

## Sequential release of bioactive IGF-I and TGF- $\beta_1$ from PLGA microsphere-based scaffolds

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### Abstract

Growth factors have become an important component for tissue engineering and regenerative medicine. Insulin-like growth factor-I (IGF-I) and transforming growth factor-beta1 (TGF- $\beta_1$ ) in particular have great significance in cartilage tissue engineering. Here, we describe sequential release of IGF-I and TGF- $\beta_1$  from modular designed poly(L,D-lactic-co-glycolic acid) (PLGA) scaffolds. Growth factors were encapsulated in PLGA microspheres using spontaneous emulsion, and *in vitro* release kinetics was characterized by ELISA. Incorporating BSA in the IGF-I formulations decreased the initial burst from 80% to 20%, while using uncapped PLGA rather than capped decreased the initial burst of TGF- $\beta_1$  from 60% to 0% upon hydration. The bioactivity of released IGF-I and TGF- $\beta_1$  was determined using MCF-7 proliferation assay and HT-2 inhibition assay, respectively. Both growth factors were released for up to 70 days in bioactive form. Scaffolds were fabricated by fusing bioactive IGF-I and TGF- $\beta_1$  microspheres with dichloromethane vapor. Three scaffolds with tailored release kinetics were fabricated: IGF-I and TGF- $\beta_1$  released continuously, TGF- $\beta_1$  with IGF-I released sequentially after 10 days, and IGF-I with TGF- $\beta_1$  released sequentially after 7 days. Scaffold swelling and degradation were characterized, indicating a peak swelling ratio of 4 after 7 days of incubation and showing 50% mass loss after 28 days, both consistent with scaffold release kinetics. The ability of these scaffolds to release IGF-I and TGF- $\beta_1$  sequentially makes them very useful for cartilage tissue engineering applications.

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### 1. Introduction

Tissue engineering has greatly benefited from growth factor based regenerative systems [1–6]. For cartilage tissue engineering in particular, growth factors have been used to differentiate progenitor or stem cells [7–10] as well as to enhance cartilage matrix production of adult cells [11,12]. Several groups have shown the importance of transforming growth factor-beta (TGF- $\beta$ ) and bone morphogenetic proteins (BMP) growth factor families on chondrogenic differentiation of mesenchymal stem cells [13]. In addition, basic fibroblast

growth factor (FGF-2) and insulin-like growth factor-I (IGF-I) have also been used to increase cell proliferation and extracellular matrix production, respectively [11]. Sequential supplementation of growth factors has been used to prevent differentiation of cells by first promoting proliferation with one specific growth factor, and then differentiation and expression of a desired phenotype with another. For example, Pei et al. promoted chondrocyte proliferation with the addition of FGF-2 and TGF- $\beta_1$ , while chondrogenesis and extracellular matrix production were obtained by subsequent addition of IGF-I [14]. Martin et al. showed that FGF-2 expansion of chondrocytes, prior to BMP-2 stimulation, enhanced chondrogenesis leading to higher and more homogeneous glycosaminoglycan (GAG) distribution in engineered cartilage constructs [15]. Similarly, Worster et al. demonstrated that mesenchymal stem cells cultured with IGF-I

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were significantly more chondrogenic when pretreated with TGF- $\beta_1$ , highlighting the importance of sequential growth factor supplementation [16].

The work presented here, focuses on two chondrogenic growth factors: IGF-I and TGF- $\beta_1$ . IGF-I is also known as somatomedin C and somatomedin A, and is a mitogenic factor enhancing growth in adult cells as well as aiding in embryonic growth and differentiation. TGF- $\beta_1$  receptors are found on just about all mammalian cells, and this growth factor is commonly used to induce chondrogenesis. These two growth factors have also contributed, in combination with dynamic loading, to increased mechanical modulus of engineered cartilage [17]. IGF-I has been encapsulated in poly(L,D-lactic-co-glycolic acid) (PLGA) microspheres [18,19] and coated onto titanium [20], showing favorable activity with surrounding cells and tissue. Similarly, TGF- $\beta_1$  has been absorbed or directly added to gelatin hydrogels [21], collagenous matrix [22], poly(ethylene glycol) gels [23] and collagen suspensions [24]. However, in these studies, precise control of release kinetics was not obtained for these growth factors. Recently, De-Fail et al. reported bioactivity of TGF- $\beta_1$  from PLGA microspheres embedded in poly(ethylene glycol) hydrogels [25]. Their work showed that TGF- $\beta_1$  stability is important as it can diminish over the 21-day release study. While the data indicate bioactive TGF- $\beta_1$  release, the authors do not provide a quantitative analysis (e.g. concentration) of release kinetics by bioassay. Mooney and collaborators have designed dual growth factor delivery PLGA scaffolds which can release vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [4], as well as alginate based scaffold which can release VEGF when mechanically stimulated [26]. Furthermore, Holland et al. have reported controlled release of IGF-I and TGF- $\beta_1$  from an oligo(poly(ethylene glycol) fumarate) hydrogel based system [27]. However, none of these scaffold systems have been shown to sequentially deliver growth factors upon hydration without external stimuli.

Here we report the evaluation of a scaffold system that can deliver two model growth factors sequentially. We show how initial burst for both IGF-I and TGF- $\beta_1$  PLGA microspheres can be controlled by formulation parameters as well as their corresponding bioactivity. In addition, we present modularly designed scaffolds fused from such bioactive microspheres that can sequentially deliver IGF-I and TGF- $\beta_1$ . These scaffolds are further characterized to understand their swelling and degradation in cell culture conditions common to engineered cartilage.

