Proceedings of the 2007 International Manufacturing Science And Engineering Conference
MSEC2007
October 15-17, 2007, Atlanta, Georgia, USA

MSEC2007-31127

PVA-BASED SCAFFOLDS FOR THE REPAIR OF MUSCULOSKELETAL SOFT TISSUE

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ABSTRACT

The objective of this study was to design a partly-degradable scaffold to repair cartilage defects. The scaffold, based on poly(vinyl alcohol), PVA, was intended to maintain long-term mechanical integrity and to facilitate cell proliferation via bioactive agent release from contained microparticles, made from either alginate, ALG or poly(lactic-co-glycolic acid), PLGA. The aim of this study was to characterize the morphological features and mechanical behaviour of composite scaffolds as a function of microparticle type and percent content. Our hypothesis was that the dynamic mechanical properties (Dynamic Modulus and Phase Angle) of the composite scaffold would not be affected by microparticle type, but that Dynamic Modulus would increase as a function of increased microparticle content. Scanning Electron Microscopy confirmed that the manufacturing process homogenously dispersed microspheres within the scaffolds. For pure PVA samples Dynamic Modulus ranged from 66±3 kPa at 0.01 Hz to 83±3 kPa at 50 Hz. As ALG microsphere content increased from 25 % to 75 %, Dynamic Modulus ranged from 92±5 kPa at 0.01 Hz to 153±19 kPa at 50 Hz. As the microsphere content increased from 25 % to 75 % PLGA, Dynamic Modulus ranged from 85±9 kPa at 0.01 Hz, to 157±16 kPa at 50 Hz. As expected, Dynamic Modulus increased with increasing test frequencies. For pure PVA specimens Phase Angle ranged from 4.3±0.8 degrees at 0.01 Hz to 12±1.2 degrees at 50 Hz. Phase Angle was not affected by microsphere content. In conclusion, the addition of microspheres affected the dynamic mechanical behavior, in particular Dynamic Modulus, of PVA scaffolds. However, the dynamic mechanical properties were not affected by the polymer from which the microspheres were manufactured. These findings suggest that microsphere type can be chosen to optimize the inclusion of bioactive factors, without detrimentally affecting the mechanical properties of the composite scaffold. It also suggests that % content of included microspheres can be used to modulate the mechanical properties of the scaffold at time zero.

Keywords: Articular Cartilage Tissue Engineering, Poly (vinyl alcohol), Microparticles, Unconfined static & dynamic compression

INTRODUCTION

Approximately 10 million Americans suffer from osteoarthritis (American Academy of Orthopaedic Surgeons)

and limited options are available to treat this painful disease in which articular cartilage is worn away. Attempts at joint resurfacing using techniques such as microfracturing, drilling, scraping, shaving and autologous grafting [1] have had variable results, but eventually lead to a total joint replacement. One method of cartilage repair involves growing replacement tissue ex vivo for subsequent implantation, so called *Tissue Engineering* [2,3].

Three components are needed to successfully engineer replacement tissue; cells to generate extracellular matrix, a scaffold to support growth and the release of bioactive factors to stimulate the desired cellular response [4]. Through the development of novel polymeric scaffolds, the ability to provide mechanical integrity to the defect site, to support to the ingrowing tissue and to release growth factors in a timed manner is paramount to the success of this approach. Ideally, a scaffold should also have a highly porous three-dimensional structure, with an interconnected porous network for cell ingress, tissue growth and mass transport of nutrients and waste.

Natural scaffold materials including chitosan [5], collagen [6], agarose [7] and alginates [8] find wide application in tissue engineering, in particular for the regeneration of soft tissues. These materials do not induce a host response, and may enhance the biological recognition in the growing neo-tissue, encouraging the normal cellular functions. Synthetic degradable polymers such as poly(lactic acid), poly(glycolic acid), combinations of the two, as well as poly(L-lactide-co-ε-caprolactone) and polyfumarate have also been used [9,10], because they are biodegradable, biocompatible and they can be easily processed into porous scaffolds. However poor mechanical properties associated with degradable scaffolds for soft tissue repair, prevent scaffolds from functioning like the tissue they are intended to replace.

