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An in vitro gel-based system for characterizing and predicting the long-term performance of PLGA in situ forming implants

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ABSTRACT

In situ forming implants are exposed to an extracellular matrix resembling a gel rather than aqueous solution upon subcutaneous administration. The aim of study was to develop a gel-based release testing system for characterizing the long-term in vitro behavior of in situ forming implants. The gel-based system consisted of an agarose gel mimicking the subcutaneous injection site and a receiver layer comprising phosphate buffer. Poly(D, L-lactide-co-glycolide) in situ forming implants containing leuprolide acetate as the model peptide and N-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO) or triacetin as co-solvent were investigated. The gel-based release testing system discriminated between the formulations. Accelerated release data obtained at elevated temperatures were able to predict real-time release applying the Arrhenius equation. Monitoring of the micro-environmental pH of the implants was performed by UV-Vis imaging in the gel-based system at 50 °C. A pH drop (from pH 7.4 to 6.7 for the NMP and DMSO implants, to pH 5.5 for the triacetin implants) within the first day was observed, followed by an increase to pH ~7.4. The gel-based system coupled with UV imaging offered opportunity for detailed evaluation and prediction of the in vitro performance of long-acting injectables, facilitating future development of in situ depot forming delivery systems.

1. Introduction

In the area of parenteral sustained release formulations, in situ forming implants (ISFIs) constitute a promising platform for local as well as systemic delivery of biopharmaceuticals since they are minimally invasive and able to provide continuous delivery for prolonged therapeutic effect (Jorgensen and Nielsen, 2009; Kempe and Mader, 2012; Nkanga et al., 2020; Packhaeuser et al., 2004). Upon injection, the liquid pre-formulations transform in the subcutaneous tissue to gels or solid depots (Kempe and Mader, 2012). Based on the factors triggering the depot formation, ISFIs are usually categorized as; i) phase separating systems based on temperature (Ruel-Gariépy and Leroux, 2004), solvent exchange (Sartor, 2003) or pH (Lu et al., 2019), ii) crosslinked systems based on photo-initiated (Burkoth and Anseth, 2000), chemical or physical (Berger et al., 2004) processes, and iii) solidifying organogels (Vintiloiu and Leroux, 2008). Compared to, for example, thermo-responsive and pH sensitive ISFIs, implant formation of solvent exchange based ISFIs can be triggered more easily, requiring only an aqueous environment (Thakur et al., 2014). The water miscible solvent present in the injectable drug polymer solution dissipates into the aqueous tissue fluid as water penetrates into the implant hereby inducing depot formation. Eligard® (Sartor, 2003) and SABER® (Okumu et al., 2002) are successful examples of solvent exchange induced ISFIs.

Depending on the phase inversion rate of PLGA implant formation, solvent induced phase inversion based ISFIs has been categorized as fast or slow forming phase inversion systems. Those incorporating a highly water soluble solvent like dimethyl sulfoxide (DMSO) (Lambert and Peck, 1995), 1-methyl-2-pyrrolidinone (NMP) (Raman and McHugh, 2005) or poly(ethylene glycol)500 dimethylether (Schoenhammer et al., 2009) undergo rapid phase inversion since the organic solvents used are completely miscible with water. In contrast, hydrophobic solvents such as triacetin (Brodbeck et al., 1999), ethyl benzoate (Brodbeck et al., 1999) and benzyl alcohol (Prabhu et al., 2005) are poorly miscible with water and exhibit slow phase inversion. Typically, drug release from ISFIs is described by i) an initial burst release of drug from the surface of the implant due to a lag between injection and coagulation, ii) a slow diffusion-dominant release of drug in the polymeric matrix, and iii) a second rapid drug release phase caused by polymer erosion (Parent...
Drug transport through the two phases is controlled by diffusion (Brodbeck et al., 1999), and thereby affected by the physicochemical properties of the incorporated drug including solubility and molecular size (Parent et al., 2013; Pitt, 1990). The interconnected network formed during matrix solidification plays a crucial role in drug release. For fast forming phase inversion implants, the high affinity between the water and the organic solvents used induces fast solvent exchange, leading to generation of “finger-like” pore structures situated close to the implant surface (Young and Chen, 1995). By contrast, slow solvent exchange for slow forming phase inversion implants results in a homogeneous sponge structure due to the finite aqueous solubility of the solvent used (Graham et al., 1999).