## 2. Materials and methods

### 2.1. Materials

Poly(L,D-lactic-co-glycolic acid) (PLGA, RG502H and 502) was purchased from Boehringer Ingelheim, Germany and used as supplied. Poly(vinyl alcohol) (PVA, 88% hydrolyzed,  $M_w \sim 25,000$ ) was purchased from Polysciences Inc, Warrington, PA. Bovine serum albumin (BSA, 98%) was obtained from Sigma–Aldrich, St. Louis, MO. The growth factors, rhIGF-I and rhTGF- $\beta_1$  were supplied by R&D Systems (Minneapolis, MN), and dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) and 2,2,2-trifluoroethanol (TFE) were reagent grade.

### 2.2. Microsphere fabrication

IGF-I and TGF- $\beta_1$  were encapsulated in PLGA microspheres via a spontaneous emulsion (SE) method [28]. Briefly, 200 mg of PLGA were dissolved in 5 mL of co-solvent ( $\text{CH}_2\text{Cl}_2$ :TFE::1:4) and mixed with 300  $\mu\text{L}$  of 3% BSA solution containing either IGF-I or TGF- $\beta_1$ , thus forming a clear, single phase solution. This was then added to 200 mL of 5% PVA or 5% PVA containing 3% BSA. The emulsion formed spontaneously and was allowed to stir at room temperature for 3 h. The microspheres were collected by centrifugation, washed three times with deionized water, and lyophilized. Microspheres were stored at  $-20^\circ\text{C}$  with desiccant until use.

### 2.3. Microsphere size distribution

Size distribution of microspheres was determined using Coulter LS 230 software (Version 3.01) on an LS 230 Coulter Counter (Beckman Coulter, Hialeah, FL).

### 2.4. Scaffold fusion

Microspheres were fused into 3D scaffolds using a recently reported dichloromethane vapor method [29]. Twenty milligrams of microspheres were added to a disc-like mold (6 mm in diameter) and placed inside a glass chamber containing 3 mL of dichloromethane. The chamber was sealed and microspheres were allowed to fuse for 8:30 min. After fusion the mold containing the scaffold was removed from the chamber and allowed to air for 10 min at room temperature, at which point the scaffold was removed from the mold and dried under vacuum overnight. All scaffolds were stored at  $-20^\circ\text{C}$  with desiccant until use.

### 2.5. Release study

Ten milligrams of microspheres were incubated with 1 mL phosphate buffered saline (PBS, pH 7.2) in capped tubes and placed on a rotator at  $37^\circ\text{C}$ . At each time point (1, 2, 4, 7, 10, 14, 21, 28, 35, 42, 49 and 56 days), the samples were centrifuged at 10,000 rpm for 5 min after which the releasate was removed. Samples were resuspended in a fresh 1 mL of PBS incubated until the following time point. To simulate cell culture conditions, scaffold release studies were carried out in cell culture media (high glucose DMEM supplemented with 1% Insulin Transferrin Selenium (ITS) Premix; 100 U/mL penicillin; 100 mg/mL streptomycin; 2 mM L-glutamine; 2.5 mg/mL amphotericin B; 0.1 mM nonessential amino acids; 0.4 mM proline) in a 24-well plate and incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Similarly, at each time point (1, 2, 4, 7, 14, 21, 28, 35, 42, 49 and 56 days), the releasate was removed and fresh 1 mL of media were added to the scaffolds. All removed releasates were stored at  $-80^\circ\text{C}$  until further analysis. Amounts of rhIGF-I and rhTGF- $\beta_1$  released from the microspheres and scaffolds were quantified using the corresponding ELISAs (R&D Systems, Minneapolis, MN). All samples were run in triplicate and data points are shown as mean  $\pm$  standard deviation. Percent cumulative release at each time point was normalized to the total growth factor released on the last day of the study. To determine significance ( $p < 0.05$ ), two-tailed *t*-test was performed assuming unequal variances.

### 2.6. IGF-I bioactivity assay

IGF-I bioactivity was quantified using an MCF-7 cell line proliferation assay [30]. The following protocol was adapted from R&D Systems (Minneapolis, MN). Briefly, MCF-7 cells (HBT-22, ATCC, Manassas, VA) were washed three times and resuspended in serum-free bioassay media (DMEM:F12::1:1; 2 mM L-glutamine; 100 U/mL penicillin; 100  $\mu\text{g}/\text{mL}$  streptomycin; 10  $\mu\text{g}/\text{mL}$  transferrin; 0.2% BSA) at a density of  $1 \times 10^5$  cells/mL. In a 96-well plate, 50  $\mu\text{L}$  of bioassay media were added to each well, followed by 25  $\mu\text{L}$  of IGF-I standards (diluted in 0.1% BSA; 500–0.5 ng/mL range) or 25  $\mu\text{L}$  of microsphere releasate. Finally, 50  $\mu\text{L}$  of MCF-7 cell suspension was added to each well and the plates were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 72 h. Wells with no added growth served as negative controls, while those supplemented

with 500 ng/mL of IGF-I served as positive controls. Cell viability was quantified with CellTiter-Blue viability assay (Promega, Madison, WI) by adding 25  $\mu$ L of reagent to each well and incubating the plates for 4 h at 37 °C with 5% CO<sub>2</sub>. Gemini XPS spectrofluorometer (Molecular Devices, Sunnyvale, CA) was used to record sample fluorescence at 560<sub>Ex</sub>/590<sub>Em</sub>. Readings were normalized to negative IGF-I control and a 4-parameter fit was used to generate a standard curve. All samples were run in triplicate and an IGF-I standard curve was run within each plate.

