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Effect of injection site on *in situ* implant formation and drug release *in vivo*

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ABSTRACT

In situ forming drug delivery implants offer an attractive alternative to pre-formed implant devices for local drug delivery due to their ability to deliver fragile drugs, simple manufacturing process, and less invasive placement. However, the clinical translation of these systems has been hampered, in part, by poor correlation between *in vitro* and *in vivo* drug release profiles. To better understand this effect, the behavior of poly(D,L-lactide-co-glycolide) (PLGA) *in situ* forming implants was examined *in vitro* and *in vivo* after subcutaneous injection as well as injection into necrotic, non-necrotic, and ablated tumor. Implant formation was quantified noninvasively using an ultrasound imaging technique. Drug release of a model drug agent, fluorescein, was correlated with phase inversion in different environments. Results demonstrated that burst drug release *in vivo* was greater than *in vitro* for all implant formulations. Drug release from implants in varying *in vivo* environments was fastest in ablated tumor followed by implants in non-necrotic tumor, in subcutaneous tissue, and finally in necrotic tumor tissue with 50% of the loading drug mass released in 0.7, 0.9, 9.7, and 12.7 h respectively. Implants in stiffer ablated and non-necrotic tumor tissue showed much faster drug release than implants in more compliant subcutaneous and necrotic tumor environments. Finally, implant formation examined using ultrasound confirmed that *in vivo* the process of precipitation (phase inversion) was directly proportional to drug release. These findings suggest that not only is drug release dependent on implant formation but that external environmental effects, such as tissue mechanical properties, may explain the differences seen between *in vivo* and *in vitro* drug release from *in situ* forming implants.

1. Introduction

The development of *in situ* forming implants (ISFIs) has been a topic of significant research interest in recent years [1,2]. The term ISFI has been used to describe a variety of polymer based systems that are initially comprised of a liquid solution that undergoes a process of solidification after placement in a site of interest. One such ISFI product first developed by Dunn et al. consists of a biodegradable polymer dissolved in water miscible organic solvent that undergoes a process of liquid de-mixing when injected into a water rich phase [3]. Upon injection into the aqueous environment, the water-insoluble polymer component of the ISFI solution precipitates as the water miscible organic solvent leaches out into the bath solution and water diffuses into the implant, a process commonly referred to as phase inversion [4,5]. The ISFI systems have several advantages compared to traditional pre-formed implant systems. Due to their injectable nature, implant placement is less invasive and painful for the patient thereby improving comfort and compliance. Additionally the manufacturing process required for fabrication is relatively mild and straightforward making ISFI systems particularly suitable for delivery of fragile protein and peptide therapies. However, due to the complexities of the phase inversion process, poor *in vitro–in vivo* correlations, potential solvent toxicity [6], and difficulty in controlling burst drug release [7], relatively few products using this delivery system have made it to market. Currently, only two FDA approved products are on the market utilizing this type of system, Eligard® and Atridox®. Eligard®, using the Atrigel® delivery system and marketed by Sanofi-Aventis in the US (Medigene in Europe), is a subcutaneously injected implant that releases leuprolide acetate over a period of 3 months to suppress testosterone levels for prostate cancer treatment [8]. Atridox® (Tolmarc Inc.) is another ISFI system that also uses the Atrigel® delivery system to deliver the antibiotic agent, doxycycline, to the sub-gingival space to treat periodontal disease [9].

With ISFI systems, the behavior of the polymer matrix has a direct effect on the release profile [10]. Typically the release profile has distinct regions of burst release, diffusion facilitated release, degradation facilitated release, and finally a period of drug depletion [11]. One of the greatest challenges in translating ISFI systems from the research phase to a clinical setting is the relatively large burst release [7]. Burst release can be defined as the percent of drug loading dose

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released during the first 1–24 h after implant injection. Since the burst drug release is largely dependent on the phase inversion process, formulation parameters that affect the phase inversion dynamics (e.g. solvent hydrophobicity, excipient additives [7], polymer type or Mw [12,13]) are all active areas of research. One particularly promising strategy uses a relatively hydrophobic solvent, such as ethyl benzoate, which reduces the rate of water influx into these systems and slows the phase inversion rate [4]. Utilizing this approach, several ISFI formulations exhibiting zero-order drug release have been created, however due to the increased viscosity of these formulations the injectability of these implant systems in a clinical setting is greatly reduced [4]. Another strategy that has been employed to reduce burst release without increasing solution viscosity is the inclusion of surfactant excipients, such as Pluronic co-block polymers in ISFI formulations [7,13]. A study by Desnoyer et al., hypothesized that Pluronic surfactants coated the surface of the PLGA matrix and reduced burst release by decreasing drug mobility through affinity based interactions [7]. In addition to reducing the burst drug release from ISFIs, certain Pluronic co-block polymers such as Pluronic P85, have also been shown to have chemo-sensitizing potential in cancer treatment [14]. Other strategies to reduce burst release without increasing implant viscosity, include using *in situ* forming microparticle based systems which are comprised of an emulsion of polymer solution and oil [15,16]. Finally, a recent study by Patel et al. has shown that adjusting the polymer Mw used in ISFI polymer formulations can help modulate burst drug release of low Mw drug molecules such as commonly used chemotherapeutic agents [13]. However, while most of these strategies were able to modulate burst drug release in an *in vitro* environment, there has been little work examining ISFI behavior in an *in vivo* environment.