Polymer degradation is a prerequisite for the erosion of PLGA based ISFIs (Gijpferich, 1996). PLGA is a poly (α-hydroxy-ester) and is degraded into shorter chain alcohols and acids upon contact with water (Ford Versyp et al., 2013). The hydrolysis reaction of the polymer can be further catalyzed by the generated acids, also known as autocatalytic hydrolysis (Ford Versyp et al., 2013; Siepmann et al., 2005). Owing to the presence of autocatalysis, PLGA degradation takes place throughout the entire process of drug release (Zomke et al., 2019). The accumulation of the generated acids leads to a significant drop in the micro-environmental pH (Houchin and Topp, 2008). In vitro experiments with PLGA based ISFIs conducted by electron paramagnetic resonance (EPR) spectroscopy have suggested that the internal pH of the depots incubated in phosphate buffer at 37 °C dropped from pH 7.4 to below pH 3 within the first 6 days (Schadlich et al., 2014). Likewise, a significant drop in in vivo internal pH was measured using EPR (Mader et al., 1996) and fluorescence imaging (Schadlich et al., 2014) for in situ forming PLGA implants.

Development of ISFIs remains challenging, partly due to the lack of suited in vitro release testing methods (Kempe and Mader, 2012; Nkanga et al., 2020; Seidlitz and Weitschies, 2012; Zhang and Fassihi, 2020). The so-called sample and separate methods and dialysis-based methods are the most commonly used release testing methods for subcutaneous injectable implants (Shen and Burgess, 2012a). However, these approaches do not take into consideration the properties of the surrounding extracellular matrix (Hernandez et al., 2016). Agarose gels (Klose et al., 2009; Kozak et al., 2021; Li et al., 2020; Solorio et al., 2010), macroporous acrylicamide phantoms (Hernandez et al., 2016; Manaspon et al., 2017) as well as hyaluronic acid solutions (Li et al., 2020; Mertz et al., 2019) have been investigated as matrices for the in vitro characterization of subcutaneously administered in situ forming dosage forms. Recently, efforts have been made to improve repeatability of the release methods, e.g., by controlling implant shape using a so-called basket in tube method (Zang and Fassihi, 2020) or PVA thin film method (Sung Suh et al., 2020). In vitro gel-based testing methods may be challenged with respect to attaining complete drug release for ISFIs (Hernandez et al., 2016; Li et al., 2020; Solorio et al., 2010), which related to lack of sink conditions.

An interesting in vitro gel-based drug release testing system consisting of a gel phase simulating the injection site using an agarose gel as the tissue mimic and phosphate buffered saline (PBS) as a receiver solution was proposed by Gietz et al. (U. Gietz et al., 1998). They demonstrated the ability to discriminate between different subcutaneous injectables containing doxorubicin and insulin. Leung et al. further explored the in vitro drug release testing approach to investigate injectable formulations comprising low and high molecular weight compounds. They used a UV–Vis spectrometer to measure continuously the drug concentration in the buffer solution, i.e., in situ measurements (Leung et al., 2017). In the present work, UV–Vis imaging was used to characterize leuprolide acetate containing PLGA in situ depot forming implants for subcutaneous injection in the gel-based in vitro setup instead of a UV–Vis spectrometer. UV–Vis imaging is used in pharmaceutical analysis and development since it can perform spatially and temporally resolved measurements in in vitro drug dissolution and release testing (Östergaard, 2018). This imaging platform has also been used for visualization and evaluation of the microenvironmental pH (pHm) in the dissolution or release medium (He et al., 2020; Östergaard et al., 2014). Here, NMP and DMSO were incorporated as the solvents of fast forming phase inversion systems and triacetin was incorporated as the solvent of a slow forming phase inversion system. The aim of the present study was to investigate whether the aforementioned gel-based drug release testing system in combination with UV–Vis imaging was suitable for in vitro evaluation of in situ forming implants comprising different organic solvents regarding drug release and change in the pHm. We present the use of the in vitro gel-based release testing system coupled with UV–Vis imaging to characterize and visualize the long-term release behavior of different ISFIs comprising NMP, DMSO or triacetin as co-solvent under real-time and accelerated test conditions. Furthermore, a relationship between real-time and accelerated drug release profiles was established. Finally, the pHm of the ISFIs was evaluated in the presence of a pH indicator by imaging performed at 520 nm under accelerated conditions.

### 2. Materials and methods

#### 2.1. Materials

Agarose (type I, gel point (0.5% (w/v) agarose in water): 36 ± 1.5 °C), poly (D, L-lactide-co-glycolide) (PLGA (EXPANSORB® DLG 75-2A), the molar ratio of lactide:glycolide: 75:25, MW 5–20 kDa), dimethyl sulfoxide (DMSO), 1-methyl-2-pyrrolidinone (NMP), and triacetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Leuprolide acetate (LA, purity > 99%) was obtained from Kajie Biopharm Co. (Chengdu, China). Trifluoroacetic acid (TFA) was bought from Carl Roth Gmbh & Co. KG (Karlsruhe, Germany). Acetonitrile and methanol (HPLC grade) were obtained from VWR International S.A.S. (Fontenay-sous-Bois, France).

