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Formulation and characterization of microspheres loaded with imatinib for sustained delivery

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ABSTRACT

The aim of this study was the development of imatinib-loaded poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres with high loading efficiency which can afford continuous release of imatinib over a prolonged period of time. Imatinib mesylate loaded PLGA microspheres with a size of 6–20 μm were prepared by a double emulsion ($W_1/O/W_2$) method using dichloromethane as volatile solvent. It was found that the microspheres were spherical with a non-porous surface; imatinib loading efficiency (LE) was highly dependent on the pH of the external water phase (W_2). By increasing the pH of W_2 phase above the highest pK_a of imatinib (pK_a 8.1), at which imatinib is mainly uncharged, the LE increased from 10% to 90% (pH 5.0 versus pH 9.0). Conversely, only 4% of its counter ion, mesylate, was retained in the microspheres at the same condition (pH 9.0). Since mesylate is highly water soluble, it is unlikely that it partitions into the organic phase.

We demonstrated, using differential scanning calorimetry (DSC), that imatinib was molecularly dispersed in the polymeric matrix at loadings up to 8.0%. At higher drug loading, imatinib partially crystallized in the matrix. Imatinib microspheres released their cargo during three months by a combination of diffusion through the polymer matrix and polymer erosion.

In conclusion, we have formulated imatinib microspheres with high LE and LC. Although we started with a double emulsion of imatinib mesylate, the obtained microspheres contained imatinib base which was mainly molecularly dispersed in the polymer matrix. These microspheres release imatinib over a 3-month period which is of interest for local treatment of cancer.

1. Introduction

Protein tyrosine kinases (PTKs) contribute in signal transduction pathways that regulate various cellular processes such as growth, metabolism, differentiation, adhesion and apoptosis. Deregulation of PTK activity has been associated with the pathogenesis of various cancers as well as other inflammatory diseases (Chute and Himburg, 2013; Dolman et al., 2012; Falke et al., 2015; Wallace and Gewin, 2013). Imatinib is a multi-targeted tyrosine kinase inhibitor (TKI) that is used as molecularly targeted therapy in different types of cancer. It acts through competitive inhibition of the ATP binding site of the PTKs, thus blocking autophosphorylation and subsequent intracellular signal transduction (Govindarajan et al., 2012; Peng et al., 2005; Ruan et al., 2013). Imatinib is now the standard treatment for patients with chronic myeloid leukemia as well as gastrointestinal stromal tumors. It is marketed as Gleevec® which is a film-coated tablet formulation that contains imatinib mesylate equivalent to 100 or 400 mg of imatinib free base for oral administration (Henkes et al., 2008).

Recently, a new way of administration of imatinib has been described in which imatinib-loaded polymeric microspheres were injected in close proximity to the tumor (Benny et al., 2009; Karal-Yilmaz et al., 2013). Benny et al. evaluated the local

Abbreviations: PLGA, poly(D,L-lactide-*co*-glycolide); M_w, molecular weight; RO, reverse osmosis; $W_1/O/W_2$, water in oil in water; W_1, inner water phase; W_2, outer water phase; LE, loading efficiency; LC, loading capacity; T_g, glass transition temperature; T_m, melting temperature; SEM, scanning electron microscopy; TGA, thermal gravimetric analysis; DSC, differential scanning calorimetry; DCM, dichloromethane; PVA, polyvinylalcohol; THF, tetrahydrofuran; PBS, phosphate buffered saline; TL, theoretical loading; GPC, gel permeation chromatography.

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tumor inhibition of imatinib-loaded PLGA microspheres in a glioblastoma xenograft mice model (Benny et al., 2009). A single dose of imatinib-loaded microspheres corresponding to 1.5 mg imatinib was injected intracranially at the site of the tumor and resulted in a 79% reduction in the tumor volume 14 days post injection. In another study of Karal-Yilmaz et al., imatinib-loaded PLGA microspheres were studied for their inhibition of angiogenesis in craniopharyngioma (a type of brain tumor) (Karal-Yilmaz et al., 2013). A more recent publication of the same group reported on the development of imatinib-loaded polystyrene-*g*-poly(lactide-*co*-glycolide) microspheres for local sustained delivery of imatinib (Kukut et al., 2014).

These studies clearly show that imatinib-loaded PLGA microspheres hold potential for local inhibition of cancer. However, the physicochemical properties of these microspheres have not been studied well. For example, an ideal microparticles formulation should have reasonably high drug loading efficiency (LE), loading capacity (LC), and sustained (preferably zero-order) release of the loaded drug for desired period of time (Ye et al., 2010). The high LE and LC are critical, especially for expensive and less potent drugs. Imatinib is one of the exceptionally expensive cancer drugs for the treatment of chronic myeloid leukemia (Experts in Chronic Myeloid Leukemia, 2013). Therefore, decreasing the drug loss during the formulation and improving its LE is of great importance. In the previous studies, maximum LE and LC which was achieved for imatinib-loaded PLGA microspheres was about 57% and 0.23%, respectively. In addition to the low LE and LC, the initial drug release (burst) was rather high in the previous studies (Benny et al., 2009; Karal-Yilmaz et al., 2013). The present study therefore aims at developing an efficient procedure for loading imatinib into PLGA microspheres and to study the effect of formulation parameters such as pH of external water phase, volume of inner water phase, and theoretical drug loading on microsphere characteristics for developing microspheres with sustained (preferably zero-order) release of imatinib.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA, 50:50 lactide:glycolide ratio, end-capped, intrinsic viscosity 0.4 dL/g) was obtained from Purac, the Netherlands. Imatinib mesylate was obtained from LC Laboratories, USA. Polyvinyl alcohol (PVA; M_w 30,000–70,000; 88% hydrolyzed) was obtained from Sigma–Aldrich, Germany. Dichloromethane (DCM), acetonitrile and tetrahydrofurane (THF) were purchased from Biosolve (Valkenswaard, The Netherlands).

