Influence of formulation parameters on encapsulation of doxycycline in PLGA microspheres prepared by double emulsion technique for the treatment of periodontitis

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ABSTRACT

Advanced therapeutics often employ controlled release of pharmaceutics by the use of microspheres. However, common techniques to produce microspheres suffer from poor encapsulation efficiency (EE) of hydrophilic drugs. The aim of this study was to assess various formulation parameters to enhance doxycycline (DOX) encapsulation into poly(D, L-lactide-co-glycolide) (PLGA) microspheres prepared by double emulsion (water/oil/water) solvent evaporation technique. The parameters included variations in co-solvents, external aqueous phase (W2) pH, and PLGA end-groups (ester- or acid-terminated). Results showed that the formulated microspheres sized <10 μm and were of low polydispersity. Furthermore, an external phase pH of 9.0 was most suitable for the incorporation of the drug. Additionally, the replacement of the polymer phase with a co-solvent system (1:5, EtOH:DCM %) significantly enhanced the drug EE using acid-terminated PLGA polymer. Drug release kinetics showed an initial burst release phase, followed by a controlled exponential phase that lasted up to 21 days. Furthermore, in vitro studies revealed concentration-dependent toxicity using periodontal ligament cells (PDLCs) and antibacterial properties against periodontal pathogens. Overall, this study demonstrated the optimal formulation parameters needed to achieve a high encapsulation of water-soluble drug into PLGA microspheres using emulsification techniques.

1. Introduction

Periodontitis is a chronic disease caused by the colonization of the gingival margin, predominantly by gram-negative bacteria, such as Porphyromonas gingivalis (Pg) and Fusobacterium nucleatum (Fn) [1,2]. A recent report described that 44.7% of adults of 30 years and older in the US had periodontitis, representing approximately 65 million people [3]. Clinical signs of severe periodontitis are associated with the development of deep periodontal pockets (~5 mm), destruction of the tooth-supporting structures (i.e. bone, gums, and ligament tissue) and ultimately tooth loss [4].

Current treatment for destructive periodontitis consists of mechanical removal of plaque from the periodontal pocket via scaling and root planning (SRP) and the use of adjunctive therapy like the administration of antibiotics [5]. Doxycycline hyclate (DOX) is classified as a broad-spectrum antibiotic and a member of the tetracycline antibiotics group, which can penetrate the bacterial membrane and inhibit bacterial protein synthesis of a wide range of gram-negative and gram-positive bacteria [6,7]. However, many drawbacks are associated with the systemic administration of DOX, including drug resistance and toxicity [8]. In periodontitis treatment, DOX has to be administered several times per day to avoid an insufficient antibiotic concentration in subgingival regions [5]. Hence, local delivery of DOX using drug carriers is a more suitable method of drug delivery for this application with fewer side-effects [1].

Antimicrobial agents are incorporated in drug delivery systems (DDS) to provide local sustained release of antibiotics to the tissue surrounding the periodontal pocket, such as hydrogels, fibers, chips and polymeric microspheres [9–12]. Local delivery of antibiotics provided improved treatment results in several clinical studies [13,14]. For example, local treatment of microencapsulated minocycline (Arestin®) showed greater probing depth reduction with SRP compared to SRP alone in patients with moderate to severe periodontitis [15,16]. The therapeutic benefit of microencapsulated minocycline delivery, however, is associated with more adverse drug reactions compared to DOX, such as nausea, weakness, and vomiting [17–19]. Considering these negative aspects, with regard to the use of minocycline delivery, the use of local delivery systems based on DOX presents advantages for the...
treatment of periodontitis. Poly(lactide-co-glycolic acid) (PLGA) microspheres are a suitable drug carrier, biodegradable and already used in many medical devices [20]. The solvent evaporation double emulsion technique (water/oil/water; W/O/W) is the most widely used technique for the encapsulation of therapeutic agents in PLGA microspheres. However, a major limitation of this technique is poor EE of hydrophilic small molecules, due to the partitioning of water-soluble molecules into the external aqueous phase (W2) before solidification of PLGA microspheres. This has led to several studies converting to the spray drying as a microencapsulation technique as a result [21–25]. Moreover, limited studies have systematically investigated the effect of formulation parameters on encapsulation of DOX in PLGA microspheres using the W/O/W double emulsion technique.