### 2.7. TGF- $\beta_1$ bioactivity assay

TGF- $\beta_1$  bioactivity was quantified using an HT-2 cell line inhibition assay [31]. Similarly, the protocol was adapted from R&D Systems (Minneapolis, MN). HT-2 cells (CRL-1841, ATCC, Manassas, VA) were washed three times and resuspended in bioassay media (RPMI 1640; 10% heat-inactivated FBS; 50  $\mu$ M  $\beta$ -mercaptoethanol; 2 mM L-glutamine; 100 U/mL penicillin; 100  $\mu$ g/mL streptomycin) at a density of  $2 \times 10^5$  cells/mL. In a 96-well plate, 50  $\mu$ L of bioassay media were added to each well, followed by 25  $\mu$ L of TGF- $\beta_1$  standards (diluted in 0.1% BSA; 50–0.0005 ng/mL range) or 25  $\mu$ L of microsphere releasate. Finally, 50  $\mu$ L of HT-2 cell suspension, supplemented with IL-4 (15 ng/mL, R&D Systems) was added to each well and the plates were incubated at 37 °C with 5% CO<sub>2</sub> for 72 h. Wells with no added growth served as negative controls, while those supplemented with 50 ng/mL of TGF- $\beta_1$  served as positive controls. Cell viability was quantified with CellTiter-Blue viability assay (Promega, Madison, WI) as described in Section 2.6.

### 2.8. Scaffold swelling and degradation study

Scaffold degradation was evaluated under *in vitro* physiologic conditions in culture media. Under sterile conditions, scaffolds were placed in a 24-well plate with 1 mL of media (high glucose DMEM supplemented with 1% ITS Premix; 100 U/mL penicillin; 100 mg/mL streptomycin; 2 mM L-glutamine; 2.5 mg/mL amphotericin B; 0.1 mM nonessential amino acids; 0.4 mM proline) and incubated at 37 °C with 5% CO<sub>2</sub>. At each time point (1, 2, 4, 7, 14, 21 and 28 days), the scaffold wet weight ( $W_{H_2O}$ ) and wet diameter were recorded. After lyophilization, scaffold dry weight ( $W_P$ ) and molecular weight were determined (Section 2.9). The scaffold swelling ratio was calculated as the wet weight divided by the dry weight ( $W_{H_2O}/W_P$ ).

### 2.9. Gel permeation chromatography

Molecular weight of scaffold polymers was determined by gel permeation chromatography (GPC). All samples were prepared by dissolving about 2 mg of dried scaffold in 1 mL of chloroform and syringe filtering through a 0.22- $\mu$ m PTFE filter. The system employed by Shimadzu (Columbia, MD) included a model LC-10AD pump, an RID-10A refractive index detector and a model DGU-14A auto-injector. Two columns (Styragel HR 4E and Styragel HR 5E, Waters, Milford, MA) were connected in series, eluted with HPLC grade chloroform and ran at 40 °C at a flow rate of 1 mL/min. Data acquisition and handling were performed using the Class-VP 7.2.1 SP1 software (Shimadzu, Columbia, MD).

## 3. Results and discussion

### 3.1. Microsphere formulations and growth factor release studies

Four microsphere formulations were prepared and are described in Table 1. IGF-I formulations (A and B) were both prepared under the same conditions except that formulation A contained BSA in the organic phase. Similarly, TGF- $\beta_1$  formulations (C and D) were identical with the exception that C and D were prepared using uncapped (502H) and capped (502) PLGA, respectively. In general, BSA was used to stabilize the growth factors since for both IGF-I and TGF- $\beta_1$  the manufacturer (R&D Systems, Minneapolis, MN) recommends reconstitution in BSA solution. Uncapped PLGA, having a carboxylic acid chain end, was used in order to enhance polymer–protein interactions and to better internalize the proteins within polymer microspheres. Capped PLGA is inert and is not expected to interact with the formulation constituents. Overall, the growth factor loadings were low (0.02% for IGF-I and 0.0005% for TGF- $\beta_1$ ) due to experimental cost and since cellular response to growth factors is highly sensitive. Fabricated microspheres had broad size distributions (Table 1), where most of the microspheres were smaller than 50  $\mu$ m. Surface morphology was smooth and no aggregation was apparent (data not shown). Additional detail on the effect of formulation parameters on microsphere size can be found elsewhere [29].

