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RESEARCH PAPER

Computer Modeling Assisted Design of Monodisperse PLGA Microspheres with Controlled Porosity Affords Zero Order Release of an Encapsulated Macromolecule for 3 Months

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ABSTRACT

Purpose The aim of this study was the development of poly(D,L-lactide-co-glycolide) (PLGA) microspheres with controlled porosity, to obtain microspheres that afford continuous release of a macromolecular model compound (blue dextran).

Methods PLGA microspheres with a size of around 40 μm and narrow size distribution (span value of 0.3) were prepared with a double emulsion membrane emulsification method. Gene expression programming (GEP) analysis was applied to design and formulate a batch of microspheres with controlled porosity that shows continuous release of blue dextran.

Results Low porous microspheres with a high loading efficiency were formed at high polymer concentrations (30% w/w in the oil phase) and were characterized with a burst release $<10\%$ and a three-phasic release profile of blue dextran. Increasing porosity (10% w/w polymer concentrations), a sustained release of blue dextran was obtained albeit with up to 40% of burst release. The desired formulation, calculated by GEP, resulted in microspheres with 72% loading efficiency and intermediate porosity. Blue dextran was indeed released continuously in almost a zero order manner over a period of 3 months after an initial small burst release of 9%.

Conclusions By fine-tuning the porosity, the release profile of PLGA microspheres for macromolecules can be predicted and changed from a three-phasic to a continuous release.

KEY WORDS controlled release · membrane emulsification · microspheres · PLGA · porosity

ABBREVIATIONS

ANNs	Artificial neural networks
GEP	Gene expression programing
LC	Loading capacity
LE	Loading efficiency
ME	Membrane emulsification
PLGA	Poly(D,L-lactide-co-glycolide)
PVA	Polyvinyl alcohol
TL	Theoretical loading

$d_{4,3}$	Volume-weighted mean droplet diameter
d_d	Droplet diameter
d_p	Membrane pore diameter
ε	Membrane porosity
F_b	The buoyancy force
F_c	The drag force of the continuous phase flow
F_d	The inertial force of the dispersed phase flow
F_γ	The interfacial tension force
γ	Interfacial tension
J_d	Dispersed phase flux
k	Fraction of active membrane pores

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η_d	Viscosity of the dispersed phase
$\overline{P_c}$	Continuous phase pressure
$P_{c,in}$; $P_{c,out}$	Pressure of the continuous phase at the inlet and outlet of the main channel
P_{ctm}	Critical transmembrane pressure
P_d	Dispersed phase pressure
ΔP_{tm}	Transmembrane pressure
q	Dispersed phase flow rate
R^2	Correlation coefficient
R_m	Hydraulic membrane resistance
r_p	Radius of the membrane pore
t_d	Period of drop detachment

INTRODUCTION

Microspheres based on biodegradable polymers such as aliphatic polyesters, e.g. poly(D,L-lactic-*co*-glycolic acid) (PLGA), are widely used for delivery of macromolecular drugs. Microspheres enable the protection and stabilization of the encapsulated drug and aim for a release profile over a desired time period (1–3). The size and size distribution of polymeric microspheres are important factors for controlling the particle degradation (4,5) and the release profile of an entrapped drug (6–9). In addition, the size also contributes to the *in vivo* fate of polymeric particles by affecting their cellular uptake (10–12). Considering the above, one can comprehend that polydispersity of polymeric particles can confound the therapeutic outcome of such delivery devices. It has been shown that the production of monodisperse microspheres results in better batch-to-batch reproducibility, also in term of release kinetics (13,14). Membrane emulsification (ME), first introduced by Nakashima *et al.* (15), is a method that makes use of a porous glass membrane with uniform pore sizes to generate monodisperse microspheres (16).

The most commonly observed release profile of macromolecular compounds from PLGA microspheres is tri-phasic, characterized by a burst release, a lag phase and a phase of sustained release (1,17–19). For macromolecules that are insoluble in the polymer matrix, their mechanism of release is mainly governed by the particle porosity in the first two release phases and by the degradation of the polymer in the third phase (1,20,21). Particle porosity is often associated with a sustained release after an initial, mostly high, burst release, and with a low drug loading efficiency (LE) (22). Several studies have reported on the physical principles of the ME process (23–25) and the influence of formulation parameters on the size and monodispersity of the microspheres (26–28). However, there is hardly information on how formulation parameters of the ME process affect the release profile of entrapped macromolecules from the obtained microspheres.

In this study, the preparation of monodisperse PLGA microspheres was pursued with control of the porosity in order to fine-tune the release of a macromolecular model compound from PLGA microspheres, eventually aiming at a continuous release profile after a low burst release. Blue dextran was chosen as a model compound, as its inertness allows to investigate the intrinsic release properties of the microspheres avoiding possible interactions between payload and polymer phase or degradation/modification of the payload.

Macromolecules such as proteins are commonly formulated in PLGA microspheres by double-emulsification. With this method a primary W_1/O emulsion, that contains the macromolecular drug in the inner water phase and the polymer in the oil phase, is emulsified in a continuous water phase to obtain a $W_1/O/W_2$ emulsion (1,29,30). In the present study, different formulation parameters of the double emulsion ME process were varied in order to evaluate their relationship with porosity of the microspheres and the release profile of blue dextran. Subsequently, different artificial intelligence tools (Artificial Neural Networks (ANNs), fuzzy logic, gene expression programing (GEP) and genetic algorithms) were used to understand the effect of formulation parameters on the microsphere characteristics and predict the ones that generate microspheres with controlled porosity, along with other preferred properties like high monodispersity and high LE.

The relationship between formulation parameters, porosity of the microspheres and the release of blue dextran was analyzed with neurofuzzy logic, which is a hybrid computational method that combines the learning capacity of ANNs with fuzzy logic technology (31,32). Fuzzy logic is a form of probabilistic logic which is able to manage linguistic variables. After a proper fuzzyfication process, a continuous variable can be transformed into a linguistic variable which is represented by a truth that ranges in degrees between 0 and 1. Following this process, neurofuzzy logic is able to detect complex relationships between variables and present them as simple rules (32). GEP is an extension of genetic programming, the soft-computing method that simulates the biological evolution process to develop algorithms. This technology is able to model empirically observed values, finding equations that fit the facts within a certain error of the correct value (33). The combination of GEP and genetic algorithms allows carrying out the process optimization to find the best selection of formulation parameters that give microspheres of preferred properties.

The aim of this study was the development of PLGA microspheres with uniform size that show sustained (preferably zero-order) release of a model macromolecule (blue dextran), while simultaneously the LE is high and the burst release is low.