Hence, the aim of the present study was to optimize the formulation parameters in the double emulsion technique, (1) to optimize encapsulation of DOX into PLGA microspheres, (2) to describe its controlled release, and (3) to evaluate its efficacy against common periodontal pathogens. Three formulation parameters were selected and investigated, i.e. the inclusion of co-solvents, manipulation of the external phase pH, and utilization of different PLGA polymer capping-groups. Different co-solvent ratios were used to facilitate faster polymer precipitation and quick solidification of the microspheres [26,27]. Additionally, the external phase pH was altered in an effort to reduce the drug's water solubility and improve the EE [28]. Furthermore, different PLGA polymer capping-groups were used to identify the best terminal-group to facilitate better drug-polymer matrix interaction [29]. To our best knowledge, the effect of these formulations has not been previously investigated on the encapsulation of DOX in PLGA microspheres using the W/O/W double emulsion technique.

Fig. 1. Schematic representation of the water-in-oil (W/O/W) double emulsion solvent evaporation technique.

2. Materials & methods

2.1. Materials

Poly(lactide-co-glycolide) (PLGA, ester-terminated, 5002, and acid-terminated, 5002A, 50:50 lactide:glycolide ratio, intrinsic viscosity 0.2 dl/g) was purchased from Corbion Purac (Gorinchem, Netherlands). Doxycycline hyclate (DOX), polyvinyl alcohol (Mw: 20,000–30,000), which was chosen as surfactant for external phase (W2), dichloromethane (DCM), and ethanol (EtOH) were obtained from Sigma-Aldrich (St. Louis, USA). Analytical grades sodium chloride (NaCl) and phosphate-buffered tablets were acquired from Merck (Darmstadt, Germany) and ThermoFisher Scientific (Waltham, USA), respectively. For the cell culture studies, Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (1% P/S), Vybrant® MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (V-12154), and Alamar Blue® reagent (all bought from ThermoFisher Scientific, Waltham, USA). All chemicals were used without further purification.

2.2. Methods

2.2.1. Preparation of PLGA microspheres

Protocol for the formulation of PLGA microspheres was adapted from Moura et al. (2015) [30]. In brief, 100 mg of PLGA was dissolved in 4 ml DCM or a co-solvent ratio composed of 0:1, 1:3 or 1:5 (EtOH: DCM). 1 ml of phosphate buffered saline (PBS) with 20% w/w DOX dissolved was added to the polymer solution and the mixture was emulsified for 4 min at 17.5 × 10³ RPM using a mixer (IKA® T25 digital ultra-Turrax®, IKA® Werke GMBH & Co. KG, Staufen im Breisgau, Germany) to form the (W1/O) emulsion. This mixture was subsequently added slowly into 200 ml of an aqueous solution containing 2% w/v polyvinyl alcohol (PVA) and 4% w/v NaCl and emulsified again at 17.5 × 10³ RPM for 6 min at room temperature to form the W1/O/W2 emulsion. Solvent removal and hardening of the microspheres were achieved by continued stirring in a magnetic stirrer overnight at 300 RPM at room temperature (23°C). Later, the microspheres were isolated by centrifugation (4000 × g for 3 min) and washed with distilled water several times to remove excess PVA. Finally, the microspheres produced were freeze-dried (BenchTop Pro, SP Scientific, Warminster,
2.2.2. Encapsulation efficiency

To determine the amount of DOX encapsulated in PLGA microspheres, 15 mg of DOX-loaded microspheres were dissolved in 1 ml DCM by continued stirring (n = 3). Once microspheres completely dissolved, 3 ml PBS was added to the mixture to precipitate the polymer and extract the drug into the aqueous layer. The suspension was vigorously mixed for 3 h and allowed to stand still to get a clear solution, followed by centrifugation for 10 min at 10,000 RPM. The supernatant was filtered and analyzed for DOX content by UV spectrophotometer at a wavelength of 360 nm using Synergy HTX multi-mode Reader (BioTek, Winooski, USA). Please note that there may be small discrepancies in the EE and LC values in Figs. 2 and 3 due to the batch-batch differences.