2.2. Preparation of imatinib-loaded microspheres

Imatinib-loaded microspheres were prepared using a double emulsion solvent evaporation technique ($W_1/O/W_2$) as described in literature (Benny et al., 2009). Briefly, inner water phase (1 mg imatinib mesylate in 250 μl reversed osmosis (RO) water) was added to 500 μl of DCM in which 200 mg PLGA was dissolved. The inner water phase (W_1) was emulsified into the polymer solution using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 s at maximum speed (30,000 rpm). This primary emulsion (W_1/O) was subsequently emulsified at maximum speed (30,000 rpm) for 30 s in 1 ml buffer with different pH (pH 5.0 [0.25 M sodium acetate buffer], pH 7.0 [0.25 M sodium potassium phosphate buffer] and pH 9.0 [0.25 M Tris hydrochloride buffer]) also containing 1% PVA. The obtained $W_1/O/W_2$ emulsion was then transferred into 40 ml of the same PVA-containing buffer and stirred for 3 h using a magnetic stirrer (500 rpm, 3 h) at room temperature (formulations, F1–F3, Table 1). For other formulations, the primary emulsion (W_1/O) was emulsified in 6 ml of Tris buffer pH 9.0 PVA 1% as external water phase. After 40 min, the emulsion was transferred into 44 ml of 1% PVA phosphate buffer (pH 7.0) under magnetic stirrer (500 rpm, 3 h). The formed microspheres were centrifuged (Laboratory Centrifuge, 4K 15, Germany) at 4000 × *g* for 3 min and washed 2 times with 100 ml tween 20 (0.025%) followed by two times washing with 100 ml RO water and lyophilized. The obtained microspheres were stored at −25 °C. Besides the pH of W_2, variables in this study were the volume of W_1 ranging from 5 to 350 μl and theoretical drug loading (weight of initial drug/weight of both drug and polymer × 100) ranging from 0.4% to 19% w/w. All the microspheres batches were prepared in triplicate.

2.3. Preparation of imatinib free base

Imatinib free base was prepared as follows: about 100 mg imatinib mesylate was added to 1 ml of pH 9.0 Tris buffer and the solution was vortexed for 10 min. The precipitated imatinib base was separated from mesylate salt by centrifugation (13,000 rpm, 5 min) and washing with 3 ml RO water. The imatinib base (precipitant) was then freeze dried overnight and stored at room temperature.

2.4. Characterization of the microspheres

The average size and size distribution of the microspheres were measured using an Accusizer 780 (Optical Particle Sizer, Santa Barbara, California, USA). The volume weighted mean diameter (vol–wt mean) of microspheres is reported as particle size and the

Table 1
Characteristics of imatinib-loaded PLGA microspheres. Microspheres were prepared using external W_2 phases of different pH values. The concentration of PLGA in DCM was 23% (w/w) and the internal W_1 volume was 20% in all formulations. Data are expressed as mean ± SD (n = 3).

Formulation[a]	W_2 (pH)	TL[b] (wt%)	Recovery[c] (%)	Particle size[d] (μm)	Span[e] value	Imatinib LC[f] (%)	Imatinib LE[g] (%)
F1	5.0	0.41	63 ± 2	20 ± 2	1.4 ± 0.1	0.042 ± 0.006	10 ± 2
F2	7.0	0.41	67 ± 3	20 ± 2	1.2 ± 0.1	0.226 ± 0.007	54 ± 2
F3	9.0	0.41	67 ± 4	22 ± 1	1.2 ± 0.2	0.378 ± 0.002	90 ± 4
F4	9.0[a]	0.41	72 ± 3	16 ± 1	1.4 ± 0.2	0.378 ± 0.006	90 ± 2
F5	9.0[a]	9.50	60 ± 4	6 ± 1	1.4 ± 0.1	8.1 ± 0.7	86 ± 7
F6	9.0[a]	19.0	59 ± 4	9 ± 1	1.6 ± 0.2	16.0 ± 0.4	84 ± 3

Mean ± SD values were calculated from the data of three independent batches and represent reproducibility between batches.
[a] The microspheres F4–F6 were prepared with the external water phase of pH 9.0 for 40 min and subsequently transferred to pH 7.
[b] TL: theoretical drug loading.
[c] Recovery of microspheres as percentage of drug and polymer starting weight.
[d] Particle size expressed as volume weighted mean diameter.
[e] Span value = (d90 − d10)/d50 which reflects the polydispersity within an individual batch.
[f] LC: loading capacity of imatinib.
[g] LE loading efficiency of imatinib expressed as free base.

span value (SP) was calculated with the following formula: SP = (d90 − d10)/d50, where d90 is the particle diameter at 90% cumulative size, d10 is the particle diameter at 10% cumulative size and d50 is the particle diameter at 50% cumulative size. The size distribution is considered as narrow for span values <0.45 (Vladisavljević and Williams, 2005). The morphology of the microspheres was studied by scanning electron microscopy (Phenom™, FEI Company, The Netherlands). Microspheres were glued on 12 mm diameter aluminum sample holder using conductive carbon paint (Agar Scientific Ltd., England) and coated with palladium under vacuum using an ion coater.

