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IMPLANTS SE FORMANT *IN SITU* POUR LE TRAITEMENT DES PARODONTITES

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"*I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale*".

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Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
AAP	American academy of periodontology
API	Active pharmaceutical ingredients
AUC	Area under the curve
BOP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment loss
CFU	Colony forming unit
DMSO	Dimethylsulfoxide
EPR	Electron paramagnetic resonance
FDA	American food and drug administration
FMSRP	Full-mouth scaling and root planing
GA	Glycolic acid
GCF	Gingival crevicular fluid
HEC	Hydroxyethylcellulose
ISFI	*In situ* forming implants
L:G	Ratio of lactic/glycolic acid
LA	Lactic acid
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
Mw	Molecular weight
NHANES	National health and nutrition examination survey
NMP	N-methyl pyrrolidone
PBS	Phosphate-buffered saline
PCL	Poly(ε-caprolactone)
PD	Probing depth
PDLLA	Poly(D,L-lactide)
PEG	Polyethylene glycol
PEG-DME	Polyethylene glycol-dimethylether
PEO	Poly(ethylene oxide)

PGA	Polyglycolic acid; polyglycolide
PLA	Polylactic acid; polylactide
PLGA	Poly(D,L-lactide-co-glycolide); Poly(lactic-co-glycolic acid)
PLLA	Poly(L-lactide)
PPO	Poly(propylene oxide)
PVP	Polyvinylpyrrolidone
R&D	Research and development
RAL	Relative attachment level
SEM	Scanning electron microscopy
SFM	Société française de microbiologie
sp55-R	P55 tumor necrosis factor receptor
SRP	Scaling and root planing

INTRODUCTION

1. Periodontal diseases

1.1. Definition

Periodontal diseases are various periodontal tissue infections including gingivitis and periodontitis [1], [2]. These diseases are caused by bacterial biofilm residing on teeth adjacent to the gingiva, leading to an inflammation of the gums. While gingivitis is the milder form, which does not harm the underlying supporting structures of the teeth and is reversible, periodontitis results in the loss of connective tissues and bone support [1].

Although the global epidemiology study of periodontal diseases is limited by the lack of standardized design, the variation of disease definition and diagnosis method, it is known that periodontal diseases are highly prevalent worldwide. Gingivitis can affect 50 to 90 % of the world population, depending on its definition [1]. Periodontitis is generally less prevalent but is a major cause of tooth loss in the world. In general, destructive periodontal disease is less common in young people than in adults. However, the incidence of loss of periodontal attachment and supporting bone increases in adolescents aged 12 to 17 when compared to children aged 5 to 11. Some epidemiologic studies indicate that in the United States, the prevalence of severe attachment loss in children and young adults is approximately 0.2 % to 0.5 % [3]. According to the 2009 and 2010 report of the National Health and Nutrition Examination Survey (NHANES), the total prevalence of periodontitis in American adults aged of 30 years and older was 47.2 %. Among that, the prevalence of mild, moderate, and severe periodontitis was 8.7 %, 30.0 %, and 8.5 %, respectively. There is a clear and significant disparity of the age and gender among periodontal population. Indeed, total periodontitis ranged from 24.4 % in 30 to 34 year old adults to 70.1 % in adults aged of 65 years and older. At the same age, the occurrence of disease is significantly higher in males than in females [4]. Periodontitis is also more common in developing countries, where dental hygiene is less controlled and dental treatment is too expensive to be afforded [5].

Periodontal diseases were recognized and treated about 5000 years ago, following ancient Egyptian and Chinese documents. From the 10th century, many authors described their observations of these diseases. However, until the 19th century, there was still

insufficient knowledge about the etiology and pathogenesis of periodontal diseases [2]. Until now, the most acknowledged classification of periodontal diseases is the American Academy of Periodontology (AAP) classification. The 1999 AAP classification, summarized in table 1, is the most recognized and implemented in the world [2], [6], [7].

Table 1. Abbreviated version of the 1999 AAP classification of periodontal diseases. *Adapted from* [7]. *(CAL = Clinical Attachment Loss)*

I	**Gingival Diseases**
	A. Dental plaque-induced gingival diseases
	B. Non-plaque-induced gingival lesions
II	**Chronic Periodontitis**
	(Slight: 1-2 mm CAL; moderate: 3-4 mm CAL; severe: > 5 mm CAL)
	A. Localized
	B. Generalized (> 30 % of sites are involved)
III	**Aggressive Periodontitis**
	(Slight: 1-2 mm CAL; moderate: 3-4 mm CAL; severe: > 5 mm CAL)
	A. Localized
	B. Generalized (> 30 % of sites are involved)
IV	**Periodontitis as a Manifestation of Systemic Diseases**
	A. Associated with hematological disorders
	B. Associated with genetic disorders
	C. Not otherwise specified
V	**Necrotizing Periodontal Diseases**
	A. Necrotizing ulcerative gingivitis
	B. Necrotizing ulcerative periodontitis
VI	**Abscesses of the Periodontium**
	A. Gingival abscess
	B. Periodontal abscess
	C. Pericoronal abscess
VII	**Periodontitis Associated With Endodontic Lesions**
	A. Combined periodontic-endodontic lesions

VIII	**Developmental or Acquired Deformities and Conditions**
	A. Localized tooth-related factors that modify or predispose to plaque-induced gingival diseases/periodontitis
	B. Mucogingival deformities and conditions around teeth
	C. Mucogingival deformities and conditions on edentulous ridges
	D. Occlusal trauma

The main cause of periodontal disease is the overgrowth of pathogenic bacteria disturbing the natural balance of host defense and commensal flora [8]. The oral cavity has a natural moist environment which provides good growth conditions for about 700 bacterial species, including normal and pathogenic bacteria [8]–[11]. These organisms grow on tooth surfaces first as microcolonies, which then secrete a sticky extracellular polymeric substance helping the bacteria to attach to the surface and to each other [10]. These complex, co-dependent colonies are called biofilms – the intense polymicrobial structure with functional heterogeneity that diversify the microbial population [1], [10]. Gingivitis often advances by inadequate oral hygiene, causing the dental plaque, so called plaque-induced gingivitis. Others factors can contribute to the cause of this disease such as genetics, tobacco, alcohol intake, nutritional deficiencies, HIV infection, osteoporosis, diabetes, stress, impaired host response and certain medication [1], [12]. The early colonization of root surfaces is known by the coaggregation of gram positive aerobes and facultative anaerobes such as *Streptococci* and *Actinomyces* species into developing biofilm. If oral hygiene is not practiced regularly, dental plaque is developed into a mature state consisting of high proportion of anaerobic organisms. Among them, the predominant microorganisms are gram negatives such as *Fusobacterium, Porphyromonas, Prevotella, Treponema* and members of the phylum *Synergistetes* [11], [13].

Untreated gingival lesions can progress to periodontitis, in which the plaque broadens and deepens below the gum, creating even better condition for bacteria colonies, especially gram negative and anaerobic bacteria [1], [14]. The transition from gingivitis to periodontitis depends not only on the presence and number of pathogenic bacteria, but also: (i) the degree of host susceptibility and (ii) the presence and number of protective bacteria. Indeed, the host defense mechanism is impaired by bacterial toxins and enzymes releasing from gram negative anaerobes such as: epitheliotoxins, endotoxins, leukotoxins,

collagenase, gellatinase, elastase, fibrinolysins and other proteolytic enzymes. On a susceptible host, these bacterial proteins irritate the gums, stimulate the inflammation response, leading to the destruction of the periodontium and alveolar bone [1], [2], [11]. By the time, the tight attachment of gingival tissues to the teeth is lost, causing the formation of periodontal pockets. The number of bacteria found in healthy shallow crevice is around 1×10^3 while in a periodontal pocket, this value increases to more than 10^5 times. As periodontitis progresses, these symptoms become more severe, resulting in occasional pain and discomfort, mastication and eventually tooth loss [1].

Normal oral microbiota (always present at a level of 10^8 bacteria/mL of saliva) contains primarily gram positive aerobes and only several pathogenic species with low virulence. Pathogenic species associated with periodontitis consists primarily of gram negative anaerobes [10]. Each type of periodontitis presents a specific subgingival flora consisting of its own microorganisms. The change in bacterial combination with the occurrence of certain specific bacterial combinations in infected root canals may be a decisive factor in causation of symptoms.

The first bacterial complex associated with periodontitis is called ‘orange complex’ and consists of the obligate anaerobe gram negative bacilli such as *Prevotella intermedia* and *Fusobacterium nucleatum*. The worse disease accompanies with ‘red complex’ microbiota including *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* [1], [2], [10], [15], [16]. The facultative gram negative *Actinobacillus* (*Aggregatibacterium*) *actinomycetemcomitans* is also commonly associated with this disease, especially in young adults [1], [2].

1.2. Treatment

The treatment of periodontal diseases aims to re-establish periodontal health by interrupting the disease progression, preventing its recurrence and preserving the teeth in a healthy state, comfort and function [1]. This objective can be achieved by various non-surgical and surgical therapies, depending on the specific disease as well as its severity.

1.2.1. Non-surgical treatment

The first and essential therapy for the treatment of periodontal diseases consists of the plaque control, which is performed by personal oral hygiene care and professional treatment called scaling and root planing. Scaling is the careful cleaning of the dental root surface in both supra and sub-gingival position to remove plaque and calculus (tartar) from periodontal pockets. Consequently, root planing is carried out to smooth the tooth root to remove bacterial toxins, which adsorb on cemental surface and limit plaque recurrence. The scaling and root planing should be managed regularly to maintain the oral hygiene and re-stabilize the normal oral flora, which will stop the gingival inflammation. Otherwise, these techniques also help the periodontist to follow the progress of the disease as well as to predict possible recurrence of inflammation. Scaling and root planing are the first choice therapies for most clinicians and are broadly considered as the ‘gold standard’ of periodontitis treatment [10]. This non-surgical therapy can achieve good efficacy in initial periodontitis such as decreased tissue inflammation, improved clinical periodontal attachment [1]. However, in severe cases, this mechanical treatment alone is not enough to attain the desired clinical outcomes. For instance, re-colonization of pathogenic species associated with disease and the recurrence of periodontitis are quite common [10].

To reinforce the non-surgical treatment of periodontitis, antimicrobial therapy is often used as an adjunct to scaling and root planing [10]. Current protocols recommend that the first phase treatment of generalized aggressive periodontitis as well as chronic periodontitis should be aimed at reducing or eliminating the pathogenic microorganisms [17]. Systemic antibiotherapy has been applied for the treatment of severe periodontitis. However, this administration route faced some disadvantages because of their side effects including hypersensitivity, gastrointestinal intolerance. Moreover, the concentration of drug at the action site (periodontal tissue) is quite low and not sufficient for an effective antimicrobial treatment [18]. These limits would be improved by the local administration of antimicrobial agents. Placing into periodontal pocket a controlled delivery system containing active agent could significantly enhance the local concentration of drug. By controlling the release of drug, the undesired second effects can also be reduced [18].

1.2.1.1. Antimicrobial choice

Generally, the choice of antimicrobial agents for the treatment of periodontitis is dependent on the bacterial etiology of the infection. Several antimicrobial agents have been tested for their efficacy against periodontitis. However, only a limited number of these substances have been used in the formulation of drug delivery systems for the treatment of periodontitis. These antimicrobial agents can be classified into 2 categories: antiseptic agents and antibiotic agents [18].

Table 2a. Antiseptic agents for the treatment of periodontal diseases.

Substance	Mechanism of action	Advantages	Disadvantages
Chlorhexidine	Reduction in pellicle formation, alteration of bacterial adherence to teeth and bacterial cell wall.	- Surface bacteriostatic action. - Improved wound healing. - Effective control of dental plaque.	- Staining of teeth. - Taste disturbance. - Increase in calculus accumulation. - Limited effects to supra-gingival area.
Sanguinarine	Reduction of bacterial aggregation and attachment due to alteration of bacterial wall.	- Plaque & gingivitis reduction in short time study.	- Low antimicrobial activity (MIC against periodontal pathogens: 1 to 32 µg/mL). - Low clinical efficacy in local controlled release system.

Table 2b. Antibiotic agents for the treatment of periodontal diseases.

Substance	Mechanism of action	Advantages	Disadvantages
Tetracyclines (tetracycline, doxycycline, minocycline)	Bacteriostatic action by interfering bacterial protein synthesis & inhibiting tissue collagenase activity.	- Broad spectrum of activity by inhibiting both gram negative and gram positive organisms. - Antiproteolytic properties due to inhibitory effect on oxygen radicals, so prevent tissue destruction. - Tetracyclines, especially doxycycline, inhibit matrix metalloproteinases, helping to reduce tissue destruction & alveolar bone loss [10]. - Tetracycline exhibits high substantivity in periodontal environment. - Doxycycline & minocycline exhibit greater oral absorption, more prolonged half-lives & enhanced lipid solubility. - Most of subgingival microorganisms are susceptible to tetracycline at MIC $\leq 1 - 2$ μg/mL.	- Bacteria may develop resistance to antibiotic. - Some strains of *Campylobacter* & *Veillonella* exhibited intrinsic tetracycline resistance (MIC ≥ 16 μg/mL).
Metronidazole	Inhibiting bacterial DNA synthesis.	- Selective efficacy against obligate anaerobes. - Adjunctive metronidazole therapy was reported more effective in adults with deep pockets than with less advanced periodontitis.	- Ineffective *in vitro* against *Actinobacillus actinomycetemcomitans.*
Clindamycin	Bacteriostatic effect by inhibiting bacterial protein synthesis.	- Broad-spectrum of activity against aerobic, anaerobic, and beta-lactamase-producing pathogens [19].	- Limited number of study. - Reported recurrence of disease after both adjunctive systemic & local therapy.
Ofloxacin	Synthetic fluoroquinolone actives by inhibiting bacterial cell division [20].	- Activity against gram positive & anaerobic bacteria. - Marked antibacterial activity against periodontopathic bacteria including *Fusobacterium* & *Actinobacillus actinomycetemcomitans.* - High chemical stability.	- Increasing ofloxacin resistance in southeast Asia.