The EE and LC were calculated using the following formulas:

Encapsulation efficiency (EE) (%) =

\[
\frac{\text{Drug loading (mg)}}{\text{Theoretical loading (mg)}} \times 100
\]

Loading capacity (LC) (%) =

\[
\frac{\text{Weight of drug in microspheres (mg)}}{\text{Weight of microspheres (mg)}} \times 100
\]

2.2.3. Doxycycline solubility in the external aqueous phase

The effect of pH on solubility was investigated by placing an equivalent of 50 mg/ml DOX in three solutions composed of the same concentration of emulsifying agent (2% PVA) and salt (4% NaCl), which were used in the formulation protocol (2.2.1). Additionally, the pH of the solution was adjusted accordingly as previously described. The control was made as DOX dissolved in distilled water without the presence of salts and stabilizers. The solutions were stirred at 300 RPM for 1 h at room temperature (23 °C). A sample was drawn and filtered through a 0.2 μm filter (Millipore, Burlington, USA) to separate the undissolved solid from the dissolved drug. DOX concentration was then analyzed using the Synergy HTX multi-mode Reader.

2.2.4. Morphologic examination and particle size distribution

The shape and surface morphology was performed using a Zeiss Sigma 300 VP-PESEM (Carl Zeiss Microscopy, Jena, Germany) after coating with 10 nm chromium. The image was acquired at 5kv. The size and particle size index (PDI) of PLGA microspheres was measured using dynamic light scattering technique (DLS), which can measure particle range from 0.3 nm to 10 μm (DLS-Zetasizer Nano ZS, Malvern Instruments, Malvern, UK), after suspending 5 mg of the microspheres in 20 ml of deionized water. Three determinations were carried out for each formulation.

2.2.5. Fourier transform infrared spectral study (FT-IR)

Chemical stability and interaction between the drug and the polymeric matrix were investigated using FT-IR spectrophotometry (Spectrum Two FT-IR Spectrometer, PerkinElmer, Waltham, USA). The samples were scanned in the 4000-650 cm⁻¹ range with an ATR accessory.

2.2.6. In vitro release study

The in vitro release kinetics of 5 mg lyophilized drug-loaded microspheres were suspended in 1 ml PBS at physiological pH (pH 7.4) and incubated in the shaker at 37 °C at 150 RPM (n = 3). During each time-point, ranging from 1 h to 21 days, microspheres were centrifuged at 10,000 RPM for 2 min and 900 μl of supernatant was removed and stored at −20 °C for later analysis and 900 μl of fresh PBS was then added to the spheres, vortexed briefly and returned to the water bath until the next time point. The dilutions were analyzed individually in the UV/VIS spectrophotometer (Synergy) at 360 nm and using their respective absorbance values and absorption curve was constructed. A standard calibration curve of DOX was constructed by plotting absorbance versus concentration. The amount of DOX release was calculated and expressed as the percentage of the amount of encapsulated drug plotted versus time.

2.2.7. Cell culture

All experiments were done in accordance with the national guidelines for working with human materials (Dutch Federation of biomedical scientific societies, human tissue, and medical research: code of
conduct for responsible use. Available at [https://www.federa.org/](https://www.federa.org/).

After informed patient consent, periodontal ligament cells (PDLCs) were harvested from an impacted third molar from one adult patient (18 years, female). The cell proliferation medium consisted of advanced Dulbecco’s Modified Eagle Medium/Ham’s F-12 (DMEM/F-12) supplemented with 10% fetal calf serum (FBS) and 1% penicillin/streptomycin (all: Gibco). The cells were cultured for a total of three passages and then frozen in medium supplemented with 10% dimethyl sulfoxide (Sigma) in liquid nitrogen. After defrosting, cells from the 5th passage were used.