The imatinib content of microspheres was investigated by dissolving 5 mg of drug-loaded microspheres in acetonitrile and measuring the absorbance at 266 nm using a UV–vis spectrophotometer (Shimadzu UV-2450). Calibration was done with imatinib dissolved in acetonitrile (concentration ranging from 5 to 40 μg/ml). Loading capacity (LC) was expressed as the encapsulated amount of imatinib divided by the total dry weight of the microspheres. Loading efficiency (LE) of imatinib in microspheres was reported as the encapsulated drug divided by the total amount of drug used for encapsulation. The microspheres recovery was calculated as percentage of the weight of the obtained product divided by the initial weight of the solid materials.

The mesylate content of the microspheres was derived from the sulfur content as determined by the Schöniger oxidation method (Schöniger, 1958). Briefly, samples were burned in a platinum-coated flask. After combustion of the samples in pure oxygen, sulfate was produced which was subsequently detected by ion chromatography (Metrohm® 883 Basic IC). Sulfanilic acid was used as standard and the method was validated with non-formulated imatinib mesylate.

Thermal gravimetric analysis (TGA) was performed using a Q Series™ (Q50) instruments to determine the percentage of residual organic solvents. Approximately 10 mg of freeze-dried microspheres were loaded into aluminum pans and heated from 0 °C to 230 °C at a heating rate of 2 °C/min.

Differential scanning calorimetry (DSC) analysis was done using a Q Series™ (Q2000) DSC, USA. Approximately 5 mg of freeze-dried microspheres were loaded into aluminum pans and heated in a single heating cycle from 0 °C to 230 °C at a heating rate of 2 °C/min and temperature modulation ±1 °C/30s. The melting temperature (T_m) was determined from the total heat flow and the glass transition temperature (T_g) was determined from the reverse heat flow. Imatinib mesylate and imatinib free base were exposed to a three cycle heating/cooling protocol. Briefly, the samples were heated from 0 °C to 230 °C with the heating rate of 2 °C/min. Next, the molten materials were rapidly cooled down to 0 °C with the rate of 100 °C/min. After being isothermal for 5 min, the samples were heated again with the heating rate of 2 °C/min and temperature modulation ±1 °C/30s. The T_m was determined from the first heating ramp and the glass transition temperature (T_g) was determined from the second heating ramp.

2.5. In vitro degradation studies

Samples of freeze dried imatinib-loaded microspheres (~10 mg) were transferred into Eppendorf tubes and dissolved in 1.5 ml of PBS (pH 7.4, 0.033 M NaH_2PO4, 0.066 M Na_2HPO_4, 0.056 M NaCl) supplemented with 0.05% (w/w) NaN_3. The microspheres suspensions were incubated at 37 °C while gently shaking. Samples of microspheres were taken out at predetermined time points, centrifuged and washed twice with 1 ml RO water and lyophilized. Dry masses were weighed and samples were dissolved in THF (2 mg/ml) while gently shaking overnight. Gel permeation chromatography (GPC) was used to analyze the change in polymer molecular weight during degradation. GPC was carried out on a Waters Alliance system, with a Waters 2695 separating module and a Waters 2414 Refractive Index detector. Two PL-gel 5 μm mixed-D columns fitted with a guard column (Polymer Labs, M_w range 0.2–400 kDa) were utilized. THF (1 ml/min) was used as mobile phase and calibration was done with polystyrene standards.

2.6. In vitro release studies

Samples of approximately 10 mg of imatinib-loaded microspheres were dispersed in 1.5 ml of phosphate buffered saline (for the buffer composition see Section 2.5) and incubated at 37 °C under mild agitation. Samples were collected at specific time points after centrifugation by removing 1 ml of the supernatant and replacing it with 1 ml of fresh buffer. The imatinib concentration in the supernatant was measured by high performance liquid chromatography (HPLC) using a C18 column (4.6 × 150 mm, 5 μm particle size; Sunfire™, Ireland). A gradient elution method was used with a mobile phase A (95% H_2O, 5% ACN and 0.1% TFA), a mobile phase B (95% ACN, 5% H_2O and 0.1% TFA) and a flow rate of 1 ml/min (Dolman et al., 2012). The eluent linearly changed from 5% to 95% ACN in 6 min; imatinib retention time was at 6.5 min. Imatinib standards (0.3–40 μg/ml dissolved in 10 μl PBS buffer) were used for calibration and detection was done at 266 nm. In vitro release data were fitted to empirical sigmoidal equations by nonlinear regression (Graphpad Prism version 4) to an empirical model for sigmoidal drug release curves. In this model according to Eq. (1), Q stands for cumulative drug release and t stands for the time since start of the release experiment, while constants A and B stand for the relative fractions of initial release phase and late release phase. K_1 and K_2 represent the rate constants of the initial release phase and late release phase. Constant T_{50} stands for the time to reach 50% drug release (Duvvuri et al., 2006).

$$Q = A \times \left(1 - e^{-K_1 \times t}\right) + \frac{B}{1 + e^{-K_2 \times (t - T_{50})}} \quad (1)$$

3. Results and discussion

3.1. The effect of external water phase pH on loading efficiency of imatinib microspheres

Imatinib mesylate (Fig. 1) is a polyvalent base with pK_a values of 8.1, 7.8, 3.7, 2.5, and 1.5. The water solubility of imatinib is highly dependent on the pH; imatinib has an excellent aqueous solubility at low pH (<200 mg/ml) but it is nearly water-insoluble at pH 8 (Peng et al., 2005). Consequently, the distribution-coefficient (log D) of imatinib increases from 1.2 at pH 5.0 to 4.3 at pH 9.0 (MarvinView software 5.11.5). Imatinib is only slightly soluble in most organic solvents (Peng et al., 2005), including DCM (<1 mg/ml). Hence, double emulsification is the preferred method for the preparation of this class of drugs (Wischke and Schwendeman, 2008). In this study, imatinib mesylate solutions of 4 mg/ml and 200 mg/ml in RO water were used as inner water phase,

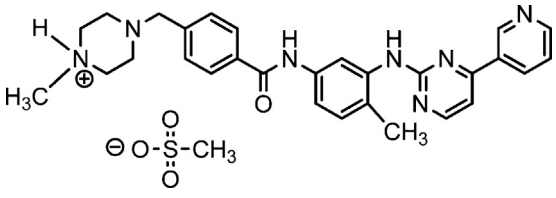

Fig. 1. Chemical structure of imatinib mesylate.

which displayed a pH of 5.3–4.9; the concentration of PLGA in DCM was 23% (w/w).

