A tunable and injectable local drug delivery system for personalized periodontal application

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ABSTRACT

In periodontal treatment, patient differences in disease phenotype and treatment responses are well documented. Therefore, therapy duration and dosage should be tailored to the requirements of individual patients. To facilitate such personalized medication, a tunable and controllable system is needed to deliver drugs directly into the diseased periodontal pockets. The current study established a system to achieve different drug release rates and periods by incorporating bioactive agents into poly(lactic-co-glycolic acid) (PLGA) microspheres dispersed into a novel thermo-reversible polyisocyanopeptide (PIC) hydrogel. Specifically, two drugs, i.e. doxycycline and lipoxin, were separately loaded into acid-terminated and ester-capped PLGA by electrospraying. Different formulations were developed by loading the two kinds of PLGA microspheres with different mass ratios in the PIC gels. The results demonstrated that the PIC-PLGA vehicle exhibited appropriate injectability, long-term structural stability, and no obvious in vivo inflammatory response for the desired clinical application. Furthermore, the release profiles of drugs could be manipulated by adjusting the loaded mass ratio of acid- and ester-terminated PLGA microspheres in the PIC gels. The more ester-capped PLGA was used, the slower the release rate and the longer the release period, and vice versa. Additionally, the released drugs still preserved their bioefficacy. This PIC-PLGA system can be further developed and tested in translational studies to demonstrate the final clinical benefit.

1. Introduction

Periodontitis was reported as the 11th most prevalent disease globally in 2016 [1]. Currently, the primary treatment option is to remove the pathogenic biofilm mechanically. However, rapid re-colonization of the biofilm after debridement often induces the recurrence of periodontitis in susceptible patients [2,3]. In such cases, adjunctive therapy is required, e.g. direct administration of antimicrobial, antiseptic or anti-inflammatory agents into the periodontal pocket [4]. Unfortunately, there is no standard protocol for every patient with refractory periodontitis, due to considerable differences in disease phenotype and treatment response between patients [5]. A solution for this problem can be found by tailoring the therapy duration and medication dosage to the requirements of the individual patient [6]. Therefore, a controllable and tunable local delivery system has to be available in order to facilitate personalized medicine in the treatment of periodontal disease.

Our previous work reported a thermo-reversible hydrogel, polyisocyanopeptide (PIC), as a periodontal drug carrier [7]. PIC is composed of a β-helical architecture stabilized by a peptidic hydrogen-bond network along the polymer backbone [8]. Notably, aqueous PIC polymer solutions can form a gel at extremely low polymer concentration of 0.05 wt% with 99.95 wt% of water [8]. The gel can be injected into the pocket in a slightly cooled liquid state and subsequently will form a gel upon reaching body temperature [8]. Besides, PIC is a strain-stiffening material [8]. A recent in vivo study on dermal healing found that PIC gel stayed in situ for at least one week, and moreover had excellent biocompatibility [9]. However, PIC hydrogel
itself does not facilitate the long-term sustained release of biomolecules [7]. A prolonged release can be obtained by incorporating another drug carrier into the hydrogel without compromising the main advantages of the hydrogel, like its injectability.

Poly(lactic-co-glycolic acid) (PLGA) spheres are commonly combined with gels, due to long-existing experience in various clinical applications and favorable degradation characteristics [10]. Importantly, drug release kinetics can be adjusted by controlling the characteristics of PLGA, mainly by modifying the molecular weight and polymer end-group [11,12]. Our previous study revealed that the molecular end group of PLGA had more effect on the release pattern of encapsulated doxycycline than molecular weight. A significant faster release rate for a shorter period was found in acid-terminated PLGA microspheres, compared to ester-capped spheres [13]. Consequently, we hypothesized that the drug release profile of PIC gel for periodontal therapy could be manipulated by adjusting the mass ratio of acid- and ester-capped PLGA microspheres loaded in the gel to achieve the desired personalized delivery system. Some examples of injectable nanoparticles/hydrogels composite for prolonged periodontal drug delivery can be found in the literature [14,15]. However, none of the investigated systems aims for the delivery of personalized medicine for periodontal application, which has been largely ignored in dentistry [6].

Therefore, this study aimed to develop a tunable and injectable local drug delivery system for periodontal application. Doxycycline (DOX) (which is a hydrophilic drug exhibiting anti-microbial as well as anti-inflammatory effects) [16,17] and lipoxin A4 (LXA4) (which is a hydrophobic drug acting as a pro-resolving inflammatory mediator) [18,19] were chosen as therapeutic model drugs in this study [20]. Both drugs were separately loaded into acid-terminated and ester-capped PLGA microspheres by using an electrospraying procedure. Subsequently, 10 different formulations, were fabricated by adjusting the release of the different kinds of PLGA microspheres in the gel. The in vitro injectability, rheological properties, and drug release patterns of the various formulations were investigated. Subsequently, the efficacy of the released DOX and LXA4 were further examined by in vitro assays. Finally, the tissue response of the various formulations was tested by subcutaneous implantation in a rat model.