### 2.2.8. Cell viability study

The AlamarBlue test was used to investigate cell viability in the presence of PLGA microspheres. PDLCs were seeded at 10,000 cells/well on a 96-well plate for 24 h before DOX using increasing concentrations of DOX ranging 0–1000 μg/ml, for 48 h (n = 3). The cells were then washed with PBS and AB reagent (10% of the well volume) was added to each well and incubated for 4 h at 37°C to allow resazurin to become converted to resorufin by metabolically active cells. PDLC viability was determined by fluorescence measurements made on Synergy HTX multi-mode Reader (BioTek, Winooski, USA) using an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The percentage of AB reduction was compared against PDLCs which received no drug (control).

### 2.2.9. Cell proliferation study

The effect of DOX release medium on PDLC proliferation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Vybrant assay kit according to ISO 10993–5. The primary human PDLCs (passage number <10) were seeded at a concentration of 10,000 cells/well in a 96-well plate (n = 3). After 24 h incubation, the medium was removed and replaced with release cell culture medium obtained from PLGA microspheres at 37°C using protocol shown in 2.2.6, including negative control (i.e. untreated cells). After 24 h of incubation, the wells were aspirated and washed twice with PBS and replaced with 100 μl of DMEM medium with 10% MTT 12 mM stock solution and incubated for 4 h at 37°C. Subsequently, 100 μl of SDS-HCL solution was added per well and incubated for 4 h at 37°C. Cell proliferation was quantified by using a spectrophotometer at a wavelength of 570 and 690 nm. The relative cell viability (%) related to the control wells was calculated by:

\[
\text{[Absorbance]test} / \text{[Absorbance]control} \times 100
\]

### 2.2.10. In vitro antibacterial activity assay

The antibacterial activity of drug-loaded microspheres was compared with that of void microspheres (control) using gram-negative anaerobic bacteria Pg (ATCC 33277) and Fn (ATCC 25586). In this assay, DOX-loaded PLGA microspheres formulated at pH 7.0 and pH 9.0 (Table 1, D11 & D12) were used. The bacterial strains were cultured in fastidious anaerobe culture agar plate (FAA) where colonies of *P. gingivalis* (Pg) and *F. nucleatum* (Fn) were inoculated at a concentration of 9.0 × 10^6 CFU/ml. 10 mg of freeze-dried DOX-loaded PLGA microspheres were placed onto 8 mm filter paper (n = 3) and added onto the surface of the agar under sterile conditions and incubated at 37°C. The size of the inhibition zone was then measured after 48 h.