To study the effect of pH of the W_2 phase on the LE of imatinib, PLGA microspheres were prepared by $W_1/O/W_2$ double emulsification. The W_2 contained PVA 1% dissolved in different buffers with the pH range of 5.0–9.0. The characteristics of the obtained microspheres are shown in Table 1. The table shows that with increasing the pH of W_2 phase, the LE increased from 10% at pH 5.0 (formulation F1) to 90% at pH 9.0 (formulation F3). Consequently, choosing a proper pH of the W_2 phase is necessary in achieving high LE of imatinib into the PLGA microparticles.

A possible concern of exposure of the microspheres to alkaline pH of the external aqueous phase is the hydroxyl anion driven hydrolysis of the ester bonds in aliphatic polyesters (Göpferich, 1996; Makadia and Siegel, 2011). To study this potential problem, the molecular weight change of the PLGA was monitored by GPC upon preparation of microspheres using W_2 with different pH (5.0, 7.0 and 9.0). However, after 3 h exposure to W_2, the M_w of PLGA remained 40 kDa for all the formulations. The morphology of the prepared microspheres is shown in the Supplementary data, Fig. S1. The figure shows that imatinib-loaded microspheres regardless of their method of preparation (different pH of W_2) had a spherical shape with a non-porous surface.

Similar LE was achieved for microspheres which had been prepared with either W_2 phase of pH 9.0 and stirred for 3 h or microspheres which had been prepared with W_2 phase of pH 9.0 and stirring for 40 min followed by additional stirring at pH 7.0 for 3 h (compare formulations F3 and F4, Table 1). It is indeed known that once primary microsphere droplets are solidified, additional drug loss hardly happens (Ng et al., 2010; Yeo and Park, 2004).

To obtain microspheres with high LC, the theoretical loading (weight of initial drug/weight of both drug and polymer × 100) was increased from 0.4% to 9.5% and 19% by increasing the concentration of imatinib in the same volume of W_1 (formulations F4–F6 in Table 1). Increasing the theoretical loading (TL) resulted in microspheres with only slightly decreased LE and thus correspondingly increasing LC of imatinib.

In the study of Benny et al., imatinib microspheres were prepared with similar $W_1/O/W_2$ emulsion method except that W_2 was PVA 0.1% containing 5% (v/v) 2-propanol solution most likely to accelerate the extraction of DCM from the particles; the pH of W_2 was not adjusted. The pH of PVA solutions can vary due to impurities such as polyvinylacetate and this may explain the highly variable LE that was observed in their study (LE of 54%, 15% and 57% were achieved for PLGA50:50, PLGA75:25, and PLGA85:15, respectively) (Benny et al., 2009). In another study of Karal-Yilmaz et al., similar polymers (PLGA50:50, PLGA75:25, and PLGA85:15) were used to encapsulate imatinib into PLGA microspheres (W_2 in this method was PVA 1%). The LE was about 36%, 21%, and 19%, respectively (Karal-Yilmaz et al., 2013). The lower LE in this case can be partly explained by their use of a lower polymer concentration in DCM (11% versus 23% w/w). High polymer concentration in dichloromethane not only increases the viscosity of the organic phase but also results in a faster solidification of the PLGA droplets, which in turn improve the LE (Chaisri et al., 2011; Katou et al., 2008).

3.2. The effect of imatinib loading on the microsphere size and size distribution

Table 1 shows that the size of microspheres with low drug loading (0.41% TL) was approximately 20 μm (F1–F4, Table 1) and decreased to around 6 μm for microspheres with high drug loading (formulations F5 and F6, respectively). A plausible explanation for this difference in size is that imatinib due to its amphiphilic character acts as an emulsifier which decreases the interfacial tension between the organic phase and water phase, which leads to a reduction in the initial droplet size of the $W_1/O/W_2$ emulsion. The span value shows that imatinib microspheres are in general polydispersed. Typically, imatinib-loaded PLGA microspheres had a smooth and non-porous surface. However, as can be seen in panel C of Fig. 2, scanning electron microscopy analysis (SEM) revealed that formulation F6 with 16% LC of imatinib contained particle aggregates which do not have the microsphere appearance that