2. Materials and methods

2.1. Materials

Two types of PLGA (Purasorb® PDLG 5002 and 5002A) were used in the experiment both with a molar ratio of lactide to glycolide of 50:50 and with a 17 kDa molecular weight (Corinon, Gorinchem, the Netherlands). Purasorb® PDLG 5002 (P2) was capped by ester end groups, whereas Purasorb® PDLG 5002A (P2A) was terminated by acid end groups. Methanol (HPLC grade), poloxamer 407, doxycycline hydrate, and trifluoroacetic acid (99%) were purchased from Sigma-Aldrich (St Louis, MO). LXA4 was supplied by Cayman chemical (Ann Arbor, MI). Acetone and acetone-tert-butanol (HPLC grade) were purchased from Macron Fine Chemicals (Center Valley, PA) and Biosolve Chimie Sarl (Dieuze, France), respectively. Phosphate-buffered saline (PBS) was from Gibco (Paisley, UK).

2.2. Electrospraying drug-loaded PLGA microspheres

P2 or P2A was dissolved in acetonitrile to a concentration of 0.16 g/mL. Then, either a solution of 24 mg DOX in 110 μL methanol or 50 μg LXA4 in 500 μL ethanol was added to 2 mL PLGA solution. The mixture was stirred until clear. In total, 4 solutions were prepared to prepare drug loaded microspheres, i.e., P2-DOX, P2A-DOX, P2-LXA4, P2A-LXA4. The electrospraying procedure was referred to as our previous work [13]. Briefly, the solution was ejected at a flow rate of 10 μL/min under a voltage of 20 kV. The electrosprayed microspheres were collected in a water bath filled with 1% Poloxamer 407 in Milli-Q. The collector was gently shaken to avoid particle agglomeration. The distance between the spinneret and collector was 17–20 cm. Thereafter, the microspheres were centrifuged and washed with Milli-Q three times to remove poloxamer. Finally, the microspheres were freeze-dried and stored at −20 °C until further use. Blank controls - P2 and P2A microspheres without loading drugs were also prepared by the same methods above for the injectability and rheology tests.

2.3. Morphology of the microspheres

The shape and surface morphology of the electrospayed microspheres were observed using a field emission scanning electron microscope (Sigma 300, Zeiss, Heidenheim, Germany). The size distribution of the PLGA microspheres was determined by a dynamic light scattering (DLS) technique using a ZetaSizer Nano-S instrument (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C for 60s (n = 3).

2.4. Characterization of PIC gel loaded with blank microspheres

The synthesis of PIC polymer (Mw ~ 635 kDa) was described previously [8]. A PIC solution was prepared by dissolving PIC polymer in cold PBS at 4 °C overnight to a final concentration of 0.5 wt%. For the various characterization assays, four different weight ratios (5%, 10%, 15%, and 20%) of blank P2 or P2A microspheres, were separately dispersed into a cold PIC solution and mixed thoroughly under vibration for injectability and rheology measurements. To visualize the spatial distribution of PLGA microspheres in PIC gels, samples were freeze-dried and cut by a surgical blade. The cross-sections of the samples were observed by scanning electron microscopy as described above.

2.4.1. Injectability test

To evaluate the effect of the added PLGA microspheres on the injectability of PIC solutions, an injectability test was performed using a tensile bench (Lloyd Instruments; Amtek, West Sussex, UK) in a compression mode [21]. In brief, 1 mL ice-chilled solution was loaded in a plastic syringe (Becton Dickinson, Plymouth, UK) with a 22G needle. The syringe was positioned in the dynamometer holder downwards, and the plunger end of the syringe was placed in contact with a loading cell. Testing was carried out at the crosshead speed of 1 mm/s, representative of manual delivery to a patient. The average force required to sustain the movement of the plunger to expel the content of the syringe was measured (N), and it was recorded whether the microspheres were thoroughly pushed out with the gel. As controls, Milli-Q water and pristine 0.5% wt PIC gel were tested. Three samples were tested in each group.

2.4.2. Rheology measurements

The samples with good injectability were further tested for viscoelastic and rheological properties using an AR2000 Advanced Rheometer (TA Instruments, Asse, Belgium) with a steel Peltier plate geometry (D = 40 mm). An aliquot of 800 μL of cold PIC-PLGA solution was loaded into the rheometer (T = 4 °C). To test the temperature of the gel-sol transition and the mechanical properties of the formed gels, a temperature sweep program was initiated at 5 °C with a heating rate of 2 °C/min until 37 °C with a load frequency of 1 Hz and strain of 2%. The sol-gel transition temperature (Tgel-sol) was determined as the onset of the increase in storage modulus G'. The value of G' and G" of each gel at 37 °C were recorded. Thereafter, the temperature sweep started by cooling down at 2 °C/min to 5 °C to confirm the reversibility of the PIC-PLGA system. Pristine 0.5% wt PIC gel were tested as the control. Three samples were tested in each group.

