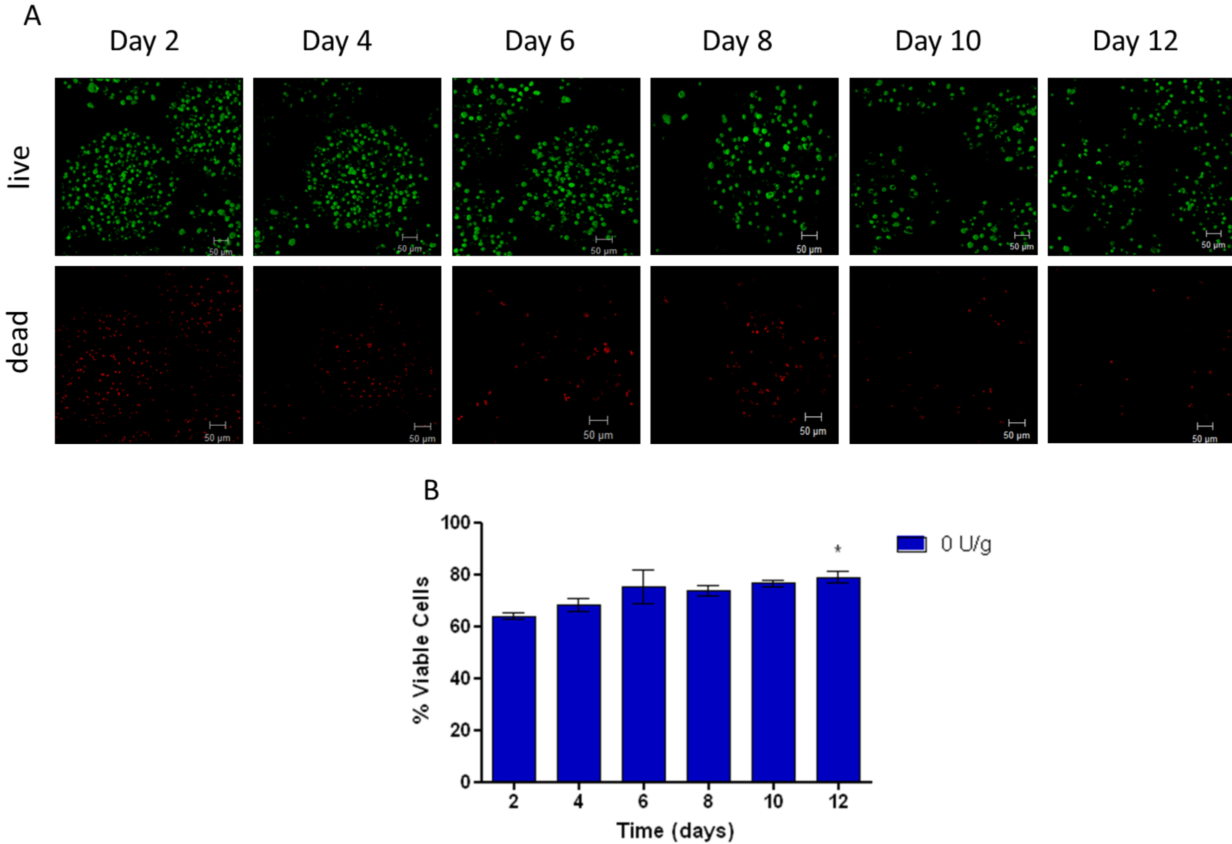

Figure 11: The viability of microencapsulated rASCs without alginate-lyase. (A) Live/dead staining of rASCs within the 0 U/g alginate (medical grade) microbeads (Scale bar = 50 µm) on days 2, 4, 6, 8, 10, and 12, where green represents live cells and red represents dead cells, and (B) the percent viable cells. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 2.