Cumulative release kinetics for IGF-I formulations A and B, as determined by ELISA, are shown in Fig. 1. For formulation A (Fig. 1a), which contained BSA in the organic phase, the release is characterized by an extremely low burst ( $6.6 \pm 1.3\%$ ) on day 1 and almost no release during the first week. This is followed by an almost 80% cumulative release over the following week. After 30 days, the release is steady at about 2% cumulative per day and remains so until the end of the study. However, for formulation B (Fig. 1b), the initial burst on the first day is  $52.1 \pm 3.8\%$  of the cumulative release. This is again followed by minimal release during the first week, followed by 20% cumulative release over the second week. Release for formulation B was carried out until the microspheres were completely degraded (total mass loss) on day 56, whereas, for formulation A, the study was stopped on day 70. The most significant result comparing these two formulations is the reduction of initial burst by almost 8-fold of the percent cumulative release by the incorporation of BSA within the formulation. This result is most likely due to the BSA,

Table 1  
Microsphere formulations with growth factor loading

Formulation	IGF-I	TGF- $\beta_1$	PLGA (100 mg)	Organic phase	Non-solvent (5% PVA)	Microsphere size	
						Mean ( $\mu$ m)	90%< ( $\mu$ m)
A	0.024% (25 $\mu$ g)	—	502H	4.3% BSA (4.5 mg)	—	11.0 $\pm$ 11.8	25.3
B	0.025% (25 $\mu$ g)	—	502H	—	—	36.8 $\pm$ 94.0	45.7
C	—	0.0005% (0.5 $\mu$ g)	502H	—	3% BSA	7.3 $\pm$ 14.8	13.5
D	—	0.0005% (0.5 $\mu$ g)	502	—	3% BSA	33.8 $\pm$ 89.2	42.2

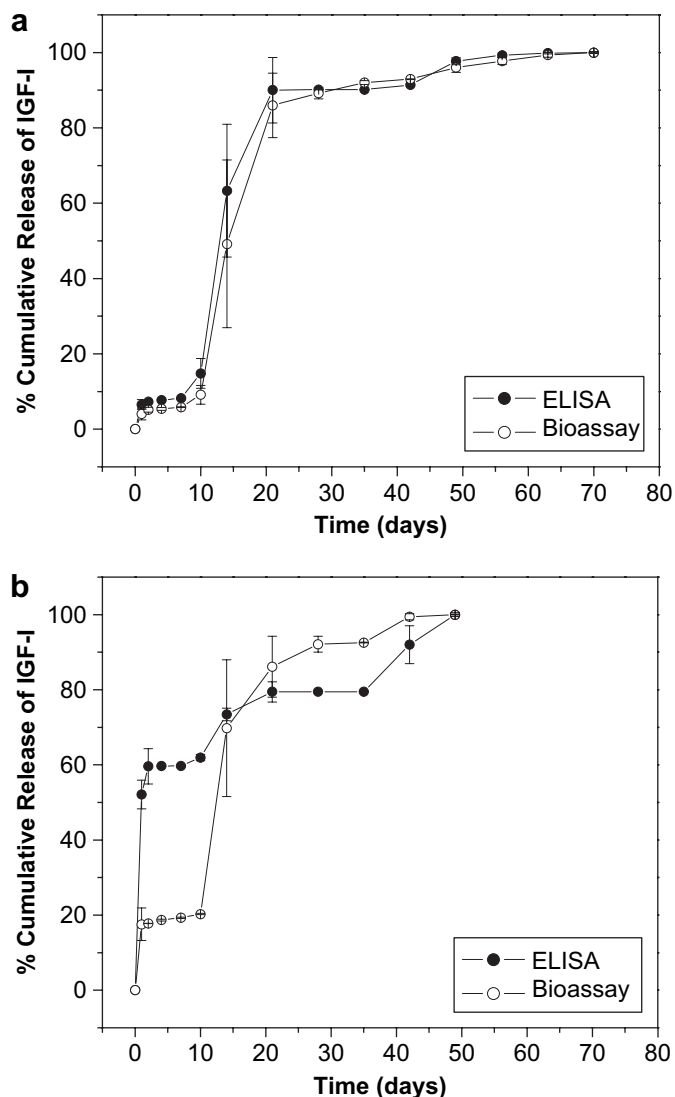


Fig. 1. Percent cumulative release of IGF-I from formulation A (a) and B (b) determined by ELISA (●) and MCF-7 bioassay (○). Error bars represent the standard deviation for  $n = 3$  samples.

IGF-I and PLGA secondary interactions, which help internalize the IGF-I as well as aid in uniformly distributing all the components within the matrix. This is further confirmed by release kinetics over the second week of the study, where 80% of IGF-I is released for the BSA formulation (B) and only 20% for formulation A. The initial burst is due to diffusion of IGF-I on the surface of the microspheres, while this second phase of release is due to polymer degradation. Therefore, for formulation A, most of the IGF-I is internalized away from the surface of the microspheres and begins releasing 1 week into the study, at which point the polymer also begins to degrade and exhibit mass loss (see Section 3.4). Besides the initial burst, the release kinetic curves are similar between the two IGF-I formulations, indicating that they are controlled by polymer degradation.