In the present study, release of a model drug agent, fluorescein, from varying Mw PLGA formulations was examined in both *in vitro* and *in vivo* environments. Fluorescein has a similar low Mw (376.28 Da) and hydrophilicity to other active chemotherapeutic agents, such as carboplatin (371.25 Da). Additionally, using a non-toxic model drug agent allowed us to test multiple implants within a single animal without incurring toxicity, thereby reducing the total number animals needed for *in vivo* experimentation. Since the composition of the external environment, in this case the tissue surrounding the ISFI, can greatly affect phase inversion and drug release, implant behavior was examined in a variety of *in vivo* environments including in subcutaneous (S.C.), non-necrotic tumor, and necrotic tumor tissue. In addition, drug release from ISFIs implanted into tumors treated with radiofrequency (RF) ablation therapy was examined. Application of chemotherapeutic drug delivery implants as adjuvant therapy for post ablative treatment has been an area of significant research interest in recent years, and the potential for ISFIs to be used for this application was explored [17–19]. To correlate the dependence of drug release with the phase inversion process in an unperturbed *in vivo* environment, a novel ultrasound implant imaging method recently described by Solorio et al. was utilized [20]. Finally scanning electron microscopy was used to examine the structure and morphology of phase inverted implants in differing *in vitro* and *in vivo* environments. Results from this study should lead to an improved understanding of the behavior of ISFI systems in a variety of environments and provide additional insight into the mechanisms leading to the discrepancy between *in vitro* and *in vivo* implant formation and drug release.

2. Methods

2.1. Materials and polymer solution preparation

ISFI polymer solutions were comprised of poly(D,L-lactide-co-glycolide) (PLGA) with a 50:50 lactide/glycolide (L/G) ratio from Lakeshore Biomaterials (Birmingham, AL), 1-methyl-2-pyrrolidone (NMP) from Sigma (St. Louis, MO), Pluronic P85 (P85, Mw: 4600 Da), a tri-block co-polymer of PEO-PPO-PEO donated by BASF Corp. (Shreveport, LA), and sodium fluorescein (Sigma), which was used as a low Mw hydrophilic water soluble model drug molecule (Mw 376.28). All solid components of the mixture were dissolved in the NMP solvent overnight at 37 °C in an orbital incubator-shaker (New Brunswick Scientific, C24) at 80 rpm and stored at 4 °C after a homogenous solution was obtained. The relative mass percentage of each component in polymer solution was 60/36.5/2.5/1 for NMP, PLGA, P85, and fluorescein respectively. To examine the effect of PLGA Mw on drug release, three different formulations comprised of PLGA were prepared with the PLGA polymer having a mean Mw of 18, 33, and 50 kDa.

2.2. In vitro drug release

In vitro drug release from varying Mw ISFI polymer formulations was examined using two different methods. The first method utilized a typical continuous, cumulative drug dissolution study which has been previously described, and shall be further referred to as the continuous time point (CTP) release method [13]. Briefly, 50 μL of polymer solution for each formulation (n = 4) was injected into a bath solution of 12 mL of PBS (pH 7.4) and placed in an orbital incubator-shaker set at 37 °C and 80 rpm. The bath solution was then sampled (100 μL) at several experimental time points (ETPs) (1 h, and 1, 2, 4, and 7 days) post injection to determine drug release profiles. The concentration of drug released into the bath solution samples was quantified using a fluorescence plate reader (Tecan Ltd., Infinite 200 series) with an excitation/emission (Ex/Em) wavelength of 485/535 nm and referenced to a standard curve. The sensitivity, accuracy, and linearity of fluorescence quantification were 20 pM fluorescein, 98%, and 99%, respectively. Cumulative drug release was calculated by normalization to the total injected drug mass determined by post experimental degradation of the implant. For this method, cumulative drug release was monitored for all time points for each trial with the same implant.

The second method was similar to *in vivo* drug release trials and shall be referred to as the discrete time point (DTP) drug release method. 50 μL of polymer solution for each formulation was injected into a 12 mL bath solution of PBS (pH 7.4) and placed in an orbital incubator-shaker set at 37 °C and 80 rpm. However instead of sampling the bath solution at each ETP the implant (n = 4 per time point) was removed from the bath solution and degraded in 2 mL of 2 M NaOH for 24 h. Degraded implant solutions were then neutralized and diluted with PBS (pH 7.4), and drug concentration was calculated using a fluorescence plate reader and standard curve for reference. Cumulative drug release was calculated by normalizing the mass of drug remaining in each implant to the initial drug loading of the implant.

2.3. In vivo subcutaneous drug release

Drug release from ISFIs in a S.C. environment was examined in fifteen 11 week old male BD-IX rats (average body weight 320 g, Charles River Laboratories Inc., Wilmington, MA). All experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). Rats were anesthetized with 2% isoflurane and an O_2 flow rate of 1 L/min (EZ150 Isoflurane Vaporizer; EZ Anesthesia, Palmer, Pa). Five 50 μL implants loaded with 1% fluorescein were injected and distributed evenly under the dorsal S.C. skin fold of each animal. For each sampling time point, implants comprised of each varying Mw PLGA polymer formulation (n = 5) were distributed across 3 animals. At each ETP post implant injection, animals were euthanized, and the implants were removed and degraded in 2 M NaOH solution. The degraded implant was neutralized, diluted in PBS at 7.4 pH, and the

fluorescence of drug remaining in the implant was quantified using a fluorescence plate reader as described in the previous section. The cumulative drug released from the implant was calculated by using the known implant drug loading mass and normalized for any small variations in injection volume as determined by experimental measurement.

2.4. Tumor inoculation and intratumoral drug release

Drug release from ISFIs in tumors was examined in ten 11 week old male BD-IX rats (average body weight 330 g, Charles River Laboratories Inc., Wilmington, MA) with S.C. rat colorectal carcinoma cell line (DHD/K12/TRb) tumors. Cells for tumor inoculation were prepared using a previously described protocol and were inoculated into animals 6 weeks prior to implant treatment [17]. Two tumors were inoculated bilaterally, approximately 1 cm inferior to the scapula and 1 cm lateral to the midline, while the other two tumors were bilaterally inoculated approximately 1 cm superior to the iliac crest and 1 cm lateral to the midline. Once tumors had fully matured, rats were anesthetized, and the tumor site was prepared by shaving and cleansing with alcohol and iodine. Tumor size was measured by calipers (R.B.P.), and the average tumor diameter was found to be 1.16 ± 0.16 cm for all groups. Any tumors that were not developed by this time or remained too small to be injected with implant were excluded from the study. Remaining animals received an injection of 50 μL of 33 kDa PLGA ISFI solution loaded with 1% fluorescein. During needle insertion if necrotic fluid leaked from the injection site, implant injection was designated as injected into a necrotic tumor cavity. To determine drug release from ISFIs in tumor tissue, implants were removed at 1 h ($n=6$), 1 day ($n=6$), 2 days ($n=6$), 4 days ($n=4$), and 7 days ($n=6$) post injection. Drug release was quantified as described in Section 2.3. Post mortem location of the implant was qualitatively examined to determine whether it was located in a necrotic cavity or within a non-necrotic tissue space was also noted and compared with earlier notations.