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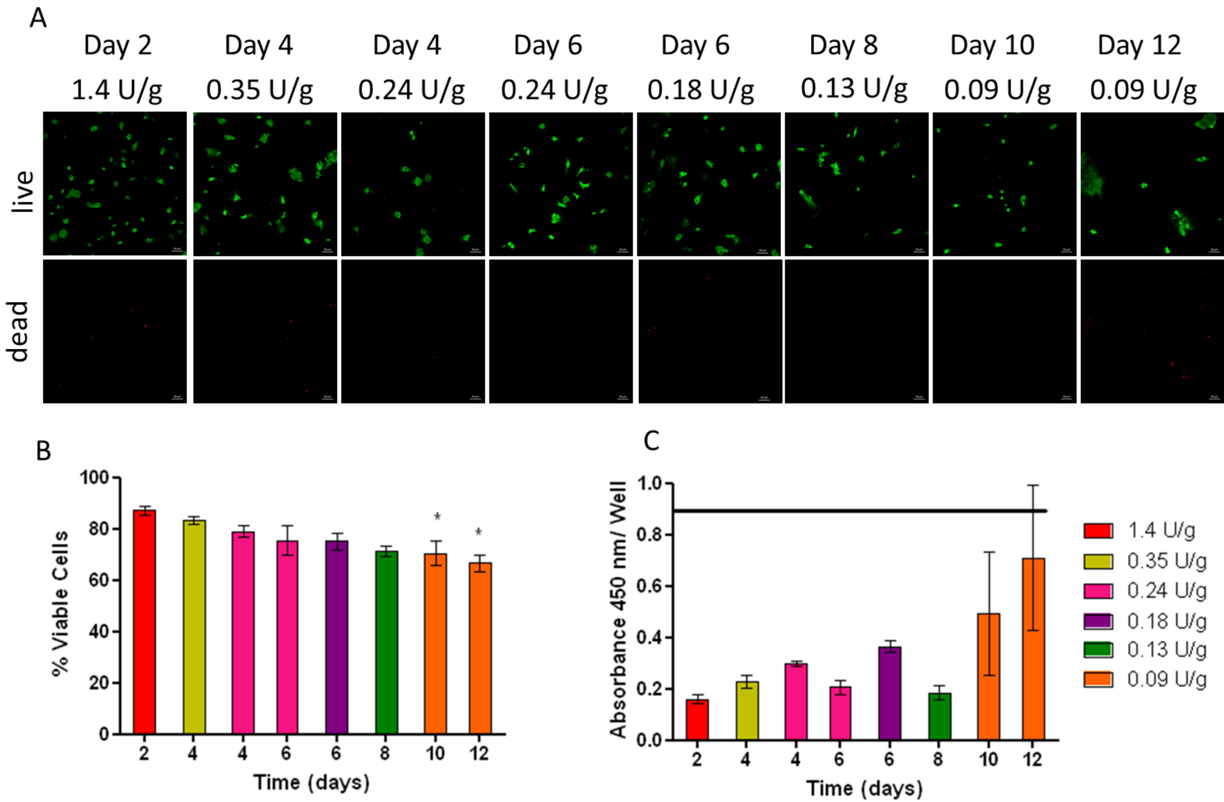

Figure 12: The viability of rASCs released from alginate (medical grade) microbeads made with different formulations of alginate-lyase (U): alginate (g) (Scale bar = 50 µm) (A) Live/dead staining of released rASCs, where green represents live cells and red represents dead cells, (B) the percent viable cells, and (C) TUNEL assay of released rASCs (the horizontal bar represents the positive control generated) at the respective times; 1.4 U/g on day 2, 0.35 U/g on day 4, 0.24 U/g on days 4 and 6, 0.18 U/g on day 8, 0.13 U/g on day 10, and 0.09 U/g on days 10 and day 12. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. day 2.