1.2.1.2. Clinical studies on adjunctive antimicrobial therapy

a. Antiseptics

Many studies have focused on the clinical efficacy of antimicrobial treatment as an adjunctive therapy to scaling and root planing. Nevertheless, only modest results have been found till now. One systematically review of 7 clinical trials has analysed the efficacy of full-mouth treatment concepts for chronic periodontitis. Meta-analysis focused on full-mouth scaling with or without the use of antiseptic chlorhexidine and quadrant scaling (control). The results showed that in adults with chronic periodontitis, only minor differences in treatment effects were observed between the treatment strategies [21]. In agreement with this, another meta-analysis concluded that the use of chlorhexidine and other antiseptics in full-mouth disinfection does not provide clinically relevant advantages over conventional staged debridement [22]. Full-mouth disinfection can never been achieved as a normal microbiota is always present at a high level. Moreover, chlorhexidine was found less effective than tetracycline and minocycline in probing depth reduction when used as local adjuncts to scaling and root planing in periodontal disease therapy [23]. In addition, antiseptics do not have the advantage of suppressing the host inflammatory response comparing to tetracyclines. Hence, antibiotics seem to be more potential in the research of adjunctive antimicrobial therapy [10].

b. Antibiotics

In the domain of antibiotics, there were numerous therapies of systemic antibiotics using alone or in combination with non-surgical or surgical periodontal treatment. Only a limited number of studies regarding the effect of antibiotic used alone have been published, for instance the 50-week term tetracycline therapy [24] or the metronidazole plus amoxicillin therapy [25]. Generally, systemic antibiotics should only be used as an adjunct to periodontal therapy, when patients do not respond to conventional mechanical therapy [26].

- *Metronidazole + amoxicillin*

One of the common adjunctive systemic antibiotic therapies, which interested clinical research, is metronidazole plus amoxicillin. Ribeiro et al. [27] evaluated the adjunctive clinical, microbiologic, and immunologic effects of the systemic administration of amoxicillin and metronidazole in the full-mouth ultrasonic debridement of patients (n =

25) with severe chronic periodontitis. For test groups, antibiotics were administered at the dose of 375 mg amoxicillin and 250 mg metronidazole, three times a day for 7 days. The outcome parameters were evaluated after 3 and 6 months of treatment. Significant clinical improvements were observed for both the test and control group. At 6 months post-treatment, the test treatment resulted in significantly lower bleeding on probing (BOP) and an additional reduction (0.83 mm) in probing depth (PD) ($p < 0.05$). Moreover, percentage of sites with PD $\geq$ 5 mm exhibiting relative attachment level (RAL) gain $\geq$ 2 mm was higher (58.03 % in test patients versus 43.52 % in control patients) ($p < 0.05$). Nevertheless, no improvement in the microbiologic or immunologic outcomes was observed with the adjunctive use of systemic amoxicillin and metronidazole. With the same objective to figure out efficacy of amoxicillin/metronidazole therapy as an adjunct to full-mouth scaling in patients with chronic periodontitis, Cionca et al. [28] designed a study on 47 patients for 6 months, with the administration of 500 mg metronidazole and 375 mg amoxicillin, three times a day for 7 days on the test group. Interestingly, positive clinical outcomes have been observed with significantly lower mean number of persisting pockets > 4 mm (0.4 ± 0.8 pockets in the test group versus 3.0 ± 4.3 pockets in the control group) and bleeding on probing that required further treatment ($p = 0.005$). Recently, a systemic review was accomplished aiming at testing the efficacy of systemic amoxicillin/metronidazole as an adjunctive therapy to full-mouth scaling and root planing (FMSRP) in the treatment of aggressive periodontitis. Meta-analysis results of six randomized clinical trials showed significant clinical attachment level gain and reduction in probing depth ($p < 0.05$) in favor of FMSRP + amoxicillin/metronidazole. These findings seem to support the efficacy and the clinical safety of FMSRP + amoxicillin/metronidazole [17]. In general, amoxicillin/metronidazole therapy in adjunction with FMSRP was proved to be efficient in the treatment of both aggressive and chronic periodontitis. However, considering the small number of included studies, future studies with larger sample size and standardized study designs are needed to confirm these results.

- *Tetracyclines*

Systemic tetracyclines may be indicated in periodontal infections due to their broad spectrum of activity and possible benefit of inhibiting matrix metalloproteinases (MMP). In particular, doxycycline and minocycline have great oral absorption and prolonged half-

life. However, the low concentration of tetracyclines in gingival crevicular fluid after systemic use (from 0 to 8 μg/mL, 50 % of samples get less than 1 μg/mL) could be the reason for variable clinical response in practice [26]. Thus, the development of local tetracyclines therapy seems to be more appropriate in the research of periodontitis treatment.

The use alone of a sustained-release, biodegradable gel containing 8.5 % doxycycline was reported to be effective on chronic periodontitis (n = 45, divided into 2 groups). Following this doxycycline administration, a significant decrease ($p < 0.01$) in total anaerobic counts in subgingival plaque was observed for 6 months after initiation of treatment. Regarding antibiotic susceptibility patterns associated with subgingival plaque and saliva, no change in the number of resistant bacteria or the acquisition of antibiotic resistance was observed [29]. When using in combination with full-mouth scaling and root planing, or full-mouth debridement, the local application of 8.5 % w/w doxycycline-loaded PLA/NMP (Atridox^{TM}) was effective in reducing clinical signs of chronic periodontitis (n = 105). After 3 month post-treatment, the proportion of pocket closure determined as probing pocket depth PPD < 4 mm was significantly increased (50 to 58 %); the clinical attachment level CAL gained from 0.5 to 0.8 mm and the proportion of sites showing a clinically significant CAL gain (> 2 mm) increased from 30 to 38 % compared to the baseline [30]. These results are quite reasonable regarding the pharmacokinetic profiles of local delivery of doxycycline gels in gingival crevicular fluid (GCF) and saliva given by the study of Kim et al. [31]. They measured local drug concentration after delivering Doxy (14 % doxycycline in PEG-PLGA copolymer gel) or Atridox^{TM} (8.5 % doxycycline in PLA/NMP polymer solution) in 10 patients with severe periodontitis. In GCF specimens, sites treated with Atridox^{TM} exhibited a faster decrease of mean doxycycline concentration (from 1085 to 274 μg/mL) than sites treated with Doxy (1388 to 804 μg/mL, measured at 2 and 24 h after application, respectively). Both doxycycline gels demonstrated pharmacokinetics of controlled-release delivery systems, with doxycycline concentration in GCF after 12 days of 8 and 19 μg/mL for Atridox^{TM} and Doxy, respectively. In contrast, another recent study investigating the effect of topical doxycycline Atridox^{TM} as an adjunct to non-surgical periodontal treatment in chronic and aggressive periodontitis patients provided negative results. 10 chronic periodontitis patients and 8 aggressive periodontitis patients were divided into 4 groups treated by scaling and root planing with or without

doxycycline gel application. The results yielded at 1, 3 and 6 months post-treatment was found not statistically different between the test and control sites in probing depth, plaque scores and bleeding on probing values. Similarly, GCF MMP-8 levels presented no significant intergroup differences [32]. These contradictory results could be explained by the complexity of periodontitis with the variety of pathogen bacteria among which many are still unknown, and host modulation is not always a feasible issue.

Among the tetracyclines, minocycline has the best absorption and tissue penetration [26]. This property provides advantages for local application in the treatment of periodontitis. Many studies on the efficacy of topical minocycline therapy have been carried out. Generally, local application of minocycline was reported inefficient when used as mono-therapy [33], but provided significant efficacy in combination with mechanical treatment. For instance, minocycline HCl 2 % ointment reported a significant reduction in microbial count and improvements in clinical parameters for the scaling with minocycline therapy versus scaling alone. With regard to the dose study, application of a 2 % ointment 3 to 4 times every 2 weeks in combination with scaling and root planing was proved to provide significant improvement in microbiological and clinical parameters versus scaling and root planing for the treatment of adult periodontitis [34]. Another study evaluated the efficacy of 2 % minocycline gel as adjuncts to scaling and root planing in the treatment of persistent periodontal lesions. The clinical parameters were also found significantly improved in the test group (n = 21) over control group (n = 20), with mean probing depth reduction at 6 months was 1.10 mm versus 0.71 mm. Thus, the benefit of adjunctive local 2 % minocycline gel was statistically significant [35]. In accordance with these previous studies, another research was performed on a total of 104 patients over 15-month period to investigate the role of subgingivally administered 2 % minocycline ointment following scaling and root planing. The administration of drug was done at baseline, week 2, and at month 1, 3, 6, 9, 12. Scaling and root planing was repeated at month 6 and 12. During the entire 15-month study period, positive results were collected in both microbiological and clinical issues. With regard to microbiological results, the number of 7 studied microorganisms reduced significantly in both treatment groups. Concerning clinical outcomes, significantly greater improvements were observed in sites treated with minocycline compared to the control sites. For instance, at the pockets with initial probing depth $\geq$ 5 mm: mean probing depth reduction was 1.9 mm in test sites versus 1.2 mm in

control sites; gain in attachment level was 0.9 mm versus 0.5 mm in the same order. Furthermore, none of the patients demonstrated hypersensitivity or any local site reactions, proving that minocycline ointment was well tolerated. This study confirmed the good efficacy of local adjunctive 2 % minocycline ointment as adjunct to scaling and root planing in chronic periodontitis over a long period of time [36]. Some other similar studies have been performed and also gained positive results [37]. Most recently, a study evaluating the efficacy of scaling and root planing with adjunctive local minocycline microspheres in the treatment of moderate to advance chronic periodontitis was performed. However, this combination therapy did not differ significantly from scaling and root planing alone in the reduction of probing depth and bleeding on probing [38]. In brief, the local administration of minocycline, especially the 2 % minocycline in gel formulation seems to be most promising when used as an adjunct to scaling and root planing in the treatment of chronic periodontitis.

1.2.2. Surgical treatment

When non-surgical treatment failed to achieve periodontal health, surgery may be indicated to restore impaired periodontal anatomy by reducing periodontal pocket depth, gaining access for debridement of residual dental plaque and stimulating the regeneration of lost periodontal support [1]. Due to excessive gingival recession, tooth roots are exposed, facilitating further recession and bone loss. Gum graft surgery can be used to cover roots and compensate the lost gum tissue. A regenerative procedure is recommended to the patients with advance periodontitis whose bone and tissue supporting the teeth has been destroyed. Membranes, bone grafts or tissue-stimulating proteins can be used to encourage the regeneration on patients. The periodontal pocket reduction procedure is necessary for the patients who have too deep pockets to be cleaned by professional care. In this case, the bacteria accumulation inside periodontal pocket should be eliminated after folding back the gum tissue. Periodontal tissue is secured to be clean before placing back into place. Last but not least, dental implants and the replacement of defective prostheses are also important for periodontal therapy on patients who have lost a tooth or teeth [1].

Briefly, the treatment therapies of periodontal diseases are various and can be tailored to individual patients depending on their etiology, severity and the associated systemic diseases. The success of the treatment depends much on oral home care,

continued efforts to control or remove risk factors, and regular maintenance or supportive follow-up therapy after active treatment. Adjunctive antibiotics re-treatment should be considered for patients with aggressive or refractory periodontitis, based on the present pathogenic microbial community and their sensitivity [1].

2. Local controlled delivery systems for the treatment of periodontitis

In patients with periodontitis, the periodontal pockets can act as a natural reservoir filled with gingival crevicular fluid (GCF) for the administration of antimicrobial agents to periodontal tissues. GCF is characterized by a typical flow, giving a flushing action that leads to a rapid removing of substances from gingival sulcus. However, this effect can be compensated by the introduction of controlled release drug delivery into periodontal environment. Moreover, the isolation effect of GCF keeps the substance within pockets separated from saliva. These characteristics makes the periodontal pockets an ideal route for local antimicrobial therapy in periodontitis treatment [39], [40]. A broad variety of local delivery systems have been developed to maintain the concentration of antimicrobial agents in GCF higher than their minimum inhibitor concentration against bacteria. These numerous systems are diversified in materials (biodegradable or non-biodegradable polymers) as well as in device form (solid or semi-solid, adhesive or non-adhesive systems). The proposed formulations include fibers, films, brushite cements, wafers, strips, microspheres, microcapsules, microparticles and gels (Table 3).

Table 3. Summary of some investigated local controlled delivery systems for the treatment of periodontitis.