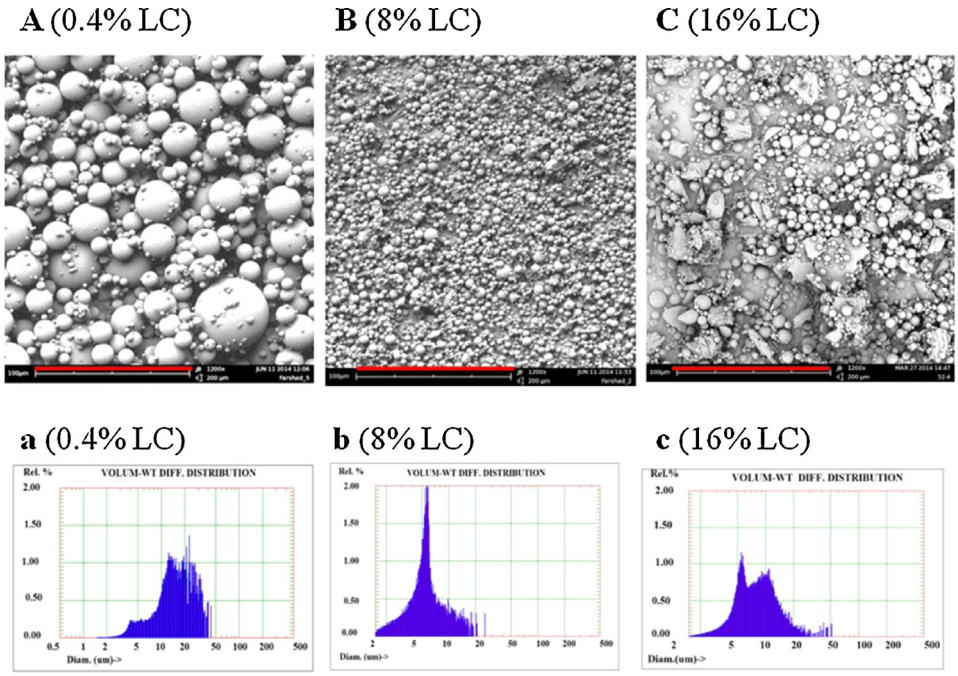

Fig. 2. SEM images and particle size distribution of microspheres with different imatinib loading. (A and a) Formulation F4 (0.4% LC); (B and b) formulation F5 (8% LC) and (C and c) formulation F6 (16.0% LC). Magnification 1200×, scale bar is 100 μm.

Table 2
The effect of inner water phase volume W_1 on the loading efficiency of imatinib and its counter ion (mesylate). Theoretical loading for all the formulation was 0.49% imatinib mesylate which corresponds to 0.41% imatinib (base) and 0.08% mesylate. The concentration of PLGA in DCM was 23% (w/w). Data are expressed as mean ± SD (n = 3).

Formulation	W_1/O[a] (μl/μl) (% v/v)	Recovery[b] (%)	Particle[c] size (μm)	Span[d] value	Imatinib LC[e] (%)	Imatinib LE[f] (%)	Mesylate LC[e] (%)	Mesylate LE[f] (%)
F7	1/500 (0.2%)	69 ± 4	20 ± 1	1.3 ± 0.2	0.30 ± 0.01	72 ± 3	0.042 ± 0.001	52 ± 2
F8	125/500 (20%)	78 ± 3	19 ± 2	0.9 ± 0.1	0.26 ± 0.01	63 ± 2	0.008 ± 0.001	10 ± 2
F9	250/500 (33%)	74 ± 4	17 ± 1	1.5 ± 0.3	0.28 ± 0.01	69 ± 2	0.003 ± 0.001	4 ± 1
F10	350/500 (41%)	71 ± 5	18 ± 1	1.0 ± 0.0	0.28 ± 0.03	69 ± 8	0.002 ± 0.001	2 ± 1

Mean ± SD values were calculated from the data of three independent batches and represent reproducibility between batches.
[a] Inner water volume% is calculated as the percentage of the following: inner water volume/(inner water volume + oil phase volume).
[b] Recovery of microspheres as percentage of drug and polymer starting weight.
[c] Particle size expressed as volume weighted mean diameter.
[d] Span value = (d90 − d10)/d50 which reflects the polydispersity within an individual batch.
[e] LC: loading capacity.
[f] LE: loading efficiency.

can be seen in the other preparations. We therefore concluded that 8% imatinib LC was optimal for this type of microspheres.

3.3. The effect of inner water phase volume on LE of imatinib and its counter ion (mesylate)

As can be calculated from the molecular formula, the 1:1 imatinib mesylate salt ratio corresponds to 83.7% w/w imatinib base and 16.3% mesylate, which further corresponds to 5.4% w/w sulfur. Hence, when 1 mg of imatinib mesylate is loaded into 200 mg PLGA (i.e., 0.498% w/w), this corresponds to a TL of 0.41% imatinib base and 0.081% w/w mesylate. Since the mesylate ion is the only source of sulfur in the imatinib microspheres (sulfur is not present in the buffer or PLGA), the sulfur content of the microspheres can be used to estimate the mesylate ion loading. The LE of imatinib was analyzed by UV spectrophotometry and the sulfur content was determined by means of the Schöniger oxidation method.

Table 2 shows that while imatinib was efficiently encapsulated at an external W_2 pH of 9.0, the encapsulation of the mesylate was poor, especially when larger inner water volumes were used in the $W_1/O/W_2$ emulsification. The table shows that formulations prepared with different volumes of W_1 resulted in microspheres with particle size of 17–20 μm and LE of 63–72%. However, increasing the volume of W_1 from 1% to 41% yielded microspheres with mesylate loading of 42% and 2%, respectively. Since mesylate is highly water soluble, it is unlikely that it partitions into the organic phase. Hence, the low LE of mesylate can only be explained when the two water phases have been interconnected during homogenization which also then elevated the pH of the inner water phase momentously to the values of the external buffer. This subsequently would favor the partitioning of imatinib into the organic phase. This latter process must have been much faster than the complete fusion of inner water phase droplets with the outer water phase, in view of the high LE of imatinib. Variation in the W_1 volume did not influence the morphology (Fig. 3) and the diameter