2.5. Tumor ablation and drug release in ablated tumors

In addition to measuring drug release from ISFIs in the S.C. and intratumoral environments, release into ablated tumor tissue that has undergone coagulative necrosis was examined [21]. DHD/K12/TRb rat colorectal carcinoma tumors were inoculated and grown to a mean diameter of 1.20 ± 0.42 cm in ten 11 week old BD-IX rats using the protocol described in the previous section. Animals were then once again anesthetized, and the tumor area was shaved and sterilized for RF ablation treatment. A modified 1 cm exposed tip 20-gauge non-internally cooled electrode was inserted into the tumor mass and a monopolar RF current was applied to the tumor using a 480-kHz RF generator (3E; Radionics, Burlington, Mass) and an abdominal grounding pad. Tumors were ablated with a power of 2–4 W with a constant electrode temperature of 80 °C for 3 min. After ablation, the electrode was removed, and 50 μL of 33 kDa PLGA ISFI solution was immediately injected into the ablated tumor volume through the ablation track. Implants were removed at each ETP post treatment and analyzed using the method described in Section 2.3.

2.6. In vivo ultrasound implant formation imaging

Implant formation was examined using a previously established ultrasound technique [20]. Briefly, animals were anesthetized, and each implant was imaged using a 12 MHz PLT-120 transducer on a Toshiba Aplio SSA–770A diagnostic ultrasound machine with the following parameters: dynamic range of 55, mechanical index of 1.1, gain of 80, and 3 cm depth (Fig. 1A). At least five ultrasound still images of each implant in their respective tissue environment were taken at each ETP.

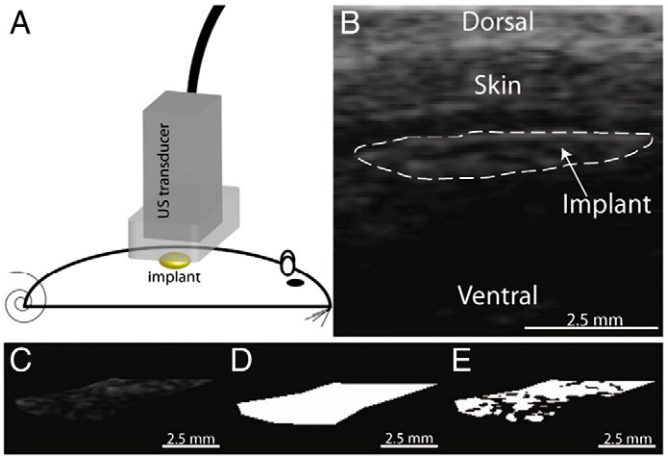

Fig. 1. Dorsal S.C. implants are imaged using US *in vivo* (A) to obtain a longitudinal cross-sectional area image of the implant under the dorsal skin fold (B). Implants are then manually segmented (C) and the ratio of the total implant area (D) to the precipitated echogenic implant pixel area (E) is calculated to determine the percent of implant precipitation.

2.7. Image analysis

Ultrasound image analysis of *in vivo* implant formation was conducted using a methodology recently developed by Solorio et al. [20]. Briefly for each condition, ROIs from five implant images (Fig. 1B) were manually segmented, and a parametric segmentation technique utilizing the method of mixed Gaussians was used to find a threshold value to remove low intensity noise from each implant image (Fig. 1C). The summation of the number of pixels in the selected ROI was used to calculate the total implant area (Fig. 1D). Next the implant area that had solidified into solid matrix, with pixels above the set threshold, was found (Fig. 1E). The percent precipitation of the implant was calculated by finding the ratio of the number of pixels above the threshold, to the total number of pixels in the implant area. The percent of implant precipitation was found for all ETPs.

2.8. Scanning electron microscopy

The structural morphology of each implant formulation *in vivo* and *in vitro* was examined using scanning electron microscopy (SEM). *In vitro* and *in vivo* implant samples were prepared by injecting 50 μL of each polymer formulation into either a PBS bath or S.C. tissue respectively. Implant samples were then removed from their location at one ($n=3$) and seven ($n=3$) days post injection, freeze fractured in liquid nitrogen, and subsequently lyophilized for 24 h. All prepared samples were then bonded to an aluminum stub and sputter coated with 5 nm of Pd. SEM micrographs were then obtained using a Quanta 200 3D ESEM with an acceleration voltage of 2 kV and a hole size of 3.5.

2.9. Statistical analysis

A one-way ANOVA test was used to determine statistically significant ($p<0.05$) differences between experimental groups. Specific differences between groups were identified using a Tukey multi-comparison test. All statistical tests were carried out with MatLab using the statistics tool box. Errors were reported as ± standard error of the mean (S.E.M.).

3. Results

3.1. In vitro drug release

The accuracy of obtaining a drug release profile by degrading several different implants at various time points after injection into a

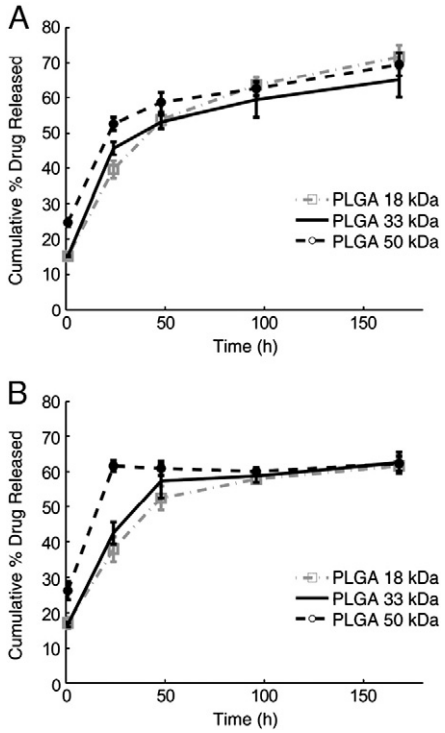

Fig. 2. The cumulative percent of the loading fluorescein mass released from varying Mw PLGA ISFIs *in vitro* is shown using the CTP (A) and DTP (B) release study methods.