MATERIALS AND METHODS

Materials

Ester terminated ("end-capped") poly(D,L-lactic-*co*-glycolic acid) with intrinsic viscosity (IV) of 0.2 dL/g (PLGA 5002)

and 0.4 dL/g (PLGA 5004), were purchased from Purac Biochem B.V., Gorinchem, The Netherlands. Blue dextran ($MW = 2 \cdot 10^6$ g/mol), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4) and sodium azide (NaN_3) were purchased from Fluka (Zwijndrecht, The Netherlands). Polyvinyl alcohol (PVA; MW = 13,000–23,000 g/mol) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide (NaOH) and sodium chloride (NaCl) were supplied from Merck KGaA (Darmstadt, Germany). Dichloromethane (DCM) and tetrahydrofuran were purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

Preparation of Monodisperse PLGA Microspheres by Membrane Emulsification

Sixteen batches of PLGA microspheres were prepared with a cross-flow emulsification process (Fig. 1a), where the continuous phase (W_2) is flowing past the membrane through which the dispersed phase (W_1/O) is pumped, resulting in the formation of emulsified droplets of uniform size. The membrane used was hydrophilic Iris-20 (microsieve^{TM} membrane technology, Nanomi B.V., Oldenzaal, The Netherlands) that generates $W_1/O/W_2$ microspheres with a mean diameter of around 40 µm. The dispersed phase, W_1/O (also known as "premix"), was prepared by mixing solutions of PLGA in DCM (1.5 mL, polymer concentrations 10, 15, 20, 25 and 30% w/w) with different volumes of blue dextran in water (0.19, 0.37 and 0.75 mL; 50 mg/mL). The inner water volumes of blue dextran are further referred to as the volume fractions, calculated as follows: inner water volume/(inner water volume + oil phase volume). The premixes were homogenized using Ultra-Turrax T8 (IKA Works, USA) with dispersing element S10N-10G, at a speed of 20,000 rpm for 30 s. Next, the premixes were passed through the Iris-20 membrane at a constant rate of 2 mL/h using a syringe pump (Nexus 6000, Chemyx, USA) into 30 mL of the continuous phase with different concentrations of PVA (2, 4 and 6% w/v). In selected formulations, W_2 was saturated with DCM (1.6%; (34)) or NaCl (1%) was dissolved, at a fixed PVA concentration of 4%. The continuous phase was pumped with a rate of 4.6 mL/min across the membrane.

At the end of the process, the formed dispersion of the emulsified droplets was left to stir for 2 h to evaporate DCM. The hardened microspheres were collected by centrifugation at 3,000 rpm for 2 min, washed three times with water, froze with liquid nitrogen and freeze-dried overnight. The yield was calculated from the weight of the microspheres recovered *versus* the weight of blue dextran and PLGA used to prepare the microspheres. The stability of the premix was evaluated using separate premix samples that were incubated at room temperature, to observe possible phase separation. The

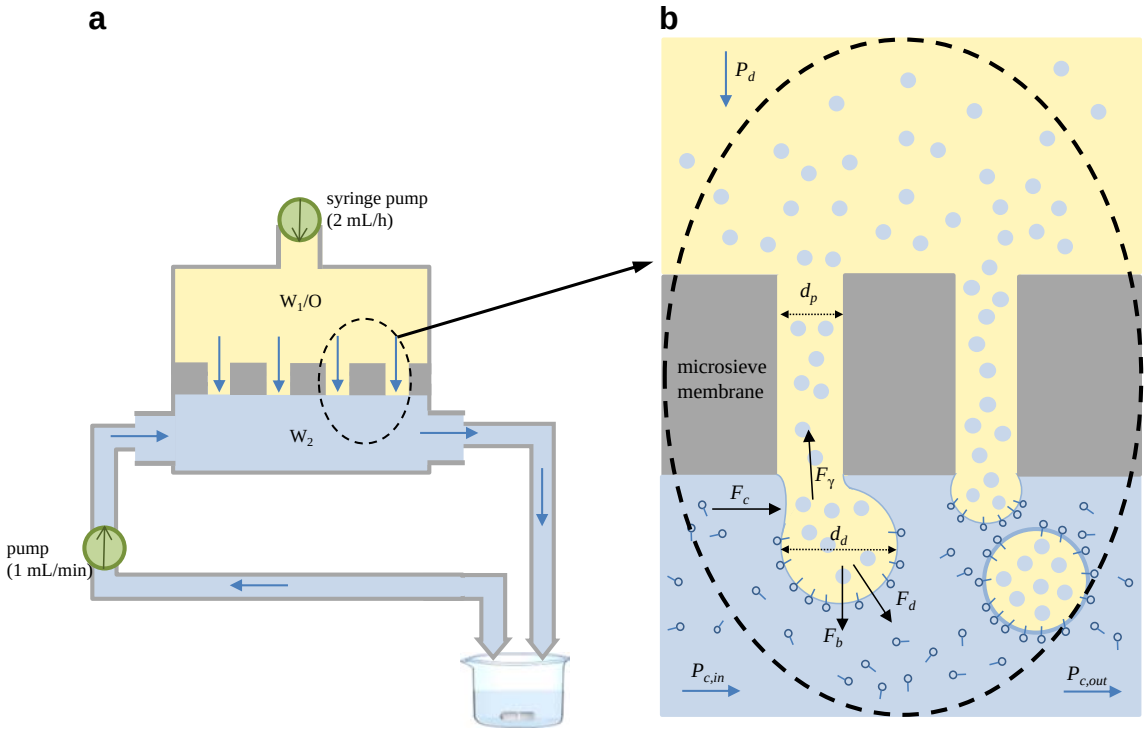

Fig. 1 Schematic diagram of the membrane emulsification method. (**a**) Membrane module; (**b**) Principle of particle preparation with microsieve membrane; (W_1/O – dispersed phase (premix), W_2 – external water phase, P_d – pressure applied on the dispersed phase, $P_{c,in}$ and $P_{c,out}$ –pressure on the flowing continuous phase at both ends of the membrane modulus, d_p – diameter of the membrane pore, d_d – diameter of the droplet formed at the membrane pore, F_γ – the interfacial tension force, F_b – the buoyancy force, F_c – the drag force of the continuous phase flow, F_d – the inertial force caused by the flow of the dispersed phase.

stability is expressed as the time until phase separation was visually observed. The stability was also measured with an Ostwald capillary viscometer and a rheometer. However, as a result of the rapid evaporation of dichloromethane, the obtained results were not reliable.

Microsphere Size and Size Distribution Analysis

The size and size distribution of the obtained microspheres were measured with an optical particle sizer (Accusizer 780, Santa Barbara, California, USA). At least 5,000 microspheres of each formulation were analyzed. The volume-weight mean microsphere diameter (vol-wt mean) is reported as particle size and the span value was calculated with the following formula: $sp=(d_{90}-d_{10})/d_{50}$, where d_x is the diameter corresponding to x vol.% on a cumulative microsphere size distribution curve. The size distribution is narrow for span values <0.45 (23).

Porosity Analysis

The morphology of the microspheres was investigated with scanning electron microscopic (SEM) analysis (Phenom, FEI Company, The Netherlands). Lyophilized microspheres were transferred onto 12-mm diameter aluminum specimen stubs (Agar Scientific Ltd., England) using double-sided adhesive tape. Prior to analysis, the microspheres were coated using an ion coater with platinum under vacuum. To determine the porosity of the microspheres, SEM pictures with similar magnification (~5,000×) were used and the porosities were visually graded by three independent individuals according to the grading scale given in Supplemental Table SI. For each formulation, at least six microspheres were scored. Representative SEM pictures of microspheres with porosity grade 1 to 5 are shown in Supplemental Fig. S1.