Drug delivery system	Antimicrobial drug	Drug load (w/w)	Vehicle	In vitro release at 24 h	Prolonged release duration	Degrad-ability of carrier	Clinical study	Refer-ence
Monolithic fibers	Tetracycline HCl	25 %	Ethylene vinyl acetate	n.a.	9 d	No	Significant probing depth reduction (n = 26)	[41] [42]
	Actisite® (Alza Corporation, Palo Alto, CA, USA)*							
Strip	Doxycycline	30 %	Polyethylmethacrylate	> 50 %	4 d	No	No	[43]
Film	Metronidazole	10 %	PLA + dichloromethane	38 % (48 h)	28 d	Yes	No	[44]
	Tetracycline HCl	25 %	PLGA (85:15) + dichloromethane	27 %	14 d	Yes	Decreasing bacterial count in intra-crevicular fluid & significant microbial inhibition for 2 weeks over placebo (n = 8)	[45]
	Chlorhexidine diacetate	20 %	Cross-linked protein (Bycoprotein + glycerol + formaldehyde)	40 %	4 d	Yes	No	[46]
Insert	Chlorhexidine gluconate	34 % (2.5 mg)	Hydrolyzed gelatin (cross-linked with glutaraldehyde)	40 %	7 – 10 d	Yes	No additional antimicrobial advantage of Periochip to thorough SRP (n = 9)	[47] [48]
	Periochip® (Dexcel Pharma, Northampton, UK)*							
	Chlorhexidine gluconate	n.a.	Oxidized-dextrin-grafted paper points	30 %	28 d	Yes	No	[49]

Drug delivery system	Antimicrobial drug	Drug load (w/w)	Vehicle	In vitro release at 24 h	Prolonged release duration	Degrad-ability of carrier	Clinical study	Refer-ence
Mucoadhesive gel	Metronidazole	5 %	HEC + Carbopol 974P + polycarbophil	n.a.	n.a.	No	No	[50]
	Tetracycline HCl	5 %	HEC + PVP + polycarbophil	n.a.	n.a.	No	No	[51]
Lipid-like gel	Metronidazole benzoate	25 %	Glycerilmono-oleate + sesame oil	n.a.	n.a.	No	Significant improvement of clinical parameters (n = 27)	[52] [42]
	Elyzol® (Dumex-Alpharma, Copenhagen, Denmark)*							
Gel	Minocycline HCl	2 %	HEC + aminoalkyl methacrylate copolymer + triacetine + $MgCl_2$ + glycerin	n.a.	n.a.	No	Significant probing depth reduction and clinical attachment gain (**Dentomycin**®**)** (n = 27)	[18] [42]
	Dentomycin® (Blackwell -Supplies, Kent, UK)* **Parocline**® (Sunstar, Levallois-Perret, France)* **Periocline**® (Sunstar, Osaka, Japan)*							
In situ gel	Meloxicam	3 %	Pluronic	n.a.	n.a.	No	Significant improvement in chronic patients	[53]
	Minocycline HCl	2 %		n.a.	3 d (85 % drug released)			
In situ implants	Doxycycline hyclate	10 %	NMP (63.3 %) + PLA (36.7 %)	n.a.	7 d	Yes	Statistically superior to oral hygiene & control (n = 822)	[54]
	Atridox® (TOLMAR Inc., Fort Colin, CO, USA)*							

Drug delivery system	Antimicrobial drug	Drug load (w/w)	Vehicle	In vitro release at 24 h	Prolonged release duration	Degrad-ability of carrier	Clinical study	Refer-ence
Brushite cement	Doxycycline hyclate	n.a.	Calcium phosphate biomaterials	50 % (12 h)	3.5 d	No	No	[55]
Wafers	Silver nitrate	12 %	PLGA (73 %) PEG (15 %)	40 %	30 d *in vitro*, 21 d *in vivo*	Yes	Significant reduction in anaerobic bacteria (n = 9)	[56]
Microspheres	Minocycline HCl	2 %	n.a.	n.a.	14 d	Yes	Reduced probing pocket depth compared to SRP in supportive periodontal therapy (n = 48)	[33]
	Arestin® (OraPharma, Horsham, PA, USA)*							
	Doxycycline HCl	9 - 25 %	PLGA 50:50 + PCL + dichloromethane	45 - 60 %	7 - 11 d	Yes	Improved clinical outcomes (30 sites)	[57]
Microcapsules	Minocycline	2, 5, or 10 %	Sodium alginate + chitosan	n.a.	7 d	Yes	Statistically significant suppression of pathogenic bacteria (n = 15)	[58]
Electrospun fibers	Metronidazole	0.1 - 40 %	PLA (70:30) + acetone	15 - 40 %	> 28 d	Yes	No	[59]

* Commercial name of the respective drug; n.a. = not available; PLA = poly(D,L-lactic acid); PLGA = poly(D,L-lactide-co-glycolide); HEC = hydroxyethylcellulose; PVP = polyvinylpyrrolidone; $MgCl_2$ = magnesium chloride; NMP = N-methyl pyrrolidone; PEG = polyethylene glycol; PCL = poly(ε-caprolactone); PLA (70:30) = poly(L-lactide-co-D/L-lactide) (70:30); SRP = scaling and root planing.

Due to the etiology of periodontal diseases and the anatomy of periodontal pockets, the local drug delivery system designed for periodontitis treatment should satisfy some criteria as followed [60]:

- It should be easy to place into the periodontal pocket and remain within the pocket during the whole treatment time to maintain the local drug concentration. The injectable delivery systems (gels, microparticles, microspheres) are convenient to be administered subgingivally. The bioadhesive systems are also preferable because of their potential adhesive force, which ensure good retention of the device after placement.

- The locally applied system must deliver drug into the periodontal pocket at a sufficient level to suppress pathogenic bacteria and sustain the drug concentration to be clinically effective for a sufficient length of time.

- To facilitate the interference of clinician and to improve the compliance of patients, the drug device should be biodegradable, so that it can erode after a certain period without any surgical procedure to remove device remnants.

- The cost of device, the facility of production technique should also be considered as factors for drug research and development (R&D).

The local controlled drug delivery systems for the treatment of periodontitis gain some advantages and also some potential disadvantages (Table 4) [18], [60].

Table 4. Principal advantages and disadvantages of local controlled delivery system for the treatment of periodontitis.

Advantages	Disadvantages
Maintenance of drug concentration in its therapeutic range.	Possible toxicity or lack of compatibility of material (solvent, polymer).
Improved drug access to local site -> Improved clinical efficacy for a long duration of time.	Mild discomfort caused by the presence of drug device within periodontal pocket.
Improved pharmacokinetics -> Benefits to short half-lives drugs.	Placement technique is needed to implant the device into target site.
Lower total drug dosage -> Reduction or elimination of undesired side effects of drug.	Expensive biodegradable polymer and high R&D cost leads to increase the price of some devices.
Improved patient compliance.	
Smaller drug device with lower excipient quantity compared to systemic systems.	

Despite numerous studies aiming at designing and developing local drug delivery system for the treatment of periodontitis, only a small number of products have been marketed. The various pharmaceutical and practical demands as well as contradictory clinical results often reported for the same system challenged the R&D of these topical formulations. The first marketed subgingival system was Actisite®, which consists of fibers of ethylene vinyl acetate containing 25 % tetracycline HCl [41]. Although Actisite® prolonged the release of tetracycline for 9 days in vitro and showed good clinical efficacy [42], this system faced some difficulties in practice. These limits include the difficult and time-consuming placement technique for clinicians. In patients, main disadvantages were anesthesia needed for fiber placement, discomfort during treatment and significant adverse effects (gingival redness, tongue pigmentation). In addition, this system has to be securely fixed by cyanoacrylate adhesive due to the lack of bioadhesiveness [18]. The next marketed product was a lipid-like gel Elyzol® containing 25 % metronidazole, which can be placed easily into periodontal pocket by a provided syringe. Nevertheless, following various clinical studies, the efficacy of this gel used in combination with scaling and root

planing is controversial. This is possibly due to the poor retention of Elyzol® gel within periodontal pocket [18]. Similarly, the clinical efficacy of Periochip®, a biodegradable insert consisting of chlorhexidine gluconate in hydrolyzed gelatin was not confirmed. This biodegradable, adhesive insert can sustain drug release over 7 days. Although, following a systematic review enrolling 5 clinical studies, the microbiological and clinical results on Periochip® in conjunction with scaling and root planing therapy are limited and controversial [48]. Besides, the 2 % minocycline gel which has been commercialized under several trademarks: Dentomycin®, Periocline® and Parocline® seems to be good in clinical therapy. Adjunctive Dentomycin® was reported to provide significant probing depth reduction and clinical attachment gain [42] as well as more advantageous outcomes in bleeding on probing [18]. However, these gels still lack of biodegradability, leading to the need of removal of the empty device after treatment. A biodegradable injectable system that was broadly studied is Atridox®. This system consists of a biodegradable polymer PLA dissolved in a biocompatible solvent NMP with 10 % doxycycline hyclate drug loading. It is an *in situ* forming system due to its change from liquid to solid state after injection into periodontal pocket. This implant can sustain drug release over 7 days. In a very large clinical study (n = 822), Atridox® performed both clinical and statistical superiority for all parameters when compared to oral hygiene and the vehicle alone [54].

Briefly, biodegradable *in situ* forming implant seems to be a very potential local drug delivery system for the adjunctive periodontal therapy. The liquid nature of drug device facilitates their placement by simple injection technique, which can reach the deep periodontal pockets. Subsequently, *in situ* formation occurs forming a hardened implant with a suitable form adapted to individual crevices. However, the retention of implant and drug release control are important issues to be solved in the research and development for such type of devices.

3. *In situ* forming implants

In situ forming implants (ISFI) are parenteral liquid drug delivery formulations generating (semi) solid depot after injection via a syringe into the body [61], [62]. ISFI was first studied in the early 1980s with the goal of developing injectable antimicrobial formulations for local treatment of periodontal diseases by Southern Research Institute, then continued by ATRIX laboratories, USA [63]. Until now, ISFI are still attracting considerable attentions from researchers because of their advantageous over the other parenteral drug delivery devices such as liquids, liposomes, emulsions, microspheres, microparticles. The principal benefits from ISFI are relatively lower production cost and simple manufacturing procedure. Moreover, ISFI (semi) solid reservoir has higher local retention and stable drug distribution, thus provides better-controlled drug release [62]. Besides dental administration, ISFI has been investigated for applications in cancer treatment, ophthalmic delivery systems, tissue engineering, three-dimensional cell culturing or cell transplantation [64]–[66].

ISFI can be classified into 3 main groups, based on their mechanisms of implant formation (Figure 1). Among the various types of ISFI, the phase separation system by solvent exchange is very attractive because of its great commercial potential.

Dunn et al. [67] invented the concept of ISFI based on polymer precipitation by solvent exchange in 1990. They dissolved a water-insoluble and biodegradable polymer poly(D,L-lactide) (PLA) or poly(D,L-lactide-co-glycolide) (PLGA) in a compatible water-miscible organic solvent N-methyl pyrrolidone (NMP). Consequently, drug was incorporated into the polymer solution forming a solution or a suspension after mixing. After injection of the formulation into the body, the organic solvent diffuses into the surrounding tissues while aqueous body fluid diffuses into organic polymeric phase. This leads to phase separation and polymer precipitation, forming a depot at injection site. The active pharmaceutical ingredients (API) entrapped within the polymer matrix are released by diffusion through the water-pores and by erosion upon polymer degradation. So far, two polymer precipitation systems based on solvent exchange have been commercialized, namely Atridox® and Eligard®. Both of these products were approved by the American Food and Drug Administration (FDA) and were prepared using Atrigel® technology.

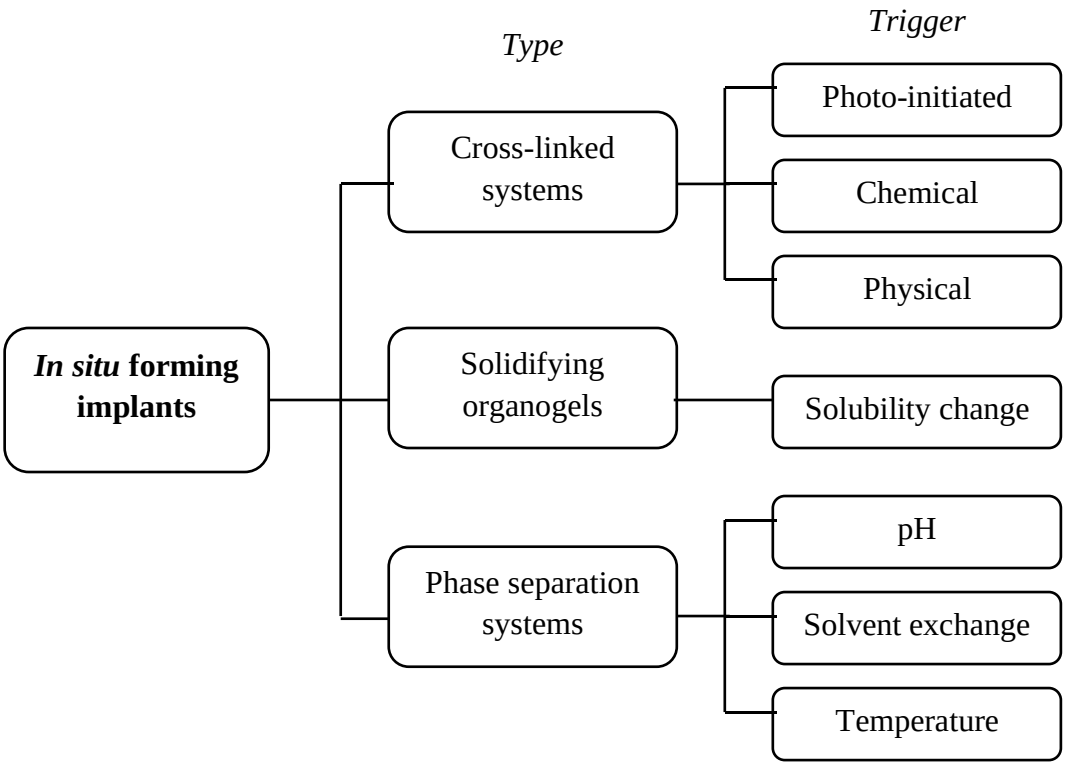


Figure 1. Classification of *in situ* forming implants.
Adapted from [66].

Atridox® is a controlled-release product used for the treatment of periodontitis, consisting of a two syringe mixing system. Syringe A contains 450 mg of 36.7 % PLA dissolved in 63.3 % NMP. Syringe B contains 50 mg of doxycycline hyclate, which is equivalent to 42.5 mg doxycycline. After mixing, the final product is a yellow viscous liquid containing 10 % of doxycycline hyclate, which is injected directly into the periodontal pocket. Upon contact with the gingival crevicular fluid, the liquid solution solidifies forming a depot allowing the controlled release of drug for a period of 7 days.

Eligard® is subcutaneous injection system providing sustained release of leuprolide acetate (7.5, 22.5, 30 or 45 mg) over a long period of time (1 month, 3 months, 4 months or 6 months, respectively), which is indicated for the treatment of advanced prostate cancer. These products also consist of 2 syringes: syringe A prefilled with PLGA dissolved in NMP; syringe B prefilled with leuprolide acetate powder. Prior to administration, two syringe parts are mixed in order to get a homogenous dispersion of drug. The controlled release of drug from Eligard® formulations is achieved by the variation of polymer type.

Clinical studies proved the high efficacy of 1-month and 3-month Eligard® formulations in reducing mean testosterone levels below the medical castration level (50 ng/dL) over 6 month treatment [68].

3.1. Compositions of *in situ* forming implants based on solvent exchange

Since this thesis focus on the *in situ* forming implants based on solvent exchange, the abbreviation ISFI will be used to denote the phase separation systems by solvent exchange. The formulation of these ISFI systems generally consists of solvent, polymer and drug.