bath solution versus sampling the bath solution from a single implant dissolution study was examined *in vitro*. The results from both methods show that low Mw PLGA implants released drug slower than high Mw PLGA implants during the burst release phase (0–24 h) (Fig. 2, Table 1). Statistically significant differences seen between implant groups are similar for both drug release methods with lower Mw 18 kDa PLGA implants having a significantly lower percent of drug release at the 1 h ($p<0.001$ and 24 h ($p<0.01$) 3 compared to higher Mw 50 kDa PLGA implants. However after 24 h, the drug release from 18 kDa PLGA ISFIs was no longer significantly different than the other implant formulations, with no further significant differences occurring for the duration of the study. The 33 kDa PLGA formulation exhibited a significantly lower ($p<0.01$ release at 1 h compared to the 50 kDa PLGA samples for both release methods. No other significant differences were noted for the 33 kDa PLGA formulation. To examine the similarity of drug release profiles obtained from each method, a correlation coefficient value correlating the drug release profile obtained from the CTP and DTP methods, R^2, was found to be greater than 0.9 for all groups.

Table 1
Fluorescein release *in vitro* (DTP) and *in vivo*.

	Time to percent release (h)		
	25%	50%	75%
In vitro			
18 kDa PLGA	9.7	44	N.A.
33 kDa PLGA	8.4	36.2	N.A.
50 kDa PLGA	1	67.7	N.A.
In vivo SQ			
18 kDa PLGA	0.4	0.8	11.4
33 kDa PLGA	0.7	9.7	67.6
50 kDa PLGA	0.7	11.5	109
Tumor—33 kDa PLGA			
Unablated	0.6	4.8	24.7
Necrotic	0.8	12.7	47.1
Non-necrotic	0.5	0.9	17.9
ABT	0.3	0.7	13.6

3.2. In vivo drug release and implant formation

Drug release from varying Mw PLGA implants was examined *in vivo* in a S.C. environment. Here, drug release displayed a trend opposite of that observed *in vitro*. Namely, low Mw PLGA implants released drug at a faster rate than high Mw PLGA ISFIs (Fig. 3A). Low Mw 18 kDa PLGA implants released a significantly ($p<0.05$) greater percent of their initial loading dose than higher Mw 33 and 50 kDa PLGA implants at 1 h, 1, 2, and 4 days. Additionally, the burst release at 24 h from *in vivo* implants was significantly greater than that of implants *in vitro* for 18 and 33 kDa PLGA polymers.

To examine the effect of implant formation on drug release, ultrasound was used to quantify implant phase change. These formation studies showed that the higher Mw 33 and 50 kDa PLGA implants have a lower rate of matrix precipitation than the lower Mw 18 kDa PLGA implants (Fig. 3B). The 33 and 50 kDa PLGA implants also have a significantly lower ($p<0.05$) percentage of solidified matrix during the burst drug release time points (1 h, 1 day). In addition when implant precipitation was correlated to drug release for all implant formulations, the correlation coefficient was found to be greater than 0.92 for all cases (Fig. 3C). Finally the total implant area was examined for each PLGA formulation, and results show that implant cross-sectional area decreased over time for all formulations (Fig. 3D).

3.3. Intratumoral drug release and implant formation

The mean drug release from implants injected into solid tumors was statistically insignificant from release observed in a S.C. environment (Fig. 4A). However, due to the large variability in tumor tissue density with several implants having been injected into a necrotic tumor cavity while others were injected into very dense tumor tissue, the release profile in tumors was quite variable. When the drug release profile was further refined into necrotic tumor and non-necrotic tumor groups, it was seen that drug release in necrotic tumors was slower than in non-necrotic tumors (Fig. 4B). Significant differences ($p<0.05$) between non-necrotic and necrotic tumor implant groups were observed at 2 and 4 days post injection.

The rate of formation in tumors was also found to be slightly faster than in an *in vivo* S.C. environment (Fig. 5). However, tissue scabbing of some tumors resulted in low image quality, resulting in a reduced number of trials and an inability to separate the data sets into necrotic and non-necrotic subgroups. Nonetheless, when the tumor formation curve was correlated to intratumoral drug release a correlation coefficient of 0.95 was found.

3.4. Ablated tumor drug release and implant formation

Drug release from implants injected into tumors treated with RF ablation was investigated as well. No significant differences were noted between drug release from ISFIs in ablated tumors in comparison to unablated tumors at time points greater than 1 h (Fig. 4A). However, the 1 h burst release in ablated tumors was significantly greater than that seen in all other *in vivo* environments. Yet within 24 h, all other trials except for necrotic tumor had reached a similar level of release as ablated tumor, and no significant differences were found between groups at time points greater than 1 h. Additionally, ISFIs implanted in ablated tumor had a higher percent of formation at the 1 h time point in comparison to unablated tumors (Fig. 5). The correlation, R^2, between drug release and implant formation in tumors was found to be 0.93.