Blue Dextran Content of Microspheres

The blue dextran content of the microspheres was determined by dissolving 50 mg of microspheres in 1 mL of DMSO. The absorption of the solution was measured with a UV microplate reader (Spectrostar Nano, BMG Labtech, Germany) at 620 nm, with a blue dextran calibration curve (from 0.01 to 3 mg/mL in DMSO). The theoretical loading (TL) of blue dextran was calculated from the amount of blue dextran in the feed divided by the sum of the amount of blue dextran and PLGA times 100%. The loading capacity (LC) was calculated from the weight of entrapped blue dextran divided by the dry weight of the microspheres times 100%. The loading efficiency (LE) was calculated as the percentage of the amount of blue dextran entrapped in the microspheres divided by the amount added during the preparation of microspheres times 100%.

In Vitro Degradation and Release Studies

Around 10 mg of the different microsphere formulations was suspended in 1.5 mL of 100 mM phosphate buffered saline (pH 7.4) containing 56 mM NaCl, 33 mM Na_2HPO_4, 66 mM NaH_2PO_4 and 0.05% (w/v) NaN_3 (added to prevent bacterial growth). The microspheres were incubated at 37°C while gently shaking. At different time-points, vials were removed and centrifuged (4,000 rpm for 5 min). The microspheres were washed three times with water, lyophilized and analyzed for residual dry weight and PLGA molecular weight.

The molecular weight of PLGA in degraded microspheres was measured with GPC (Waters Alliance system), consisting of a Waters 2695 separations module and a Waters 2414 refractive index detector. Two PL-gel 5 μm Mixed-D columns fitted with a guard column (Polymer Labs, Mw range 0.2–400 kDa) were used. For calibration, polystyrene standards (PS-2, Easi Cal, Varian, USA) with narrow molecular weight distributions (MW = 580–377,400 g/mol) were used. Tetrahydrofuran was used as the mobile phase at a flow rate of 1 mL/min. Standards and samples were dissolved in tetrahydrofuran overnight and filtered through 0.2 μm filters prior to the analysis. The data acquisitions and analysis were performed using Empower Pro software (Waters Corporation).

The release of blue dextran was studied in triplicate. At indicated time points, samples were centrifuged and 0.5 mL of the supernatant was removed and replaced with the same volume of fresh buffer. The amount of released blue dextran was measured with a UV microplate reader at 620 nm. Two main points were applied for characterization of the release curves: *the burst release*, calculated as the percentage of the loaded blue dextran released within 24 h, and the slope of the release curve from day 1 till day 20 referred to as the *release rate*$_{(1–20\ d)}$ (expressed in % of the loading released per day (%/day)).

The Software Tools: Neurofuzzy Logic and GEP

The database of 16 formulations was modeled using two different artificial intelligence approaches. A commercial neurofuzzy logic software, FormRules® v3.31 (Intelligensys Ltd, 2008, UK), was used to generate information and knowledge related to the influence of different parameters to the particle outcome, and a commercial software, INForm ® v4.11 (Intelligensys Ltd, 2008, UK), implementing GEP and genetic algorithms, was used to model and optimize the system obtaining suitable ingredients and process conditions to achieve a microsphere batch with the desirable characteristics. The polymer type, polymer concentration in the oil phase, inner water volume and excipients in the continuous phase were introduced as *input* parameters, whereas size of the microspheres, span value, LE and porosity were selected as *output* parameters (Table I). In order to model release properties (i.e. burst release and release rate$_{(1\text{-}20d)}$), two extra inputs

Table I Selected Formulation Parameters Studied and the Results Obtained

Formulation #	PLGA	PLGA in the oil phase (%)	W_1[a] (%)	Continuous phase (W_2)	PVA (%)	TL (wt%)	Stability[b] W_1/O (min)	Yield (%)	Vol-wt mean diameter (μm)	Span value	LC (%)	LE (%)	Porosity[c]	Burst release[d] (%)	Release rate$_{(1\text{-}20d)}$[e] (%/day)
1	5002	10	20	PVA	4	7.8	30	72	40 ±13	0.9	1.5	19	4	0	0
2	5002	15	20	PVA	4	5.0	60	65	43 ±8	0.4	2.0	58	4	25	1.25
3	5002	20	20	PVA	4	3.5	80	67	40 ±6	0.3	1.9	52	2	9	0.08
4	5002	25	20	PVA	4	2.7	120	60	47 ±7	0.2	2.0	71	3	5	0.43
5	5002	30	20	PVA	4	2.1	120	31	59 ±5	0.1	1.9	88	1	0.4	0
6	5004	10	20	PVA	4	7.8	90	44	41 ±9	0.5	5.1	64	5	39	0.98
7	5004	15	20	PVA	4	5.0	100	45	45 ±9	0.3	2.6	50	5	21	1.16
8	5004	20	20	PVA	4	3.5	130	40	63 ±15	0.6	2.6	71	3	11	0.32
9	5004	25	20	PVA	4	2.7	180	40	75 ±19	0.7	2.5	91	3	11	0.55
10	5004	30	20	PVA	4	2.2	185	39	76 ±15	0.5	2.2	100	2	10	0.33
11	5002	20	11	PVA	4	1.8	40	46	58 ±23	1.1	1.0	55	2	0	0
12	5002	20	33	PVA	4	6.8	20	64	46 ±14	0.8	3.1	45	3	34	0.30
13	5002	20	20	PVA	2	3.5	80	65	45 ±6	0.2	1.9	53	3	39	0.50
14	5002	20	20	PVA	6	3.5	80	68	48 ±12	0.5	2.1	60	3	27	0.83
15	5002	20	20	PVA + 1% NaCl	4	3.5	80	63	52 ±20	1.0	2.2	60	1	0	0
16	5002	20	20	PVA + 1.6% DCM	4	3.5	80	59	42 ±10	0.4	1.5	43	2	26	0.32

Fields in grey indicate the parameters varied in those particular formulations which were used as inputs for statistical analyzes with ANN and GEP, whereas the results were used as outputs

PVA polyvinyl alcohol, *TL* theoretical drug loading, *LC* loading capacity, *LE* loading efficiency, *DCM* dichloromethane

[a] Inner water volume is calculated as the percentage of the following: inner water volume / (inner water volume + oil phase volume)

[b] Time after which visual phase separation occurred

[c] Porosity was graded by three independent individuals according to the procedure given in Supplemental Table SI. The results do not differ for more than one point

[d] Release of blue dextran in 24 h

[e] Slope of the release curve calculated between days 1 and 20

were included: porosity and theoretical loading. The common training parameters used by FormRules v3.31 were the following: ridge regression factor of 1 e^{-6}, number of set densities: 2, set densities: 2.3, maximum inputs per submodel: 4, maximum nodes per input: 15, adapt nodes: true. Specific training parameters selected for each property are given in Supplemental Table SII. FormRules v3.31 contains various statistical fitness criteria including Cross Validation (CV), Minimum Description Length (MDL), Structural Risk Minimisation (SRM), Leave One Out Cross Validation (LOOCV) and Bayesian Information Criterion (BIC). All were investigated to obtain the model that gave the best predictability together with the simplest and more intelligible rules (35).