2.5. Drug release test of PIC gel loaded with drug incorporated microspheres

After injectability and rheology test, the maximum loading weight
of microspheres in the PIC gel was decided. Within the loading range, for the drug release test, 15 mg of drug-loaded PLGA microspheres was dispersed in 200 μL cold PIC solution with the formulations shown in Table 1. For each drug, five different weight ratios of P2/P2A microspheres, 100/0, 75/25, 50/50, 25/75, and 0/100 were mixed. PIC solution mixed with free DOX or LXA4, but without PLGA microspheres, served as control.

The drug release test was performed at 37 °C as described before [7]. Briefly, the cold samples at ~4 °C were injected into 1.5 mL Eppendorf tubes and then gelled at 37 °C (n = 3). Subsequently, 800 μL 37 °C PBS was laid over the gels. Samples were then incubated at 37 °C with agitation at 200 rpm/min. At each predetermined time point, 600 μL supernatant was withdrawn carefully and 600 μL fresh PBS was refilled. The amount of released LXA4 and DOX in the supernatant was measured at the same time to monitor the degradation of PLGA microspheres. Finally, light photographs of samples were also recorded for visual observation.

2.6. Efficacy test of the released DOX and LXA4

2.6.1. Efficacy test of DOX

The efficacy of the released DOX was assessed by the ability to inhibit the gram-negative anaerobic bacteria Porphyromonas gingivalis (ATCC 33277) [22]. The release medium from Group D3 (P2/P2A = 50/50) collected at day 3, day 8, and day 14, was chosen for this experiment. Release medium from blank microspheres (P2/P2A = 50/50) without LXA4 was collected at the same time point as a vehicle control. Three samples were tested in each group. RAW264.7 cells were seeded in 96-well plates at a density of 2 × 10^4 cells/cm² in α-minimum essential medium eagle (Gibco) and 10% fetal bovine serum (Gibco) and left for 24 h for attachment prior to experimentation. Thereafter, each macrophage was exposed to approximately 100 Nile-red fluorescent latex beads (D = 2 μm; Invitrogen, Carlsbad, CA). Then, 50 μL LXA4 releasate (concentration calculated by HPLC results) or freshly prepared LXA4 (PC) was added into the culture to reach a final LXA4 concentration of 100 nM. The same volume of NC and vehicle control medium was added accordingly. After incubation for 4 h, the fluorescence of non-phagocytosed beads was quenched with 0.4% trypan blue. The fluorescence intensity of each well was read in a Synergy HTX multimode reader (BioTek Instruments, Winooski, VT), with excitation/emission wavelengths at 535/575 nm.

Phagocytosis results were also visualized by fluorescent microscopy. RAW 264.7 cells were seeded on an 8-well μ-slide (ibidi GmbH, Gräfelfing, Germany) at a density of 15,000 cells/cm². The cells were treated using LXA4 releasate and negative control medium with the same procedure mentioned above. After 4 h of incubation, the cells were washed with PBS and then fixed in 4% paraformaldehyde for 10 min and rinsed in PBS three times. Cell nuclei were stained with 0.5 g/mL 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 min. Fluorescence was visualized under an argon-laser-powered confocal fluorescent microscope (Olympus FV1000, Zoeterwoude, the Netherlands).

2.7. In-vivo inflammatory response test

2.7.1. Experimental design and procedure

The animal experiment was approved by the Dutch Central Animal Experiment Committee (project license AVD10300 2015–241) and the work protocol (No. 2015–0072-004) was approved by the Animal Experiments Committee of Radboud University according to the legal regulations as stipulated in the amended Animal Testing Act in the Netherlands and Directive 2010/63/EU of the European Parliament and of the Council.

Ten 8-week-old male Wistar rats (~250 g) were used. The experimental material was injected into a tissue cage made of stainless steel wire mesh, left to gelation and then implanted subcutaneously (Fig. 1) for 4 weeks to obtain histological and exudates samples. Three formulations, (1) P2: 1 mL PIC gel loaded with 75 mg P2 microspheres, (2) P2A: 1 mL PIC gel loaded with 75 mg P2A microspheres, and (3) P2/P2A: 1 mL PIC gel loaded with a mixture of 37.5 mg P2 and 37.5 mg P2A (mass ratio of 50:50), were tested. Blank cages were implanted as controls.