For TGF- $\beta_1$  formulations, no BSA was added to the organic phase; however, 3% of BSA was added to the non-solvent for both formulations (Table 1). BSA is known to adsorb onto

polymer surfaces, especially PLGA [32], thus it was used here to coat the outer surface of the microspheres and diminish the initial burst of TGF- $\beta_1$ . Percent cumulative release, determined by ELISA, for TGF- $\beta_1$  formulations is shown in Fig. 2. The difference between the two microsphere formulations is that C was made with uncapped PLGA containing a carboxylic acid terminal group, while D was made with capped PLGA. For formulation C the release is characterized by essentially no release over the first 4 days followed by rather steep release (up to 80%) over the following 10 days (Fig. 2a). Thereafter, the release is steady ( $\sim 0.3\%$  per day) over the next 50 days. However, for formulation D,  $61.6 \pm 6.2\%$  of TGF- $\beta_1$  is released on day 1 and up to 100% by day 4 (Fig. 2b). This result is most likely due to secondary interactions between uncapped PLGA, BSA and TGF- $\beta_1$ . Increased interactions of these components in the organic phase will lead to a better distribution within the microspheres [28]. Once again, for TGF- $\beta_1$  we

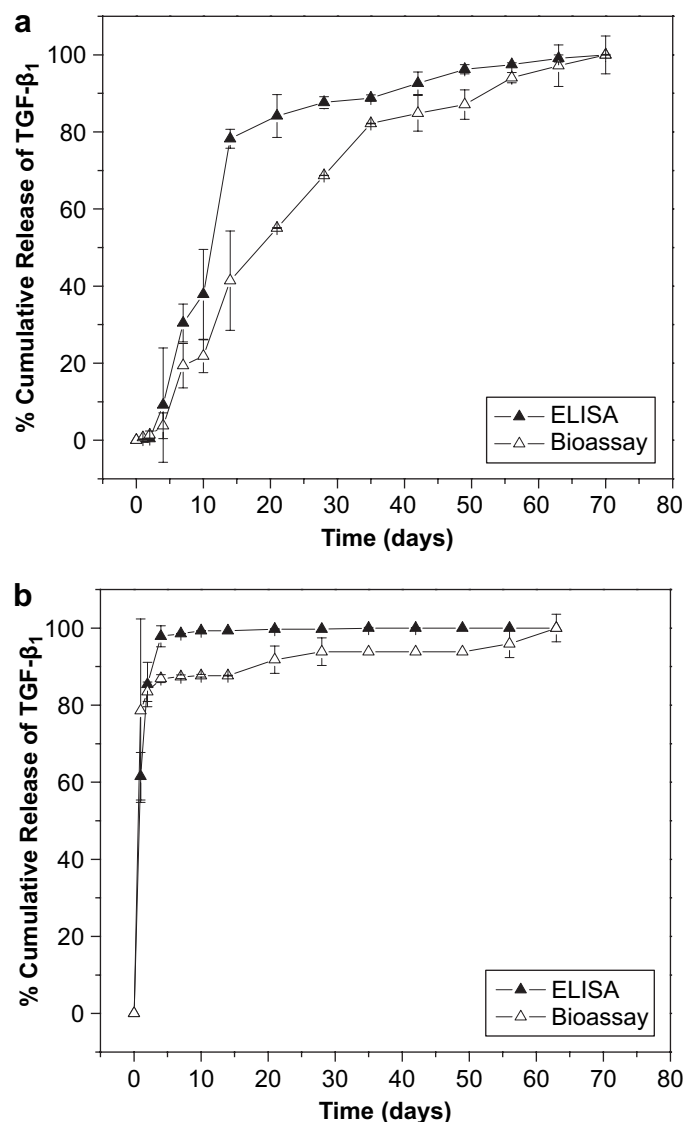


Fig. 2. Percent cumulative release of TGF- $\beta_1$  from formulation C (a) and D (b) determined by ELISA (▲) and HT-2 bioassay (△). Error bars represent the standard deviation for  $n = 3$  samples.

have shown that by controlling the formulation parameters we can modulate the growth factor release kinetics.

### 3.2. IGF-I and TGF- $\beta_1$ bioactivity

Since these microspheres will eventually be used for tissue engineering applications, it was necessary to evaluate their bioactivity using established cell lines. IGF-I bioactivity for formulations A and B is shown in Fig. 1 and is compared to ELISA results. Overall, there is good agreement between the ELISA and bioactivity data for formulation A (Fig. 1a). Release samples at each time point were compared, and there is statistical difference ( $p < 0.05$ ) for samples on days 28, 35, 49 and 63. Specifically, the bioactivity is higher on days 28, 35 and 63, while the ELISA reading is stronger on day 49. Diminished IGF-I bioactivity as compared to ELISA is likely the result of growth factor degradation due to storage and repeated freeze/thaw cycles [25]. Stronger IGF-I bioactivity than ELISA can be caused by IGF-I epitope damage, where the antibody binding in ELISA is compromised, but the bioactivity receptor binding is still functional. In addition, for ELISAs the IGF-I standards used were those supplied by the manufacturer, which have bioactive equivalence by ELISA but are not bioactive themselves. Whereas, for the bioassays, the standards used were freshly thawed rhIGF-I. For formulation B, there is increased signal for ELISA as compared to bioactivity on day 1 and 49. This formulation contains no BSA, which aids in growth factor stability and increases macromolecular crowding in solution [33,34]. Thus, the 40% decreased activity on day 1 could be due to the storage/thaw IGF-I degradation and/or damage during the encapsulation process. The increased bioactivity evident on days 4, 7, 14, 28 and 35 can be explained as above for formulation A.

Similarly, TGF- $\beta_1$  bioactivity was determined and compared to the ELISA data (Fig. 2). Again, for both formulations C and D, the release kinetics follows the same trend when comparing ELISA versus bioactivity. For formulation C, there is no statistical difference ( $p < 0.05$ ) between the results except for time points on day 28, 35 and 56, where the bioactivity data are higher. For formulation D, statistical difference in data occurs on days 2 and 4, where the bioactivity is lower than the ELISA results. This is probably due to TGF- $\beta_1$  degradation and the discussion is analogous to that described above for IGF-I.