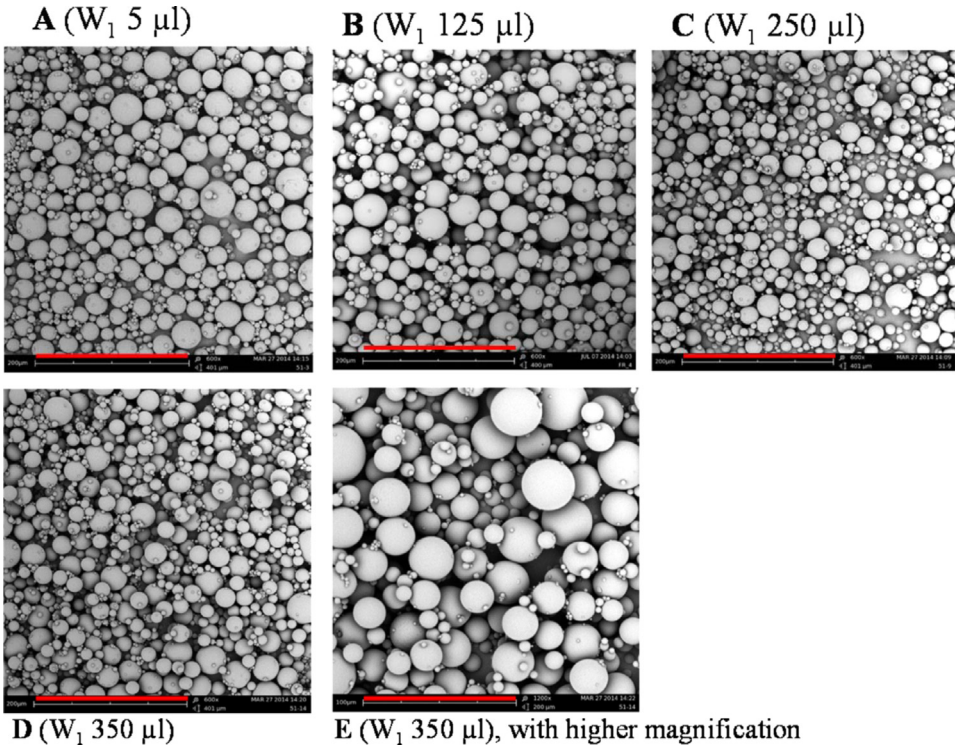

Fig. 3. SEM images of imatinib-loaded PLGA microspheres. (A) Formulation F7 (1% W_1), (B) formulation F8 (20% W_1), (C) formulation F9 (33% W_1), and (D and E) formulation F10 (41% W_1). A–D magnification 600×, scale bar 200 μm; E magnification 1200×, scale bar 100 μm.

Table 3
Thermal analysis of imatinib mesylate, imatinib base and imatinib-loaded PLGA microspheres with different drug loading. Data are expressed as mean ± SD ($n = 3$).

Sample	LC	[a]T_g (°C)	[b]T_g (°C)	T_m (°C)		Enthalpy (J/g)	
Imatinib mesylate	–	109.2 ± 0.1	–	214.2 ± 0.3	224 ± 0.0	9.5 ± 2.5	75.0 ± 2.1
Imatinib base	–	79.1 ± 0.2	–	206.6 ± 0.1		104.0 ± 3.0	
Blank microspheres	–	43.9 ± 0.1	–	–		–	
F4	0.4	44.0 ± 0.7	44.1	–		–	
F5	8.0	47.9 ± 0.0	46.5	181.6 ± 1.6		4.4 ± 0.8	
F6	16	48.1 ± 0.2	49.2	191.0 ± 0.8		8.8 ± 0.5	

[a] Experimental T_g using DSC ($n = 3$).
[b] Calculated T_g using Fox's equation $1/T_g = m_1/T_{g,1} + m_2/T_{g,2}$, where m_1 and m_2 are the mass fractions of PLGA and imatinib base and $T_{g,1}$ and $T_{g,2}$ are the individual T_g values of PLGA and imatinib base, respectively.

(17–20 μm) of imatinib microspheres and non-porous microspheres with smooth surface were obtained. These results appear to contradict previous studies in which increasing the W_1 volume resulted in porous particles of bigger particle size (Parikh et al., 2003; Yushu and Venkatraman, 2006). It is worth to mention that the diameter and the morphology of the particles is not only dependent on the volume of W_1 but also other parameters are relevant, such as emulsification energies, polymer concentration, surface active agent concentration, phase volumes and phase viscosities (Rosca et al., 2004). For example, it was shown that decreasing the polymer concentration in organic phase, increasing the speed of homogenization and increasing the volume of external water phase resulted in smaller microparticles (D'Aurizio et al., 2011).

3.4. Physical state of imatinib in PLGA microspheres

Imatinib-loaded in PLGA microspheres can be molecularly dispersed or it can be present as crystalline form. The physical state of the encapsulated drug influences its release kinetics from the microspheres (D'Aurizio et al., 2011; Mao et al., 2008). In the current study modulated DSC was applied to study the physical state of imatinib in PLGA microspheres. The melting temperature (T_m) of imatinib and the glass transition temperature (T_g) of the polymer were determined from the thermogram recorded in the total heat flow and reversing heat flow, respectively. We used the first heating cycle for this purpose, since heating and cooling in multiple cycles can change the history of the mixed materials (drug and polymer). TGA analysis was done prior to the DSC to determine the amount of residual organic solvent as it may affect the T_g of the polymer. The percentage of weight loss as function of temperature (from 20 °C to 100 °C) was about 0.04% w/w for all of the prepared microspheres.

To understand the physical state of the encapsulated drug and the changes in the drug crystallinity upon formulation one need a suitable reference materials for comparison. It was already shown that during the formulation only imatinib stayed in the microspheres and the majority of its counter ion, mesylate, was not encapsulated. Therefore, the encapsulated drug was imatinib free base and not imatinib mesylate. In contrast to the microspheres for which we used a single-heating ramp, we used a multicycle heating/cooling protocol for determination of the reference T_g value of amorphous imatinib base since this prevented formation of imatinib crystals.