GEP training parameters selected with INForm v4.11 for modeling included Mean Squared Error as fitness criteria and the following general operation parameters: number of populations:10, number of generations: 1,000–10,000, gene headlength: 5–7, number of genes: 2–3 and random seed: 1–10. Equations included the mathematical functions +, −, x, /, exp when necessary (33).

Separate models were developed with FormRules and INForm for each property, the accuracy of which was assessed using correlation coefficient (R^2) and ANOVA *f*-ratios for each output:

$$R^2 = \left(\frac{1 - \sum_{i=1}^{n} \left(y_i - y_i' \right)^2}{\sum_{i=1}^{n} \left(y_i - y_i'' \right)^2} \right) \times 100\% \quad (1)$$

where y is the actual point in the data set, y' is the value calculated by the model and y" is the mean of the dependent variable. The larger the value of the train set R^2, the more the model captured the variation in the training data. Values for $R^2 > 70\%$ are indicative of reasonable model predictabilities (35). The ANOVA is used to assess whether the values of a quantitative variable predicted by the model within several pre-defined groups differ from the corresponding experimental values. The ANOVA *f*-ratio is calculated with the variation due to an experimental treatment or effect divided by the variation due to an experimental error. ANOVA *f*-ratios higher than *f*-critical values (36) for the degrees of freedom of the model mean that there are not statistical significant differences between those groups.

RESULTS AND DISCUSSION

Membrane Emulsification Process: General Features

In this study, different PLGA microspheres with a narrow size distribution, loaded with blue dextran, were prepared by a membrane emulsification (ME) process. The ME module consists of a specially developed microsieve™ membrane with uniform pore sizes that acts as an emulsifying element (Fig. 1). In ME, the dispersed phase (premix) is pressed through the membrane pores with a diameter d_p (m) into the continuous phase which flows past the membrane in a recirculating loop. Small droplets are formed at the pore openings near the membrane surface, which are detached once they reach a certain size d_d (m). The minimum pressure that has to be applied in order to make the dispersed phase flow through the porous membrane is known as the critical transmembrane pressure, P_{ctm} (Pa), or the capillary pressure. Calculated from the Laplace equation, P_{ctm} is proportional to the interfacial tension γ ($N{\cdot}m^{-1}$) between the oil phase (W_1/O) and the water phase (W_2) divided by d_p, $P_{ctm}=4\gamma/d_p$ (16,37–39). The transmembrane pressure, ΔP_{tm} (Pa), is used to overcome flow resistances in the pores and interfacial tension forces and is defined as the difference between the pressure of the dispersed phase, P_d (Pa), and the average pressure of the continuous phase $\overline{P}$ (Pa), $\Delta P_{tm}=P_d-\overline{P}$, where $\overline{P}=(P_{c,in}+P_{c,out})/2$ ($P_{c,in}$ and $P_{c,out}$ refer to the pressure of the continuous phase at the inlet and outlet of the main channel) (40–42). For the production of monodisperse emulsions, ΔP_{tm} should be 2–10 times higher than P_{ctm} (43).

The flux with which the dispersed phase flows through the membrane, J_d ($m{\cdot}s^{-1}$) can be calculated from the Hagen-Poiseuille law (44,45), $J_d=\Delta P_{tm}/(\eta_d R_m)$, where, η_d (Pa·s) is the viscosity of the premix and R_m (m^{-1}) is the hydraulic membrane resistance. The hydraulic membrane resistance is a constant that can be calculated using the formula $R_m=\Delta P_{tm}/\eta_w J_w$, in which J_w is the clean water flux through the membrane and η_w is the viscosity of water (44,45). The flow rate through a pore, q, can be related to the period of drop detachment, t_d (s) using the following equation (38)

$$q=\frac{\eta_d r_p}{\gamma t_d} \tag{2}$$

where, r_p (m) is the radius of the membrane pore ($d_p=2r_p$). According to Eq. (2), with increasing η_d the formed droplets retain longer at the membrane before they detach resulting in an increase in size. The droplet formation time can be expressed as a function of J_d and d_d using the following equation (46),

$$t_d=\frac{2}{3}\frac{k\varepsilon}{d_p^2}\frac{d_{4,3}^3}{J_d} \tag{3}$$

where, k is the fraction of active pores, ε is the membrane porosity and $d_{4,3}$ (m) (47) is the volume-weighted mean droplet diameter. The factor k is introduced as during ME not all pores are permeated with liquid. It has been shown that between 2 and 40% of the pores are active (39,40,46).

Once a droplet is formed at the membrane pore, the d_d upon detachment is governed by the balance between four different hydrodynamic forces (Fig. 1b): the drag force generated by the continuous phase flow (F_c), the interfacial tension force (F_γ), the inertial force caused by flow of the dispersed phase (F_d) and the buoyancy or gravitational force (F_b) (37,40,42). In microfluidics flow, the buoyant force is insignificant as it is very small compared to F_c and F_γ (37). From these forces, F_γ is the attaching force while the others are detaching forces. Hence the droplet is detached from the pore when the detaching forces are greater than the attaching force (42).

The drag force of the continuous phase flow affects the size and the size distribution of the formed emulsion droplets by generating a shear stress along the membrane surface, which detaches the formed droplet. An increase in flow velocity of the continuous phase causes a larger shear force along the membrane which in turn results in deformed droplets with increased polydispersity (37). The surfactant (PVA) in the continuous phase has an important effect on particle size as it reduces the interfacial tension force (F_γ) by adsorbing onto the interface between the immiscible water and oil phases. The adsorption kinetics determines the size of the droplets because when PVA adsorbs quickly onto the interface of the formed droplets and the continuous aqueous phase, it causes a quick reduction of the interfacial surface tension which in turn results in earlier droplet detachment from the membrane surface and thus in smaller droplets (40,44).

In this study, different formulation parameters were varied, mainly polymer molecular weight, polymer concentration in the oil phase, inner water volume and excipients in the continuous phase. The dispersed phase flux and the continuous phase flow rate were kept constant at 2 mL/h and 4.6 mL/min, respectively. The yield of the microspheres was around 60% for most of the formulations and 40% for formulations with high viscosity of the dispersed phase (Table I: 30% PLGA 5002 and all formulations with PLGA 5004). In these latter formulations, as a result of higher viscosity, a significant amount of the premix remained in the module as a void volume.

Formulation parameters and the obtained microsphere characteristics that were used as training parameters for the computational modeling are shown in Table I. Values of training parameters used by FormRules v3.31 are given in Supplemental Table SII together with R^2 values and their corresponding ANOVA f values, and the inputs selected as significant by the fuzzy logic software to express the variability of each parameter. From this table it can be seen that the values of R^2 are higher than 77% and the f-ratios are higher

than the critical *f*-values (36) for the corresponding degrees of freedom, indicating a successfully developed model. A more detailed discussion regarding the observed microsphere properties of the different formulations is given below.