Even though for both IGF-I and TGF- $\beta_1$  microspheres there is some discrepancy between ELISA and bioactivity analysis, it is clear that both growth factors are released in bioactive form for up to 70 days. In addition, the data presented in Figs. 1 and 2 show that by modulating the encapsulation parameters, the release kinetics of IGF-I and TGF- $\beta_1$  microspheres can be controlled. This yields two sets of release profiles for each growth factor, one with high initial burst and one with delayed release.

### 3.3. Scaffold release studies

Scaffolds were fabricated using a recently developed method where microspheres are used as building blocks,

yielding a porous three-dimensional structure [29]. This method utilizes dichloromethane vapor to fuse adjacent microspheres to each other, thus allowing essentially any combination of microspheres to be fused into a 3D structure. To illustrate the versatility of this scaffold system, we designed three different scaffolds (Table 2), two of which can release growth factors sequentially. Scaffold 1 contains equal amounts of all four formulations described in Section 3.1 and is designed to begin releasing IGF-I and TGF- $\beta_1$  upon hydration. Scaffold 2 is designed to release TGF- $\beta_1$  immediately, followed by IGF-I about 10 days later. Scaffold 3 releases IGF-I first, followed by TGF- $\beta_1$  about a week later. The percent cumulative release profile for scaffold 1, as determined by ELISA, is shown in Fig. 3a indicating that more than 20% of IGF-I and TGF- $\beta_1$  are released on day 1 and the scaffold continues to release the two growth factors at almost identical rates up to 56 days. Scaffold 2 eliminates the IGF-I formulation with high initial burst, thus creating a scaffold profile with TGF- $\beta_1$  being released from day 1 and IGF-I beginning around day 10 (Fig. 3b). Similarly, scaffold 3 is the inverse of scaffold 2. By eliminating the TGF- $\beta_1$  high burst formulation, the scaffold releases IGF-I starting on day 1 followed by TGF- $\beta_1$  on day 7 (Fig. 3c). Thus, scaffold 1 releases the growth factors simultaneously, while scaffolds 2 and 3 release them sequentially. Therefore, we are able to formulate scaffolds with control over which growth factor is released first and which is delayed. Moreover, tissue development and repair are regulated by gradients of growth factors and cell competencies, and our scaffolds permit growth factor delivery in ways that may mimic such biological events. Therefore, the delivery systems presented here may be useful for optimizing therapeutic interventions in tissue repair and restoration.

The scaffold release studies were evaluated by ELISA, and we feel confident that the results shown are also representative of the bioactivity for several reasons. The ELISA and bioactivity comparisons presented in Section 3.2 indicate that there is general agreement between the two analysis methods for both IGF-I and TGF- $\beta_1$ . Furthermore, the only modification done to the bioactive IGF-I and TGF- $\beta_1$  PLGA microspheres is fusion into scaffolds with brief exposure to dichloromethane vapor. As described in Section 2.2, encapsulation of the growth

Table 2  
Scaffold composition and growth factor loading

	Microsphere formulation	% Microsphere loading		% Scaffold composition
		IGF-I	TGF- $\beta_1$	
Scaffold 1	A (delayed)	0.05	—	25
	B (burst)	0.05	—	25
	C (delayed)	—	0.001	25
	D (burst)	—	0.001	25
Scaffold 2	A (delayed)	0.05	—	50
	C (delayed)	—	0.001	25
	D (burst)	—	0.001	25
Scaffold 3	A (delayed)	0.05	—	25
	B (burst)	0.05	—	25
	C (delayed)	—	0.001	50