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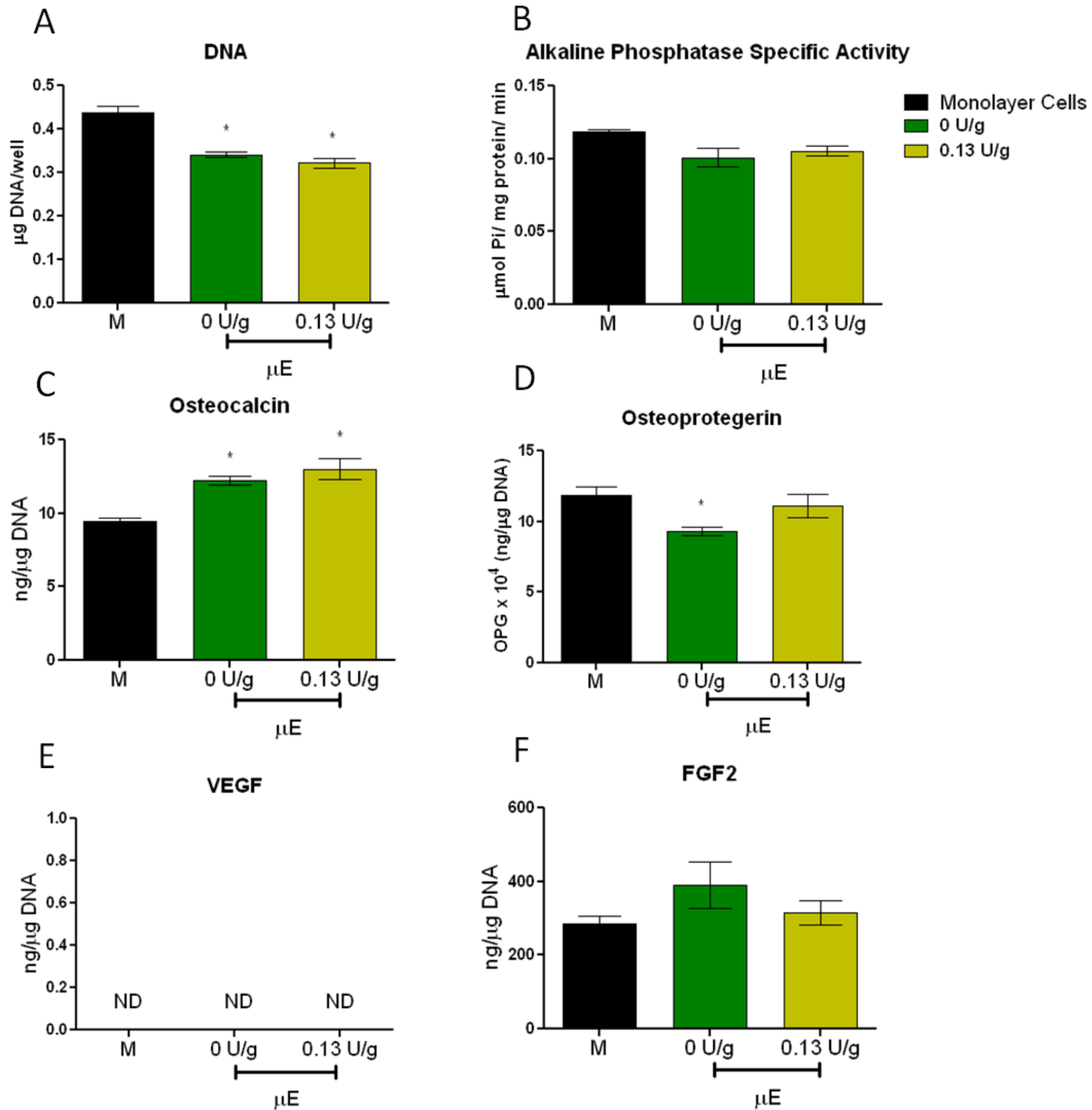

Figure 13: The response of rASCs to osteogenic media, that were not encapsulated (monolayer cells) (M), microencapsulated (μE) and released from 0 U/g and 0.13 U/g alginate (medical grade) microbeads. (A) DNA content, (B) alkaline phosphatase specific activity in the cell lysate, (C) osteocalcin (OCN) levels, (D) osteoprotegerin (OPG) levels, (E) vascular endothelial growth factor (VEGF) levels, and (F) fibroblast growth factor 2 (FGF2) levels. Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. M