During the surgery, the animals were anesthetized with isoflurane. After 4 subcutaneous pockets had been created, four cages from different groups were separately inserted into the pockets of each rat according to a computerized random sequence generator (www.random.org). All rats were housed under standard conditions in groups and euthanized on day 28 using CO2 suffocation. After retrieval of the cages, exudate samples were collected from the cages of 7 randomly selected rats with 18G needles and then stored at ~80 °C until further cytokine analysis (n = 7). Tissue-covered specimens were retrieved from the remaining 3 rats for histology (n = 3).

2.7.2. Measurement of cytokines in exudates and histological evaluation

Before analysis, the collected exudates were centrifuged at 2000 g at 4 °C to isolate supernatants and then tested for inflammatory cytokines with a Bio-Plex Pro™ assay (Bio-Rad, Hemel Hempstead, UK) using a rat cytokine magnetic bead assay kit (Bio-Rad). Seven cytokines were measured after incubation of 72 h at 36 °C.

### Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>P2/P2A (%/%)</th>
<th>Mass of PLGA microspheres (mg)</th>
<th>Volume of 0.5% PIC solution (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>DOX</td>
<td>100/0</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>D2</td>
<td>DOX</td>
<td>75/25</td>
<td>15</td>
<td>200</td>
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<tr>
<td>D3</td>
<td>DOX</td>
<td>50/50</td>
<td>15</td>
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<tr>
<td>D4</td>
<td>DOX</td>
<td>25/75</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>D5</td>
<td>DOX</td>
<td>0/100</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>D6</td>
<td>DOX</td>
<td>0/0</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>L1</td>
<td>LXA4</td>
<td>100/0</td>
<td>15</td>
<td>200</td>
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<td>L2</td>
<td>LXA4</td>
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<td>L3</td>
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<td>L4</td>
<td>LXA4</td>
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<td>L6</td>
<td>LXA4</td>
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</table>
tested, including the chemokine growth-regulated oncogene-KC (GRO/KC or CXCL1), monocyte chemotactic protein-1 (MCP-1), and cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10). Cytokine concentrations (pg/mL) were determined from fluorescence intensities compared to the standard curve.

The samples for histology were first fixed in 10% formalin, dehydrated in a graded series of ethanol and embedded in polymethylmethacrylate (PMMA). Thereafter, samples were split from the mid of the long axis of the cages into two halves and cross-sections of 350 μm perpendicular to the long axis of the cages were prepared using a microtome (Leica Microsystems SP 1600, Nussloch, Germany). Three sections of each specimen were stained with methylene blue and basic fuchsin. The sections were cut from at least three arbitrary chosen regions.

2.8. Statistical analysis

For drug release results, a linear mixed model analysis was used to determine the difference of the release patterns. For the other in vitro results, a one-way analysis of variance with Tukey’s multiple comparisons was used. For the in vivo cytokine test, different statistical analyses were used. To detect the overall difference between the experimental groups (P2, P2A and P2/P2A) and the cage control group, a multilevel model with random intercepts was used. To detect the differences between each group, either a simple paired t-test only when the cytokine concentrations of all samples could be detected, or a Wilcoxon signed-rank test was performed followed by Bonferroni correction for multiple comparisons. The significance level was set at α = 0.05 for the entire set with a Bonferroni correction value to α/n, n representing the number of comparisons. All analyses were done on SPSS 26.0 (IBM, Armonk, NY) or the lme4 library of the package R (version 3.6.1).

3. Results

3.1. Morphology and size distribution of drug-loaded microspheres

Drug-loaded electrosprayed PLGA microspheres exhibited a round shape and smooth surface (Fig. 2A-D). Drug loaded P2 and P2A microspheres had similar shape and diameter. LXA4 loaded P2 and P2A microspheres were around 2 μm, while DOX-P2 and DOX-P2A were around 1 μm in size (Fig. 2E). The blank microspheres, which were used in the injectability and rheology test, were observed to have a round form but were bigger in diameter (4-5 μm) than drug-loaded ones (Fig. S1). The difference in sizes was likely caused by the increased electrical conductivity of the formulation after the incorporation of drugs: higher electrical conductivity leads to smaller particle size [24].

3.2. Injectability and rheology

When P2 or P2A microspheres were dispersed into PIC solution, all formulations appeared visually as a homogenous milky suspension. The freeze-dried gel samples (Fig. 3A) showed that the microspheres were homogenously embedded in the PIC polymer. With the addition of 5% and 10% wt% P2 microspheres, the flow of the mixture was easy and continuous, and the mixtures were squeezed completely out the syringe. The average glide force required to sustain the movement of the plunger for those samples was comparable to that for water and pure PIC solution (Fig. 3B). With the increase of the number of PLGA microspheres in PIC solutions, i.e. 15% and 20% wt% formulations, microspheres sometimes agglomerated and clogged the needle inducing a filter pressing effect. Some microspheres remained in the syringe. The
samples loaded with P2A (Supplement-Fig. S2) showed similar results as those with P2, indicating that PLGA type had no influence on the injectability of the mixture formulations.