3.1.1. Solvent

Relatively high amounts of solvent are used in ISFI to dissolve the polymer, forming a polymeric solution. As this carrier is then injected into the body and solvent diffuses into surrounding tissues, the employed solvent must meets some requirements. It must be non-toxic and biocompatible, hence it does not cause any severe tissue irritation or necrosis at injection site. Moreover, the solvent should be water miscible to diffuse quickly into the body fluid and allow water to diffuse into the polymeric solution, leading to polymer precipitation. Suitable solvents meeting those criteria includes N-methyl pyrrolidone, 2-pyrrolidone, acetone, dimethyl sulfoxide, methyl acetate, ethyl acetate, methyl ethyl ketone, ethanol, propylene glycol, dimethylformamide, tetrahydrofuran, caprolactam, decylmethylsulfoxide, oleic acid, and 1-dodecylazacycloheptan-2-one. The four first solvents are preferred due to their solvating ability and their compatibility [67].

N-methyl pyrrolidone (NMP) is the most frequently used organic solvent because of its solvating ability; allowing to dissolve a wide range of polymers. This solvent has good properties such as low volatility, low inflammability and relatively low toxicity. Following the European chemicals agency, NMP is classified as toxic for reproduction.

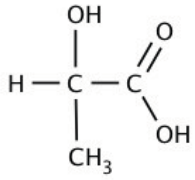
3.1.2. Polymer

Biodegradable polymers which can be used in ISFI includes polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyamides, polyurethanes, polyesteramides, polyorthoesters, polydioxanones, polyacetals, polyketals, polycarbonates, polyorthocarbonates, polyphosphazenes, polyhydroxybutyrates, polyhydroxyvalerates,

polyalkylene oxalates, polyalkylene succinates, poly (malic acid), poly (mino acid), polyvinylpyrrolidone, polyethylene glycol, polyhydroxycellulose, chitin, chitosan and copolymers, terpolymers, or blends of the materials mentioned above. Polymers with low degree of crystallinity and more hydrophobicity are preferable because of their high solubility in organic solvents. Examples of such polymers are polylactides, polycaprolactones, and poly(lactide-co-glycolide). They present more amorphous regions to enhance solubility [69]. These polymers are also widely studied because of their safety approved by FDA and long history of clinical use.

Lactic acid (LA) and glycolic acid (GA) are organic acids found in the nature, which have the molecular structure as below:

Lactic acid (α-hydroxypropionic acid), $CH_3CH(OH)COOH$



Glycolic acid (α-hydroxyacetic acid), $HOCH_2COOH$

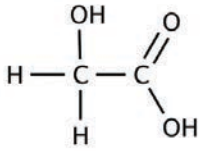


Figure 2. Molecular structure of lactic acid and glycolic acid.
Adapted from [70].

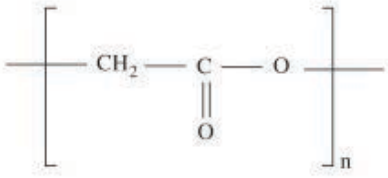
Lactic acid exists in two active forms: L(+)-lactic acid and D(-)-lactic acid. It was first isolated from milk in 1780, and polylactic acid (PLA) was reported since 1932, although its applications in medical research has attracted interest since 1960s [71]. Poly(L-lactide) (PLLA) is a crystalline polymer (37 % crystallinity) presenting good tensile strength compared to poly(D,L-lactide) (PDLLA), which is an amorphous polymer [72].

Glycolic acid can also be found in natural products such as sugar beets, unripe grapes, and wheat [70]. The polyglycolic acid (PGA) has been known since 1954 to be a potentially low cost fibre-forming polymer and was developed as the first synthetic

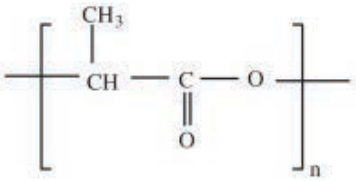
absorbable suture in 1962 [73]. PGA is a highly crystalline polymer (45-55 % crystallinity), hence exhibits a high tensile strength. Both PGA and PLA undergo hydrolytic degradation via the bulk erosion mechanism by the non-specific scission of the ester backbone. They break down into glycolic acid and lactic acid, which can be excreted in the urine or converted into water and carbon dioxide via the citric acid cycle [72]. These two monomers can be found in the human body under normal physiological conditions, as by-products of various metabolic pathways and can thus be considered as non-toxic.

Poly(lactic-co-glycolic acid) PLGA is a copolymer of PLA and PGA.

Polyglycolic acid (PGA)



Polylactic acid (PLA)



Poly(lactic-co-glycolic acid) (PLGA)

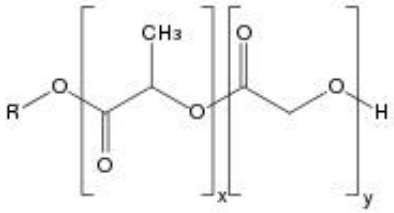


Figure 3. Molecular structure of PGA, PLA and PLGA.
(x is the number of lactic acid units, y is the number of glycolic acid units)
Adapted from [72], [74].

The product range of PLGA is large, due to the copolymerization ability of both PLLA and PDLLA with various ratios of monomers. PLGA biodegrades in water by hydrolysis of its ester linkages. In controlled release drug delivery applications, the choice of a PLGA with suitable degradation kinetics is important to achieve desired release kinetics. PLA is more hydrophobic than PGA due to the presence of methyl group, therefore the lactide-rich PLGA copolymers are more hydrophobic, hence absorb less

water and degrade more slowly. For instance, 50/50 poly(D,L-lactide-co-glycolide) degrades in 1-2 months, 75/25 in 4-5 months and 85/15 in 5-6 months. These time frames also depend on the PLGA molecular weight, the shape and structure of polymer matrix [72], [74].

Since 1970s, PGA, PLA and PLGA interested researchers as biodegradable materials in dental, orthopaedic and drug delivery application [73]. There were various studies investigating their biocompatibility and toxicity, especially for use as materials in wound suture and fixation device in orthopaedic fracture. Generally, the animals, human and in vitro tests proved that PGA and PLA provide satisfactory biocompatibility without significant toxicity neither inflammatory reaction [75]. In a cytological analysis, PGA has also been considered as immunologically inert implant material [76]. Approved by the FDA for the use in human, PLGA is considered as the best-defined biomaterial available for drug delivery with respect to design and performance so far [72].

3.1.3. Drug

The choice of active substance depends on ISFI application. For the treatment of periodontitis, chosen drugs are antiseptics or antibiotics with suitable antibacterial spectrum [18]. In prostate cancer treatment, the peptide agonist hormone receptor leuprolide acetate was chosen as active drug [64]. Besides, numerous ISFI devices has been studied using drug varying from small molecules such as diclofenac sodium [77], aspirin [78] to big molecule of proteins, namely bovine serum albumin [79], human growth hormone [80]. The properties of drug (molecular weight, solubility, affinity to the solvent) and its content in the formulation can affect the drug release profile of ISFI systems.

3.2. Mechanism of drug release from PLGA-based *in situ* forming implants

Numerous studies have been performed to investigate the release mechanism of drug from PLGA-based drug delivery system, especially in films, microspheres, microparticles, preformed implants [81]. PLGA-based implants relying on *in situ* polymer precipitation by solvent exchange, however, have not yet been extensively studied. The main differences of ISFI are: (i) the shape of ISFI can only be defined after injection of polymeric solution; (ii)

during the transformation from liquid to solid state, a complex physico-chemical process takes place, affecting the depot structure and hence the following drug release profile. Therefore, the knowledge of these characteristics is essential to understand the drug release mechanism and develop controlled-release ISFI.

3.2.1. Release mechanism

Drug release from PLGA-based ISFI results from a complex physico-chemical process occurring within PLGA matrix, from the injection of polymeric solution until the end of matrix degradation. These processes begin by the solvent exchange causing the polymer precipitation, subsequently leading to the formation of a solid depot. Within the PLGA matrix, the presence of water triggers the hydrolysis of PLGA, hence cuts the ester bonds and increases polymer chain mobility. The decrease in polymer molecular weight finally leads to the erosion of the polymeric matrix, which in turn might affect drug release. In brief, the underlying drug release mechanism can be resumed in two principal processes: diffusion and erosion. These mechanisms can occur concomitantly and are influenced by formulation parameters as well as the surrounding environment of the injection site.

3.2.1.1. Diffusion

Diffusion has been described as one of the main release mechanism controlling drug release from PLGA-based drug delivery system. It is directly related to the porosity of the polymer matrix, and thus on the processes of pore formation [81]. In the case of ISFI, the solvent exchange occurring upon contact of the polymeric solution to the aqueous environment leads to a liquid-liquid phase separation. The polymer solution transforms to a mixture of gel phase located on the surface and solution phase downside, namely two-phase, gelled structures. It was suggested that the initial drug release occurs mainly by diffusion through the interconnected polymer-lean phase that exists in gel region. Thus, fast gelling system has high burst release compared to low gelling system [82].

The resulting solid depot consists of polymer matrix with a negligible or significant quantity of water-filled pores, depending on the type of solvent and polymer. The solvents with high affinity to water (NMP, DMSO) have been reported to create highly porous structure, in contrast to the dense sponge like morphology of systems based on low water

miscible solvents (triacetin, ethyl benzoate) [83], [84]. The high density of water-filled pores provides multiple diffusion pathways to drug molecule, therefore improves the drug release rate. The biodegradable polymer, PLGA is hydrolyzed in the presence of water, resulting in shortened polymer chain length. As water uptake is faster than polymer degradation, PLGA generally undergoes bulk erosion. Erosion starts when the polymer degradation products can be dissolved in water and thus diffuses into the surrounding aqueous solution. Hydrolysis and erosion increase the pore size, hence accelerate the drug release. These effects are more pronounced on systems based on less hydrophobic PLGA (low molecular weight, low lactic:glycolic acid ratio and un-capped polymer end groups), which have greater water absorption, hydrolysis and erosion rate [81].

The diffusion coefficient of drug from PLGA-based ISFI is dependent on the diffusion coefficient in the fluid filled the pores, the porosity and the tortuosity. Consequently, this parameter is not constant but time-dependent due to altered depot structure induced by polymer degradation [81].

3.2.1.2. Erosion

Erosion has been reported to start as the polymer molecular weight goes below a threshold of 15 kDa. This process can be considered as a rate-controlling release mechanism as well as a true-release mechanism. In the first case, erosion increases pore formation, and thus increases the rate of diffusion [81]. Besides, erosion can be considered as a true-release mechanism, inducing directly drug release in the mean time of polymer mass loss. In a study investigating the influence of the organic salt deoxycholate in the medium bath on the lysozyme release from PLGA/ethyl benzoate depot, Brodbeck et al. [83] have found a significant increase of protein release rate. Interestingly, the addition of this organic salt did not impact the bulk water absorption and the phase inversion dynamic of the system. Instead, the increased release rate was found as the result of increased PLGA erosion at the surface of the injected depot. The erosion is considered as a release mechanism in this case.

Other mechanisms might be involved in the control of drug release from PLGA-based drug delivery systems including diffusion through the polymer network and osmotic pumping, which are well described in the literature. In the case of PLGA-based in situ

implants, the absorption of water leads to pronounced polymer swelling. This phenomenon can compensate the osmotic pressure within systems. Therefore, diffusion through water-filled pore is usually the dominant true release mechanism [81].

3.2.2. Burst release and phase inversion dynamic

Burst release can be defined as a high amount of drug released in the first hours up to 24 hours, which is often reported for PLGA-based ISFI. This phenomenon occurs due to the fact that solid depot formation can last from a few minutes to several hours. The rate of diffusion of the drug substance from the coagulating polymeric solution may be more rapid than the release rate from solid matrix. Consequently, a high leakage amount of API can be observed during implant formation, namely burst release [85]. The burst release is hence related to the liquid-liquid phase separation process, which is characterized by the phase inversion dynamic.

Phase inversion dynamic is the dynamic of the interactions that takes place between the polymer solution and an aqueous (non-solvent) environment, causing the formation of polymer membrane at the interface. Accordingly, the solvent/non-solvent diffusion lowers the polymer solubility, thus leading the polymer solution to phase separate into a polymer-rich matrix surrounding dispersed polymer-lean droplets. The arrangement of this two-phase structure determines the separation characteristics, the device morphology and thus the drug release kinetics [82]. The phase inversion dynamic is influenced directly by the properties of organic solvent.

There were a few studies investigating the role of organic solvent type on the phase inversion dynamic and drug release profile of a polymeric solution. It was concluded that the solutions based on strong, hydrophilic solvents that are miscible with water (NMP, DMSO…) generally cause a fast phase inversion. The solidification of these systems often takes place in the order of seconds to minutes, eventually forms a highly interconnected network with large finger pores, leading to a high burst release. In contrast, weaker solvents with lower water solubility (triacetin, ethyl benzoate…) leads to slower phase inversion resulting in a uniformly dense structure with few pores, and hence slower release rates, as illustrated in Figure 4 [84]. Brodbeck et al. [83] investigated the role of solvent type on the phase inversion, depot morphology and resulting release profile of lysozyme

from 50 wt. % PLGA-based polymeric solutions. The solvents used were NMP, triacetin and ethyl benzoate, which have solvent strengths reducing in the same order. The resulting data showed lower water diffusion and thus phase separation rate when decreasing the solvent/non-solvent affinity. For instance, water absorption of PLGA/NMP solution increased up to 20 % during the first day whereas it was almost zero in triacetin or ethyl benzoate solutions. Up to 14 days, significant differences could be clearly seen with water absorption reaching almost 80 %, 20 % and less than 10 % in NMP, triacetin and ethyl benzoate systems, respectively. This is in good agreement with the depots structure observed by scanning electron microscopy (SEM) (Figure 4). Indeed, NMP system showed a highly porous structure whereas pores were less visible in triacetin systems and even much less pronounced in ethyl benzoate systems. Interestingly, lysozyme release rate was much faster in NMP system (≈ 35 % drug release in 24 h) compared to the others (≈ 2 %). The subsequent release of triacetin system was faster than that of ethyl benzoate system due to the finite but higher water solubility of triacetin compared to ethyl benzoate (7 % versus 0.4 %, respectively).