Phosphate buffered saline (PBS) used in the study consisted of 0.01 M phosphate, 0.137 M NaCl and 0.002 M KCl adjusted to pH 7.40. A stock solution of bromothymol blue (6.7 × 10^{-3} M) was prepared by dissolving 0.418 g of the pH indicator in 50 ml of 96% ethanol and 1.86 ml of 0.1 M sodium hydroxide, and adding deionized water to 100.0 ml.

#### 2.2. Preparation of PLGA pre-formulations

Four pre-formulations incorporating the organic solvents NMP, DMSO or triacetin with different drug loading were prepared. PLGA was dissolved in the organic solvents, followed by addition of LA to form the pre-formulations as listed in Table 1. For the triacetin containing formulation, LA was suspended in the PLGA solution. The mixture was then subjected to 1 min of ultrasonication in an ice bath to ensure dispersion of LA. The obtained pre-formulations were stored at 4 °C prior to use.

#### 2.3. Modulate temperature differential scanning calorimetry (mDSC)

The PLGA was analyzed using a Discovery DSC (TA Instruments, New York, USA).

### Table 1

<table>
<thead>
<tr>
<th>Pre-formulation</th>
<th>Solvent (mg)</th>
<th>LA (mg)</th>
<th>PLGA (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>NMP, 690</td>
<td>30</td>
<td>280</td>
</tr>
<tr>
<td>F2</td>
<td>NMP, 690</td>
<td>50</td>
<td>260</td>
</tr>
<tr>
<td>F3</td>
<td>DMSO, 690</td>
<td>30</td>
<td>280</td>
</tr>
<tr>
<td>F4</td>
<td>Triacetin, 690</td>
<td>30</td>
<td>280</td>
</tr>
</tbody>
</table>

DMSO, dimethyl sulfoxide; LA, leuprolide acetate; NMP, N-methyl-2-pyrrolidinone; PLGA, poly (D, L-lactide-co-glycolide).
heated from Z. Li et al. (version 2.0.60624, Paraytec Ltd., York, UK). A 52.4.3. Image analysis were examined to determine the glass transition temperature (Tg) (midpoint of the reversing heat flow signal) using Trios software (TA Instruments, New Castle, USA). The measurements were conducted in triplicate and the results were reported as mean ± standard deviation (SD).

2.4. In vitro release studies

2.4.1. Drug release in gel-based release testing system

In vitro release studies were conducted in 10 mm-light path cuvettes made of Quartz SUPRASIL® (type No. 100-QS, Helima GmbH & Co. KG, Müllheim, Germany) filled with agarose gel as a diffusion barrier and PBS as receiver solution (Figure 1 and S1), resembling the previously reported procedures (Leung et al., 2017; U. Gietz et al., 1998). Briefly, agarose gel (0.5% w/v) was prepared by suspending 250 mg of agarose in 50 ml of PBS. The suspension was incubated at 95 °C for 30 min allowing the agarose to dissolve. Hot agarose solution (300 μl) was added into the cuvette and allowed to cool until the temperature was around 40 °C, which was below the glass transition temperature (Tg) of the PLGA. The pre-formulation (100 μl) was injected into the center of the agarose solution/gel using a automate pipette, while the quartz cuvettes were weighed before and after the injection allowing determination of the administered amount of LA. An additional agarose layer was subsequently added by pipetting 300 μl of agarose solution on top of the existing layer. Upon 30 min until complete gelation of the agarose, 2.00 ml of PBS as the receiver solution was added to the cuvette on the top of the gel layer. The cuvettes were sealed and placed in an incubating hood (TH 30, Edmund Bühler GmbH, Bodelshausen, Germany) at 37.0 °C. Prior to measurement of the released LA, the samples were gently shaken to attain a homogenous LA containing receiver medium. An Actipix D200 UV–Vis imaging system having a 25 × 25 mm² (H × W) CMOS chip with an effective pixel size of 13.75 × 13.75 μm² (Paraytec Ltd., York, UK) at 280 nm was used to quantify the released LA based on the calibration curve (Figure S2). The receiver PBS solution was withdrawn and replenished with fresh PBS at the selected time points (Figure S3). The time intervals for PBS replenishment varied between formulations to keep the absorbance of LA within the linear range of the calibration curve (Leung et al., 2017). Samples were protected from exposure to light during storage.

2.4.2. Drug release in PBS system

One hundred microliters of LA pre-formulation was transferred into the cuvette followed by addition of 3.00 ml PBS (0.01 M, pH 7.4) at 37.0 °C. At selected time intervals, the samples were gently shaken and analyzed using the D200 imaging system. The PBS receiver solution was withdrawn and replenished with fresh PBS as mentioned above (n = 3).