In the rheology test, there were no significant changes on the $G'$ and $G''$ after loading of 10% P2 microspheres into PIC gel (Fig. 3C). The gelation temperature was not significantly affected either. The PIC-PLGA system was still thermo-reversible. The result of P2A was similar to P2 (supplement-Fig. S3).

### 3.3. Drug release profile and degradation

#### 3.3.1. DOX release

DOX release was assessed for all D1 to D6 formulations (Fig. 4 A-C).

The plain PIC gel (D6) facilitated DOX release for about 4 days. The 100% P2A (D5) formulation sustained DOX release for about 1 week with less burst release compared to pristine PIC gel. In the first 4 days (Fig. 4B), initial burst release was gradually decreased from D5 (~95%) to D1 (~70%). From D5 to D1, the release rate gradually decreased and resulted in a longer release period. Eventually, D1 released DOX for 6 weeks (Fig. 4C). Comparison of the release data for all formulations indicated that the observed differences were significant ($P < .05$).

Examination of the pH changes in the release medium (Fig. 4D) showed that after 4 days, the pH of all groups containing P2 or P2A particles started to drop, but with a faster rate for the P2A compared to P2. But plain PIC gel group constantly kept pH ≈ 7. These data corroborated with the visual observation: the volume of PIC-PLGA groups
(D1 to D5) decreased and the erosion rate was positively associated with the amount of P2A (Fig. 4E).

3.3.2. LXA₄ release

LXA₄ was released from the plain PIC gel (L6) for about 4 days, while PLGA prolonged release to 7–10 days depending on the end-group composition (Fig. 5A, B). Both dominant acid-terminated microsphere formulations (L5 and L4) showed that release of LXA₄ was completed at day 7, and no significant differences were found compared to L6. In contrast, for the dominant ester-capped microsphere...
formulations (L1 and L2), and also for the L3 group, the release of LXA4 lasted up to 10 days, and the difference with L6 was significant. Furthermore, L2 and L3 significantly differed from L1. Notably, the initial burst release was reduced in L1, L2 and L3 compared to L4, L5 and L6.

The pH changes in the release medium of LXA4 (Fig. 5C) followed a similar trend as described above for DOX (Fig. 4D). An obvious pH drop started at day 4, and a higher percentage of acid-terminated PLGA resulted in faster pH decrease. The visual observation indicated similar results as compared to those loaded with DOX (Fig. 5D).

3.4. In vitro efficacy test of released DOX and LXA4

3.4.1. Bioactivity of released DOX

The efficacy of released DOX from group D3 was evaluated through the antibacterial test against P. gingivalis, by comparing the diameters of the inhibition zones from freshly prepared DOX versus released DOX on day 3, day 8 and day 14 (Fig. 6A, B). After curve fitting, the diameters of the inhibition zone showed a positive correlation with DOX amount. Furthermore, the released DOX demonstrated a comparable inhibition zone compared to the fresh DOX (Fig. 6C).

3.4.2. Bioactivity of released LXA4

The efficacy of released LXA4 from group L3 was evaluated through a phagocytotic activity test (Fig. 7). The quantitative analysis showed that LXA4 released from PIC-PLGA system significantly enhanced the phagocytotic activity compared to the NC and pristine PIC-PLGA vehicle (Fig. 7A). The efficacy of the released LXA4 was equivalent to the fresh prepared LXA4. Microscope inspection showed that more fluorescent beads were internalized in the released LXA4 group compared to PIC-PLGA vehicle (Fig. 7B).

3.5. In-vivo evaluation of tissue response

3.5.1. Descriptive histology of host tissue response

All incisions were fully healed after 7 days. No signs of adverse tissue response or increased stress behavior of the rats were observed during the observation period. Light microscopical analysis of the empty cages (blank control group) revealed that the cages were surrounded by a thin connective tissue capsule (Fig. 8A). The capsule was connected to the struts of the cage and had the appearance of a suspension bridge (Fig. 8B). A seroma-like structure could be observed in the cavity of the cage, which was characterized by the presence of fibrous tissue that showed a very loose structure, as illustrated by the absence of dense collagen fibers (Fig. 8C). Fibroblasts and (micro)capillaries could be recognized. There was no evidence of an inflammatory response and almost no inflammatory cells were present.

The histological sections of the cages that were filled with the various types of PIC-PLGA gel, showed a striking similarity. The cages were all surrounded by a thin collagen capsule with suspension bridge like connections between the struts of the cage. The gels had shrunk to about 75% of its original size. The resulting seroma-like space was filled small cavities and fibrous tissue (Fig. 8E). Around all remaining PIC-PLGA gels, a layer of inflammatory cells could be observed (Fig. 8F). The surface of the gels seemed to erode and occasionally even small portions of gel were loosened from the gel surface (Fig. 8G). Small pores could be recognized inside the body of the PIC-PLGA gel, which might be formed by the residual aggregations of PLGA particles; infiltration of inflammatory cells was also observed (Fig. 8H). No evident difference in inflammatory response existed between the various PIC-PLGA materials.