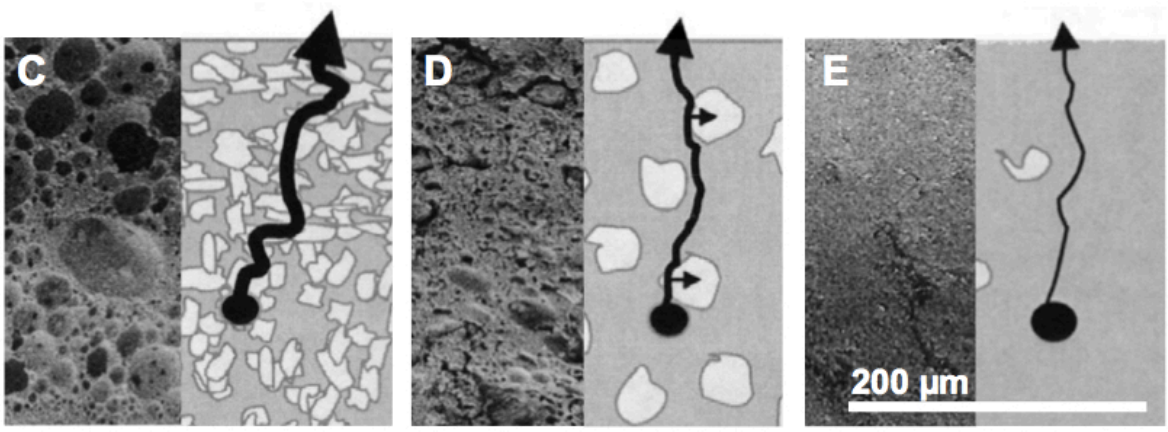


Figure 4. SEM images and scheme of matrix structure and postulated preferred pathway of protein release for NMP (C), triacetin (D), and ethyl benzoate (E) as solvents. *In schemes: grey regions indicate the polymer-rich phase, bright areas represent water filed pores, and arrow thickness indicates release rates. Adapted from* [86].

Briefly, the phase inversion dynamic can be considered as an important characteristic of ISFI, which is essential for the gelation rate, depot morphology, and thus burst release as well as the overall drug release profile. Solvent type is important but is not

the only factor influencing the phase inversion dynamic of an in situ polymer precipitation system.

3.3. Impacts of various parameters on the drug release of PLGA-based *in situ* forming implants

Many factors might influence drug release of PLGA-based ISFI, including the formulation parameters as well as the properties of the surrounding environment at the injection site.

3.3.1. Solvent

As mentioned above, the organic solvent has a significant impact on the formation of the polymer matrix and subsequent drug release properties. Depending on the *solvent strength* (water miscibility) of solvent (Table 5), the resulted polymer matrix can be porous or almost dense without any pores. The solvents with high water miscibility (NMP, DMSO…) promote fast liquid-liquid phase separation and thus a porous structure, which can increase drug release. In contrast, the more uniform structure created by weaker solvents (triacetin, ethyl acetate, ethyl benzoate…) results in slower drug release [83], [87]. If all the solvents are miscible with water, the differences in drug release profiles of the systems depend on the *polymer-solvent affinity*. Comparing the 40 % PLA/NMP or DMSO systems, Kranz and Bodmeier [88] found that NMP has higher solvating power for PLA compared to DMSO. Therefore, the solution of PLA in NMP performed a slower polymer precipitation and subsequent less porous implant structure with slower drug release.

Table 5. Aqueous solubility of different solvents used for *in situ* forming implants. *Adapted from* [86].

Solvent	Solubility in water (%)
N-methyl pyrrolidone (NMP)	Miscible
Dimethylsulfoxide (DMSO)	Miscible
Polyethylene glycol (PEG)	Miscible
PEG-dimethylether (PEG-DME)	Miscible
Glycofurol	Miscible
Triacetin	≈ 7
Triethyl citrate	≈ 5.7
Ethyl benzoate	≈ 0.4
Benzyl benzoate	≈ 0.15

Besides, the *polarity of solvent* has also an impact on the drug release, due to its influence on polymer degradation. Slower degradation rate of PLGA has been observed in polar aprotic solvents (DMSO, NMP, triacetin) than in polar protic solvent (PEG 400, 2-pyrrolidone, triethyl citrate). It was suggested that the polar protic solvents could donate hydrogen and possibly form hydrogen bonds with PLGA. Thus, the ester bonds of polymers can be exposed to residual water of solvent, and as a consequence enhance hydrolysis. In contrast, the ester bonds of the polymer might be shielded inside the polymer chains in the case of polar aprotic solvents, hence get less access to water [89]. This phenomenon has been confirmed in the study of Schoenhammer et al. [90], where they found block-copolymers during the degradation of PLGA in PEG 300. By capping the solvent with an alkyl-end group (PEG-dimethyl ether), the degradation rate of PLGA significantly reduced. Furthermore, the degradation of PLGA increased when increasing the water content of both protic and aprotic solvents [89]. Accordingly, the type of solvent and its *water content* should be taken into account for the design of PLGA-based ISFI, especially for the controlled-release devices over prolonged period of time.

3.3.2. Polymer

The biodegradable polymer PLGA has been widely used in the formulation of in situ polymer precipitation systems. The biodegradation of PLGA provides great benefits to

the drug delivery system because of its capacity to modulate drug release profile. The effect of physico-chemical properties of PLGA on the resulting drug release kinetics has been extensively investigated in the literature.

3.3.2.1. Molecular weight

The molecular weight (Mw) is a substantial property of PLGA, which is proportional to the polymer chain length and thus also proportional to its inherent viscosity. According to Ahmed et al. [91], low Mw PLGA results in a less hydrophobic polymer with increased rate of water absorption and matrix degradation, thus provide faster drug release rate compared to the higher Mw. The release profiles of the protein p55 tumor necrosis factor receptor (sp55-R) from an injectable implant system significantly reduced when increasing PLGA 50:50 Mw (inherent viscosity η increased from 0.24 to 0.38, 0.47 and 0.55 dL/g, respectively). This was explained by the fact that higher Mw polymers tend to solidify faster than low Mw polymers, leading to a smaller burst release and a higher amount of drug entrapped into polymer matrix. Regarding the degradation, it was found that low Mw polymer (η = 0.24 dL/g) degrades in a shorter time (5 days) to produce oligomers of 10 kDa compared to high Mw polymer (η = 0.59 dL/g) (over 40 days). This trend is in agreement with slower release observed for high Mw polymer systems.

In another study comparing the leuprolide acetate (LA) release profiles from PLGA/NMP systems, the PLGA RG 502 H led to a much lower initial release than RG 503 H (18.8 vs. 48.1 %). Subsequently, a fast release phase was observed with RG 502 H, whereas a much slower release rate was observed with RG 503 H [92]. These trends were further evaluated and clarified later by Astaneh et al. [93]. It has been found that the morphology of PLGA-based solid depot correlated with the drug release profile. Among 3 types of PLGA different in Mw, PLGA RG 502 H (Mw = 12 kDa) and PLGA RG 504 H (Mw = 48 kDa) presented thin skin and finger-like pore structure and thus similar shape of release profile. Due to the higher Mw, system based on RG 504 H released drug in a slower rate compared to RG 502 H. However, the PLGA RG 503 H (Mw = 34 kDa) depot exhibited a cellular-based surface with a sub-layer presenting a sponge-like structure. The cracking of cells containing dissolved LA leads to the highest content of initial drug

release compared to the others. After this burst, drug release from RG 503 H formulation was very slow because of the lack of diffusion path in such spongy structure.

3.3.2.2. Polymer concentration

The effect of polymer concentration on the drug release kinetic from in situ polymer precipitation devices has widely been studied in the literature. Higher polymer concentration leads to increased viscosity and hydrophobicity of polymer solution compared to lower polymer concentration. Consequently, water influx rate, phase separation rate and diffusion of drug into release medium were limited, leading to slower release rates [82]. For instance, PLGA 50:50/glycofurol systems with increasing polymer concentration from 10 %, 15 % to 20 % reduced the bovine serum albumin (BSA) burst release. Similarly, the initial protein sp55-R release from 20 % PLGA matrix was smaller than from 10 % PLGA matrix. However, in both case, the long-term release kinetics after the burst were independent on the polymer concentration [91], [94].

Moreover, it is suggested that higher polymer concentration conducts to a dense polymer matrix structure, hence increases the required time for degradation of the solid implant [95]. Graham et al. [82] reported the change in morphology of PLGA/NMP depots from finger to sponge transition when PLGA concentration increased from 40 % to 50 % and 60 %. As a result, the initial release was slowed down. In a recent study, it has been shown that *in situ* implant formulations with increasing PLGA concentration (20 %, 30 % and 40 %) sustained the release of haloperidol more effectively, regardless the type of solvent. For instance, in DMSO, the initial burst of drug was reduced (20 %, 17 % and 15 % within the first 24 hours) and the release was extended over 24, 31 and 45 days, respectively [87].

The degradation of PLGA matrix is known as a hydrolytic process resulting in the formation of carboxyl end groups which are able to catalyze the hydrolysis of other ester bond, namely autocatalysis [96]. Depending on matrix size, the diffusion pathways of degradation products are different; altering the neutralization of generated acids [97]. Thus, the local degradation rate and subsequently the erosion characteristic of PLGA matrices are varied on system morphology. This dependence was pronounced in the case of PLGA-based films or PLGA-based microparticles, where degradation rate increased

with increasing device size [97], [98]. Similar effect can also be observed in the case of PLGA-based ISFI, hence altering the underlying drug release profiles.

3.3.2.3. Functional-end group

The end group of polymer PLGA can be uncapped (carboxyl-end group) or capped with a hydrophobic ester group (ester-end group), leading to change in its chemical properties. Polymers with carboxyl-end group are more hydrophilic, thus increase the rate of water absorption and subsequent hydrolysis and erosion [81], [87]. Therefore, the functional-end group has a considerable impact on the drug release rate.

In some prior studies, PLGA with carboxyl-end group has been reported to slow down the drug release rate compared to PLGA with ester-end group because of polymer-protein interaction. For instance, the interaction of PLGA RG 503 H (carboxyl-end group) and the protein L-Asparaginase reduced the burst and subsequent release of drug compared to PLGA RG 503 (ester-end group) [99]. An ionic interaction between the terminal carboxylic end groups of PLGA and the basic amino acids of leuprolide acetate possibly occurred, thereby hindered drug diffusion [92].

3.3.2.4. Ratio of lactic/glycolic acid (L:G)

PLGA copolymers can be prepared in any ratio of lactic to glycolic acids. This proportion affects the polymer cristallinity and thus, the water uptake and degradation rate [91]. Due to the more hydrophilic property of PGA compared to PLA, low L:G ratio PLGA polymers are less hydrophobic than high L:G ratio. Consequently, they absorb more water and degrades more quickly [87], [95]. Since primary hydrolysis site of PLGA are the G-G or L-G linkages, the ester linkages of PLGA 50:50 are more accessible to water than those of the PLGA 75:25, causing faster degradation. Especially, the PLGA 50:50 polymers are hydrolyzed and degrade much faster than those with higher proportion of either monomer. Consequently, both the burst release and the overall release rate can be reduced by increasing L:G ratio of PLGA. In practical results, Eliaz and Kost [91] have found that at high Mw, the PLGA 50:50 (η = 0.55 dL/g) leaded to higher protein release rate than PLGA 75:25 (η = 0.59 dL/g). Whereas at low Mw (η = 0.24 dL/g), the initial release reduced in increasing L:G ratio from 50:50 to 75:25.

3.3.3. Drug

3.3.3.1. Drug properties

In ISFI systems, drug can either be dissolved or dispersed into the polymeric solution, depending on its solubility in organic solvent. Since diffusion is considered as the main drug release mechanism, in which the drug must be dissolved in water before being release, the *solubility of drug* in the release medium is also an important parameter [81]. For example, risperidone and paliperidone present good solubility in NMP (69 mg/mL and 40 mg/mL, respectively) but low solubility in DMSO (< 10 mg/mL) and poor solubility in phosphate buffer (< 0.2 mg/mL). These drugs were either dissolved in NMP or dispersed in DMSO (20 % drug loading). The resulting drug release kinetics were significantly different. Both ISFIs exhibited fast extraction of solvent in the surrounding aqueous medium. This led to a rapid release of the dissolved drugs from NMP-based ISFI but a sustained release in DMSO-based ISFI because most of the dispersed drug particles were encapsulated in the matrix after fast polymer precipitation [100]. On the other hand, the solubility of drug can alter the solvent exchange of the polymer solution, thus modify the drug release rate. Generally, hydrophilic drug leads to higher diffusion (swelling) rate and degradation rate than hydrophobic drug. It was assumed that the practical insoluble drug haloperidol could inhibit water diffusion into the matrix, thereby slowed down the erosion of implant and caused further decrease in drug release [87].

The release of drug from PLGA matrix is also dependent on the *chemical property* of drug. The basic drugs were found to create a strong ionic interaction with the polymer, keeping drugs dissolved in the matrix. This interaction shields the polymer terminal carboxyl groups, therefore declines the matrix erosion. Consequently, the drug diffusion through the matrix is restrained. In contrast, due to weak interaction with PLGA, the acidic and neutral drugs quickly precipitate out as crystals in the matrix during release time. Therefore, the solubility of these drugs in the hydrated matrix becomes the dominant parameter affecting drug diffusion [101], [102].

On the other hand, drug can accelerate polymer degradation, thus leading to its faster release. The free acid N-acetyl cysteine encapsulated in PLGA 50:50 implant led to plasticization, increased catalytic degradation of polymer matrix, resulting in faster drug

release [103]. Another acidic drug, aspirin was found to facilitate the degradation of PLGA polymers and consequently faster release of drug from PLGA-based in situ gel system [78]. The plasticization effect was also observed in the case of ketoprofen, where hydrogen bonding of this drug and PLGA caused a lubricant effect on polymer chain and thus an accelerated drug release [104], [105].