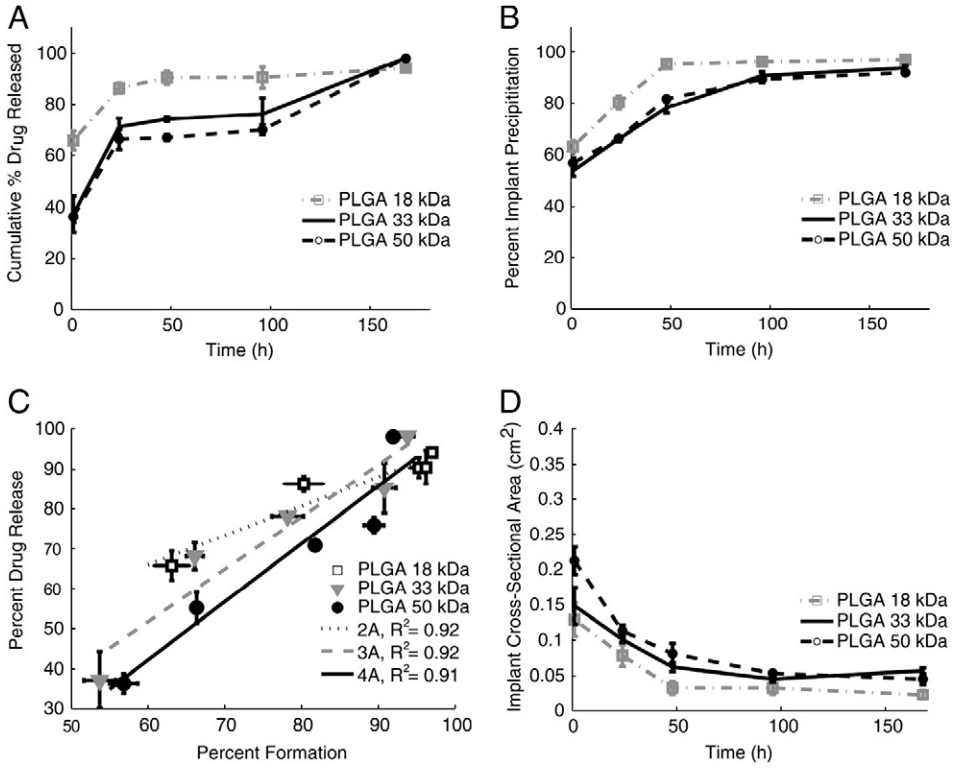

Fig. 3. Cumulative percent release of the loading fluorescein mass (A), percent implant precipitation over time (B), the correlation between the drug release and implant precipitation curves (C), and the change in implant cross-sectional area over time is shown for varying Mw PLGA implants (D).

3.5. Scanning electron micrographs of in vivo and in vitro implants

In vitro and *in vivo* implant samples were prepared for SEM using implant samples injected into their respected environments and retrieved 1 day and 7 days post injection. Due to incomplete implant precipitation and likely a large residual solvent concentration after one day post injection, *in vitro* lower Mw 18 and 33 kDa PLGA implants were destroyed during the freeze–drying process of SEM sample preparation. However, *in vitro* higher Mw 50 kDa PLGA implants were sufficiently precipitated after this same time period and their structure could be compared to *in vivo* implant samples at day 1. Qualitative observation of these implants shows that matrix morphology of *in vitro* implants is more homogenous than what is observed *in vivo* (Fig. 6). In addition the overall porosity of *in vitro* implants is greater than *in vivo* 50 kDa PLGA implants at one day. By day 7, all *in vitro* and *in vivo* implant samples had sufficient time to precipitate enough to survive sample preparation. In contrast to the 1 day 50 kDa PLGA samples, *in vitro* implants had a comparable porosity to *in vivo* implants. Moreover the *in vitro* lower 18 kDa Mw PLGA implants had a much greater porosity than the higher Mw 50 kDa PLGA implants, however the *in vivo* implants of both these formulations had a similar level of porosity (Fig. 7).

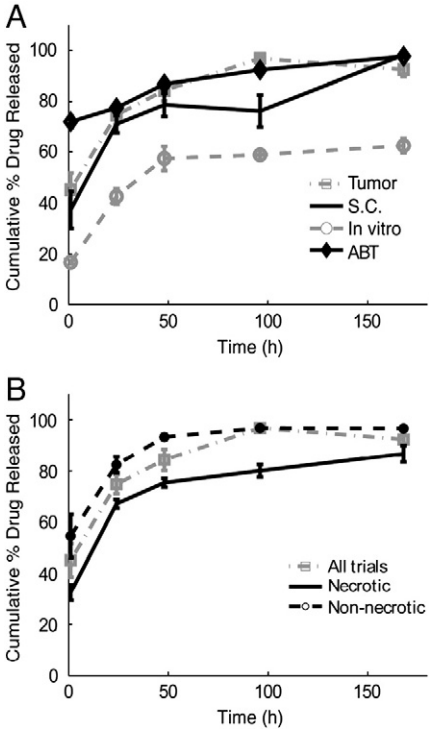

Fig. 4. The cumulative percent of the loading fluorescein mass released from ISFIs in varying *in vitro* and *in vivo* environments (A) is shown. Differences in drug release from implants in necrotic and non-necrotic tumors is also displayed (B).

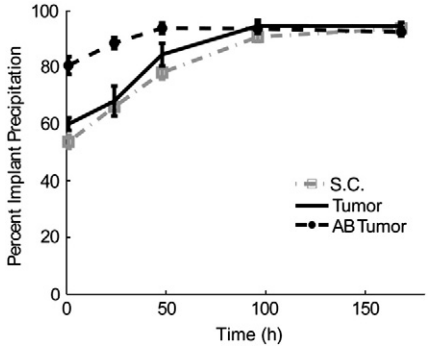

Fig. 5. Implant precipitation over time as determined by US formation studies in varying *in vivo* environments is shown.

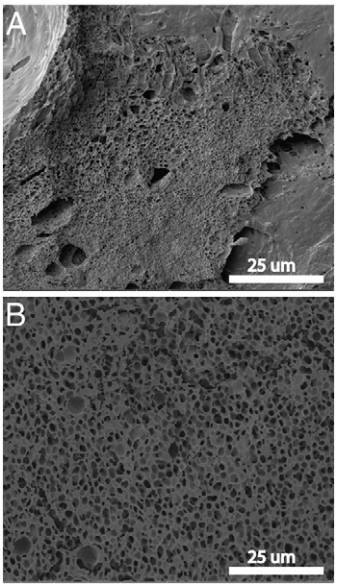

Fig. 6. SEM images of the implant matrix of 50 kDa PLGA implants at day 1 post injection in *in vivo* (A) and *in vitro* (B) environments.