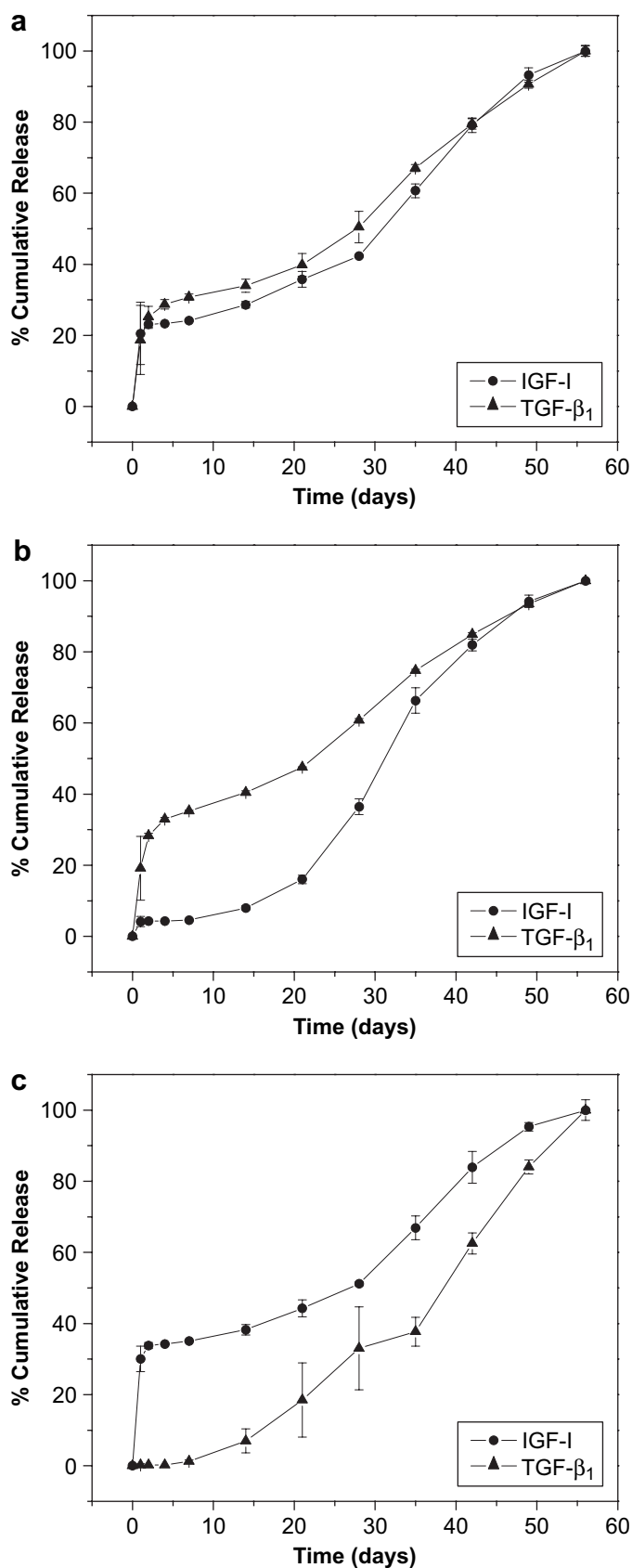


Fig. 3. Percent cumulative release determined by ELISA of IGF-I (●) and TGF-β<sub>1</sub> (▲) from scaffold 1 (a), scaffold 2 (b) and scaffold 3 (c). Error bars represent the standard deviation for  $n = 3$  samples.

factors is done by dissolving the growth factors in an organic solution containing dichloromethane. Thus it seems that protein degradation would be most significant during this processing step, rather than during fusion where the growth factor is in solid form. In fact, one way to stabilize protein formulations is to utilize micronized proteins in solid form in order to avoid interfacial tension and degradation [35]. If growth factor activity is present for the microspheres, it is likely that it is retained in the fused scaffolds. In addition, the bioactivity of scaffold releasate was tested, however, because the scaffold release studies were carried out in cell culture media rather than the PBS, there was significant background in the assays. For both the MCF-7 and HT-2 bioassays the media had a strong background, masking the growth factor activity. Dilution of samples helped diminish some of this effect but not to the point where quantitative analysis could be performed. However, samples from scaffolds containing IGF-I and TGF-β<sub>1</sub> had a significantly higher effect as compared to the controls (data not shown), confirming that IGF-I and TGF-β<sub>1</sub> bioactivities are present after scaffold fusion.

### 3.4. Scaffold swelling and degradation

To further characterize the growth factor releasing scaffolds, the swelling ratio and wet scaffold diameter were recorded over a 2-week incubation period for scaffold 1 (Table 2). Within the first 24 h of hydration in cell culture medium, the scaffolds swelled 2-fold (Fig. 4a). This infusion of medium into the scaffold supports the scaffold release data (Fig. 3a), where we see at least a 20% initial burst. Over the next two time points, the swelling ratio increases only slightly ( $<0.05$ ), which is similar to growth factor release ( $<10\%$ ). On day 7, the swelling ratio peaks at 4, and we also begin to see an increase in the release rate from the scaffolds. For the rest of the time points, we see a decrease in swelling ratio which is due to scaffold mass loss as indicated in Fig. 5a. Fig. 4b shows the change in wet scaffold diameter over time. The overall wet diameter data coincides with scaffold swelling results, both of which peak at day 7. These results not only reinforce the swelling data, but also give important information pertinent to the scaffold's application in tissue engineering. Most likely, these scaffolds will be used to fill a tissue defect of a particular size, so understanding their swelling behavior is essential for secure and stable implantation for the timeframe of the study.

Percent scaffold mass loss and molecular weight distribution over a 4-week period are shown in Fig. 5. There is no significant mass loss until after day 7, when the degradation begins at about 2% mass loss per day (Fig. 5a) and reaches about 50% at 28 days. Polymer degradation occurs by hydrolysis and the onset of degradation is confirmed by peak swelling on day 7 (Fig. 4a). Moreover, the molecular weight peak remains constant over the first four time points (days 0, 1, 2 and 4), and begins to continually shift toward lower molecular weight starting on day 7 (Fig. 5b). The significant degradation observed on day 28 is clearly apparent in the scaffold release data (Fig. 3a) where there is an increase in the slope of the