2.4.3. Image analysis

Imaging files were analyzed using the SDI Data Analysis software (version 2.0.60624, Paraytec Ltd., York, UK). A 5 × 5 mm² measuring zone was utilized to obtain the average absorbance. The conversion of absorbance values to drug concentrations was performed based on the calibration curve (Figure S2).

The cross-sectional area of the in situ forming implants was evaluated from the Vis images as described previously (Li et al., 2020). Briefly, the grayscale values were defined as the absorption of the formed implants using Image J and the number of pixels with a grayscale value > 50 (n) was determined using MATLAB. The following equation was used for the calculation of the cross-sectional area of the implants (Acc):

\[ A_{cs} = n \times A_p \]  \hspace{1cm} (1)

where \( A_p \) (13.75 × 13.75 μm²) is the effective pixel area of the UV–Vis imaging camera chip.

2.4.4. Data analysis

For comparison of real-time and accelerated release profiles, the in vitro release data were modeled using non-linear regression analysis in Excel. Apparent release rate constants for estimation of the energy of activation were determined by linear regression analysis of selected data points from the release profiles during the initial, lag and zero-order release phases.

2.5. Microenvironmental pH (pHM) imaging of the implants

The local pH during accelerated release testing at 50 °C in the gel-based release system was monitored employing the release medium containing 1.34 × 10⁻⁴ M bromothymol blue in PBS. Hot agarose solution (300 μl) was added to the 10 mm light path quartz cuvette and a tube with 5.4 mm in diameter was placed into the center of the solution in the upright position. After 30 min, the tube was removed and a gel with a cylindrical reservoir perpendicular to the light path was formed. An Agilent Cary 60 UV–Vis spectrometer (Santa Clara, California, US). The samples were protected from exposure to light during storage.

2.6. Quantification of leuprolide acetate by HPLC

The LA containing bromothymol blue solution was analyzed by HPLC using an isocratic method. The LaChrom Elite HPLC system (VWR International, Tokyo, Japan) consisted of a pump L-2130, a column oven L-2300, a diode array detector L-2450, and an autosampler L-2200. A reverse phase column (Gemini, C18, 150 × 4.6 mm, Phenomenex, Torrance, CA) with a Phenomenex Gemini C18 guard column was employed for the LA analysis. The injection volume was 10 μl and the flow rate was 1 ml/min. The column oven was set at 30 °C. The retention time of LA was 3.3 min and detection was performed at 240 nm. The eluent consisted of 30% (v/v) acetonitrile and 70% (v/v) deionized water containing 0.1% TFA. The LA concentration in the samples was calculated from peak areas using a calibration curve (2.5, 5, 10, 50, 100, 250, and 500 μM LA) constructed in PBS.

2.7. Statistical analysis

A one-way ANOVA test was used to determine statistically significant differences (p < 0.05) between experimental groups conducted using GraphPad Prism 8. Data are expressed as mean ± SD (standard deviation).
3. Results and discussion

In this study, a drug release testing system consisting of an agarose gel as the injection site and PBS above the gel layer as receiver phase was used to mimic the in vivo situation after subcutaneous administration of ISFIs. LA diffused through the agarose gel layer upon release from the PLGA based implant into the PBS receiver solution (Fig. 1). The solubility of LA in water at 37 °C has been estimated to >400 mg/ml (Stevenson, 2008). By visual inspection, we found that the solubility of leuprolide acetate in phosphate buffer, pH 7.4, at room temperature was ≥10 mg/ml. In case all LA was released, the LA concentration in the PBS receiver solution would be maximum 2.5 mg/ml (in case of 100 μl preformulation F2, see Table 1). Thus, sink condition would be maintained in the receiver phase throughout the release testing experiment without replacement of PBS. To be within the linear range of the LA calibration curve (Figure S2), i.e., ≤1.2 AU (absorbance unit) corresponding to ≤250 μM (317 μg/ml) of LA at 280 nm, the PBS was exchanged at selected time points as shown in Figure S3.

The D200 UV–Vis imaging system was used in this study rather than a UV–Vis spectrophotometer applied in the previous studies by Leung et al. (2017). It served as a UV–Vis spectrophotometer when measuring the LA concentration in the receiver solution at 280 nm. The imaging functionality was exploited to visualize swelling during in situ implant formation and local pH distribution in the vicinity of the in situ formed implants at 525 nm.