Non-degradable materials, the mechanical properties of which can be defined by controlling manufacturing processes, are potentially attractive as scaffold materials. Poly(vinyl alcohol), PVA, for example, is biocompatible and non-toxic, and has been proposed for a wide range of biomedical applications including contact lenses, corneal implants and as a substitute for cartilage [11,12] and meniscus [13]. PVA can be used to form a hydrogel, which retains water within its three-dimensional network of polymer chains. Furthermore, PVA hydrogels can be manufactured using freeze-thaw treatments thereby avoiding the use of potentially toxic chemical crosslinking agents such as glutaraldehyde and formaldehyde. The ability to manufacture PVA hydrogels with provisions for the release of included bioactive agents for tissue engineering applications, however, has been less characterized [14,15].

The most widely employed strategy to local deliver growth factors is via their direct incorporation within the scaffold. One limitation to this approach is that growth factor release occurs via enzymatic degradation, which rapidly occurs when the macromolecule is exposed to the *in vivo* environment [16]. Furthermore, local protein concentration as a function of time cannot be controlled easily. Another delivery strategy is the incorporation of growth factors in micron-sized particles, which are then embedded in a scaffold. This approach protects the bioactive agents from degradation. Accordingly, growth factors have been included in micron-sized particles [17] and in nano-sized particles [18] and made from materials such as gelatin, alginates, chitosan, poly(lactic-co-glycolic) acid (PLGA) [19].

Among naturally-derived polymers, alginates have been extensively studied as a hydrophilic-drug delivery system because of their biocompatibility and biodegradability [20,21]. In particular, Alginic acid sodium salt, ALG, is a water-soluble polysaccharide that can be crosslinked by the use of bivalent cations such as Ca^{+2}, Ba^{+2} or Sr^{+2} [22].

PLGA, a Food and Drug Administration approved synthetic copolymer, has been widely investigated as drug delivery system, owing to its biodegradability and biocompatibility. PLGA copolymers have been used for both hydrophilic and non-hydrophilic drug release [15,23,24] because their release properties can be easily tailored by varying composition (lactide/glycolide ratio), molecular weight and chemical structure (i.e. capped and uncapped end-groups).

However it is unclear which polymer most efficiently encapsulates growth factors. Furthermore, it is unknown what effect microparticle polymer type and percent microparticles might have on the mechanical performance of the scaffold.

This study describes the development of a partly-degradable scaffold to repair cartilage defects. A non-degradable PVA scaffold is intended to maintain long-term mechanical integrity, and degradable microparticles made of either ALG or PLGA are intended for the incorporation of hydrophilic and non-hydrophilic bioactive agents respectively.

The objective of this study was to investigate morphological features and mechanical response of a PVA-based composite scaffold as a function of microparticle polymer type and content. Our hypothesis was that the dynamic mechanical properties (Dynamic Modulus, E*, and Phase Angle, δ) of the scaffold would not be affected by the polymer from which the microparticles were fabricated. Furthermore, we hypothesized that E* would increase as a function of increased microparticle content.

MATERIALS & METHODS

Manufacture of ALG microparticles

ALG (Alginic acid sodium salt, low viscosity, Fluka, Italy) microparticles were manufactured using a standard "Water in Oil" emulsification technique. The Oil phase was prepared by mixing 110 ml of isooctane (Carlo Erba, Italy) with 4 ml of Span 85 (Sigma, Italy). The Water phase was prepared dissolving 400 mg of ALG powder in 20 ml of double distilled water. The crosslinking solution was prepared dissolving 2 g of Calcium Chloride (Carlo Erba, Italy) in 20 ml of double distilled water. All the components were cooled to 4 °C.

The Water phase was added drop-wise to the Oil phase, while the solution was stirred at 1000 rpm. The solution was stirred for a further 10 min then 12 ml of Tween 85 (Sigma, Italy) was added as surfactant. Calcium Chloride solution was added drop-wise to crosslink the particles, and the solution was stirred for a further 15 minutes. The emulsion decanted for 45 min and the organic phase (upper liquid) was discarded. All the steps were performed at the constant temperature of 4 °C. The solution underwent 3 centrifugation cycles at 5500 rpm for 5 min; each cycle was composed of centrifugation, supernatant elimination and addition of fresh isooctane/ethanol (Carlo Erba, Italy) mixture. ALG microparticles were collected, freeze-dried and stored at room temperature.

Manufacture of PLGA microparticles

PLGA (Medisorb® 5050 DL 3.5A, iv=0.4 dl/g, Lakeshore Biomaterials, USA) microparticles were manufactured using a standard "Oil in Water" emulsification and solvent evaporation technique. The Oil phase was prepared by dissolving 1 g of PLGA powder in 20 ml of dichloromethane (Carlo Erba, Italy) and the Water phase was prepared dissolving 4 g of PVA powder (MW 22000, 97.5-99.5% hydrolyzed, Fluka, Italy) in 200 ml of double distilled water at 90 °C. Both the Oil phase and the Water phase were cooled to 4 °C.