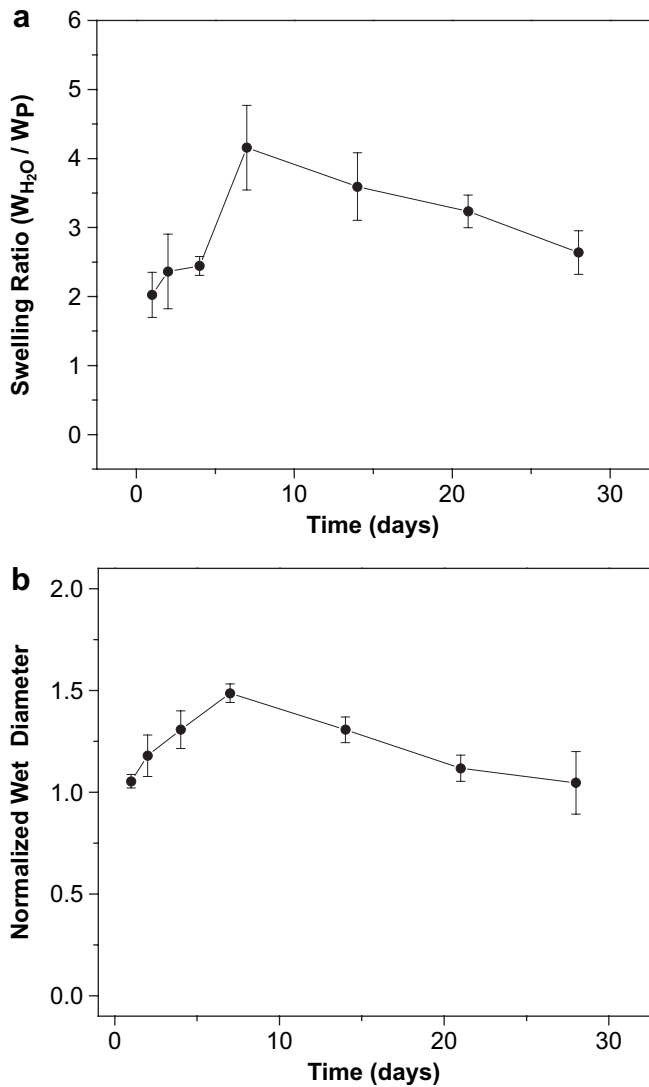


Fig. 4. Scaffold 1 swelling ratio (a) and normalized wet diameter (b) over time. Error bars represent the standard deviation for  $n=3$  samples.

percent cumulative release for IGF-I and TGF- $\beta_1$ . This is more pronounced for the sequential or latent release profiles of IGF-I and TGF- $\beta_1$ , where the kinetics is controlled by polymer degradation (see discussion in Section 3.1).

#### 4. Conclusion

We have developed a scaffold system that can deliver growth factors in a sequential manner. We have shown that the onset of release for IGF-I and TGF- $\beta_1$  can be controlled by formulation parameters and that both can be released in bioactive form for up to 70 days. Using modular design, we fabricated scaffolds that can release IGF-I and TGF- $\beta_1$  either at the same rate or sequentially with at least a 1-week lag-time upon hydration. The ability of these scaffolds to release IGF-I and TGF- $\beta_1$  sequentially makes them extremely valuable for tissue engineering and drug delivery applications.

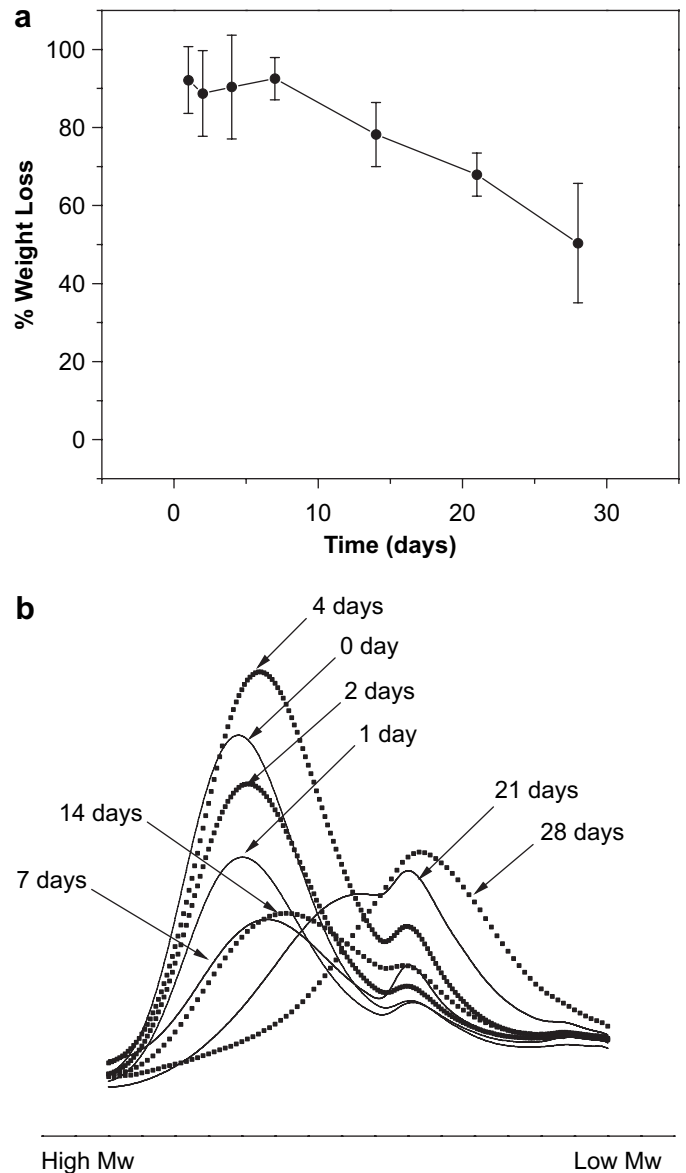


Fig. 5. Scaffold 1 percent weight loss (a) and molecular weight distributions as determined by GPC (b).

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