3.1. In vitro release testing of the leuprolide acetate in situ forming implants

The implants released all LA over a period of ~50 days in the gel-based release testing system (Fig. 2A) and ~40 days in the PBS release testing system (Fig. 2B) with the same rank ordering of the four formulations. All the formulations exhibited triphasic release characteristics with an initial burst release, a lag phase eventually followed by an apparent zero-order release phase. The release profiles obtained using the gel-based system were characterized by good repeatability with <6% relative standard deviation at all the time points. The method was able to discriminate between the formulations comprising the different organic solvents. In contrast, a relatively poor discriminatory ability was observed when LA released from formulations F2 and F4 in the PBS release testing system (Fig. 2B).

Differences with respect to the initial release phase were observed for the formulations. For formulations F1, F2, and F3, burst release terminated at day 4 in the in vitro gel-based release testing system. Here, the amount of LA released corresponded to 60%, 38% and 52%, respectively. Formulation F4 had a shorter initial burst, which lasted for approximately 2 days, and released 13% of the LA in the in vitro gel-based release testing system during this initial phase. In the PBS release testing system, the duration of the burst release phase was unchanged for formulations F1, F2, and F3 but was increased for formulation F4 (4 days). Formulations F1, F2, F3 and F4 released 61%, 42%, 49% and 40% of LA, respectively, during the burst release phase. More drug was released from the NMP and DMSO containing ISFIs (formulations F1, F2 and F3) than that from the triacetin containing ISFIs (formulation F4) during the initial burst, which was consistent with existing literature (Ahmed et al., 2014; Zhang et al., 2015).

Apparently, the use of an agarose gel layer reduced initial LA release. Vis imaging at 525 nm was utilized to get a simple estimate of implant swelling through measurement of changes in cross-sectional area (Fig. 3). The in situ forming implants exhibited different water absorption in agarose gel and PBS as estimated from different increases in cross-sectional area. Within the first two weeks, increments of the cross-sectional area were ~25 mm² for formulations F1, F2 and F3, and ~12 mm² for formulation F4 in the PBS release testing system, which were higher than the cross-sectional area increases observed in the gel-based release testing system (~18, ~16, ~15 and ~10 mm² for formulations F1, F2, F3 and F4, respectively). Previous studies by McHugh and co-

Fig. 1. Schematic representation of the in vitro release testing system showing agarose gel encapsulating a leuprolide acetate loaded in situ forming implant and PBS receiver solution in a quartz cuvette.

Fig. 2. Leuprolide acetate release from the PLGA based in situ forming implants at 37 °C in the in vitro gel-based (A) and PBS (0.01 M, pH 7.4) (B) release testing systems. The solid lines represent zero-order fits of the post-lag release phase. Data are mean ± SD (n = 3).
workers showed that a relatively high increase in water uptake was associated with rapid phase separation and high initial drug (lysozyme) release in vitro (Brodbeck et al., 1999; Graham et al., 1999). Compared to PBS, the agarose gel may lead to a larger mechanical force exerted on the formulation, limiting implant water uptake and lowering burst release (Kozak et al., 2021; Li et al., 2020; Patel et al., 2010).

In addition to the initial release, differences with respect to the LA lag and zero-order release phases were observed between the two release testing approaches. Fig. 3 shows a remarkable decrease in the cross-sectional area in the PBS release testing system for formulation F1, F2 and F3 after ~18, ~14 and ~24 days, while a gradual reduction in the cross-sectional area was observed in the gel-based release testing
system. This indicated that a faster erosion of the ISFIs containing NMP and DMSO occurred in the PBS release testing system than in the gel-based release testing system. However, for formulation F4, the triacetin containing ISFI, no significant differences (p > 0.2) were found with respect to the implant erosion behavior based on the comparison of the cross-sectional changes in the two release testing systems. The calculated zero-order release rates for the post-lag release phase (Fig. 2) formulations F1, F2, F3 and F4 were 1.5, 2.0, 1.5 and 4.2 %/day in the gel-based release testing system, and 6.1, 5.9, 2.7 and 4.4 %/day in the PBS release testing system, respectively. Thus, application of PBS as release medium lead to pronounced increases in the rate constants for the formulations incorporating the completely water miscible organic solvents (NMP and DMSO) as compared to the gel-based release testing system. The results suggested that the NMP and DMSO implants were more sensitive to alteration in the aqueous release medium than the system. The solid lines represent zero-order fits of the post-lag release phase.

In terms of the commercial LA product, Eligard® 7.5 mg, the initial (first 3 days) serum exposure of LA accounted for 40–50% of the total LA exposure (AUCburst phase/AUCTotal dosing interval) after injection (Sanofi-Aventis Canada Inc., 2018). Eligard® 7.5 mg comprises 3% (w/w) LA, 33% (w/w) PLGA with the lactide to glycolide ratio of 50:50 and 64% (w/w) NMP. Formulation F1 resembled the Eligard® 7.5 mg with respect to cosolvent content and LA concentration (Table 1). It is interesting to note that at day 3, the amount of LA released from formulation F1 was 53% ± 0.8% and 60% ± 2.2% using the gel-based and the PBS release testing system, respectively. These percentages of LA released were found to be statistically significantly different (p-value of < 0.01), with the former being closer to the in vivo data.