Stability of the Premix

The stability of the premix plays an important role in generating monodisperse microspheres with ME (27). For most formulations given in Table I the premix was stable during the processing time. The premixes of formulation 1 with 10% of PLGA 5002 in the oil phase and formulations 11 and 12 with 11% and 33% of the inner water volume had marginal stability, as phase separation occurred in less than 40 min. This may have affected droplet formation especially at the end of the processing period, and thus can explain the relatively higher polydispersity of the obtained microspheres (span value > 0.8) (27). Neurofuzzy logic showed a good correlation between the formulation parameters and premix stability with R^2 of 97% (Supplemental Table SII). The most important parameter contributing to the premix stability was the concentration of PLGA in the oil phase, by yielding more stable premixes with increased concentrations. Likely, high PLGA concentrations in the oil phase result in high viscosity of the premix that in turn retards phase separation.

Effect of Formulation Parameters on the Size Characteristics of Microspheres

Table I shows that using the same pore-size membrane, microspheres ranging from 40 to 76 μm were produced, with different size distributions (span value between 0.1 and 1.1) and different porosities. The size of the droplet that detaches from the membrane depends on several factors, as discussed above. However, as during the preparation of the formulations in this study the flow rate of the dispersed phase and the flux of the continuous phase were kept constant, the size of the droplet depended mainly on the composition of the dispersed phase and the continuous phase. Thus, the final microsphere size depended on the size of the detached droplets as well as the polymer concentration, as presented in Figs. 2 and 3. In accordance with neurofuzzy logic analysis (Supplemental Table SII), the mean diameter of the microspheres is dependent on the PLGA type and concentration in the oil phase, both influencing the viscosity of the dispersed phase (η_d). The diameter of the microspheres doubled with an increase of PLGA molecular weight and its concentration, reaching 76 μm for formulation 10. This is in accordance with Eq. (2) that when η_d increases the t_d increases as well and consequently the droplets retain longer at the membrane pores before detaching, leading to an increased size of the final droplets, which in turn will yield bigger microspheres after evaporation of the solvent. For the formulations with low or intermediate viscosities, the t_d was <1 s, as observed by microscope, whereas a longer time (t_d > 3 s) was noted for the droplets with the highest viscosities of the premixes. Bigger droplets at the membrane pores can give coalescence when present at neighboring pores resulting in bigger particles and broadening of the distribution (Table I, formulations 8, 9, 10) (38,48). In addition to the size, the distribution became bimodal with increased viscosity of the dispersed phase (Fig. 2) as a result of the coalescence of the formed droplets (48).

The span value was also affected by the inner water volume and the PVA concentration in the continuous phase (Fig. 3), as supported by the neuroffuzy logic analysis (Supplemental Table SII). Indeed the narrowest size distribution of microspheres (span value of 0.3) was obtained for the formulation with 20% inner water phase (Table I, compare formulations 3, 11, 12). The external phase with 2 and 4% PVA resulted in almost monodisperse microspheres with span value <0.3 (formulations 13 and 3). Increased span value of 0.5 was seen for 6% PVA (formulation 14) likely as a result of increased viscosity of the continuous phase which decreases the diffusion rate of PVA molecules from the bulk to the newly formed droplets leading to slow reduction rate of the interfacial tension (44). This in turn results in an increase of the coalescence probability of the droplets formed at the membrane surface causing broader size distribution and slightly larger microspheres (48 μm compared to 40 μm; formulations 14 and 3) (44). Lower PVA concentrations (<1%) were previously shown by Liu *et al.* (24) to yield insufficiently stable emulsion droplets and were therefore not used in this study. The addition of 1% NaCl to the continuous phase (formulation 15), increased the mean microsphere size to 52 μm and the span value to 1.0, most likely because NaCl reduces the zeta-potential of the emulsified droplets resulting in their fusion which in turn yields microspheres of increased average size and broader size distribution (28). Saturation of the continuous phase with DCM slightly increased the span value from 0.3 to 0.4, while the mean microsphere size remained around 41 μm (compare formulations 3 and 16).

Effect of Formulation Parameters on the Porosity of the Microspheres

Microsphere porosity was dependent on the PLGA molecular weight, PLGA concentration in the oil phase and the composition of the continuous phase, with PLGA concentration being the most important variable (Table I, and Supplemental Fig. S2.A). Highly porous microspheres with grade 4 and 5 were formed with 10 and 15% of PLGA in the oil phase (Table I; formulations 1, 2, 6, 7). Likely, in these formulations with relatively low viscosity, the inner water phase moves relatively easily through the emulsified droplet during solidification and comes in contact with the external phase, resulting in a more porous structure (49,50). On the contrary, increase of the PLGA concentration to 30% resulted

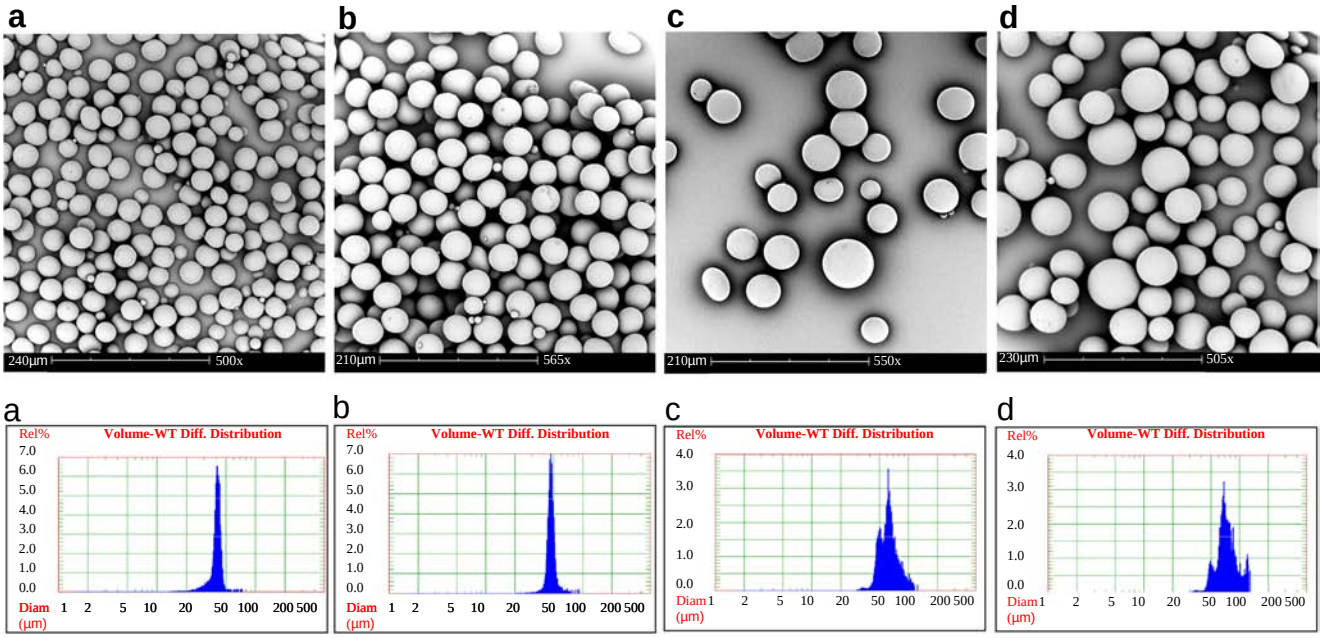

Fig. 2 SEM photographs and volume weight mean diameter of microspheres from different formulations showing the influence of the dispersed phase viscosity; (**a**) and *a*. Formulation 3 (20% PLGA 5002, 20% W_1, 4% PVA); (**b**) and *b*. Formulation 4 (25% PLGA 5002, 20% W_1, 4% PVA); (**c**) and *c*. Formulation 8 (20% PLGA 5004, 20% W_1, 4% PVA) and (**d**) and *d*. Formulation 9 (25% PLGA 5004, 20% W_1, 4% PVA) (magnification 500×).