The Oil phase was added drop-wise to the Water phase and the solution was stirred at 10500 rpm for 8 min using a high-speed homogenizer (Art. Miccra-D8, Falc Instruments, Italy). 400 ml of double distilled water was added to the emulsion, and the solution was magnetically stirred for 4 h at 400 rpm to promote dichloromethane evaporation. All the steps were performed at the constant temperature of 4 °C. The solution underwent 3 centrifugation cycles at 5500 rpm for 7 min; each cycle was composed of centrifugation, supernatant elimination and addition of fresh water. PLGA microparticles were collected, freeze-dried and stored at room temperature.

Manufacture of PVA-based scaffolds

A 10 wt% PVA (MW 85-146000, 99+ % hydrolyzed, Sigma, Italy) aqueous solution was prepared by autoclaving at 120 °C for 1 h. The solution was then allowed to cool to room temperature. ALG microparticles were added where the amount of ALG microparticles was varied as a weight percent of PVA to produce the following groups: 0 %, 25 %, 50 %, and 75 % ALG (n=8 per group). After adding the microparticles to the PVA solution the mixtures were magnetically stirred for 5 min at 300 rpm at room temperature.

PLGA microparticles were added to PVA as follows: A 12.5 wt% PVA aqueous solution was prepared by autoclaving at 120 °C for 1 h. The solution was then allowed to cool to room temperature. PLGA microparticles were initially dispersed in double distilled water by magnetic stirring and then the mixture was added to the PVA solution to manufacture batches of novel partly-degradable PVA-based composite scaffolds with the same content of PVA (10 wt%). The amount of PLGA microparticles was varied as a weight percent of PVA to produce the following groups: 0 %, 25 %, 50 %, and 75 % PLGA (n=8 per group). After adding the water-dispersed microparticles to the PVA solution, the mixtures were magnetically stirred for 5 min at 300 rpm at room temperature.

All solutions were poured into wells of a 24-well polystyrene plate, sealed with parafilm and subjected to the following freeze-thaw treatment: 1 cycle of 16 h at –20 °C, followed by ½ h at 30 °C, and 7 cycles of 1 h at –20 °C, followed by ½ h at 30 °C. Samples were stored at -20 °C.

Morphology

ALG and PLGA microparticles were sputtered with gold under vacuum and morphologically analyzed using a scanning electron microscope, SEM (Jeol JSM-5600). Two magnifications, 1000x and 4000x, were used for a qualitative analysis of size and surface appearance, respectively.

Discs of ALG/PVA and PLGA/PVA samples approximately 5 mm thick were sliced from the middle of each composite scaffold for morphological analysis (n=2 per group). The discs were placed in an environmental chamber of a scanning electron microscope, Environmental SEM (FEI Philips). Images were taken at 500x magnification and were used for the qualitative analysis of microparticle dispersion within the scaffolds.

Mechanical Testing

Scaffolds (n=6 per group) were sliced to a thickness of ~4 mm on a freezing-stage microtome to ensure flat parallel surfaces and then cored using a 10 mm diameter biopsy punch connected to a hand-actuated press. Double distilled water was added to each specimen one day prior to testing. Sample diameter and thickness were measured with a digital caliper.

Unconfined compression tests were performed under displacement control using an ElectroForce® test instrument (EnduraTEC, Bose Corporation, USA) using Dynamic Mechanical Analysis (DMA) software. Samples were positioned between two 50 mm diameter non-porous stainless-steel platens and some double-distilled water drops were placed around the samples to ensure that they remained hydrated throughout testing. The unconfined samples were compressed at a rate of 0.0167 mm/s to a strain of 20 % and allowed to relax for 300 s. After relaxation a sinusoidal displacement of ±5 % was superimposed at frequencies, ranging from 0.01 to 50 Hz (n=8 different frequencies), applied consecutively from the lowest to the highest.

The data were analyzed using the DMA software using Fourier analysis. The Dynamic Modulus, E*, which is calculated as the ratio of the force amplitude to the displacement amplitude divided by the shape factor (for a cylindrical specimen is $(\pi/h)*(d/2)^2$, where h is the height and d is the diameter) and Phase Angle, δ, which is a function of the internal friction of the material, were computed for each frequency tested and for each specimen.