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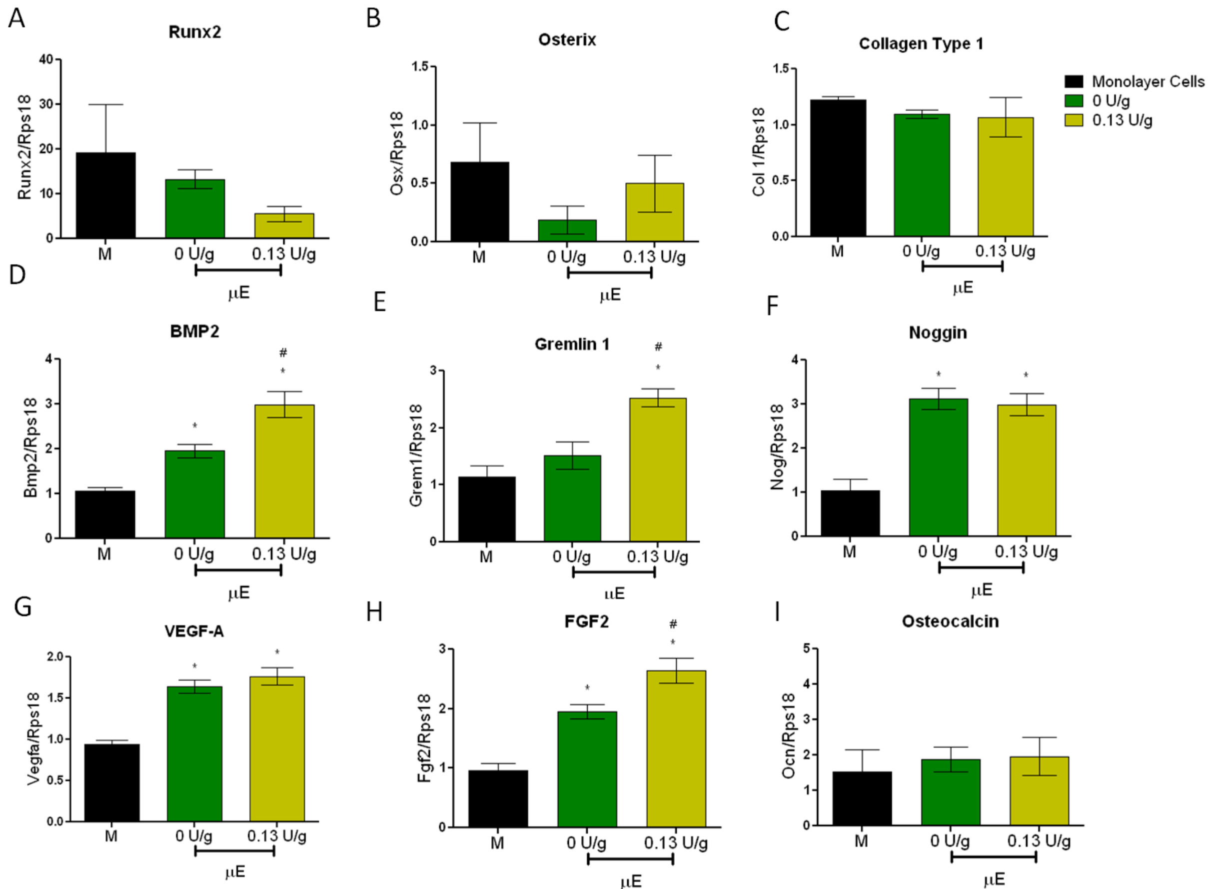

Figure 14: The mRNA levels in rASCs treated with osteogenic media that were not encapsulated (monolayer cells) (M), microencapsulated (μE) and released from 0 U/g and 0.13 U/g alginate (medical grade) microbeads. (A) Runt-related transcription factor 2 (Runx2), (B) Osterix (Osx), (C) collagen type 1 (Col1), (D) bone morphogenetic protein 2 (Bmp2), (E) gremlin 1, (F) noggin, (G) vascular endothelial growth factor -A (Vegfa), (H) fibroblast growth factor (Fgf), and osteocalcin (Ocn). Data were analyzed using one-way ANOVA followed by Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. M.

DISCUSSION

The viability of cells post-injection is critical to the success of injectable cell-based therapies. Injection of stem cells is known to result in low viability ranging from 1% to 32% because of the harsh environment at the injury site and the mechanical disruption of the cell membrane by extensional flow during ejection from the needle [25]. Delivering cells in crosslinked alginate by injection has been shown to improve cell viability because of its protective effects during ejection via "plug flow". This plug flow occurs when the hydrogel near

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the wall acts as a lubricant, protecting the rest of the hydrogel by allowing it to stay intact and move through the needle [25].

Once the cells are injected, the alginate can provide further protection while retaining them at the injury site. Our results indicate that cells encapsulated in the microbead formulations tested in this study are viable in the microbead environment, indicating favorable mass transfer of nutrients. The small increase in the number of viable cells noted in microbeads produced without enzyme may have been due to proliferation or to retention of secreted factors within the microbead [26] or to limited apoptosis. Moreover, inclusion of alginate-lyase in the alginate did not have a negative impact on viability of ASC released from the microbeads. Indeed, viability of cells released from microbeads containing alginate was greater than from microbeads without enzyme. This suggests that the weakened structure of the alginate microbead due to cleavage of glycosidic bonds may have improved nutrient transfer.