The obtained calorimetric data are listed in Table 3. Thermograms can be found in the Supplemental information (Figs. S2 and S3). The T_m of imatinib base was 206 °C with an enthalpy of 104 J/g while a T_g was observed at 79 °C. Imatinib PLGA microspheres with low drug loading (0.4% w/w LC, formulation 4) did not show a melting peak that could be attributed to imatinib base, while the formulation with 8% w/w imatinib LC (formulation 5) showed a small melting peak around 181 °C with enthalpy of (4.4 J/g). This peak was more prominent in formulation 6 with higher drug loading (16% LC), which however did not display the typical microsphere appearance by SEM (Fig. S3). The exact percentage of crystallinity could not be calculated from these data as the T_m of the imatinib-loaded microspheres (181–191 °C) is lower than that of imatinib free base (206 °C). Shifting the T_m of imatinib-loaded microspheres to lower temperature than that of imatinib free base can be attributed to the polymeric environment after encapsulation, in agreement with the data reported in the literature (Bragagni et al., 2013).

The T_g of PLGA (empty microspheres) was 44 °C (Table 3 and Fig. S3). Imatinib-loaded microspheres showed single T_gs that were elevated as compared to blank microspheres. The observed increase in T_g corresponded to the expected changes in T_g according to Fox's equation (Gedde, 1995). From this result, we conclude that imatinib is primarily molecularly dispersed in the amorphous polymeric PLGA particles.

3.5. In vitro release of imatinib from PLGA microspheres

Microspheres with different imatinib LC (0.4% and 8% w/w) were evaluated for their release characteristics by incubating them at 37 °C in PBS. In parallel, aliquots of the microspheres were incubated to study polymer degradation and particle erosion.

Fig. 4 shows that imatinib microspheres with 0.4% LC showed a sigmoidal release profile with an initial burst release of approximately 16% in the first 24 h, followed by a diffusion and erosion controlled release phase that accelerated from day 50 toward the end of the experiment. In case of imatinib microspheres with 8.0% LC, burst release was around 23% which was followed by sustained release which reached completion around day 80 after the start of in vitro incubation. Since the experimental data were only poorly

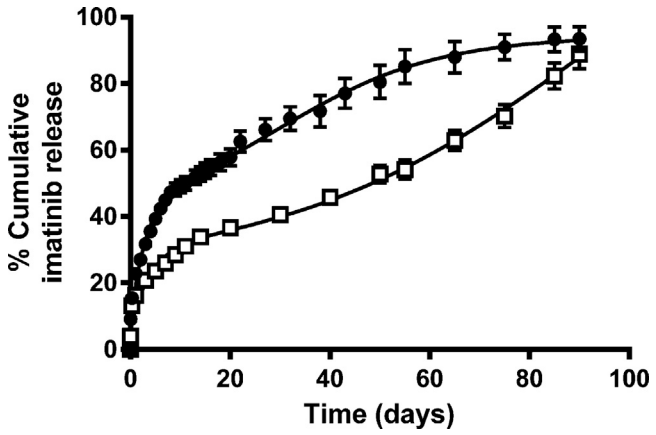

Fig. 4. In vitro release study of imatinib microspheres in PBS, 37 °C. Open squares: formulation with imatinib 0.4% LC (F4), closed circles: formulation with imatinib 8.0% LC (F5). Data are expressed as mean ± SD ($n = 3$). Lines drawn represent non-linear regression fit of the data.

Table 4
Best-fit values of cumulative drug release from imatinib-loaded microspheres (curves are shown in Fig. 4).

Fitted parameter	0.4% imatinib LC	8.0% imatinib LC
A (% release)	24 ± 2	38 ± 2
K_1 (day^{-1})	0.38 ± 0.08	0.29 ± 0.04
B (% release)	80 ± 5	59 ± 3
K_2 (day^{-1})	0.044 ± 0.004	0.060 ± 0.006
T_{50} (day)	64 ± 3	33 ± 3
R^2	0.9755	0.9709

fitted by mechanistic models like power-law or Higuchi based formulas, cumulative release curves were fitted by an empirical equation for sigmoidal release curves (Duvvuri et al., 2006), as summarized in Table 4. In this empirical model for sigmoidal drug release, constants A and B represent the relative proportion of drug release during phase I and phase III of the curves, T_{50} represents the time point at which 50% of the drug has been released and time constants K_1 and K_2 reflect the drug release rate during phase I and phase III. A more mechanistic understanding of the processes that contributed to imatinib release was deduced from the data on in vitro degradation of the microspheres (Fig. 5). During the first 20 days of incubation, imatinib microspheres with either low (0.4%) or high (8.0%) LC showed no polymer erosion (dry mass loss, Fig. 5A), although PLGA chain scission (PLGA M_w,Fig. 5B) was