3.6. Implant shape in varying in vivo environments

In addition to the bath side components such as dissolved proteins, salts, and acids present in the external tissue surroundings, another factor that likely affects implant formation and drug release is the final shape of the injected implant in different environments. For example, in an *in vitro* environment our implants conformed to a uniform spherical shape (Fig. 8A), while in an *in vivo* S.C. environment the injected implant had a flat disc like shape upon removal (Fig. 8B). Intratumoral implants had a variable shape that was globular (Fig. 8C + D) and sometimes consisted of multiple lobes. Additionally the cross-sectional area of implants in a necrotic cavity (Fig. 8C) was greater than that of implants in a non-necrotic tumor location (Fig. 8D). Finally, since implants in ablated tumors were injected directly into the ablation track formed during RF treatment, these implants conformed to a rod-like shape that filled the ablation track cavity (Fig. 8E). Ultrasound images of the cross-sectional area of implants in each environment also show how different environmental effects have on implant shape (Fig. 8F–J).

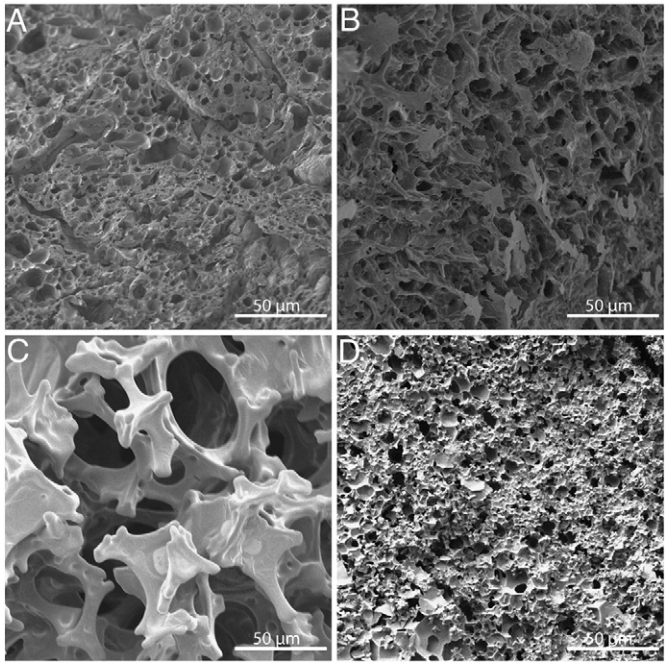

Fig. 7. SEM images of the implant matrix of 18 kDa (A, C) and 50 kDa (B, D) PLGA implants at day 7 post injection in *in vivo* (A, B) and *in vitro* (C, D) environments.

4. Discussion

In vitro drug dissolution studies have shown that release from ISFIs is dependent on a variety of factors such as solvent type [4], polymer Mw [13], excipient factors [7], and external bath side effects [5]. However the effect these differing variables have on the rate of implant formation may hold the key to understanding the variability of drug release from ISFIs. Implant formation or precipitation occurs by a process of solvent exchange whereby as water influx into the implant occurs and the water miscible solvent leaves the system, the hydrophobic polymer will precipitate out of solution. Previous studies by Graham et al, have shown that *in vitro* implant formation examined using dark ground imaging (DGI) correlated to observed drug release from differing ISFI formulations with slower forming implants releasing drug slower than faster forming implants [5]. The proposed reason for this effect as hypothesized by McHugh et al., is that the mobility of the drug agent is reduced in the more viscous interior non-phase inverted core than in the porous water rich precipitated matrix shell [1]. Therefore as solvent efflux increases and implant precipitation increases, drug dissolution and release would thereby be increased. In our current study, we also found the rate of implant precipitation to be correlated, ($R^2 > 0.90$) for all cases, to the rate of drug release. Because of this relationship, ultrasound imaging of the implant formation process can be a useful technique to noninvasively monitor and predict drug release in a variety of *in vivo* environments [20]. This technique may be particularly beneficial for monitoring ISFIs injected into cancerous lesions due to the high degree of inter-patient tumor variability and differences in tissue density and architecture. In the current study, the external environmental effects on ISFIs in a variety of *in vitro* and *in vivo* surroundings were examined.

First, drug release from ISFIs in varying *in vivo* environments was compared to their release *in vitro*. For all *in vivo* tissue environments, drug release from ISFIs was found to be significantly greater than their corresponding *in vitro* release. In addition, the rate of implant formation was found to correlate with the rate of implant drug release with *in vivo* ISFIs solidifying much quicker than what has previously been shown *in vitro* [20]. While this finding is consistent with previous studies using ultrasound to examine implant formation [20,22], other studies using electron paramagnetic resonance (EPR) to determine the rate of implant formation have found little difference between *in vivo* and *in vitro* implant formation [22]. One reason for the difference seen between our method and the one used for EPR may be due to the use of a spin-probe in EPR technique that may affect the phase inversion dynamics of the ISFI polymer solution [22]. *In vitro* implant formation studies using DGI have also shown similar results to ours with slower forming implant formulations releasing their drug slower than faster forming ISFIs [4]. However while implant formation has been correlated *in vitro* and *in vivo* using EPR and ultrasound, studies directly correlating drug release from these ISFIs in a similar manner have not been conducted to our knowledge. Previous examination of similar implants types *in vivo* have only measured serum plasma drug levels with several studies concluding that *in vivo* release was in good agreement with release profile seen *in vitro* [23,24]. Since serum plasma drug concentrations are dependent on several physiological factors such as drug adsorption, metabolism, and clearance that occur after the drug is released, a direct comparison between *in vitro* and *in vivo* environments is difficult. Therefore this may account for any discrepancies seen between our results and previous findings in the literature [22].