in microspheres with low porosity, grade 1 and 2 (Table I; formulations 5 and 10). Increasing the inner water volume to 33% resulted in microspheres with porosity grade 3 (formulation 12) and with additional presence of fissures on their surface (Supplemental Fig. S3). During the formation of these microspheres, there is a higher volume fraction of inner water droplets which results in less dense and thus more porous microspheres upon solidification (49,51). The addition of 1% NaCl to the continuous phase (formulation 15) resulted in the formation of denser microspheres (porosity grade 1), likely because of the reduced outflow of the inner water phase to the continuous phase of higher osmotic pressure (52). The addition of DCM to the continuous phase had no influence on the microsphere porosity (formulation 16). However, the

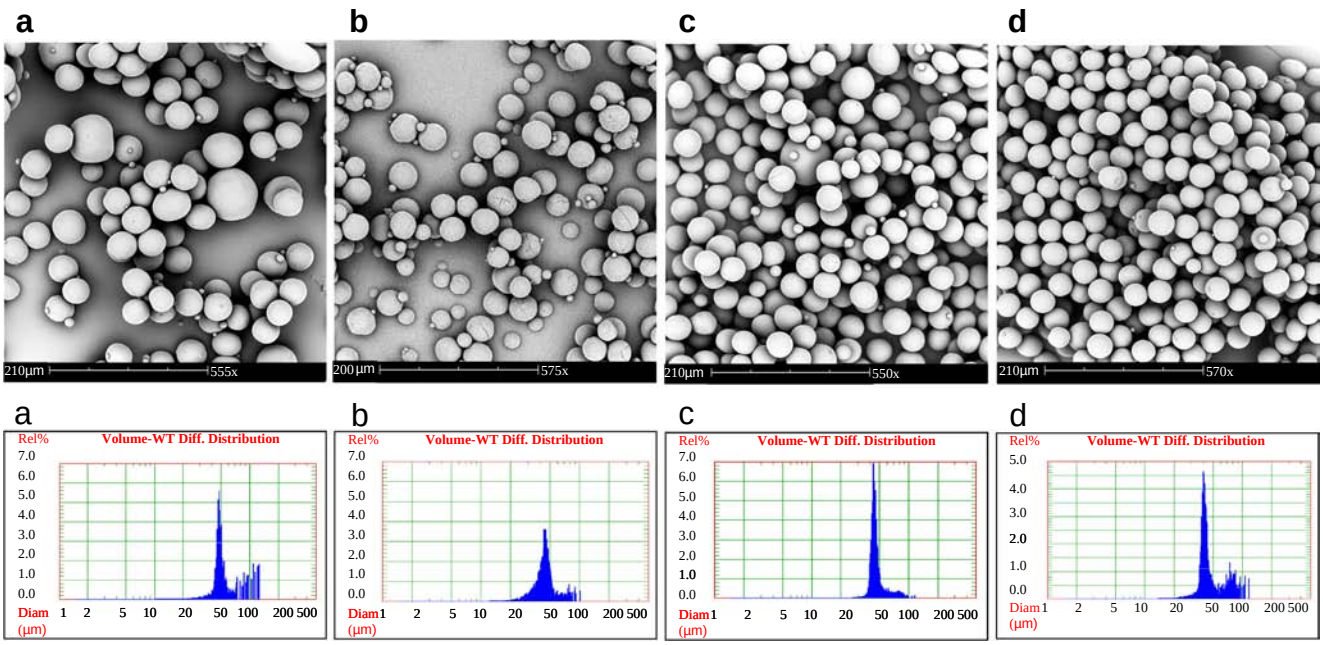

Fig. 3 SEM photographs and volume weight mean diameter of microspheres from different formulations showing the influence of the inner water phase and external phase content; (**a**) and *a*. Formulation 11 (20% PLGA 5002, 11% W_1, 4% PVA); (**b**) and *b*. Formulation 12 (20% PLGA 5002, 33% W_1, 4% PVA); (**c**) and *c*. Formulation 14 (20% PLGA 5002, 20% W_1, 6% PVA) and (**d**) and *d*. Formulation 15 (20% PLGA 5002, 20% W_1, 4% PVA and 1% NaCl) (magnification 500×).

SEM pictures of this formulation showed the presence of a fissured surface, similar to formulation 12, probably as a result of a slower solidification process when the continuous PVA phase was saturated with DCM.

Effect of Formulation Parameters on LC and LE

Table I and Supplemental Table SII show that both LC and LE were dependent on the PLGA molecular weight and concentration in the oil phase. With PLGA 5002 and a polymer concentration of 10% in the oil phase, the LC was relatively low (1.5%, formulation 1), whereas with 10% of PLGA 5004 in the oil phase the LC increased to 5.1% (formulation 6). The variability of LE is due to PLGA concentration and molecular weight, with PLGA concentration having the highest effect. A maximum LE of 100% was obtained with a high concentration of high molecular weight PLGA (30% PLGA 5004; formulation 10), whereas the lowest LE of 19% was obtained with low concentration of the low molecular weight PLGA (10% PLGA 5002; formulation 1). Microspheres prepared with higher PLGA concentration have shorter solidification times of the emulsified droplets and thus a lower probability that the inner water droplets come in contact with the external phase which in turn results in a higher LE. In addition, a higher viscosity of the oil phase decreases the transport of the blue dextran from the inner aqueous phase to the outer phase leading to the formation of microspheres with high LE (21).

A slight increase from 53 to 60% in LE was seen with increasing PVA concentration from 2 to 6% (formulation 13 and 14, respectively). An increase from 52 to 60% was seen with the addition of 1% NaCl (compare formulations 3 and 15), in line with previous experiments (27,28,52). The addition of NaCl to the continuous phase changes the osmotic pressure between the inner and the outer water phase, suppressing the leakage of blue dextran (28). Higher concentrations than 1% of NaCl were not tested in this study, as it was previously shown that this did not improve the LE (27).

Effect of Formulation Parameters on Degradation and Release Profile

The microspheres of Table I were evaluated for their release and degradation characteristics by incubating them at 37°C in 150 mM phosphate pH 7.4 buffer. Figure 4 shows the degradation characteristics (molecular weight and weight loss) of the microspheres prepared with different concentrations of PLGA (either 5002 or 5004) in the oil phase. It is shown that the microspheres degrade via bulk degradation, characteristic for end-capped PLGA (53,54), as no weight loss occurred during the first 20 days followed by a decrease thereafter, while the weight average molecular weight (M_w) gradually decreased in time. The degradation was followed for 90 days, in which period 90% weight loss occurred.