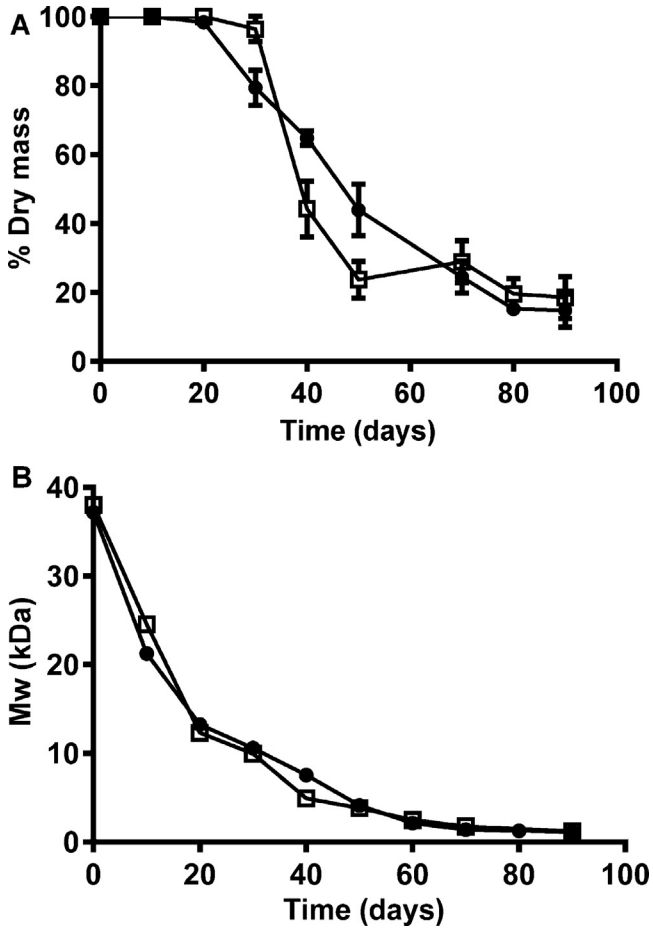

Fig. 5. Degradation study of imatinib PLGA microspheres prepared with different drug loading in PBS pH 7.4, 37 °C. (A) Dry mass loss versus time and (B) change in the M_w (kDa) versus time. Open squares: imatinib 0.4% LC (F4), closed circles: imatinib 8.0% LC (F5). Data are expressed as mean ± SD (n = 3).

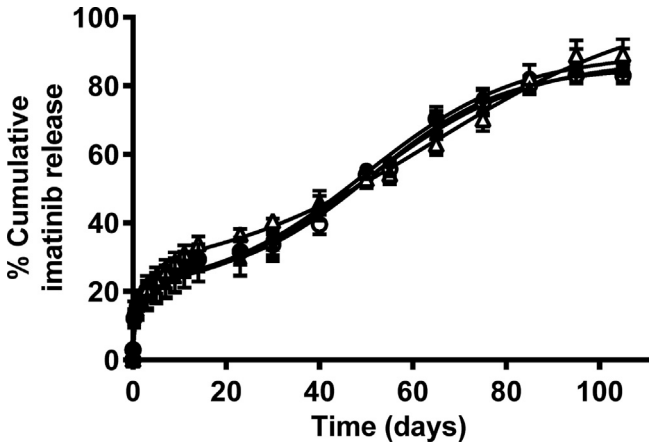

Fig. 6. In vitro release study of imatinib microspheres in PBS pH 37, formulations F7–F10, Table 2. Formulation F7 (W_1 volume 1% open circles); formulation F8 (W_1 volume 20% closed circles); formulation F9 (W_1 volume 33% open triangles) and formulation F10 (W_1 volume 41% closed triangles). Data are expressed as mean ± SD (n = 3). Lines drawn represent non-linear regression fit of the data.

ongoing from start of the incubation. Hence, we can expect that the drug release in phase I is mainly driven by diffusion and dissolution of imatinib base in the polymeric phase. From day 30 particle erosion is prominent in both types of microspheres, which is in good agreement with the accelerated release rate observed from this latter time point. Degradation results suggest that imatinib-loaded microspheres degrade via bulk degradation, characteristic for end-capped PLGA (Kazazi-Hyseni et al., 2014; Samadi et al., 2013). It has been highlighted that for small molecule drugs which are molecularly dispersed in the PLGA, the burst release is most likely due to drug diffusion through polymer matrix unless the microspheres are porous (Allison, 2008; Gaignaux et al., 2012; Huang and Brazel, 2001). The higher initial release observed for imatinib-loaded microparticles with 8% LC could be due to their smaller size (6 μm compared to 17 μm for microspheres with low LC) and hence the larger specific area of these microspheres, as well as the shorter diffusion distance to the surface of the microspheres.

Fig. 6 shows that similar release profiles were obtained by imatinib-loaded microspheres prepared with different volume of W_1 suggesting that this parameter or the resulting differences in mesylate encapsulation did not affect release of imatinib. Fitting results of these particles are summarized in Table S1 in the Supplemental information.

Compared to the previous studies, the initial burst was much lower in the current study (16% and 23% versus 63%) (Benny et al., 2009) and it was comparable with the study of Karal-Yilmaz et al., (2013). However, the duration of drug release in our study was much longer (90 days versus 30 days) likely due to the higher polymer concentration that we have used (23% w/w versus 11% w/w), although we cannot exclude that also other differences between the studies like differences in the polymer molecular weight and the size of particles will have affected the drug release profiles.

4. Conclusion

Our results demonstrate that the amphiphilic nature of imatinib highly contributes to its successful encapsulation in PLGA microspheres. Partitioning of imatinib between organic solvent and water in the $W_1/O/W_2$ leads to its entrapment as free base and not as the initially formulated mesylate salt. Crystallization of imatinib within the particles only occurred when high

amounts of imatinib were used but this composition did not provide regular microspheres. It was demonstrated that imatinib release from microparticles was governed by the combination of diffusion and polymer degradation and that PLGA microparticles, regardless of their imatinib LC, released imatinib continuously for approximately three months. The previous studies showed that local delivery of imatinib-loaded microspheres was promising in the treatment of brain cancer in a mice model. This form of sustained-release depot can be applied for other cancer models as well. For example, intraperitoneal administration of imatinib-loaded microspheres may open a new avenue for the treatment of gastrointestinal tumors in the future.

Conflict of interest

Authors declare that there is no conflict of interest in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2015.01.043.

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