RESULTS

Morphology

The yield was 81.0±2.4 % (n=15 preparations) and 90.0±1.8 % (n=5 preparations) for ALG and PLGA microparticles, respectively. SEM images showed a relatively smooth surface for both microsphere types, and a maximum diameter of ~12 μm for ALG microspheres (Fig. 1) and ~7 μm for PLGA microspheres (Fig. 2).

Pure PVA samples were non-porous and had a relatively smooth surface (Fig. 3). Environmental SEM images confirmed that the manufacturing process dispersed microspheres evenly throughout the scaffolds for all % contents of ALG (Fig. 4). Less homogeneous composite scaffolds were obtained when PLGA microspheres were incorporated into the PVA scaffolds (Fig. 5).

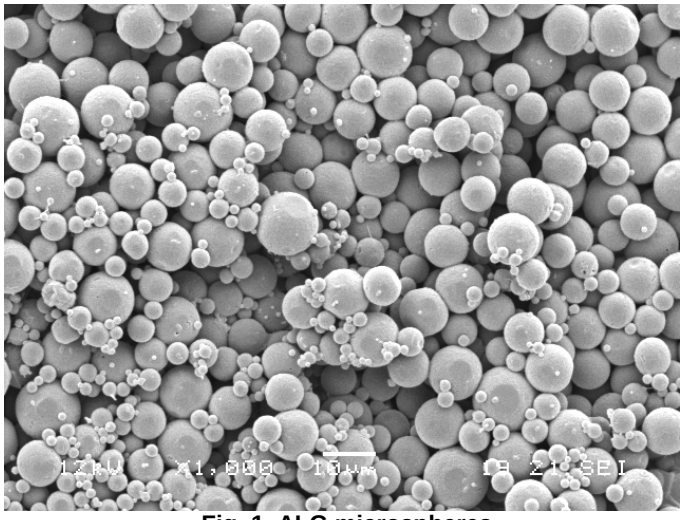

Fig. 1, ALG microspheres

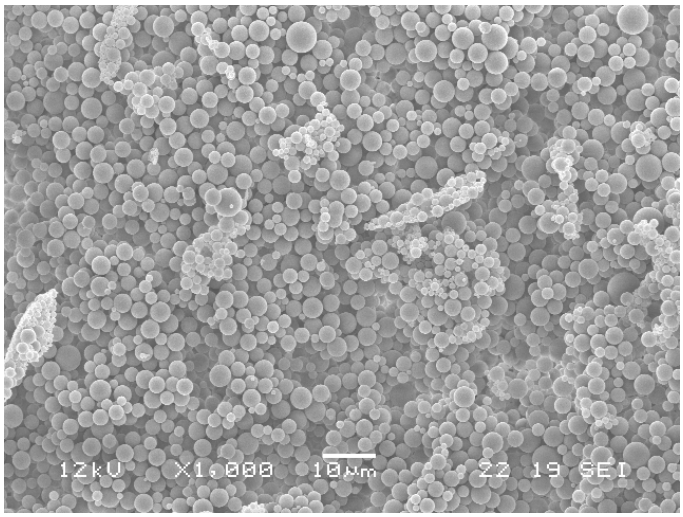

Fig. 2, PLGA microspheres

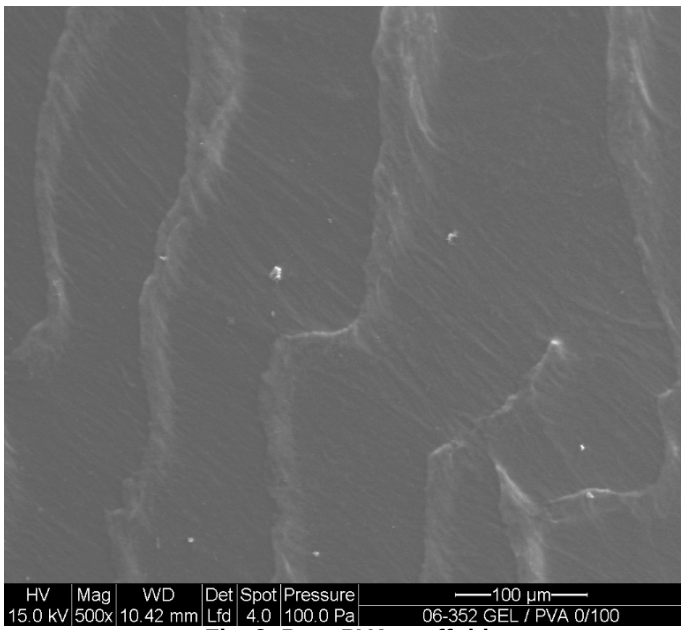

Fig. 3. Pure PVA scaffold

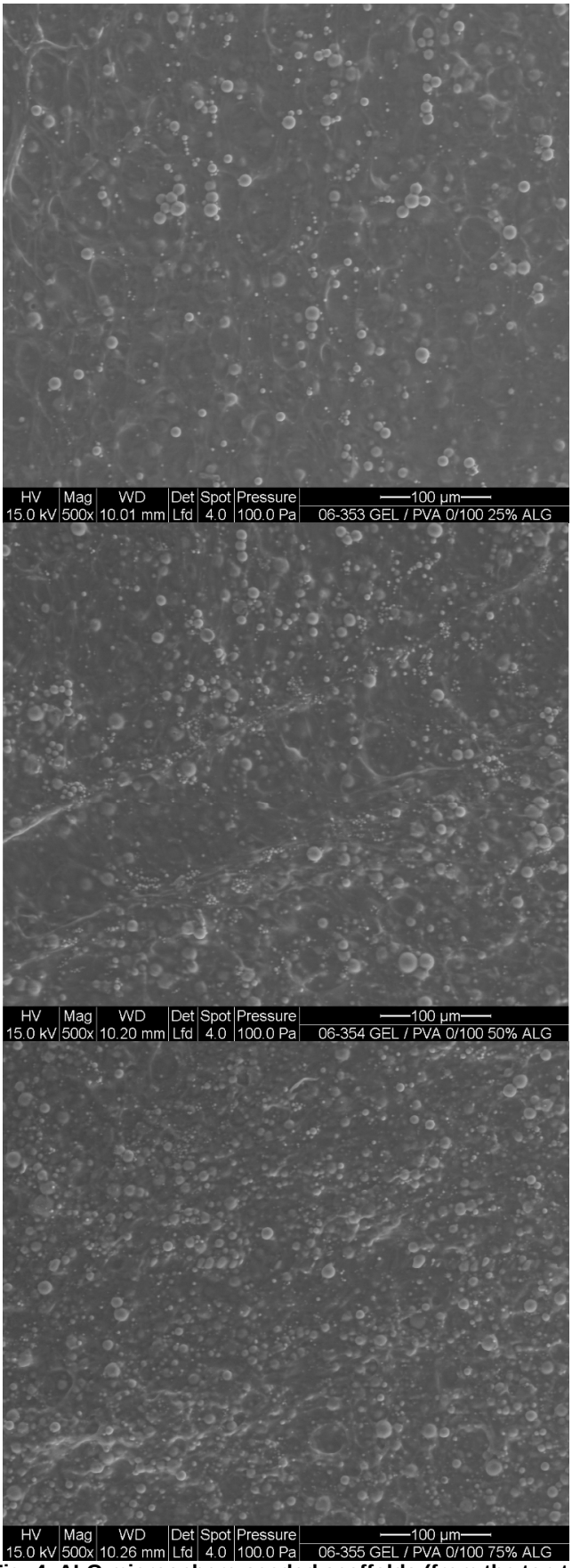

Fig. 4. ALG microsphere-seeded scaffolds (from the top to the bottom, 25%, 50% and 75% ALG)