Figures 5, 6, 7 and 8 show the release of blue dextran from the different microsphere formulations. During degradation, a total release of blue dextran was reached, albeit with striking differences in the release profiles between different formulations. Microspheres with low porosity presented a three-phasic release profile characterized by (I) burst release, (II) lag phase and (III) sustained release, which is frequently observed for the release of macromolecules from PLGA microspheres (22). For this type of microspheres, as exemplified by formulation 5 in

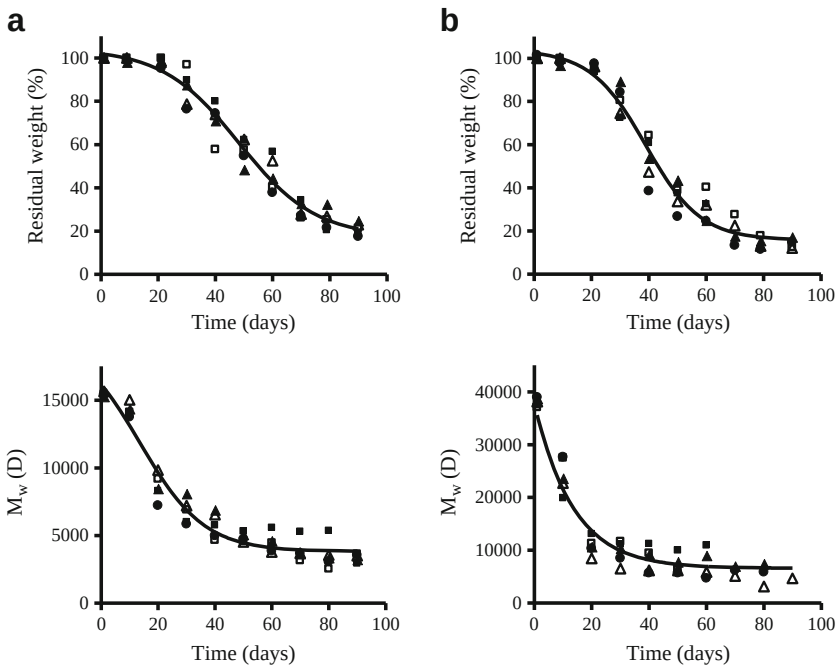

Fig. 4 Residual weight of the microspheres (%) and PLGA weight average molecular weight (M_w) over time. The microspheres were prepared with different polymer concentration in the oil phase. (**a**) PLGA 5002 (formulations 1–5) and (**b**) PLGA 5004 (formulations 6–10); (PLGA 10%, *closed squares*; PLGA 15%, *open squares*; PLGA 20%, *closed triangles*; PLGA 25%, *open triangles*; PLGA 30%, *closed circles*).

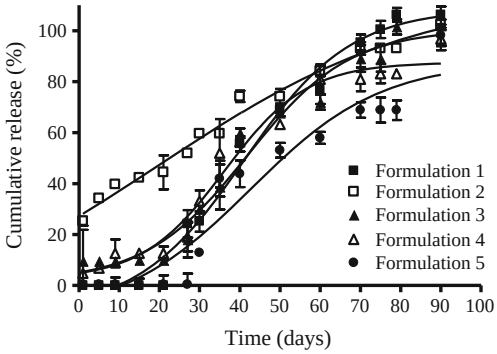

Fig. 5 Cumulative release of blue dextran from microspheres prepared with PLGA 5002 and different polymer concentrations in the oil phase. Formulation 1: PLGA 10%, Formulation 2: PLGA 15%, Formulation 3: PLGA 20%, Formulation 4: PLGA 25% and Formulation 5: PLGA 30%. The detailed formulation variables are listed in Table I.

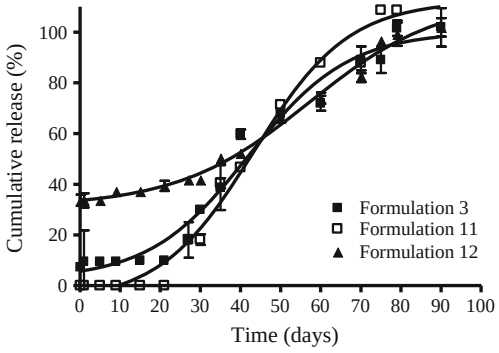

Fig. 7 Cumulative release of blue dextran from microspheres prepared with PLGA 5002 and different inner water phase volumes. Formulation 3: 20%, Formulation 11: 11% and Formulation 12: 33%. The detailed formulation variables are listed in Table I.

Fig. 5, the burst release was rather low (0.4%) and there was no blue dextran release during phase II (a typical lag phase from day 1–20). The release of blue dextran started around day 20 together with the onset of microsphere erosion that was shown in Fig. 4. In contrast, the microspheres with porosity grade 4 and 5 showed a different release pattern, characterized by a high burst release (up to 39%) and continuous release of blue dextran from day 1 until day 90, with a release rate (day 1–20) of around 1.0%/day (see, for example Fig. 6, formulation 6). Porosity of PLGA microspheres has been shown to play a major role for the burst release by creating a pathway for diffusion of the loaded macromolecules present in pores that are connected with the external medium (55). As the hydrodynamic radius of blue dextran with molecular weight of $2{\cdot}10^6$ Da is substantially smaller (~27 nm) (56) than the radius of the pores in microspheres with porosity grade 4 and 5 (>1 μm), this may give rise to the initial burst release of blue dextran in highly porous microspheres. One exception was seen for formulation 1 (Fig. 5) which showed a three-phasic release profile with no burst, although the porosity grade was 4. This formulation had a low LE of 19%, and most probably the majority of the payload was lost during the washing while the remaining blue dextran was localized in the core of the microspheres rather than in pores near the surface (17,57). It has been shown that pore closure occurs in PLGA microspheres and films, once they are hydrated during degradation studies (58–60). The kinetics of closure depends on the size of the pores, the temperature of the degradation medium and the glass transition temperature of the matrix. In line with the results published, the pore closure is rather slow for the PLGA microspheres of this study (T_g in dry state is around 45°C and when hydrated it is depressed to about 30°C (9,61,62), allowing diffusion of entrapped blue dextran through these water-filled pores.

The 3D graphs in Supplemental Fig. S2 (B and C) present the relationship between porosity and inner water volume as input parameters, and burst release and release rate (day 1–20) as output parameters. The inner water volume has the most significant effect on the burst release, whereas porosity has a significant role on the release rate at day 1–20 (Supplemental Table SII). Increasing the inner water volume from 11 to 33%, substantially increased the burst release from 0% up to 34% (see Table I, formulations 3, 11, 12) as a result

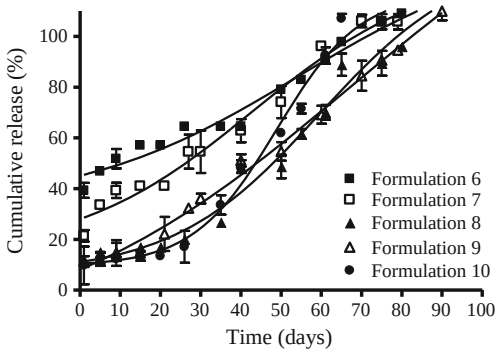

Fig. 6 Cumulative release of blue dextran from microspheres prepared with PLGA 5004 and different polymer concentrations in the oil phase. Formulation 6: PLGA 10%, Formulation 7: PLGA 15%, Formulation 8: PLGA 20%, Formulation 9: PLGA 25% and Formulation 10: PLGA 30%. The detailed formulation variables are listed in Table I.