3.5.2. Expression of inflammatory cytokines

Regarding the cytokine measurements, IL-6 was not detectable in any sample and therefore excluded. Overall, the blank cages showed the significantly lower level of all cytokines than PIC-PLGA groups (Fig. 9). Both pro-inflammatory (GRO/KC, MCP-1, TNF-α, IL-1α, IL-1β, and IL-10) and anti-inflammatory (IL-10) cytokines were up-regulated by PIC-PLGA groups. However, no significant difference could be found after Bonferroni correction for multiple comparisons was further performed to compare any two groups.

4. Discussion

Topical administration of anti-infectious and anti-inflammatory medication is regarded essential in the treatment of refractory
periodontitis. Although many types of delivery systems have been
described before, i.e. micro-or nano-spheres [25], polymer chips [26],
fibers [27], and gels [28], there is still lack of efficient ways to apply
and retain drugs topicaly, especially in combination with a tunable
period of drug release. In this study, we investigated a thermo-re-
versible PIC hydrogel mixed with drug-loaded PLGA microspheres
to serve as a personalized delivery system. The results showed that this
system possessed the desired properties for our envisioned clinical ap-
lication, such as good injectability, a tunable release of bio-active
drugs, and not inducing severe inflammation in vivo.

The first prerequisite for successful gel therapy is the ease of ad-
ministration. Several of the commercially available gels, such as
Periocline® (Sunstar Inc) and ATRIDOX® (Tolmar GmbH), are very
viscous and not freely flowable, which makes penetration into deep
pockets difficult. The currently used thermo-reversible PIC hydrogel has
the advantage of water-like consistency. When slightly cooled, the gel
is viscous and not freely flowable, which makes penetration into deep
pockets difficult. The results showed that this system possessed the desired properties for our envisioned clinical ap-
lication, such as good injectability, a tunable release of bio-active
drugs, and not inducing severe inflammation in vivo.

A second requirement is the adequate in-situ retention of drug de-
pots. The hydrogel was first formed at the bottom of an Eppendorf tube.
The volume of 200 μL was chosen according to the volume of com-
mmercial Emdogain® periodontal gel (Straumann, USA). Thereafter, it
was incubated in the medium with agitation, which mimicked the mi-
croflow in the periodontal pocket. It is worth mentioning that the
material inside the periodontal pocket will always experience me-
chanical forces from mastication, constant turnover of epithelium and
gingival crevicular fluid [31], which was not attainable in the in vitro
model. Several thermosensitive hydrogel products, mostly based on
poloxamers, have previously been developed for periodontal applica-
tions [32,33]. Poloxamer gel has much higher storage modulus than PIC
gel. However, the structural integrity of hydrogels is not directly related
to the value of G. Based on our previous structural stability test, po-
loxamer gel was found to disintegrate totally in just a few days [7]; in
contrast, the rigid polysiloxane backbone of PIC gives rise to fibrous
semiflexible networks, which maintain structural integrity over weeks
[7]. Op ‘t Veld et al. applied PIC gel on mice for dermal wound dressing
and traced the gel by radiolabeling. They found that PIC gel stayed in
situ for at least one week, which further supported the structural in-
tegrity of PIC [9].

After loading 10% microspheres into PIC gel, the rheology results
suggest that the blank microspheres with a relatively larger size did not
significantly influence the mechanical properties, nor did they affect
the gelation temperature. It indicates that the rheological property of
the mixture is determined only by the PIC gel and not affected by the
addition of PLGA microspheres by physical mixing. In the drug release
test, it was observed that the PIC-PLGA gel was gradually eroded. To
investigate the effect of PLGA degradation on the stability of the PIC
gel, we carried out additional rheology experiments at pH = 3 (hy-
drogen chloride, the lowest pH tested in the drug release experiment)
and in the presence of the ultimate PLGA degradation products, lactic
and glycolic acid (LA/GA, 5% wt each) (Fig. S4). For the freshly pre-
pared PIC gels, the low pH has no effect, whereas an increased transi-
tion temperature and a decreased storage modulus G’ were observed
(0.8 vs 0.27 kPa) in the presence of LA/GA. This phenomenon is in line
with the well-known Hofmeister effect [34,35]. After incubation at 37 °C
for 3 days, the gel in acidic solution slowly and heterogeneously
disassembled, indicated by the broad transition, which is attributed to
the acid-induced disruption of the hydrogen bonds that stabilize the
helical polymer. However, the gel in the presence of LA/GA aged much
faster at 37 °C (G’ = 0.004 kPa), due to the combination of the low pH
and the aforementioned Hofmeister effect. It has to be noticed that in an
in vivo environment, this erosion process will probably proceed slower
because the acidic degradation byproducts will be removed constantly
by body or gingival crevicular fluid [36]. Nevertheless, a clinically re-
levant retention period of the PIC-PLGA gel inside periodontal pockets
should be further explored.