### 2.2.11. Statistical analysis

One-way analysis of variance (ANOVA) with a Tukey post-test was used to detect differences in all the tests which were run in triplicates (n = 3) and presented as the mean ± standard deviation (SD). A student’s t-test was used to compare the in vitro cytotoxicity between treated and untreated cells. Statistical analyses were carried out using GraphPad Prism software. The level of significance in the statistical analysis was determined at p < 0.05.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Polymer type</th>
<th>W2 (pH)</th>
<th>Particle Size (μm) ± SD</th>
<th>PDI ± SD</th>
<th>LC ± SD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>5002</td>
<td>5.5</td>
<td>3.16 ± 0.26</td>
<td>0.63 ± 0.49</td>
<td>0.47 ± 0.26</td>
</tr>
<tr>
<td>D2</td>
<td>7.0</td>
<td>4.59 ± 0.05</td>
<td>0.84 ± 0.27</td>
<td>0.55 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>9.0</td>
<td>2.17 ± 0.23</td>
<td>0.63 ± 0.54</td>
<td>0.44 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>5002 + 5.5</td>
<td>7.78 ± 3.15</td>
<td>0.38 ± 0.25</td>
<td>0.72 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>7.0</td>
<td>5.63 ± 0.62</td>
<td>0.67 ± 0.43</td>
<td>1.64 ± 0.59</td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>solvent</td>
<td>9.0</td>
<td>6.55 ± 0.13</td>
<td>0.18 ± 0.06</td>
<td>0.99 ± 0.21</td>
</tr>
<tr>
<td>D7</td>
<td>5002A</td>
<td>5.5</td>
<td>5.09 ± 0.03</td>
<td>0.73 ± 0.37</td>
<td>0.85 ± 0.37</td>
</tr>
<tr>
<td>D8</td>
<td>7.0</td>
<td>6.44 ± 2.99</td>
<td>0.39 ± 0.40</td>
<td>1.68 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>D9</td>
<td>9.0</td>
<td>4.53 ± 0.61</td>
<td>0.69 ± 0.53</td>
<td>3.87 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>5002A + 5.5</td>
<td>5.5</td>
<td>6.16 ± 0.44</td>
<td>0.15 ± 0.13</td>
<td>2.25 ± 0.47</td>
</tr>
<tr>
<td>D11</td>
<td>co-</td>
<td>7.0</td>
<td>8.00 ± 1.61</td>
<td>0.47 ± 0.16</td>
<td>5.11 ± 0.13</td>
</tr>
<tr>
<td>D12</td>
<td>solvent</td>
<td>9.0</td>
<td>7.76 ± 1.66</td>
<td>0.26 ± 0.08</td>
<td>9.05 ± 1.26</td>
</tr>
</tbody>
</table>

### 3. Results

#### 3.1. Effect of co-solvent on the encapsulation efficiency

Fig. 2a shows the effect of utilizing different EtOH: DCM co-solvents ratios (i.e. 0:1 control, 1:3 and 1:5) in manufacturing 5002A PLGA microspheres using an external phase pH of 9.0, under otherwise identical conditions. The EE increased significantly from 19.33% ± 4.93 for the control to 42.12% ± 4.66 using a co-solvent system composed of a ratio of 1:5 (p < 0.05). However, an increase in ethanol concentration to 1:3 (EtOH: DCM, %) did not result in a change in drug EE (p > 0.05). The same significant effects were measured for the LC. Hence, a co-solvent system in the ratio of 1:5 (EtOH: DCM, %) was used for the remainder of the study.

#### 3.2. Effect of pH and terminal group on the encapsulation efficiency

To improve the EE of the microspheres further, two additional parameters were investigated, i.e. the pH of the external aqueous phase (W2) and the polymer terminal group. Fig. 2b shows that increasing the alkalinity of the W2 from pH 5.5 to pH 7.0 or pH 9.0 resulted in a significant increase in EE of DOX (p < 0.05). Moreover, the choice of polymer terminal-group also influenced the EE and LC. Ester-terminated PLGA using co-solvent at pH 9.0 resulted in a maximum EE of 21% ± 4.06 (LC = 1.64% ± 0.59). In contrast, acid-terminated 5002A, under identical conditions, resulted in a maximum EE of 45% ± 6.31 (LC = 9.05% ± 1.26). Hence, acid-terminated PLGA formulations utilizing a co-solvent system were selected for further in vitro studies.

#### 3.3. Doxycycline solubility

To explain the pH effects on EE, DOX solubility was measured at different pHs. Fig. 3 shows the effect of pH of the external phase (W2) on drug solubility. The figure demonstrates that drug solubility decreased significantly with the inclusion of our external phase conditions (2% PVA and 4% NaCl compared to control (p < 0.001). Moreover, a significant decrease in drug solubility was observed between W2 pH 5.5 and pH 9.0 (p < 0.01).