3.2. Selection of elevated temperatures for accelerated release testing

Accelerated drug release is commonly achieved by elevating temperature to increase molecular mobility, polymer degradation and drug diffusion (Mohammed Shameem et al., 1999; Zolnik et al., 2006). PLGA polymer with a molar ratio of lactide: glycolide: 75:25 and 5 – 20 kDa MW was utilized for preparing the pre-formulations. The glass transition temperature (Tg) of the polymer was determined to 46.8 ± 0.4 °C using mDSC (Figure S4). However, the Tg of PLGA has been reported to decrease significantly during release testing due to the plasticizing effect of water (Kozak et al., 2021). Release studies were conducted at 50, 55 and 60 °C to accelerate LA release from the four ISFIs in the gel-based release testing system.

The shape of the profiles of drug release under the accelerated conditions was overall similar to those obtained at 37 °C as they exhibited a triphasic behavior; burst release followed by slow release, and finally a second rapid release (Fig. 4). The amounts of LA released were significantly (p < 0.0001) higher at each time point at the elevated temperatures as compared to the corresponding real-time studies at 37 °C. The time to complete drug release from the four formulations was reduced from ~50 days at 37 °C to ~14 days, ~7 days and ~7 days at 50, 55 and 60 °C, respectively, which represented a 4 to 7 – fold reduction in the time required for complete release. However, the discriminatory ability of the in vitro gel-based release testing system appears to decrease with increasing temperature (Fig. 2A and Fig. 4). First, the effect of increasing temperature on the release rate constants calculated from the apparent zero-order release phase of the release profiles (% cumulative LA release versus time as indicated in Fig. 4) were compared. For formulations F1, F2, F3 and F4, the obtained rate constants were 8.5, 8.3, 7.9 and 10.3 %/day at 50 °C, 9.9, 9.7, 8.9 and 14.6 %/day at 55 °C, and 16.7, 14.9, 14.6 and 17.3 %/day at 60 °C, respectively. The release of LA from the four formulations increased with the increase in temperature.

Under the ideal accelerating conditions, the release mechanism remains unchanged with establishment of a 1:1 correlation between real-time and accelerated release data (Martinez et al., 2008; Nkanga et al., 2020; Shen and Burgess, 2012a). In order to investigate whether the release profiles obtained at the elevated temperatures can be used to

Fig. 4. Leuprolide acetate release from the prepared in situ forming implants at 50 °C (A), 55 °C (B) and 60 °C (C) in the in vitro gel-based release testing system. The solid lines represent zero-order fits of the post-lag release phase. Data are mean ± SD (n = 3).
predict real-time release, an Arrhenius relationship between the real-time zero-order release rate constants and the corresponding accelerated release rate constants was investigated for the four formulations. The rate constants were plotted (Fig. 5) according to the Arrhenius equation,

$$\ln k = \ln A - \frac{E_a}{R} \times \frac{1}{T}$$  \hspace{1cm} (2)

where $k$ is the zero-order release rate constant, $A$ is the Arrhenius factor, $E_a$ is the energy of activation, $R$ is the gas constant and $T$ is the absolute temperature.

The phenomenological activation energies for the LA zero-order release phase for formulations F1, F2, F3 and F4, as calculated from the slopes in Fig. 5, were 83.3, 76.0, 78.1 and 46.3 kJ mol$^{-1}$, respectively. The activation energy is associated with several parameters such as formulation composition, polymer properties and dissolution conditions (Jalil and Nixon, 2008). In this study, the formulations used the same polymer and release method, but different organic solvents or different drug to polymer ratios, resulting in varying phenomenological $E_a$ values. The $E_a$ values for formulation F1, F2 and F3 were close to previously reported $E_a$ value (98.8 kJ mol$^{-1}$) for PLGA degradation (Shen and Burgess, 2012b), indicating that the drug release of the zero-order release phase was driven by PLGA polymer erosion for formulations F1, F2 and F3. Different $E_a$ values represent different release mechanisms (Zolnik et al., 2006). By comparison, the lower $E_a$ value found for formulation F4 might be attributed to an alteration of the release mechanism.

The Arrhenius plots (Fig. 5) obtained by fitting the experimental data at 50, 55 and 60 °C to Eq. (2) were further used to predict the zero-order rate constants of the real-time release experiments at 37 °C. Thus, the predicted zero-order rate constants ($k$) for formulations F1, F2, F3 and F4 were 1.8, 2.2, 1.8 and 5.1 %/day at 37 °C, respectively, which were relatively close to the experimental values obtained (1.5, 2.0, 1.5 and 4.2 %/day for F1, F2, F3 and F4, respectively). The similarity between the predicted and experimental values indicated that the elevated temperatures appeared to be appropriate for the formulations. The temperature of 50 °C was selected for the subsequent investigation because the in vitro gel-based release system appeared to have a better discriminatory ability at this temperature than at 55 or 60 °C.