A third aim was to facilitate a tunable and sustained drug release.
Acid-terminated PLGA degrades faster than ester-capped PLGA, which
is unable to be ascribed to both its higher hydrophilicity as well as to the more
speedy accumulation of acid in the polymers [13]. As a result, acid-
terminated PLGA facilitates a much faster drug release [13,37]. Our
previous study reported that the acid-terminated PLGA microspheres
alone facilitated DOX release for 1w, while ester-capped microspheres
displayed triphasic release, i.e. an initial burst phase (0-7d), a lag phase
(7-20d) and an accelerated release phase(20d ~ 6w) [13]. In the cur-
rent study, due to the PIC gel as the secondary encapsulation vehicle,
the lag phase was transformed into a sustained release. Furthermore,
the PIC-PLGA delivery system was found to have the capacity to ma-
nipulate the range of the DOX release period from 1 week to 6 weeks,
by simply decreasing the amount of P2A and increasing the amount of P2 microspheres. When more ester end-capped PLGA is used, release will be slower and longer, and vice versa. Thus, in a future practical application, the DOX dose and release period can be tailored based on the individual characteristics of patients. Still, all formulations displayed an initial burst release in the first 4 days, even though for composition D1, this burst release was ~25% less than D5. However, it can also be argued that such a burst release is necessary for any type of patient, regardless of their characteristics, in order to achieve an initial bactericidal effect. Thereafter, the long-term sustained DOX release can be adjusted. When the DOX level in gingival crevicular fluid remained well above the minimum inhibitory concentration for periodontal pathogens, it will have a constant antimicrobial effect. On the other hand, its level can be tuned to the sub-antimicrobial dose, which has been proven to have an anti-inflammatory effect as the adjunctive treatment for periodontitis in large randomized clinical trials [16,38]. Many studies suggested the sustained release of doxycycline (or tetracycline or minocycline) did not induce resistance among the normal flora or among periodontal pathogens [39–42] or only a transient increase in resistant bacteria with no permanent change to the microbiota [43,44]. Still, bearing in mind the global rise of antimicrobial resistance, the usage of antibiotics should only be limited for clear indications. The PIC-PLGA system can be tailored later according to the clinical need. The effect of the PLGA end groups influenced the LXA4 release in a similar manner as for DOX. However, the effect size seemed much smaller. To achieve an extension of the release of LXA4, the mass ratio of P2 microspheres has to be ≥50%. This observation corroborates with other studies. For example, Reis et al. encapsulated LXA4 in PLGA microspheres by single emulsion, which resulted in a controlled release of the LXA4 over two days [45]. Likewise, E. Cianci et al. reported that

Fig. 8. Histological sections of the cages after 4-weeks of subcutaneous implantation (methylene blue and basic fuchsin staining). Fig. A provides a low magnification overview of blank control cages. The cage is surrounded by a fibrous capsule (yellow arrows) with a suspension bridge-like configuration. The black dots are the struts of the cage. The inner space of the cage, shown at higher magnification in fig. B and C, was filled with loose structured fibrous tissue and capillaries (as indicated by red arrows). Fig. D shows a low magnification overview of a cage filled with PIC-PLGA gel. The gel did shrink during implantation and the created space became filled with fibrous tissue. This was confirmed at higher magnification (Fig. E), which also shows that the remaining PIC-PLGA gel was surrounded by a fibrous capsule. Frequently, a thin layer of macrophages/giant cells (green arrows) were present at the interface between fibrous tissue and PIC-PLGA gel (Fig. F). Occasionally, the fibrous tissue contained adipose cells (#). Further, it was noticed that the gels often eroded (Fig. G) and small portions of gels (black arrows) were loosened from the gel surface. Higher magnification (Fig. H) confirmed that small pores (*) were formed inside the body of the PIC-PLGA gel, which might be the residual aggregations of PLGA particles. Inflammatory cells were also seen inside the gel (green arrows in Figs. G and H). Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 9. The concentrations of detected cytokines in the exudates after 4 weeks in all different groups. Empty cage was used as blank control. Data were presented as 0 when below the detection limit. Data are shown as the median with 25 to 75 percentiles (Boxes) and 5 and 95 percentiles (Whiskers). No significant difference was detected when compared every two groups separately. However, the overall effect of P2, P2A and P2/P2A groups showed significant higher level of all cytokines compared to blank cage group. *, P < .05; ***, P < .001.