#### 3.4. Morphological examination and size distribution

The surface morphology of DOX-loaded microspheres was examined by SEM at 5000x magnification (Fig. 4). Typically, all the microspheres prepared under various conditions had an acceptable size (<10μm) and were suitable for further in vitro studies. It can be observed that hollow PLGA microspheres were formulated with different surface morphologies. Additionally, SEM micrographs revealed a smooth
surfaced spherical morphology without aggregation at pH 5.5. Increased porosity and surface imperfections were observed at higher pH, particularly for acid-terminated 5002A (Fig. 4, g-i). The mean particle size (z-average) of all the samples are shown in Table 1. The size of the microspheres was the smallest and largest when they were prepared using monosolvent and co-solvent, respectively. The mean microsphere size for DOX-loaded microspheres with ester- and acid-terminated PLGA (without co-solvent) were found to range 2.17–4.59μm (D1-D3) and 4.53–5.09μm (D7-D9), respectively. The mean microsphere size for ester- and acid-terminated microspheres (with co-solvent) was found to be significantly higher (p < 0.05), which ranged 4.53–5.09μm (D4-D6) and 6.16–8.00μm (D10-D12), respectively. Statistical evaluation revealed that no significant difference were obtained by increasing the external aqueous phase pH.

3.5. Fourier transform infrared (FT-IR) studies

The FT-IR spectra of placebo PLGA microspheres, pristine DOX, and DOX-loaded microspheres with the highest EE (Table 1, D12) are depicted in Fig. 5. The bands observed with the placebo microspheres were almost identical to the DOX-loaded microspheres with the additional bands due to DOX with minor shifting. DOX-loaded in PLGA microspheres can easily be identified by the broad IR stretching vibration regions of functional groups -OH/-NH at 3000-3500 cm⁻¹. Furthermore, bands at 1610 and 1459 cm⁻¹ correspond to carbonyl (C=O) stretching and methylene (-CH₂) bending vibrations of the drug, respectively. Band observed between 1450 and 850 cm⁻¹ corresponds to C-H bending vibrations of the PLGA polymer. This result clearly indicates the chemical stability of the drug and polymer during the formulation process.

3.6. In vitro drug release study

The in vitro release profiles of DOX-loaded PLGA microspheres after 21 days are displayed in Fig. 6. These formulations were selected because they contained the highest EE compared to other formulations. Generally, all formulations showed an initial burst release followed by a controlled phase in which the remaining of the drug is released. Overall, a total of 27.87 ± 1.4, 36.98 ± 0.2 and 40.81μg ± 0.8 of the drug was released from PLGA microspheres formulated with a W₂ of pH 5.5, 7.0 and 9.0 after 21, respectively. The release rate was also higher for the formulation with the lowest amount of drug (i.e. pH 5.5) and became slower when the incorporated amount of drug was higher i.e. pH 7.0 and 9.0.

Fig. 4. SEM images of DOX-loaded PLGA microspheres prepared using external phase conditions. (a–c) PLGA 5002 microspheres formulated using an external phase (W₂) of: (a) pH 5.5, (b) pH 7.0 and (c) pH 9.0. (d–f) PLGA 5002 with co-solvent (1:5, EtOH: DCM); (g–i) PLGA 5002A without co-solvent; (j–l) PLGA 5002A with co-solvent. Magnification is 5000x, scale bar is 10 μm.
3.7. Cytocompatibility and antibacterial studies

3.7.1. Free drug cytotoxicity

The effect of DOX concentration (0–1000 μg/ml) on PDLC viability is shown in Fig. 7a. DOX at a concentration of 5 μg/ml showed no toxic effects on PDLC in vitro, with a mean reduction of AB reagent equal to that of control (p > 0.05). The percentage of AB reduction decreased significantly at concentrations greater than 25 μg/ml (p < 0.0001) with the greatest difference observed at the highest concentrations of DOX tested (500 & 1000 μg/ml).

3.7.2. Cytotoxicity of released drug

The cytotoxicity of DOX released from PLGA microspheres as determined by MTT cell proliferation assay is shown in Fig. 7b. In this assay, the release medium was obtained at 37 °C at three different time-points: 0–1, 3–4 and 6–7 days. The data determined that there was no significant effect between drug release obtained at different time intervals and cytotoxicity of PDLCs (p > 0.05).