3.3.3.2. Drug concentration

Drug loading can play a considerable role in modifying the release mechanism and resulting release rate of ISFI systems. Eliaz and Kost [91] investigated the impact of drug loading in injectable implant containing the protein sp55-R as an active agent and bovine serum albumin (BSA) acted as a carrier for sp55-R. They demonstrated that for both proteins, the release rate from the devices depended on the drug loading. At BSA loading of 3 % or lower, similar drug release profiles were observed, suggesting that the release mechanism was degradation dependent. At BSA loading of 10 % or higher, the drug release rate increased with increasing BSA content, indicating that both the diffusion and degradation affected the drug release. It was assumed that the high protein loading led to a more porous structure matrix, providing interconnected diffusion pathways, thus increasing matrix degradation and protein release. This correlates well with the *in vivo* results, in which more extended sp55-R serum concentration was measured in the case of 10 % BSA loading compared to 3 % (35 days vs. 20 days) [94].

For low Mw drug, the effect of drug loading was also found to be in agreement with these previous studies. When altering the fluorescein content of PLGA/NMP systems from 0.5 to 5 %, two different trends were observed. At low drug content (0.5 % or 1 %), there was no significant difference in drug release rate at 1 hour, 1 day and 1 week. However at higher drug content (2 % and 5 %), significantly higher release rates were observed [106]. Consequently, the increased drug loading did not only increase the initial drug release concentration but also the total release rate.

3.3.4. Additives

The presence of a *second polymer* is often reported to modify drug release kinetics from *in situ* forming polymer matrices. It was suggested that the addition of a hydrophilic polymer into NMP-based depots could be a valuable tool to adjust their release properties.

For example polyvinylpyrrolidone (PVP) is known to influence the morphology of phase inversion membranes. Graham et al. [82] showed that the addition of only 3 wt. % PVP increased dramatically (8-fold) the liquid-liquid phase separation rate of PLGA/NMP solution. However, neither the water influx rate nor the overall depot morphology did change significantly. In contrast, the drug release rate, especially during the gel formation period ($t < 1$ day) was much higher for the solution containing PVP.

The amphiphilic copolymer Pluronic also altered the release of lysozyme from PDLA/NMP solution but under different mechanisms. Pluronic triblock copolymers consist of blends of poly(ethylene oxide) (PEO)/poly(propylene oxide) (PPO)/poly-(ethylene oxide) (PEO). In polymer blend systems, the hydrophobic PPO segments anchor in the polymer matrix, while the hydrophilic PEO segments extend into the surrounding aqueous phase. Due to the increased water uptake related to hydrophilic PEO blocks, the phase inversion rate of polymer solution increased. Nevertheless, the preferential segregation of Pluronic (PEO segments) into the hydrophilic polymer-lean phase primarily affected the release characteristic of the system. Increasing the Pluronic concentration (from 3.6 to 7.2 %) resulted in a decrease in the initial release rates as well as a change in the overall release profile. On the other hand, at the same Pluronic concentration (5.4 %), increasing PEO block length led to a reduced burst release but did not affected the drug release rate [84], [107]. Patel et al. [106] however found that Pluronic only reduced the burst release of fluorescein form PLGA/NMP system at 2.5 %. No significant difference in release profiles was reported when using Pluronic at lower neither higher concentration.

Another way in which the drug release can be influenced by the change of phase inversion rate is by adding a *co-solvent*. Co-solvents with low water miscibility were reported to restrain the phase inversion process, leading to less porous structure and subsequently slower burst release. Such investigated co-solvents were glycerol, ethyl heptanoate [108] or triacetin [82], [109].

Some other authors have investigated the effect of *plasticizer additive* on the release profile of the semi-cristalline polymer PLLA matrix. As expected, the high Tg of polymer PLLA reduced from 67.3 ^{o}C to 59.4 ^{o}C after adding 5 wt. % PEG 4000 into the matrix, thus accelerated the polymer degradation onset. Consequently, the burst release of heparin from plasticized matrix was suppressed, followed by a faster drug release

compared to the matrix without plasticizer. This could be explained by the increased hydrophilicity of PEG-plasticized PLLA that improved the solubility of heparin in the matrix [110]. Similarly, the addition of PEG 400 into the PLGA/NMP solution led to the suppression of the initial release of aspirin. However, the subsequent release of aspirin did not change significantly [78].

Finally, the addition of an *excipient that can interact with the drug* is also an effective way to modify drug release profile from PLGA system. For instance, the cationic chitosan can interact with negatively charged drugs to hinder drug diffusion out of the matrix. It has been found that the re-encapsulation of thymosin alpha 1 in chitosan before charging into PLGA/NMP ISFI systems effectively slowed down drug release. Not only a much lower initial release was reported but the overall continuous release period was also prolonged [109].

3.3.5. Injection site

In vitro – in vivo correlation is an important issue for any drug delivery systems, due to the fact that *in vitro* conditions cannot always imitate the real *in vivo* condition. Especially for ISFI systems, there is no standardized *in vitro* release method so far, the *in vitro* release set-ups are very different and can lead to significant variations in the obtained results. To understand this correlation, there were some studies investigating the influence of injection site on ISFI drug release kinetics, including the impact of composition of body fluid *in vitro* as well as the real release profiles *in vivo*.

3.3.5.1. Bath composition

It is known that there are many potential reactions between the injectable drug solution and the surrounding aqueous solution at injection site. For example, acids and bases can have pronounced effects on PLGA degradation, subsequently influencing the drug release rate. In vitro condition normally employs the phosphate-buffered saline (PBS) pH 7.4 as the physiological solution for testing. However, there are other compositions existing in the body fluid (enzyme, ester lipid, organic salts…), among that, triglyceride and organic salts are found in subcutaneous space.

The impacts of triglyceride (triacetin) and deoxycholate (an organic bile salt) on the

phase inversion and drug release dynamics of lysozyme on PLGA/solvent systems were investigated by Brodbeck et al. [83]. It was found that the addition of 6 % triacetin in PBS solution resulted in a decrease in the burst release of PLGA/NMP, but still presented as a rapid phase inversion, high burst system. Nevertheless, in the PLGA/ethyl benzoate system, a much higher initial release and significant elevation in overall protein release rate was reported. This could be explained by the fact that the strong, hydrophilic solvent NMP-based system was insensitive to the weak, less hydrophilic solvent triacetin presented in the bath. Thus, the phase inversion and drug release dynamic of NMP systems was not significantly influenced. In contrast, for the hydrophobic solvent ethyl benzoate, triacetin could diffuse from the aqueous solution and therefore increased the hydrophilicity of the polymer solution. Consequently, overall depot viscosity decreased and water uptake increased, leading to faster phase inversion dynamic and faster drug release. Regarding the impact of deoxycholate on the PLGA/ethyl benzoate system, a steep increase of protein release was reported after addition of this organic salt from 0.5 to 3 wt. %. Whereas no change in phase inversion dynamic and water absorption was observed, this alteration was supposed to be the result of PLGA erosion at the surface of the injected depot.

3.3.5.2. *In vitro – in vivo* correlation

To understand the correlation between *in vitro* and *in vivo* behavior of PLGA-based ISFI, Patel et al. [111] conducted a study, in which implant formation and drug release were measured in both conditions. The administration of ISFI *in vivo* was varied from subcutaneous injection to necrotic, non-necrotic and ablated tumor (in rat). The obtained results showed that the burst release from ISFI *in vivo* was significantly greater than *in vitro* for all formulations. Varying *in vivo* environment led to variations in drug release with fastest release in ablated tumor followed by implants in non-necrotic tumor, in subcutaneous tissue and finally in necrotic tumor. In addition, ultrasound implant imaging method revealed that *in vivo* ISFI solidified much quicker than what has previously been shown *in vitro*. Thus, the rate of implant formation correlated with the rate of implant drug release.

However, these results are discordant with a previous study, in which little difference between solvent exchange and implant precipitation *in vitro* and *in vivo* (subcutaneous in rat) was found by electron paramagnetic resonance (EPR) method [112].

Regarding the release kinetics, some previous studies reported good agreement between *in vitro* and *in vivo* release. Liu et al. [109] found that the release of thymosin alpha1 from PLGA/NMP/triacetin *in vitro* was slightly slower than *in vivo* (subcutaneous in rat). Nevertheless, both *in vitro* and *in vivo* release followed Higuchi kinetics and the linear correlation coefficient between them was 0.9, showing a good correlation. In a precedent study, the sustained release of human growth hormone *in vivo* by injection into subcutaneous tissue of rat was obtained from PLGA/ethyl benzoate solution, as in the case of *in vitro* experimentation [80].

Since there existed controversial results on the correlation between *in vitro – in vivo* release of ISFI, it is so still challenging to conclude about the difference in the behavior of ISFI systems in these two environments. Besides the effect of different composition of release medium *in vitro* and *in vivo*, its amount is also a factor altering implant formation and subsequent drug release. Generally, the amount of body fluid *in vivo* is low, thus the depot structure can be varied. The shape of hardened depot is also not similar *in vivo* to *in vitro*, possibly leading to different degradation rates of PLGA matrix. Eliaz et al. [94] showed that increased injection speed of polymer solution into subcutaneous tissue can result in the formation of a more compacted structure of implant, thus burst release can be reduced *in vivo*. Therefore, artificial effects should also be taking into consideration when comparing the results from *in vitro* and *in vivo* tests.

4. Research objectives

The present study aimed to develop new in situ forming implants (ISFI) for the treatment of periodontitis with improved adhesive properties. These systems were prepared from the biodegradable polymer, polylactic acid (PLA) or poly(lactic-co-glycolic acid) (PLGA) dissolved in the biocompatible solvent N-methyl pyrrolidone (NMP). With the addition of bioadhesive agents and plasticizers, the developed ISFI would have bioadhesiveness and convenient mechanical properties to avoid the risk of premature expulsion from periodontal pocket. The release of antibiotic from depot system would be controlled for a prolonged period of at least 7 days, simultaneously with the degradation of PLGA matrix. However, the underlying drug release profile would be modified due to the presence of these additives. Therefore, their effects on the drug release kinetics were investigated to compromise both mentioned requirements.

The main objectives of this research includes:

(i) Preparation and characterization of the mechanical properties of ISFI.

(ii) Investigation of the effects of formulation parameters (the type and content of PLGA polymer, bioadhesive agent, plasticizer) on resulting drug release kinetics.

(iii) Elucidation of underlying drug release mechanisms based on the physico-chemical techniques such as: optical microscopy, gel permeation chromatography (GPC), electron paramagnetic resonance (EPR), proton nuclear magnetic resonance (^{1}H NMR).

(iv) Evaluation of antimicrobial activity of developed ISFI by microbiological tests on the complex periodontal samples and on isolated bacteria from gingival crevicular fluid of periodontitis patients.

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CHAPTER I

IN SITU FORMING IMPLANTS FOR PERIODONTITIS TREATMENT WITH IMPROVED ADHESIVE PROPERTIES

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Abstract

Novel *in situ* forming implants are presented showing a promising potential to overcome one of the major practical hurdles associated with local periodontitis treatment: limited adhesion to the surrounding tissue, resulting in accidental expulsion of at least parts of the implants from the patients' pockets. This leads to high uncertainties in the systems' residence times at the site of action and in the resulting drug exposure. In the present study, the addition of different types and amounts of plasticizers (acetyltributyl citrate and dibutyl sebacate) as well as of adhesive polymers (e.g., cellulose derivatives such as hydroxypropyl methylcellulose) is shown to allow for a significant increase in the stickiness of poly(lactic-co-glycolic acid)-based implants. The systems are formed *in situ* from N-methyl pyrrolidone-based liquid formulations. Importantly, at the same time, good plastic deformability of the implants can be provided and desired drug release patterns can be fine-tuned using several formulation tools. The antimicrobial activity of this new type of *in situ* forming implants, loaded with doxycycline hyclate, was demonstrated using the agar well diffusion method and multiple *Streptococcus* strains isolated from the oral microflora of patients suffering from periodontitis.

Keywords: *in situ* forming implant; periodontitis; local drug delivery; PLGA; doxycycline

1. Introduction

"Periodontitis" can be defined as "a disease that affects the periodontal structures and, as a result of interactions between periodontopathogens and the host immune response, leads to the destruction of the tooth supporting tissues, periodontal ligament and alveolar bone" [1–3]. Briefly, microorganisms colonizing the patients' periodontal pockets are considered as a major factor, causing inflammation and tissue destruction [4]. It seems that the microflora in the disease state is different from that in healthy subjects. For example, the number of gram negative anaerobic bacteria is likely to be increased and certain clinical forms of periodontitis might be associated with specific microbiota [5]. Recently, Silva-Boghossian et al. [1] reported that *Streptococcus* species (such as *Streptococcus sanguinis*) are also associated with suppuration in periodontitis subjects. Up to date, the exact mechanisms underlying this disease are not yet fully understood. It is hypothesized that: (i) suspected periodontal pathogens produce biologically active molecules, which directly attack the host tissue, and/or that (ii) the immune response of the host to these pathogens results in the tissue destruction. The consequence of the tissue loss is the deepening of the periodontal pockets, and -once the mechanical anchorage of the tooth becomes insufficient -the latter is lost. Periodontitis is indeed the main cause for tooth loss in adults [6].

At present, the standard treatment method of periodontitis is the mechanical removal of the bacteria (especially of the bacterial biofilms): a procedure, which is also called "root planing". However, the geometry of the patients' pockets can be very disadvantageous, hindering complete bacterial removal and in various cases the pathogens re-colonize the cavities after the treatments. To minimize the risk of this re-appearance of the pathogenic microorganisms, the use of different antimicrobial drugs has been proposed [4,7,8], in combination with root planing. This includes antibiotics (e.g., tetracycline [9,10], doxycycline [11–14], minocycline [15–17], and metronidazole [18,19]) as well as antiseptic agents (e.g., chlorhexidine) [20–22]. A major challenge for this type of drug treatment is the appropriate administration: (i) Systemic administration leads to the exposure of the entire organism to the respective drugs, resulting in potentially severe side effects and development of bacterial resistances. (ii) Mouth rinsing does not allow achieving sufficient drug concentrations in the periodontal pockets. (iii) Local drug

delivery systems, releasing the active agent in a time-controlled manner over a pre-determined period of time directly at the site of action, are currently considered as the most promising approach.