Importantly, ASCs released from the microbeads retained their ability to differentiate along an osteoblastic lineage, not only to the same extent as ASCs that were never exposed to microencapsulation, but for some markers, to a greater extent. At the time of release, cellular alkaline phosphatase specific activity was comparable for all formulations of microbead. Released cells cultured in monolayer to confluence and then for 7 days in osteogenic medium had comparable alkaline phosphatase activity to ASCs that had never been previously microencapsulated, two fold greater than seen in ASCs immediately following release. Similarly, mRNA levels for the osteogenic transcription factors Runx2 and Osx were not different compared to the monolayer cells, nor were mRNA levels for Col1.

Our results also suggest that microencapsulation may enhance the osteogenic potential of the ASCs with respect to their ability to produce paracrine factors that stimulate bone formation.

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The released ASCs exhibited reduced DNA content and increased osteocalcin production compared to the never-encapsulated ASCs after 7 days in OM. This was correlated with increased levels of Bmp2 mRNA, suggesting that BMP2 might act as an autocrine/paracrine regulator of the microencapsulated cells. Interestingly, expression of Bmp2 was accompanied by expression of its inhibitors, Grem1 and Nog, but in a differential manner. The significance of this is not known at this time, but other studies have shown that these proteins are regulated independently [27].

There was not complete convergence between mRNA levels at 8 hours post-treatment with osteogenic medium and protein levels at 24 hours for a number of factors, including osteocalcin, VEGF and FGF2, which may reflect normal cellular transcription and translational controls. Interestingly, mRNA expression was more sensitive to differences in microbead formulation. Cells encapsulated in alginate containing alginate-lyase had greater expression of Bmp2, Grem1 and Fgf2 and lower expression of Opg than cells that had been encapsulated in alginate without enzyme. Similarly, for some mRNAs, having the experience of encapsulation caused greater expression than seen in ASCs that had not been encapsulated, even though the same starting population of ASCs was used for these experiments. Previous research has shown that glucocorticoids can decrease production of angiogenic factors [28], suggesting that the dexamethasone present in osteogenic medium may have inhibited VEGF production by the cells. The fact that Vegf mRNA was not affected and that the ASCs produced both Fgf2 mRNA and FGF2 protein argues against this, however.

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CONCLUSION

In conclusion we have developed a degradable and injectable delivery system for stem cells. The controlled degradation was achieved through the incorporation of alginate-lyase into alginate microbeads. This system has shown that it can be used with other cell types. The cells released remained viable and maintained the ability to differentiate into osteoblasts. These microencapsulated cells can be directly injected to the site of injury. Therefore, in cases where surgery is usually required for fractures or nounions, this stem cell delivery method can be used, allowing controlled release of cells and the production of factors thus enhancing tissue regeneration.

FUTURE WORK and CLINICAL IMPLICATIONS

Creating smaller microbeads that are less than 200 μm in width and achieving the controlled release of viable cells is a series of studies that will be done. This would enhance the mass transfer properties of this injectable delivery system and possibly increase the viability of the released cells. Smaller alginate microbeads would also lead to the use of a smaller needle for delivery. Following this study, an *in vivo* study will be carried out to test the ability of the delivery system to aid in the regeneration of bone and determine which ratio of alginate-lyase to alginate is optimal for bone regeneration. For example, the microbeads could be implanted into a metaphyseal defect and after several weeks, asses the bone growth in the fracture through micro CT amongst other methods.

The factors produced by the microencapsulated cells are critical to the bone regeneration process. Therefore the conditions that best lead to the production of these local factors should be explored. This may take the form of investigating the effects of the various components of

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osteogenic media and identifying which combinations illicit the greatest production of osteogenic local factors. Another study will be done looking at the various osmolytes used in the crosslinking solution and identifying an optimal osmolyte that leads to the greatest production of these same local factors.

As previously suggested, an *in vivo* study should be done to determine the ideal ratio of alginate-lyase to alginate that is ideal for bone regeneration. Alternatively, a more extensive study should be done to determine when most cells migrate to the fracture. Based on this information, one could determine which ratio is best for the regeneration of bone.

Clinically, the findings from this study would add to the knowledge for personalized therapies for fractures. Here the patient's ASCs could be isolated, microencapsulated and delivered to the fracture. On a broader scale the parameters identified in this study can be applied to other areas of tissue regeneration apart from bone.

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