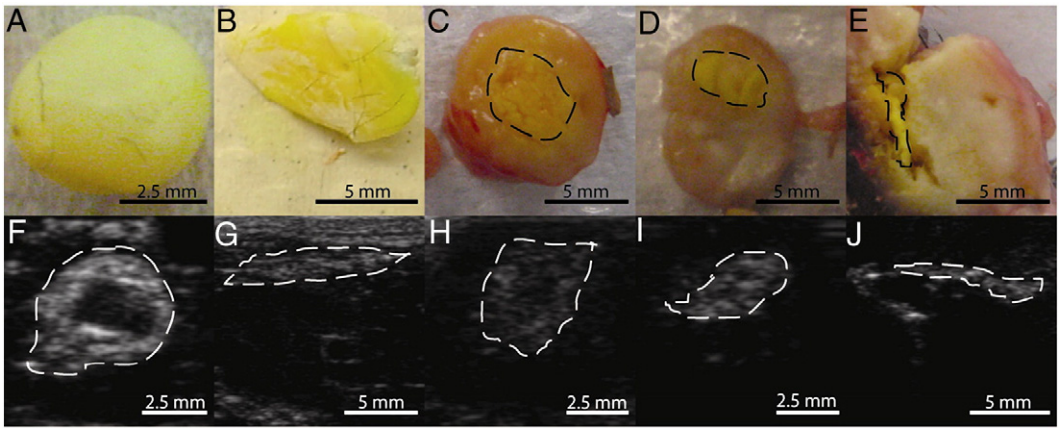

Fig. 8. Photographs (A–E) of implants 7 days post injection in *in vitro* (A, F) and *in vivo* S.C. (B, G), necrotic tumor (C, H), non-necrotic tumor (D, I), and ablated tumor (E, J) environments and their corresponding cross-sectional area using US imaging (F–J). Scale bar represents 2.5 mm for A, F, H,and I. Scale bar represents 5 mm for B,C,D,E,G, and J.

Concurrently, the effect of polymer chain Mw on drug release was investigated in both an *in vitro* and a S.C. *in vivo* environment. However since there are significant difficulties in performing a continuous release study *in vivo*, first the accuracy of an adapted method (discrete time point method, DTP) was tested in comparison to a traditional continuous cumulative release study. For the DTP method, the release profile was obtained by determining the percent of drug remaining in implants removed from bath solution and degraded at each time point. The *in vitro* results described in Section 3.1 show that the DTP method has a release profile comparable to that obtained using a more traditional continuous release method. The results from these experiments also corroborate previously established findings whereby lower Mw PLGA ISFI formulations release drug slower than higher Mw ones [13]. Interestingly, the opposite trend was seen when these same formulations were injected into an *in vivo* S.C. environment with higher Mw PLGA formulations releasing drug slower than lower Mw ones. While data in the literature on the effect of polymer Mw on *in vivo* drug release is limited, a study by Ravivarapu et al. has found a similar trend whereby lower Mw PLGA ISFIs injected into the S.C. skin fold of canines released drug faster than higher Mw implants as measured by plasma drug concentration [24].

The contradictory effect of polymer Mw on drug release *in vivo* and *in vitro* is a perplexing finding; however examination of implant formation in these two environments may provide some answers. *In vivo* (Section 3.2), the higher Mw 33 and 50 kDa PLGA formulations precipitate significantly slower than the lower Mw 18 kDa PLGA formulation. However, a study by Solorio et al. using similar US formation methods found that *in vitro*, 18 kDa PLGA implants formed slower than 50 kDa PLGA implants [20]. Moreover the authors found that 18 kDa PLGA implants underwent greater swelling resulting in a significantly greater volume expansion over time compared to higher Mw 50 kDa PLGA implants *in vitro*, and in some cases would even burst through agarose tissue phantoms when embedded inside them [20]. This *in vitro* swelling effect suggests that there is greater water influx than solvent efflux, and lower Mw 18 kDa PLGA implants may retain a greater percent of their solvent and drug than higher Mw 50 kDa implants which have a faster formation and release rate. Conversely, this expansion effect was not seen *in vivo* with all implants having a declining total cross-sectional implant area over time as determined by US imaging. These findings suggest that there is a decoupling between the rate of water influx and solvent efflux from the implants, with solvent ejection occurring faster than water movement into the implants resulting in the decrease in total implant cross-sectional area.

Implants that retain solvent will likely precipitate slower and thereby have slower drug release than implants which form faster as described above. Lending credence to this hypothesis is the fact that *in vivo* implants survived SEM preparation, while 18 and 33 kDa *in vitro* implants dissolved in the residual solvent once the water had been removed from the implant following lyophilization. Meanwhile, the *in vitro* PLGA 4A sample and all of the 1 day *in vivo* samples were able to withstand processing for SEM suggesting that these implants had a low concentration of residual solvent at this time point and were more fully precipitated. The greater porosity of *in vitro* implants in comparison to their corresponding *in vivo* counterparts shown by SEM in Section 3.5 also suggests that *in vitro* implants expand and swell more than *in vivo* implants. Moreover, previous studies examining phase separation of varying Mw PLGA polymeric implant solutions have shown that lower Mw PLGA polymers undergo a two-part phase inversion process whereby these polymers first form into a soft hydrogel and later solidify into a stiffer polymer matrix [5]. Studies by Patel et al. have also shown that *in vitro* higher Mw PLGA polymers have a lower solubility than lower Mw PLGA polymers, and a lower critical water concentration threshold is required to induce their precipitation out of solution [13]. The ability of low Mw PLGA implants to retain their solvent, solubilized polymer, and drug *in vitro* may lead to a persistent osmotic effect that results in implant swelling.

In vivo, the ability of the implant to expand is likely limited due to interstitial pressure or compressive forces exerted on the implant by the surrounding tissue. Therefore due to a mechanically induced convective force, caused by the *in vivo* environment, solvent and drug efflux are prone to be increased, and the rate of implant formation and drug release are greater than the *in vitro* counterparts. This hypothesis is further supported by comparing the burst release of the *in vitro* and *in vivo* for the high Mw implants. During the first hour post injection the *in vivo* high Mw PLGA implants show only a modest increase in the amount of drug released relative to the *in vitro* implants (Fig. 9). This effect is presumably due to limited swelling tendency of the higher Mw implants during this time [18]. In contrast, lower Mw PLGA

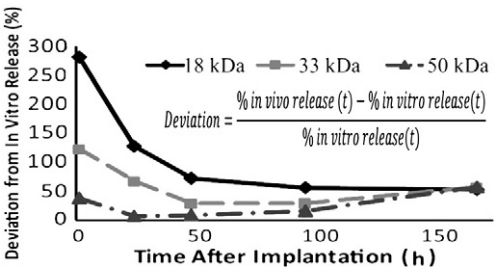

Fig. 9. Deviation of *in vivo–in vitro* drug release from varying Mw ISFIs over time.