A variety of local controlled drug delivery systems for periodontitis treatment have been proposed, including fibers [23,24], films and strips [25–28], inserts and wafers [29–32], microparticles [32–34], gels and other semi-solid formulations [35–41], and biodegradable *in situ* forming implants [42]. The latter type of systems provides important advantages, namely: (i) Relatively easy administration using standard syringes. (ii) Efficient spreading within the periodontal pockets. Consequently, the geometry and size of the resulting implants is adapted to each individual cavity ("personalized"). (iii) The resulting drug release rate can be time-controlled. (iv) There is no need to remove empty remnants. (v) Biocompatible excipients can be used.

Atridox® is such an *in situ* forming implant formulation, which is commercially available. It consists of the biodegradable and biocompatible matrix former poly(D,L-lactic acid) (PLA, 36.7 %), the organic solvent N-methyl pyrrolidone (NMP, 63.3 %), and is loaded with 10 % doxycycline hyclate. The PLA is dissolved in the NMP. Upon injection into the periodontal pocket, the NMP diffuses into the surrounding environment and water penetrates into the formulation. Consequently, the solubility of the PLA decreases and the polymer precipitates, entrapping the drug [43–46]. The latter is released at the site of action through the degrading polyester matrix during about 1 week. A multicenter clinical trial has shown the superior efficacy of Atridox® compared to oral hygiene in patients with chronic adult periodontitis [47]. However, a major practical disadvantage of this type of systems is the limited adhesion of the *in situ* formed implants to the environmental tissue. Consequently, parts of the devices, or the entire implants can accidentally be expulsed from the periodontal pockets. This is in part caused by the non-negligible flow of gingival crevicular fluid in these cavities: a few to dozens of microliters per hour have been reported in the literature, depending on the severity of disease [48]. Thus, there is a considerable uncertainty how much drug really reaches its target site. To reduce this uncertainty, the administration of Atridox® is recommended to be accompanied with the placement of a periodontal dressing or cyanoacrylate dental adhesive. But this additional procedure complicates the administration of the system (increasing the costs and

prolonging the time of the intervention), and the efficacy of such “in-place holders” is not guaranteed.

The major aim of the present study is to help overcoming this crucial practical hurdle of limited bioadhesion of the *in situ* formed implants. The idea is to add different types of compounds, namely plasticizers and bioadhesive polymers, to the liquid formulations in order to improve the adherence of the devices to the surrounding tissue, resulting in prolonged and more reliable residence times in the periodontal pockets. In addition, the impact of these additives on other key properties of the systems was to be investigated, namely their plasticity/elasticity and drug release kinetics. For reasons of comparison, the commercially available drug product Parocline® was studied [a “dental gel” consisting of hydroxyethylcellulose, magnesium chloride, Eudragit® RS, triacetin, glycerol and minocycline (2 %)].

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA, Resomer® RG 502 H; Evonik, Darmstadt, Germany); acetyltributyl citrate (ATBC) and dibutyl citrate (DBS) (Morflex, Greensboro, NC, USA); hydroxypropyl methylcellulose (HPMC, Methocel® E5, E50; Colorcon, Dartford, UK); poloxamer (Lutrol® micro 68, 127) and polyvinylpyrrolidone (PVP; Kollidon® 25) (BASF, Ludwigshafen, Germany); hydroxypropyl cellulose (HPC; Klucel® LF Pharm) and hydroxyethyl cellulose (HEC; Natrosol® 250 G Pharm) (Hercules, Wilmington, DE, USA); N-methyl pyrrolidone (NMP, 99 %), glucose and cysteine hydrochloride (Acros organics, Geel, Belgium); doxycycline hyclate (Fagron, Colombes, France); sodium metabisulfite (Merck, Darmstadt, Germany); agarose (GenAgarose® LE; Genaxxon BioScience, Ulm, Germany); Columbia agar base and agar (Oxoid, Basingstoke, UK); defibrinated horse blood (E&O Laboratories, Burnhouse, UK); Parocline® (2 % minocycline; Sunstar France, Levallois-Perret, France).

2.2. Preparation of the liquid formulations

PLGA (28 %, 32 % or 37 % w/w, based on the total liquid formulation without drug) was dissolved in NMP at 25 °C for 30 min under stirring in a glass vial. Optionally, a

plasticizer (ATBC or DBS) and a second polymer (10 % or 20 % w/w, based on the PLGA mass) was/were added and the mixture vortexed for 3 min, followed by standing for 3 h at 25 °C. Subsequently, doxycycline hyclate (2 %, 5 % or 10 % w/w, based on the total liquid formulation without drug) was added, and the mixture vortexed for 3 min, followed standing for 3 h at 25 °C. To eliminate air bubbles, the formulations were ultrasonicated for 10 min. The formulations were stored at -20 °C and protected from light to avoid drug degradation.

2.3. *In situ* implant formation and drug release measurements

One hundred microliters of the respective formulation was injected at the bottom of an Eppendorf vial using a standard syringe. One and a half milliliters preheated (37 °C) phosphate buffer pH 7.4 (USP 35) was carefully added using a pipette, initiating solvent exchange and implant formation. The vials were horizontally shaken at 37 °C at 80 rpm (GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, the bulk fluid was completely withdrawn and replaced with fresh phosphate buffer pH 7.4. The drug content in the samples was determined UV-spectrophotometrically (λ = 325 nm; UV-1650PC, Shimadzu, Champs-sur-Marne, France). For reasons of comparison, the commercially available formulation Parocline® was also studied (note that this product contains a different drug: minocycline). In this case, the 100 µL of formulation was injected using the supplied syringe and 0.01 % sodium metabisulfite was added to the release medium to improve the drug's stability. The drug content was determined UV-spectrophotometrically at λ = 324 nm (UV-1650PC). Each experiment was conducted in triplicate, and the results are presented as mean values ± standard deviation.

2.4. Monitoring of dynamic changes in the implants' mass

Implants were prepared and treated as described in *Section 2.3. In situ implant formation and drug release measurements*. At pre-determined time points, implants were weighed (after removal of excess water by careful blotting with precision wipes) [*mass (t)*]. The *mass change in percent* was calculated as follows:

$$mass\ change\ (\%) = 100 * [mass\ (t) - mass\ (t=0)] / mass\ (t=0) \quad (1)$$

where *mass (t=0)* is the initial weight of the formulation used for implant preparation.

2.5. Mechanical and adhesive properties

The mechanical and adhesive properties of the investigated *in situ* forming implants were determined with a texture analyzer (TA.XT.Plus; Stable Micro Systems, Surrey, UK) using the experimental set-up schematically illustrated in Figure 1.

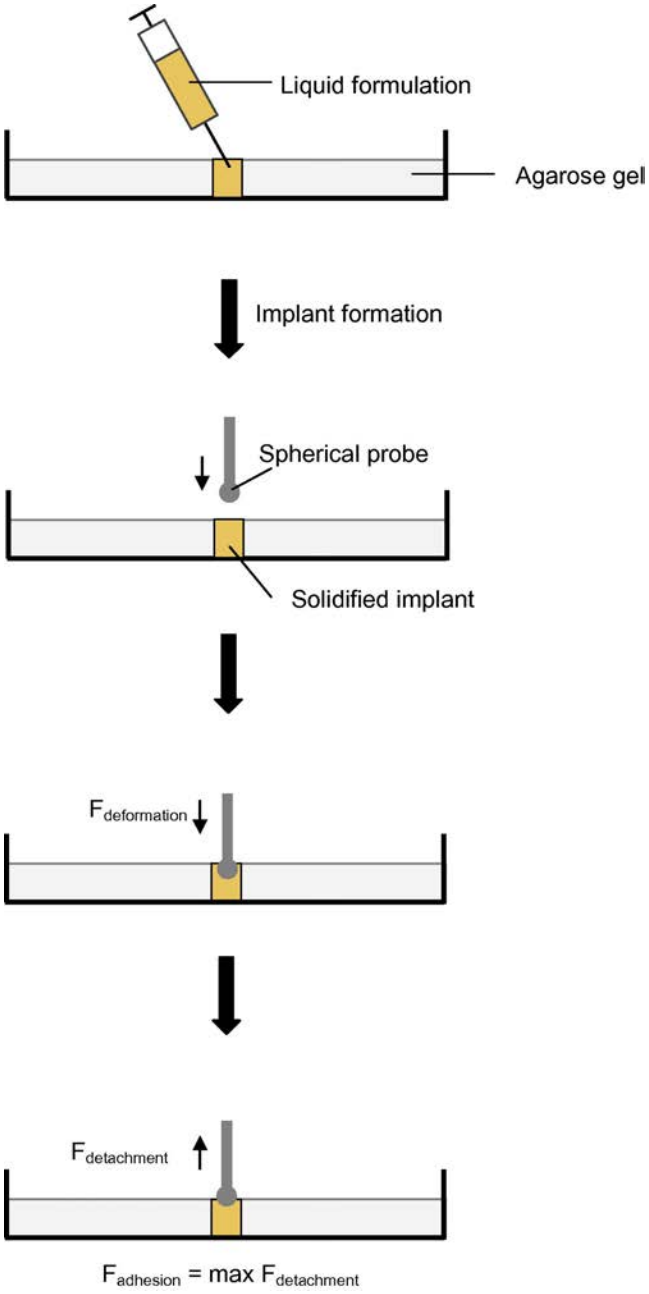


Figure 1. Schematic presentation of the experimental set-up used to determine the mechanical and adhesive properties of the investigated *in situ* forming implants.

Agarose was dissolved in boiling water (0.6 % w/v), and the solutions were cast into Petri dishes (diameter = 9 cm). Upon cooling to room temperature, gels formed. At the center of the gels, cylindrical holes (diameter = 6 mm) were made and filled with 100 µL liquid formulation using a standard syringe and a drop of distilled water. Upon solvent exchange, the implants formed. At pre-determined time points, a spherical probe (diameter = 5 mm) was driven downwards (at a speed of 0.5 mm/s). Once in contact with the implant, the applied force and displacement of the probe were recorded as a function of time. When the penetration depth was 1.5 mm, this position was held for 60 s. Then, the probe was driven upwards at a speed of 10 mm/s.

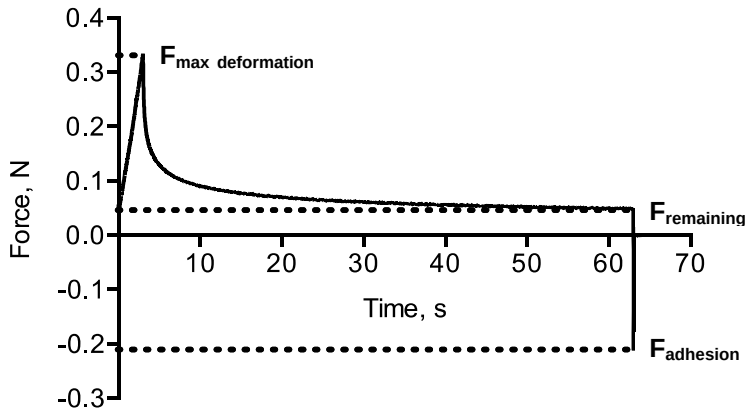


Figure 2. Typical "force-time" diagram obtained with the applied experimental set-up to determine the mechanical and adhesive properties of the investigated implants. The different forces are explained in the text.

Figure 2 shows a typical force-time diagram obtained with this type of measurements. Here, the maximum deformation force ($F_{max\ deformation}$) is the force measured at maximum probe penetration into the implant. The force measured after the 60 s holding time is called "remaining force" ($F_{remaining}$). In this study, the "adhesion force" is defined as the maximum force measured with this set-up during the upward movement of the probe, accounting for the negative sign/direction of the force ($F_{adhesion}$). The ratio "$F_{remaining}/F_{max\ deformation}$" is used as a measure for the elasticity/plasticity of the implant. High values indicate high elasticity, low values indicate high plasticity. Each experiment was conducted in triplicate, and the results are presented as mean values ± standard deviation.

2.6. Antibacterial activity

The *in vitro* efficacy of the investigated implants against bacteria associated with periodontitis was assessed using the agar well diffusion method. Columbia agar was prepared from Columbia base, glucose, cysteine hydrochloride, and agar. The systems were sterilized in an autoclave (121 ^{o}C for 15 min). Prior to plating, Columbia agar was enriched with defibrinated horse blood (5 % v/v) and cast into Petri dishes. After cooling to room temperature, 0.1 mL of the following bacterial suspensions were inoculated onto the agar surface: *Streptococcus* sp. (1) (D36A12), *Streptococcus* sp. (2) (D20B9), *Streptococcus salivarius* (D28A9), *Streptococcus sanguinis* (D28A11), and *Streptococcus cristatus* (D18A2) (which were isolated from samples from periodontal pockets from patients suffering from periodontitis). A cylindrical hole (diameter = 6 mm) was subsequently made at the center of the agar, and filled with 30 µL of liquid formulation using a standard syringe. Upon solvent exchange, the implants formed *in situ*. The Petri dishes were incubated for 4 d under optimum culture conditions (35 °C, anaerobic atmosphere) (Whitley A85 workstation, Don Whitley Scientific, West Yorkshire, UK). The diameter of the observed bacteria growth inhibition zones around the center of the Petri dishes was measured using a ruler. Each experiment was conducted in triplicate, and the results are presented as mean values ± standard deviation.