Electrospun poly(ethylene oxide) and poly(D,L-lactide) membranes facilitated the release of LXA4 for 4 days [46]. We prolonged the release period up to 10 days in the current study. The two-layer encapsulation by the PIC-PLGA vehicle may synergistically contribute to the more sustained release. It is important to note that the clinical effective amount of LXA4 to treat periodontitis still remains to be determined, which should be done next before further studies on loading, release, and translation can be regarded. Thereafter, the loading dose of LXA4 in the PLGA microspheres can be adjusted based on the therapeutic requirement. Nevertheless, adjusting the loaded mass ratio of acid- and ester- capped PLGA microspheres in the PIC gels can be used to control the release of LXA4 to a limited extent. Moreover, all gel carriers maintained integrity longer than the actual drug release period in all formulations, meaning that the carrier system is stable enough for long-term drug release. After release, the antibacterial and phagocytosis tests further confirmed the bioactivity of the released DOX and LXA4.

Finally, it is important that the vehicle material does not evoke an obvious inflammatory response. For the in vivo application of PLGA materials, the local accumulation of acidic degradation products (i.e., lactic acid and glycolic acid) is the main cause of inflammation [47]. A rat subcutaneous implantation model has long been used to explore the inflammatory response of the dental biomaterials in an early stage of translational research [48]. This model is well-developed and standardized. Also, it does not cause much discomfort for animals. We found that both pro-inflammatory and anti-inflammatory cytokines were up-regulated by PIC-PLGA groups compared to empty cages. The empty cage did not evoke an inflammatory response. The multiple comparison test did not find any statistical differences between any two groups, indicating the inflammation induced by the PIC-PLGA groups was not severe. The histological observation corroborated with the cytokine measurements. PIC-PLGA gel remnants surrounded or infiltrated by only a layer of inflammatory cells were observed, which explains the presence of inflammatory cytokines. In comparison, in an earlier comparable cage model study involving PLGA/PCL electrospun nanofiber membranes [23], we observed a pronounced inflammatory response. A likely explanation for the limited inflammatory response of the PIC-PLGA vehicle is that the inclusion of PLGA microspheres in a very high-water content like the PIC hydrogel in combination with the presence of the body fluid flow results in an enhanced dilution and drainage of the acidic degradation products. Finally, the limited shrinkage or erosion of the PIC-PLGA after 28 days of implantation corroborates with the in vitro study after 28 days. But the erosion rate was less compared with the in vitro results again due to faster drainage of the acidic degradation products.

Although promising results have been acquired in this study, the in vitro evaluation is still far different from the in vivo situation, especially with the special periodontium. This study mainly explored and discussed the tunable drug release model, which composed the early stage of the translation. Herein, the limitations of this study should be addressed. During electrospaying, using water bath to collect the electrospayed microspheres could prevent particle agglomeration but unavoidably resulted in the drug loss. By reducing the collecting period and quick freeze-drying could decrease the loss of unencapsulated drug to some extent. Future studies can explore the effect of using different collecting methods or surfactants to minimize drug loss. Besides, the rat subcutaneous implantation model could only provide information on the inflammatory response of the PIC-PLGA vehicle. Since the periodontium is a peculiar tissue, a reliable in vivo periodontitis model is of great value to the evaluation of the actual feasibility, retention, drug release, and efficacy of this delivery system. The previous study showed that PIC hydrogel labeled with Indium-111 could be traced by single-photon emission computed tomography [9]. This technique can be used to non-invasively monitor the distribution of PIC gel in the periodontal pockets after topical application. Moreover, the concentration of drugs in the gingival crevicular fluid could be detected to reflect the real-time drug release. The carrier retention and drug release should be, therefore, tailored and further optimized. For the drug efficacy, whether the long-term low-dose release is sufficient to meet the diverse needs of periodontitis patients should be investigated in the future study. Accordingly, the loading amount of the drug in the PLGA microspheres could be adjusted.
5. Conclusion

In the present study, we have designed and formulated a tunable and injectable local delivery system by loading the PLGA-drug microspheres into the PIC hydrogel. This system exhibited appropriate injectability, long-term structural stability, and no obvious in vivo inflammatory response. Furthermore, the release of drugs could be controlled by adjusting the loaded mass ratio of acid- and ester- end capped PLGA microspheres. As evidenced in these results, we envision that upon further optimization, this novel and simple thermo-responsive PLGA-PIC carrier system may have the potential to be translated as an effective therapy for penalized medicine in periodontal clinics.

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Authors’ contribution

B.-W. and J.W. designed and conducted the experiments and wrote the manuscript. J.S. and P.H.J.K. provided technical and material support, helped with the experiments and revised the manuscript. E.M.B. performed statistical analysis and revised the manuscript. J.A.J., X.F.W. and F.Y. designed, supervised and supported this study and critically revised the manuscript.

Declaration of Competing Interest

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References

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References

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References