3.7.3. Morphologic examination of PDLCs

Microscopic images of PDLCs exposed to increasing concentrations of DOX are shown in Fig. 7c. The images obtained showed cells treated with DOX at high concentrations (≥25 μg/ml) displayed greater morphological signs of apoptosis compared to healthy cells. Microscopic images revealed that the cells exposed to high concentrations of DOX were less adherent (Fig. 7c, iii–viii) compared to untreated cells (Fig. 7c, i). Overall, the morphologic images showed that DOX is toxic in a dose-dependent manner.

3.8. Antibacterial potential of PLGA microspheres

Drug-loaded PLGA microspheres with the highest EE (i.e. pH 7.0 and 9.0 using 5002A + co) were used in the antibacterial tests with anaerobic bacteria found in periodontal pockets of periodontitis patients (i.e. Pg and Fn). The results displayed in Fig. 8 showed no inhibition zone surrounding the void microspheres (control). However, drug-loaded PLGA microspheres showed an inhibition zone surrounding all the spheres tested using the inhibition zone test. A significant difference was obtained between pH 7.0 and 9.0 microspheres for P. gingivalis (p < 0.05) where spheres formulated at pH 9.0 resulted in a larger inhibition zone. However, no significant difference was observed between pH 7.0 and pH 9.0 microspheres for F. nucleatum bacteria (p > 0.05).

4. Discussion

The objective of this study was to identify the optimum formulation parameters required to achieve a maximum encapsulation efficacy for DOX-loaded PLGA microspheres using the double emulsion technique. We hypothesized that poor encapsulation of DOX was attributed to high drug water solubility of the drug, which favors partitioning into the external phase before solidification of the polymeric microspheres. To prevent drug partitioning, we adjusted specific formulation parameters which we hypothesized were essential for improving drug encapsulation, i.e. the inclusion of co-solvents, the pH of the external phase, and the variation of the PLGA terminal group (acid or ester-terminated). Overall, the data suggested that drug encapsulation was strongly influenced by the formulation factors investigated.

Where adjusting the pH of the external phase resulted in a significant increase in drug encapsulation and loading efficiency. The increase in encapsulation efficiency was found to be mainly due to a reduction in DOX solubility in the external phase in response to increasing the alkalinity of the W2. DOX is an amphoteric compound with three pKₐ values corresponding to pKₐ1 3.02 ± 0.02 (tricarbonyl system), pKₐ2 7.97 ± 0.15 (ketophenolic system), and pKₐ3 9.15 ± 0.3 (dimethylammonium group). When the pH of the solution is below the pKₐ
(pH < pK_a), DOX is ionized resulting in increased solubility in the W_2.

However, when the pH is greater than the pK_a (pH > pK_a), DOX is unionized resulting in a decreased solubility in the W_2. Furthermore, under acidic pH conditions, the addition of hydrochloric acid causes the formation of doxycycline hydrochloride (DOX·HCl·H_2O) [7]. However, under alkaline conditions, drug neutralization with sodium hydroxide occurs resulting in the insoluble form of the drug, doxycycline monohydrate (DOX·H_2O). The monohydrate variant lacks a hydrogen bond donor or acceptor and is, therefore, unable to interact with water molecules [31]. Hence, a reduction in water solubility was observed.

Experimentally, it was not possible to increase the alkalinity of the W_2 phase beyond 9.0, due to increased polymer erosion as observed after the close morphologic examination.

In addition to the external phase pH, PLGA polymer capping-group played a vital role in facilitating drug encapsulation. Acid-terminated PLGA encapsulated significantly more drug under the same conditions compared to ester-terminated PLGA. This can be due to better interaction between the drug molecule and carboxylic acid end groups of PLGA. A higher drug-polymer interaction leads to a slower drug diffusion from the polymer microspheres into the W_2 prepared with an acid-terminated polymer [21]. Furthermore, the inclusion of co-solvents resulted in a higher EE due to the quick solidification of PLGA polymer into polymeric droplets by transitioning from the organic phase into the water phase [32]. Quick solidification results in a fast mass transfer of the polymer from a semi-solid to a solid microsphere, which facilitates a higher encapsulation of hydrophilic drugs [33]. In the present study a maximum ratio of 1:5 (EtOH: DCM) was used. However, it was not possible to increase the concentration of co-solvent (EtOH) beyond this range due to polymer stability issues.