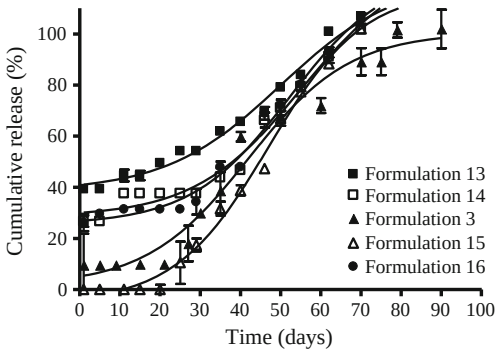

Fig. 8 Cumulative release of blue dextran from microspheres prepared with PLGA 5002 and different excipients in W_2. Formulation 13: 2% PVA, Formulation 3: 4% PVA, Formulation 14: 6% PVA, Formulation 15: 4% PVA and 1% NaCl and Formulation 16: 4% PVA and 1.6% DCM. The detailed formulation variables are listed in Table I.

of more porous structure of the microspheres formed with the increased inner water volume, as explained earlier. A continuous release of blue dextran was obtained from the formulation prepared with an inner water volume of 33% (Fig. 7, formulation 12) as a result of increased porosity and presence of fissures on the surface of microspheres (Supplemental Fig. S3).

Figure 8 shows that the release characteristics also depended on the composition of the continuous phase. The burst release increased to 39 and 27% for formulations prepared with 2% and 6% PVA, respectively, as compared to the formulation with 4% PVA which had only a 9% burst (Table I, compare formulations 13 and 14 with 3). Formulations 13 and 14 with porosity grade 3 had a continuous release of blue dextran with release rate of 0.5 and 0.8%/day (day 1–20), respectively. Interestingly, the addition of 1% NaCl to the continuous phase resulted in microspheres with no burst release (9% for the formulation without NaCl; compare formulation 15 with 3).

Preparation and Characterization of the Formulation with Controlled Porosity and Desired Release Profile

For controlled release applications, a zero-order release of macromolecular drugs from microspheres is often desired (1). In addition, the presence of the initial burst release is also undesirable as it is often not reproducible, and it can also be associated with toxic side effects due to the resulting high drug plasma levels (19). Further, a high encapsulation efficacy of the particularly expensive biotherapeutics is required. In order to achieve a formulation that has a low burst release and a continuous release without a lag phase, the input and output parameters given in Table I were modeled by GEP and genetic algorithms technology, to find the best combination of inputs for producing microspheres with the desired properties. The following constrains were used in the model to calculate the optimal formulation: mean microsphere size of 40 μm, span value lower than 0.70, LE higher than 60%, burst release lower than 20% and release rate in days 1–20 higher than 0.7%/day. The training R^2 obtained for the outputs were greater than 70% (Supplemental Table SII), indicating an acceptable prediction for each of the output parameters (35). For a continuous release of blue dextran a controlled and intermediate porosity is needed, and thus porosity grade equal or higher than three was used for modeling. GEP combined with genetic algorithm analysis proposed the following formulation characteristics: 15% PLGA 5004 in the oil phase with the inner water volume of 16% and 3% PVA in the continuous phase. This premix was stable for 85 min (GEP predicted a value 88.2 min). This formulation was processed with ME and three independent batches were prepared. Figure 9 shows the release profiles of these microspheres and Table II reports the predictions of the output

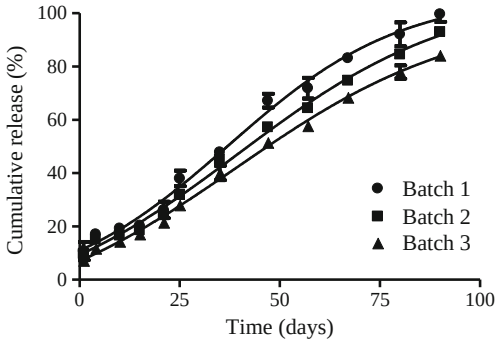

Fig. 9 Cumulative release of blue dextran from microspheres prepared using the formulation parameters calculated by GEP (see Table II) to yield microspheres with the following predicted release characteristics: porosity grade 4, burst release of 9% and release rate$_{(1\text{-}20d)}$ of 0.8%/day.

parameters and the resulting values obtained for these batches. Advantageously, the prepared batches showed a smaller span value (0.25) and higher LE (70±8%) compared to the prediction (0.53 and 54%, respectively). Microsphere size was around 41 μm and the average porosity grade was 3.6±0.4. By controlling the porosity of this formulation, a zero-order release profile was achieved for blue dextran with a complete release in a period of three months. A low burst of only 9±2% was achieved together with a continuous release of blue dextran from day 1 till 20 (release rate = 0.8±0.1%/day), a period during which the release is mainly governed by the porosity of microspheres. The continuous release from day 20 till day ~90 is governed by degradation of the microspheres. Thus, these prepared nearly monodisperse microspheres showed the desired continuous release profile of blue dextran with high LE and low burst release, demonstrating the power of GEP and genetic algorithm to design microspheres with predictable and tailorable characteristics.

Table II Results of the Defined Formulation (Microsphere Size 40 μm, Span Value <0.7, Porosity ≥3, LE >60%, Burst Release <20% and Release Rate$_{(1\text{-}20d)}$ >0.7%/day) Predicted with GEP

Output parameters	Predicted values	Batch 1	Batch 2	Batch 3
Stability W_1/O (min)	88.2	85	nd	nd
Vol-wt mean diameter (μm)	47	41±9	40±5	43±7
Span value	0.53	0.24	0.25	0.25
Porosity	4	4	4	3
Loading efficiency (%)	54	62	78	75
Burst release (%)	9	11	10	7
Release rate$_{(1\text{-}20d)}$ (%/day)	0.8	0.9	0.8	0.7

The formulation was produced in triplicate with the following formulation parameters: PLGA 5004, 15% PLGA in oil phase, inner water volume of 16% and 3% PVA in W_2

nd not determined

CONCLUSIONS

This article reports a systematic approach for preparation of PLGA microspheres with controlled porosity that showed continuous and almost zero-order release of a macromolecular model compound for three months together with a high LE and low burst release. To understand the relation between formulation parameters (polymer molecular weight, polymer concentration in the oil phase, inner water volume and excipients in the continuous phase) and microsphere characteristics, an experimental design approach was followed in which the porosity was correlated to the release profiles of blue dextran. This study successfully predicted the formulation conditions that are required to prepare microspheres that release the macromolecular model compound in a sustained manner, with low burst release.

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