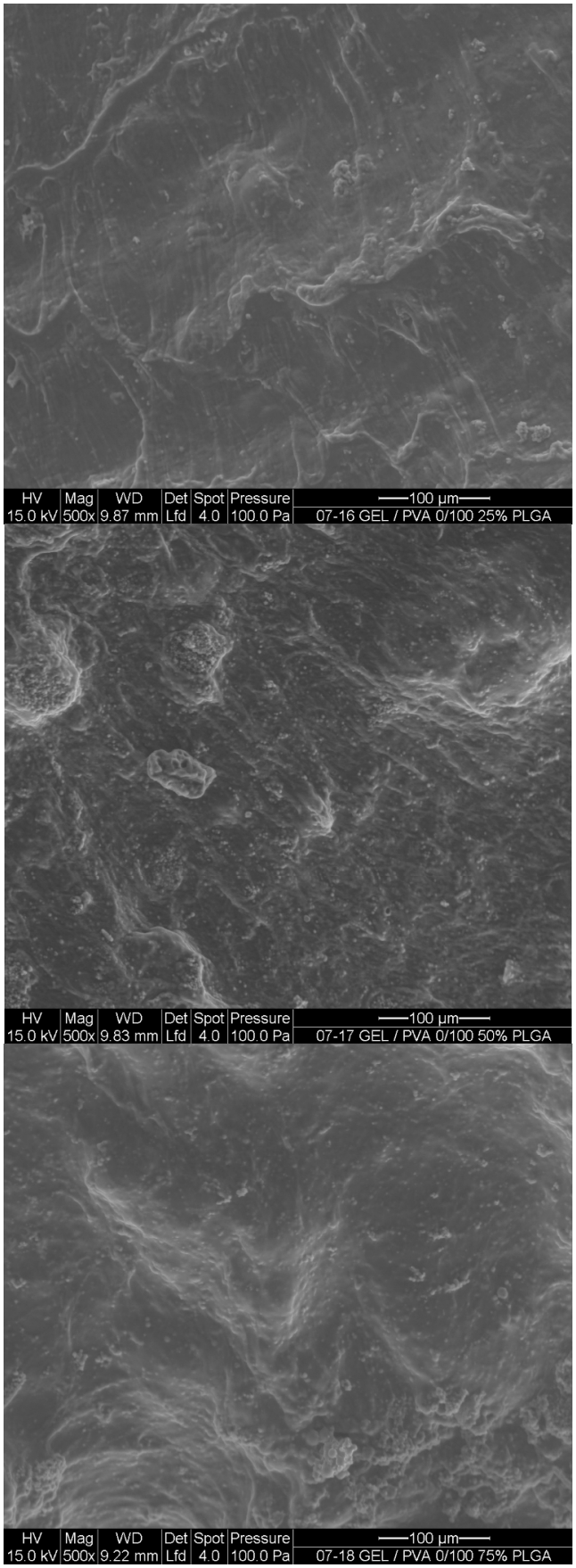

Fig. 5. PLGA microsphere-seeded scaffolds (from the top to the bottom, 25%, 50% and 75% PLGA)

Mechanical Testing

The samples that were mechanically tested showed no evidence of surface damage or failure.

E* for pure PVA samples ranged from 66±3 kPa at 0.01 Hz to 83±3 kPa at 50 Hz. E* ranged from 92±5 kPa at 0.01 Hz to 113±8 kPa at 50 Hz for 25 % ALG-PVA samples (Fig. 6) and from 85±9 kPa at 0.01 Hz to 120±15 kPa at 50 Hz for 25 % PLGA-PVA samples (Fig. 7). At the highest microsphere content of 75 wt%, E* ranged from 120±8 kPa at 0.01 Hz to 153±19 kPa at 50 Hz (ALG/PVA scaffolds) and from 123±14 kPa at 0.01 Hz to 157±16 kPa at 50 Hz (PLGA/PVA scaffolds). In all cases, E* increased with increasing test frequencies. There was no difference between E* of 50 % and 75 % ALG/PVA composite scaffolds, nor between the 25 % and 50 % PLGA/PVA composite scaffolds, in the tested frequency range.

The Phase Angle was not affected by microsphere content and type (Fig. 8, Fig. 9). δ for the pure PVA specimens ranged from 4.3±0.8 degrees at 0.01 Hz to 12±1.2 degrees at 50 Hz, with a peak of 14±0.9 degrees at 10 Hz. δ peaked to a value of 14 degrees at 10 Hz for ALG/PVA composite scaffolds, while peak δ occurred at 5 Hz for all PLGA/PVA formulations. δ at 50 Hz of the PLGA/PVA samples was lower than the δ of ALG/PVA ones at the same frequency.