At the accelerated release testing, only the zero-order release was assessed using the Arrhenius equation in the study. The increase in temperature obviously shortened the lag phases of the four formulations (Fig. 2A and 4) and attempts to predict real-time release for the lag phase resulted in large deviations. The predicted apparent zero-order rate constants ($k$) for the initial phase of formulations F1, F2, F3 and F4 were 18, 11, 8.0 and 7.6 %/day at 37 °C, respectively, while the experimental values were 12, 7.9, 10 and 7.1 %/day, respectively. The predicted rate constants for the lag phase of formulations F1, F2, F3 and F4 were 0.88, 0.32, 2.1 and 0.30 %/day at 37 °C, respectively, while the
experimental values measured were 0.96, 0.80, 0.48 and 0.52 %/day, respectively. Apparently, testing at elevated temperature was also unable to give an accurate prediction of the burst release phase. Variable release mechanisms of ISFIs during the initial and lag phases might be the main reason for the poor prediction. A previous study on drug release from PLGA microspheres reported two competing factors at elevated temperature, i.e., increased polymer mobility giving rise to surface morphological changes eventually leading to decreased drug release and enhanced drug diffusivity and polymer degradation leading to increased drug release (Zolnik et al., 2006). These phenomena might also occur for the PLGA ISFIs, however, further studies in this area are needed.

3.3. Real-time and accelerated release studies

Fig. 6 shows a comparison between the accelerated (at 50 °C) and real-time (at 37 °C) release profiles of the four formulations. A high degree of similarity between the accelerated release profiles and real-time release profiles for formulations F1, F2 and F3 was found, suggesting that the drug release mechanism might be the same at both temperatures. However, a significant increase in drug release from formulation F4 was observed within the initial and lag phases at 50 °C as compared to that at 37 °C (Fig. 6).

The degree of correlation between the accelerated (50 °C) and real-time (37 °C) release profiles after time scaling was assessed in Fig. 7A-D. The applied scaling factors for formulations F1, F2, F3 and F4 were 4, 4.4, 4.4 and 3.4, respectively, which were determined based on Fig. 6. The correlation coefficients (R^2) obtained were 0.997, 0.970, 0.970 and 0.948 for formulations F1, F2, F3 and F4, respectively. The correlations for formulations F1, F2 and F3 were considered acceptable. Accelerated release testing for formulation F4 displayed a relatively poor prediction for real-time release. In Fig. 7E, the points for all formulations are distributed around the reference line with the equation y = x, which was regarded as an ideal correlation. Deviations from the ideal correlation are observed for all formulations, in particular to the burst release phase. The cause of poor correlation obtained for formulation F4 comprising triacetin is not obvious. It appears that more work focusing on the initial and lag phases when conducting accelerated tests is required for attaining adequate prediction of drug release from slow forming phase inversion based ISFIs. It has previously been suggested that the initial burst can be determined under real-time conditions and accelerated drug release studies should be conducted for the lag and the zero-order release phases (Burgess et al., 2004).

3.4. Microenvironmental pH (pH_{M}) measurements

To gain insights into the changes in the microenvironmental pH (pH_{M}) during drug release, the pre-formulations were injected into an agarose gel containing 1.34 × 10^{-4} M bromothymol blue in the gel-based release system followed by monitoring the absorbance of the surrounding matrix at 525 nm. Unfortunately, upon direct injection of the pre-formulation into the agarose gel, the interface between the agarose
gel and the implants was difficult to identify at 525 nm (Figure S5). A cylindrical reservoir in the agarose gel was made to control the implant geometry and facilitate identification of the implant-gel interface. The influence of the cylindrical reservoir on the in vitro behavior of the four formulations was evaluated by comparison of their release profiles. The presence of bromothymol blue impeded the use of UV imaging for measuring the LA release due to the UV absorption of bromothymol blue at 280 nm. Hence, LA quantification in the PBS receiver solution was done by HPLC. Comparison of the release data obtained from the implants in a confined geometry (Figure S6) with their accelerated release profiles (Fig. 4A) at 50 °C is presented in Figure S7. Slight differences were found in terms of the release profiles, which might be attributed to the shape of the formed implants and/or the presence of the pH indicator.