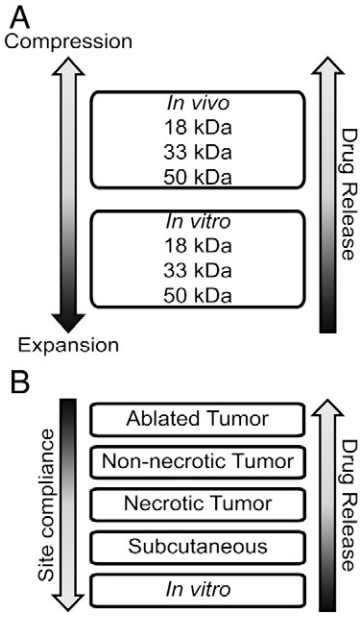

Fig. 10. The tendency of varying Mw PLGA implants to expand or compress *in vitro* and *in vivo* (A) and the effect of *in vivo* tissue compliance on drug release from ISFIs (B) is summarized.

implants have been shown to swell and expand much more significantly than high Mw PLGA implants post injection into an aqueous phase [18]. Consequently, the convective force created by the mechanical pressure of the dorsal skin fold pushing on the swelling implant causes a larger mass of drug to be released immediately after injection *in vivo*. This trend is lost over time as the implants continue to precipitate and the osmotic forces inducing implant expansion are reduced. The tendency of varying Mw PLGA implants to expand or compress in *in vitro* and *in vivo* environments is summarized in Fig. 10A.

Finally, drug release and implant precipitation of ISFIs in different *in vivo* tumor environments was examined. Due to differences in the external tissue environment of necrotic, non-necrotic, and ablated tumors a different drug release profile was seen for each. The drug release from necrotic tumors was very similar to drug release in subcutaneously placed implants. Although the implant shape and likely composition of the external interstitial fluid are different for both environments, both have a limited tissue density allowing the implant to expand. The mechanical pressure exerted by the dorsal skin fold on the implants resulted in their compression and flattening. Similarly, necrotic tumors have been shown to have a high interstitial fluid pressure [25], which can constrain implant swelling or expansion similar to the force generated by the dorsal skin fold. In contrast, implants placed in non-necrotic and ablated tumors, which have a greatly increased tissue density and have been found to be much stiffer than normal tissue [26–28], exhibited a faster drug release (Fig. 10B). The space available for these implants to swell and expand was even more restricted and resulted in smaller implant sizes. Surprisingly, implants placed in ablated tumors had a greater 1 h burst release than in non-necrotic tumor. However this may be explained by increased tissue stiffness, even in comparison to non-necrotic tumor tissue, and edema that has been shown to occur post ablative treatment [27]. In addition, since implants were injected into ablated tumors only 1 min post ablation, residual heat at the injection site may play a role in implant formation and drug release behavior. By the 24 h time point, the drug release observed in non-necrotic tumors was similar to ablated tumor implants. Examination of implant precipitation for each *in vivo* environment demonstrated that faster precipitating implants released drug faster than slower forming counterparts.

While the role of tissue mechanical forces remains our primary hypothesis to explain differences between *in vitro* and *in vivo* drug release from ISFIs, various other factors may play a role including the water content of the surrounding medium, implant shape and surface area, and tissue perfusion. However, if the water content of the surrounding medium was playing a major role in the observed drug release profiles, it would be expected that implants placed in more water rich environments would precipitate and release drug faster. Therefore, *in vitro* ISFIs would be expected to precipitate the quickest and have the fastest drug release since this environment has the greatest water content. Similarly, if differences in tissue perfusion were the primary factor affecting drug release, release in the relatively avascular necrotic tumor cavity would be expected to be slower than in the more vascular subcutaneous space due to increased drug elimination and a larger gradient driving force for drug release. However in our studies, we found *in vitro* implant precipitation and drug release to be the slowest. In addition, no differences in behavior were found between implants placed either subcutaneously or in a necrotic tumor cavity. As for implant surface area, qualitative observation of implant shape (Fig. 8) did confirm that implants in S.C. tissue and ablated tumor tissue which were shaped as a thin flat disk and long thin rod had a greater surface area to volume ratio in accordance with their faster drug release in comparison to spherically shaped *in vitro* implants. However implants in S.C. tissue (thin flat disc shaped) which exhibited similar behavior to intratumoral ISFIs (spherical or globular shaped), would be expected to have a greater surface area to volume ratio. Therefore while implant shape and surface area may contribute to differences in drug release from ISFIs placed in different tissue environments, our findings suggest that implant shape is constrained by the available space at the injection site. While the data presented in these studies has only hinted at this phenomenon, future work comparing release from ISFIs of similar shape in various constrained environments should be conducted to validate this hypothesis. Another limitation of this study is that release of only one agent, fluorescein was examined, and release behavior of other agents with differing solubility, Mw, or charge may be quite different. Since the focus of these studies was on environmental effects on drug release, only release of one agent fluorescein was examined, however release of other agents should be considered in future studies.

5. Conclusion

While modulation of drug release from ISFIs has incited significant research interest over the past decade, most *in vitro* drug release experiments have focused on the effects of drug type, polymer solution formulation components, and external bath side effects. Our findings suggest that in an *in vivo* environment, local compressive mechanical forces and interstitial fluid pressures may play an even greater role in determining drug release from ISFIs. This is especially true in the case of low Mw polymer implants that have a greater tendency to swell and are inclined to precipitate slower *in vitro*. Higher Mw polymer implants which have been shown to precipitate faster with a stiffer matrix and tend to swell significantly less than smaller Mw PLGA polymers can be used to mitigate the local environmental mechanical effects. Another interesting byproduct of ISFI behavior *in vivo* that has not yet been explored but may be of significant interest for future studies is the potential use of these implants for convective drug transport. The tissue mechanical forces and interstitial fluid pressure acting on the implant increase solvent and drug efflux as well as implant precipitation and may also increase local tissue drug penetration. This process in a tumor volume could be of significant interest for local chemotherapeutic drug delivery.

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