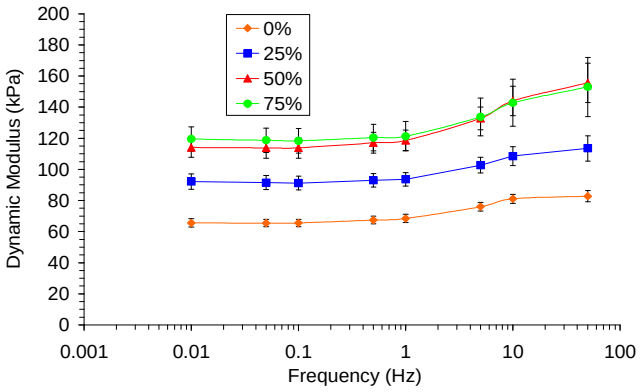

Fig. 6. Dynamic Modulus (E*) for ALG/PVA scaffolds

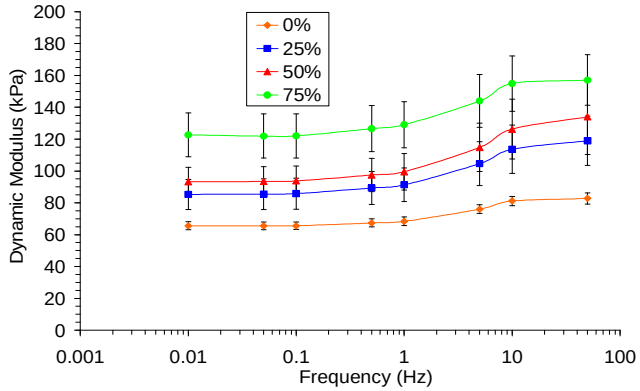

Fig. 7. Dynamic Modulus (E*) for PLGA/PVA scaffolds

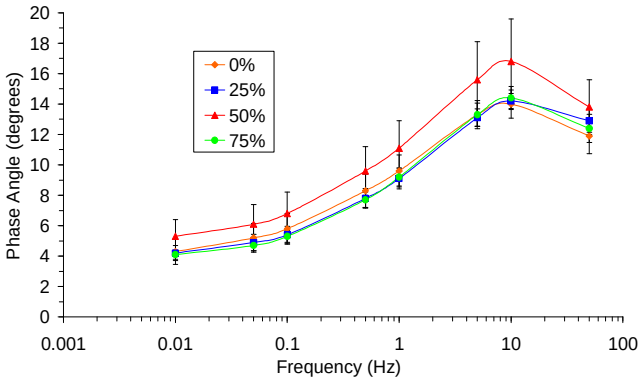

Fig. 8. Phase Angle (δ) for ALG/PVA scaffolds

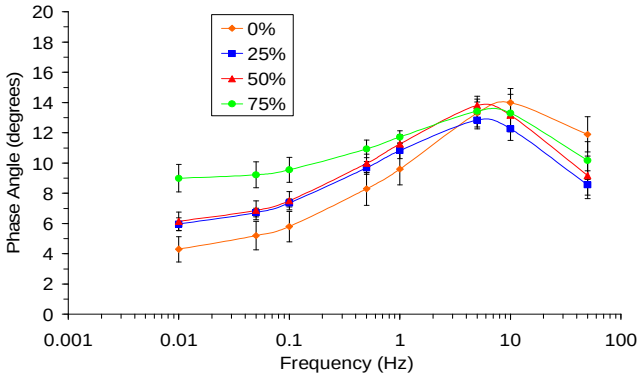

Fig. 9. Phase Angle (δ) for PLGA/PVA scaffolds

DISCUSSION

Clinically used techniques to repair articular defects, such as debridment or microfracture of the subchondral plate, or the transplantation of autogenous osteochondral plugs from non-weight bearing areas provide short term relief of pain [25], but long-term performance is unclear. One challenge that faces these approaches is that the tissue that grows to fill the defect is fibrocartilage and, as such, has poor mechanical properties [26].

The focus of our research is to stabilize a cartilage defect using a non-degradable scaffold. The scaffold will ideally provide mechanical integrity to the defect site and will allow for controlled bioactive agent release via the degradation of microspheres. It is envisaged that growth factor release will encourage cell proliferation and matrix generation, while the release of anti-inflammatory drugs will help to avoid an inflammatory response after implantation.

A non-degradable polymer, PVA was chosen as the scaffold material to provide long-term mechanical stability to the defect site. PVA is biocompatible and can be crosslinked without the use of potentially harmful crosslinking agents. As a hydrogel, PVA has been proposed for a wide range of biomedical applications including, contact lenses, corneal implants and substitute for cartilage [11,12] and meniscus [13]. PVA has also been investigated as a drug delivery system for the release of hydrophilic agents such as proteins [27] and for the release of bioactive agents via inclusion in the scaffold [14] and seeded within carriers in the scaffold [15]. Furthermore, in preliminary data generated in our laboratory (unpublished data) bovine articular chondrocytes injected into PVA-based scaffolds remained viable over 4 weeks in culture.

Microparticles have been shown to protect included bioactive agents from premature degradation [23,28]. Accordingly, microparticles were manufactured from either ALG or PLGA and included in the scaffolds. ALG and PLGA were chosen because of their known biocompatibility and biodegradability [20,21]. In this study, ALG was intended for the incorporation of hydrophilic bioactive agents such as growth factors and PLGA was intended for the incorporation of non-hydrophilic bioactive agents, such as anti-inflammatory drugs.