Fig. 8A and B visualize the local apparent pH distribution in the vicinity of formulations F1, F2, F3 and F4 by color. Due to the non-
transparency of PLGA based depots, UV–Vis imaging was not capable of capturing the pH change inside the formed implants. At 525 nm, the changes in absorbance reflect changes in pH since the organic solvents did not absorb light at this wavelength. The absorbance-pH profile (Figure S8) for bromothymol blue was determined as described previously (Østergaard et al., 2014). Using the obtained absorbance-pH profile, the pHM estimation was performed by measuring the absorbance of three regions at the surface of the implants at 525 nm. Bromothymol blue was found to be sufficiently stable over 3 weeks at pH 4.2 and 7.4 at 37 and 55 °C to be used as pH indicator (Figure S9).

The time dependency of the estimated pHM for all formulations at the three zones, marked in Fig. 8A (at F1 at 0 day), are shown in Fig. 9. Significant differences with respect to the measured pH were not found between the three zones. A pH drop was observed for all formulations within the first day, followed by an increase to around pH 7.4 at the end of the experiments. This pH drop has been ascribed to the accumulation of acidic degradation products in the implants followed by their diffusion out of the formed implants when pore formation increased during implant formation (Schadlich et al., 2014; Steppmann et al., 2005). Formulations F1, F2 and F3 exhibited similar pHM characteristics, i.e., a slight decrease in pH from 7.4 to around 6.7 within the first 12 h. However, the local apparent pH at the surface of formulation F4 decreased below pH 5.5 at 12 h.

The pHM measurements were conducted at the elevated temperature. Based on Fig. 6, it is tempting to speculate that the pH minimum at approximately 12 h in the accelerated test may correspond to days 4–6 in real-time release testing as this would be consistent with previous reports on internal pH changes of PLGA based ISFIs in vitro (Schadlich et al., 2014). A more pronounced drop in pH was found for formulation F4 than the other formulations, indicating that an acceleration in polymer degradation occurred and a greater number of acidic products accumulated in the vicinity of the implant. Triacetin, the short-chain triglyceride of acetate, generates acetic acid during its hydrolysis. For a formulation like F4 comprising triacetin as the solvent, acids generated from the hydrolysis reaction of both PLGA and triacetin are able to accelerate the hydrolysis of the polymer (Zhang et al., 2019). Zhang et al. compared PLGA molecular weight loss and found an acceleration in polymer degradation when using triacetin as the organic solvent of in situ forming implants as compared to formulations comprising NMP and DMSO (Zhang et al., 2019). A previous study reported that the pH inside PLGA in situ forming implants decreased below pH 3 as measured by EPR spectroscopy (Schadlich et al., 2014), which was lower than the pHM reduction measured in the current study. This might be associated with the diffusion of the generated acidic products into the release media.

4. Conclusions

The in vitro real-time release of leuprolide acetate containing PLGA in situ forming implants using different organic solvents was characterized using a gel-based and a PBS release testing method at 37 °C. The gel-based release testing system displayed a better discriminatory ability in terms of the in vitro behavior of the in situ forming implants obtained as compared to the PBS release testing system. The swelling behavior of the formed implants in the two release testing approaches was different, i.e., a more extensive swelling was found in the PBS release system as compared to that in the gel-based release system. The in vitro behavior of the four ISFIs was investigated in the gel-based release testing system at elevated temperatures. Similarity between the predicted zero-order release rates of the post-lag release phase obtained from Arrhenius plots and the experimental values indicated that the release mechanisms of the formulations at the selected temperatures were consistent. A good correlation between accelerated and real-time release data was only found for the implants incorporating NMP and DMSO. Compared with the NMP and DMSO containing formulations, a more pronounced pH decrease was observed at the surface of the triacetin containing formulation during the initial phase. This might be attributed to increased hydrolysis rates of the PLGA due to the catalytic effects of triacetin derived hydrolysis products. The application of UV imaging in the characterization of long-term behavior of formulations, so far, has been limited by the need for referencing measurements and the large imaging files obtained. This study, for the first time, used the in vitro gel-based release testing approach in combination with UV–Vis imaging to obtain and compare the entire in vitro behavior of long-acting injectables over a period of months. The feasibility of the in vitro gel-based release testing system for long-term characterization and prediction of the in vitro performance in relation to peptide containing in situ forming
implants at elevated temperatures was demonstrated. In combination with UV–Vis imaging, in situ assessments of drug release, implant swelling and erosion, and the local pH change for extended-release injectable formulations can be achieved in a non-destructive manner.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Zhuoxuan Li reports financial support was provided by China Scholarship Council.].

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Appendix A. Supplementary data

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References


Fig. 9. Time dependent pH change obtained on the top (A), left (B) and right (C) zones at the surface of all the formulations using UV–Vis imaging measuring at 525 nm. The measurements were performed at 50 °C (n = 3, mean ± SD).


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