SEM analysis showed that PLGA microspheres manufactured using acid-terminated PLGA displayed a porous morphology compared to ester-terminated PLGA, which contained a smoother surface appearance. Acid-terminated polymers have a higher water solubility compared to ester-capped PLGA, which is related to the orientation of the carboxylic end groups [29]. The porous morphology is due to autocatalysis by the carboxylic end groups, which translated into faster biodegradation of the polymer matrix as a result of hydrolysis of the ester bonds in the polymer chain [28].

The mean particle size and distribution data showed that dependent on the formulation parameters, PLGA microspheres of different sizes...
(< 10 μm) were obtained. Microsphere size is an important factor to consider because it affects the encapsulation efficiency, product injectability, and in vivo biodistribution [34]. In the context of periodontitis, smaller spheres facilitate better penetration of periodontal tissue [35]. For example, smaller sized spheres can penetrate areas that are not accessible to other devices, such as alveolar bone trabeculae and the underlying connective tissues of the periodontal pocket. Hence, a higher concentration of drug can reach these inaccessible areas with the added benefit of a controlled release of antibiotic. Additionally, the particle size is important for controlling microsphere degradation and release kinetics of the encapsulated drug [36]. Ultimately, the purpose of these microspheres is intended for injection to the periodontal pocket in an aqueous or gel suspension.

The in vitro release kinetics revealed a sustained release profile for up to 21 days. The release of the encapsulated drug from PLGA microspheres occurs via diffusion and/or erosion of the polymer matrix [34]. More noticeably, formulations which encapsulated the highest amount of drug (Table 1, D12) displayed the slowest release compared to formulations having the lowest amount of drug (D10). At higher drug loading, there is a greater interaction between the microspheres leading to the formation of aggregates, which extends into the polymer matrix and needs more time to dissolve [37].

Cytotoxicity studies revealed concentration-dependent toxicity. Higher concentrations displayed greater cell cytotoxicity compared to the lower concentrations tested. Interestingly, the concentrations of DOX from 500 to 1000 μg/ml displayed similar values identical to the lower concentrations tested. Also, our findings imply that concentrations greater than 25 μg/ml should be avoided in the treatment of PD to maximize therapy. The minimum inhibitory concentration (MIC90) of DOX required to inhibit the growth of 90% of periodontitis-causing bacteria (i.e. Pg) is 0.12–1.56 μg/ml [38]. From our data, the maximum concentration of DOX that had little adverse effect in PDLC is 5 μg/ml. Thus, little cellular damage should occur with topical administration of DOX to the periodontal pocket at the dose equivalent to the MIC90. According to our in vitro release study, just 1 mg of DOX can provide sustained release of therapeutic concentrations of DOX (i.e. above its MIC90) and maintain these levels for several weeks, which easily fits most of the periodontal pockets (supplementary material, Table S1) [39]. The amount of DOX remaining in microspheres after the duration of the release study is supplied in the supplementary material (Table S2). Moreover, sterilization techniques should be considered for the PLGA-loaded microspheres to meet industry guidelines for pharmaceutical products. As such, gamma(γ)-irradiation (~5–25 kGy) is accepted for the sterilization of pharmaceutical products in accordance with good manufacturing practices [40].

In summary, the microspheres formulated should induce a controlled release of DOX equivalent to the MIC90 without inducing cellular cytotoxicity. It is important to note, however, that in vivo studies have to be done to confirm the current in vitro data.

5. Conclusions

An overview is provided of the effect of key formulation parameters, i.e. the pH of the W2, polymer end-group and inclusion of co-solvent, on the encapsulation of DOX using the double emulsion technique. The effect of these parameters was found to be highly important for the encapsulation of DOX. Overall, the formulated microspheres were robust with controlled drug release kinetics and antibacterial properties against periodontitis causing pathogens.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdddt.2019.04.031.

References