The yield for both microsphere types was high and observations using SEM confirmed that microsphere size and shape were controllable and reproducible. On average, smaller microspheres were produced using PLGA when compared to that produced using ALG. This size difference can be attributed to several factors such as the difference in the density of the solutions used and the difference in the ratio between the Oil Phase and the Water Phase. The faster stirring speed at which the PLGA microspheres were manufactured also contributed to their smaller size.

SEM analysis revealed that the manufacturing process dispersed microspheres evenly throughout the ALG/PVA composite scaffolds. Less homogeneous PLGA/PVA composite scaffolds were obtained due to the low hydrophilicity of PLGA. It is envisaged that degradation of included microspheres will leave pores in the scaffold. If interconnected, these pores could result in channels in which incorporated cells can proliferate and generate extracellular matrix [29].

Although microparticles have been used in a variety of scaffolds for tissue engineering [17,30,31], the effect of polymer type and microparticle content on the mechanical properties of the scaffold has not been characterized. The samples tested in this study were subjected to unconfined compression, over a span of frequency ranges, similar to test configurations used by others [32-34].

The E* values measured in this study ranged from 66±3 to 157±16 kPa. These values were higher than that measured by Hunter et al. [35] who measured E* of chondrocyte-seeded fibrin gels of 12 kPa at 0.1 Hz (14 kPa at 1 Hz) after 10 days of free swelling culture. But, our values were lower than that measured by Kisiday et al. [32], who showed that for chondrocyte-seeded peptide gels, E* ranged from 0.5 MPa at 0.1 Hz to 0.7 MPa at 1 Hz at 39 days of free swelling culture. Similarly, our values were lower than that measured by Buschmann et al. [33] who found that for chondrocyte-seeded agarose gels E* was 1.75 MPa at 0.1 Hz and 2 MPa at 1 Hz after 35 days of culture (E* was 0.25 MPa at 0.1 Hz at time zero). Of note, Kisiday and Buschmann tested samples in confined chamber, which can explain the differences between our test results and theirs. For both ALG/PVA composite scaffolds and PLGA/PVA composite scaffolds the Dynamic Modulus increased with increased microsphere content. However, beyond 50% ALG, no further increase in Modulus was found. Modulus also increased with increasing test

frequencies, which is a characteristic of poroelastic tissues, such as cartilage [32,33].

The Phase Angle for both ALG/PVA composite scaffolds and PLGA/PVA composite scaffolds was not affected by the addition of microspheres and it increased with increasing test frequencies. The polymer from which the microspheres were manufactured did not affect the change of δ as a function of frequency; however the PLGA/PVA composite scaffolds reached a peak δ at 5 Hz and the ALG/PVA composite scaffolds reached a peak δ at 10 Hz. The Phase Angle of articular cartilage decreases with increasing frequency. Sah et al. [36], for example, reported a decrease in δ from 15 to 8 degrees when calf cartilage explants were tested at 0.1 Hz and 1 Hz, respectively. Huang et al. [37], also reported a decrease in δ from to 30 degrees at 0.01 Hz to 15 degrees at 1 Hz for bovine cartilage plugs.

A limitation of this study is that the effect of microsphere degradation on the mechanical properties was not explored.

CONCLUSION

We successfully developed a novel partly-degradable PVA-based scaffold with variable mechanical properties. The manufacturing process dispersed microspheres evenly throughout the scaffolds. The Dynamic Modulus of the scaffold increased as a function of increased microsphere content, but microsphere type did not affect the Modulus. These findings suggest that microsphere type can be chosen to optimize the inclusion of bioactive factors, without detrimentally affecting the mechanical properties of the scaffold. It also suggests that % content of included microspheres can be used to modulate the mechanical properties of the scaffold at time zero. The dynamic mechanical behavior of these composite scaffolds will be further investigated by subjecting the novel scaffolds to confined compression tests. The morphological features (total porosity, pore diameter, pore interconnectivity) of the scaffold will now be modified, tailoring it to optimize cell matrix generation within the scaffold.

ACKNOWLEDGMENTS

The authors would like to thank Nicole Davis for her assistance with mechanical testing (EnduraTEC, Bose Corp) at the Hospital for Special Surgery, USA. We would also like to thank Piero Narducci for his assistance with SEM at the University of Pisa, Italy and Anthony Labissiere for his assistance with Environmental SEM at the Hospital for Special Surgery, USA.

This research was supported by BIOS – Research Doctorate School in BIOmolecular Sciences, course in Biomaterials, University of Pisa, Italy. Part of this research was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06-RR12538-01 from the National Center for Research Resources, NIH. The authors would like to acknowledge support from NIH AR046121.

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