3. Results and discussion

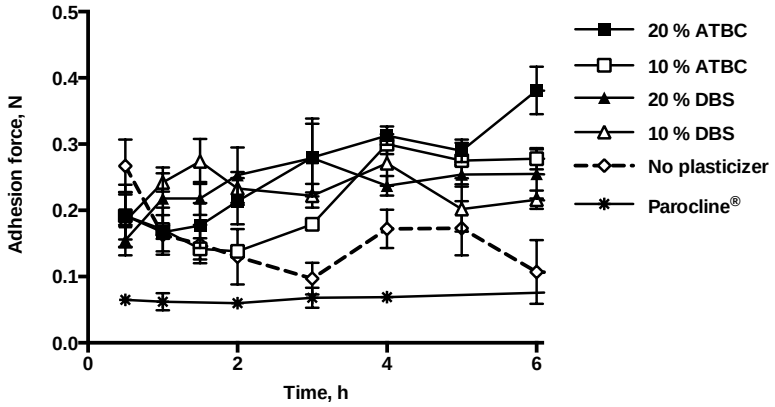
3.1. Effects of the addition of plasticizers

Figure 3a shows the impact of adding 10 % or 20 % ATBC or DBS on the adhesion forces of *in situ* forming implants, based on PLGA RG 502 H (37 % w/w) and loaded with 10 % doxycycline hyclate. The adhesion force was measured as a function of the exposure time to phosphate buffer pH 7.4. For reasons of comparison, also plasticizer-free implants (dotted curve) as well as the commercially available product Parocline® were studied. Very clearly, the addition of the plasticizers significantly increased the adhesion forces of the systems (probably due to the increased mobility of the macromolecules, allowing for facilitated interaction with the environment). This can be expected to be a great benefit in practice, since accidental expulsion of the implants (or parts thereof) from the periodontal pockets of the patient is a major source of uncertainty for all currently available

formulations. This can at least partially be attributed to the non-negligible flow of gingival crevicular fluid [48]. Consequently, it is uncertain how much drug is delivered for which period of time at the site of action. The plasticizer-containing systems show a promising potential to overcome this crucial practical hurdle: As it can be seen in Figure 3a, all ATBC/DBS containing implants showed much higher adhesive forces than the commercially available product Parocline® during the entire observation period. The observed time-dependent changes can at least partially be explained by the dynamic changes in the systems' composition (e.g., decreasing NMP content, increasing water content), which are particularly pronounced at early time points. Furthermore, it can be seen in Figure 3a that increasing the plasticizer level generally leads to increased adhesion forces, irrespective of the type of plasticizer.

In addition to the adhesion/stickiness of the *in situ* formed implants, also their elasticity/plasticity can be expected to play a major role for their residence times in the patients' periodontal pockets: If the system is difficult to deform plastically, the implant is unlikely to be able to adapt its geometry to dynamic changes in the periodontal pocket' size and shape with time. A fully elastic implant would force the periodontal pocket to keep its geometry and dimensions, which is not desirable. As a measure for the "plasticity" of the investigated *in situ* forming implants, the ratio of the "force remaining at the end of the 60 s holding time" ($F_{remaining}$) to the "maximum deformation force" ($F_{max\ deformation}$) was used in this study (Figures 1 and 2). A value of "1" indicates that the system is ideally elastic (does not change its inner structure during the holding time in a permanent manner and fully recovers, once the pressure is released), whereas a low value indicates that the implant structure at least partially changes in a permanent manner during the holding time. As it can be seen in Figure 3b, all the investigated *in situ* forming implants exhibit much lower $F_{remaining}/F_{max\ deformation}$ ratios than Parocline®, indicating that they are much more easy to deform in a permanent manner and can more likely adapt to changes in the patients' pockets' geometry.

a)



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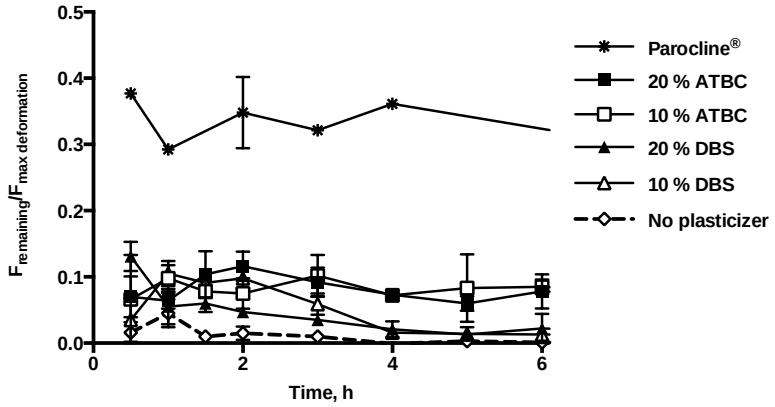


Figure 3. Effects of the addition of different types and amounts of plasticizers on the: a) adhesion force, and b) mechanical properties of *in situ* forming implants, based on PLGA RG 502 H (37 % w/w) and loaded with 10 % doxycycline hyclate. For reasons of comparison, also Parocline® was studied.

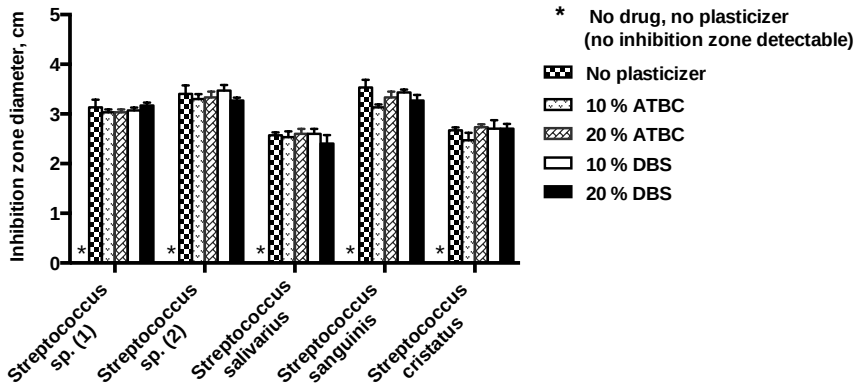
Based on these results, it can be expected that adding ATBC or DBS to PLGA based *in situ* forming implants is likely to allow for a substantial increase in the residence time in the periodontal pockets of the patients (due to increased adhesion), while the systems provide good deformability. This can be expected to help overcoming a crucial current hurdle for efficient local periodontitis treatment. However, the addition of the plasticizers might also affect the resulting antimicrobial activity of the implants. For this reason, the capability of the different systems to inhibit the growth of bacterial strains isolated from periodontal pockets has been studied.

3.2. Antimicrobial activity

Figure 4a shows the inhibition zones observed with *in situ* forming implants based on PLGA RG 502 H (37 % w/w), loaded with 10 % doxycycline hyclate and containing 10 % or 20 % ATBC or DBS (as indicated). For reasons of comparison, also "plasticizer-free" and "plasticizer-free and drug-free" systems have been studied. The investigated bacteria (isolated from periodontal pockets from patients suffering from periodontitis) were 5 *Streptococcus* strains, two strains could not be identified to the species level, the others were *Streptococcus salivarius*, *Streptococcus sanguinis* and *Streptococcus cristatus*. Very clearly, all drug-loaded implants were effectively limiting the growth of all these bacteria. The measured inhibition zone diameter varied from 2.4 to 3.5 cm, depending on the specific bacterial strain. Importantly, the addition of 10 % or 20 % ATBC or DBS did not alter the antimicrobial activity of the systems. As an example, Figure 4b shows a picture of a Petri dish incubated with *Streptococcus sanguinis* for 4 d. The white circle in the middle shows an *in situ* formed implant based on PLGA RG 502 H (37 % w/w), loaded with 10 % doxycycline hyclate and containing 10 % ATBC. Figure 4c is a zoom on the center of this Petri dish, highlighting the inhibition zone and the implant. The negative controls (implants free of drug) did not show any inhibition of the proliferation of the bacteria (Figure 4a).

Based on these results, 10 % ATBC was selected for further experiments, showing a substantial increase in the adhesive force, while providing good deformability as well as a clear antimicrobial activity, and requiring only the addition of a limited amount of substance. To further improve the implants' properties (especially, adhesion to the periodontal pocket and plasticity), a second type of polymer was added to the systems.

a)



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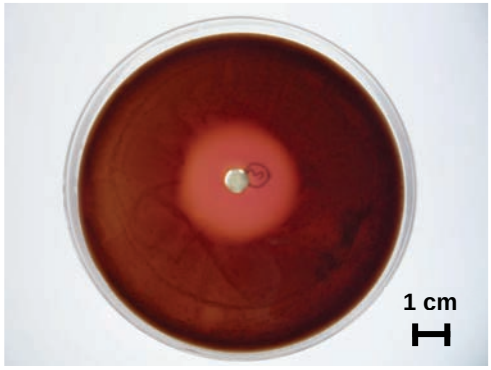

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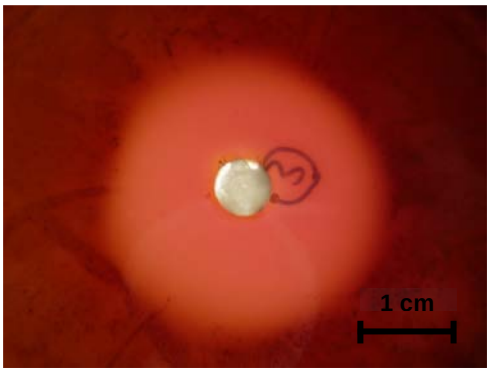
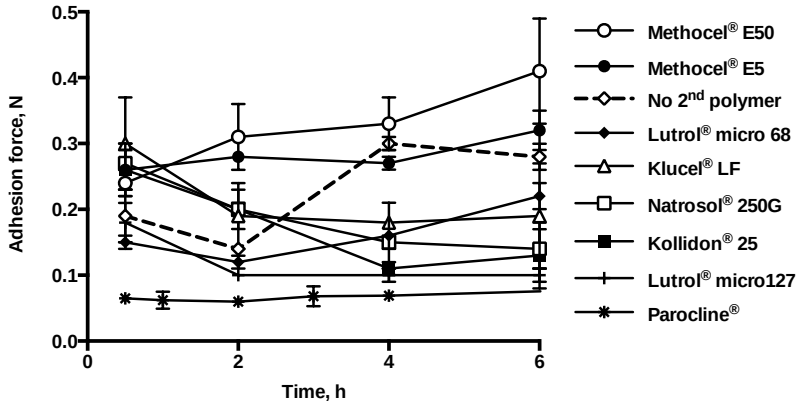


Figure 4. Antimicrobial activity of *in situ* forming implants based on PLGA RG 502 H (37 % w/w), loaded with 10 % doxycycline hyclate and containing different types and amounts of plasticizers: a) inhibition zone diameters for several bacterial strains isolated from periodontal pockets after 4 d incubation, b) picture of a Petri dish incubated with *Streptococcus sanguinis* for 4 d; the white circle at the center is an *in situ* formed implant based on PLGA RG 502 H (37 % w/w), 10 % ATBC, initially loaded with 10 % doxycycline hyclate, c) higher magnification of the inhibition zone and implant shown in b).

3.3. Effects of the addition of a second type of polymer

Figure 5a shows the adhesive forces (and dynamic changes thereof with time) of doxycycline hyclate-loaded (10 %) *in situ* forming implants based on PLGA RG 502 H (37 %), ATBC (10 %) and 10 % hydroxypropyl methylcellulose (Methocel® E5 or E50), hydroxypropyl cellulose (Klucel® LF), hydroxyethyl cellulose (Natrosol® 250 G), polyvinylpyrrolidone (Kollidon® 25), or poloxamer (Lutrol® micro 68, 127). These polymers have been reported to show a promising potential for bioadhesion, since they can be expected to be able to attract water from the gingival crevicular fluid, hydrate and facilitate adhesive interactions [49-54]. For reasons of comparison, also implants free of these polymers as well as the commercially available product Parocline® were studied. As it can be seen, all implants showed much higher adhesive forces than Parocline®. Interestingly, some of the 2nd polymers further improved the systems' adhesion during the observation period, namely Methocel® E5 and E50, whereas others decreased the implants' stickiness, namely Lutrol® micro 68 and 127. This might at least partially be attributable to differences in the interactions between these compounds with PLGA, ATBC, NMP and water. The observed time-dependent changes in the adhesive forces of the systems can at least partially be attributed to the time-dependent changes of the implants' composition: NMP leaches out into the surrounding environment and water penetrates into the systems. Figure 5b shows the $F_{remaining}/F_{max\ deformation}$ ratios of the respective *in situ* forming implants as well as time-dependent changes thereof. Again, the two Lutrol® types had a negative effect on the systems' properties with respect to expected prolonged residence times in the patients' periodontal pockets, whereas an increase in plasticity was observed at later time points with Natrosol® 250G and Kollidon® 25. The mechanical properties of Methocel®-free and Methocel®-containing implants were rather similar. In all cases, the plastic deformability was significantly superior to that of the commercial reference product Parocline®. Since the most promising adhesion results were obtained with the two hydroxypropyl methylcellulose types (differing in their molecular weight), while not affecting the plasticity of the implants, Methocel® E5 or E50 were selected for all further studies.

a)



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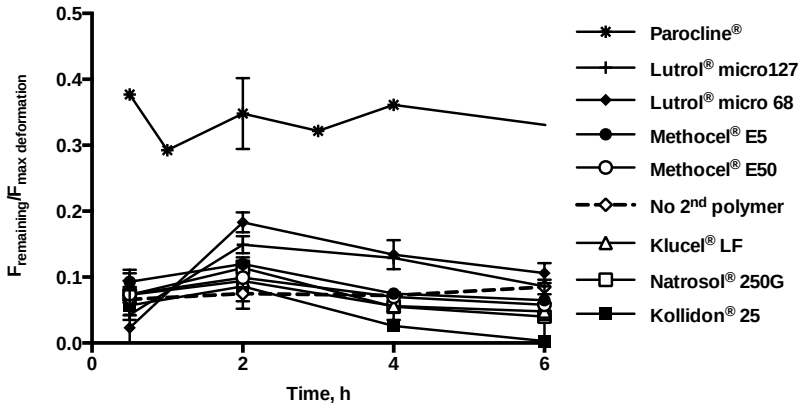


Figure 5. Impact of the addition of a second type of polymer (indicated in the diagrams, 10 % w/w) on the: a) adhesion force, and b) mechanical properties of *in situ* forming implants based on PLGA RG 502 H (37 %), ATBC (10 %), and loaded with 10 % doxycycline hyclate. For reasons of comparison, also Parocline® was studied.

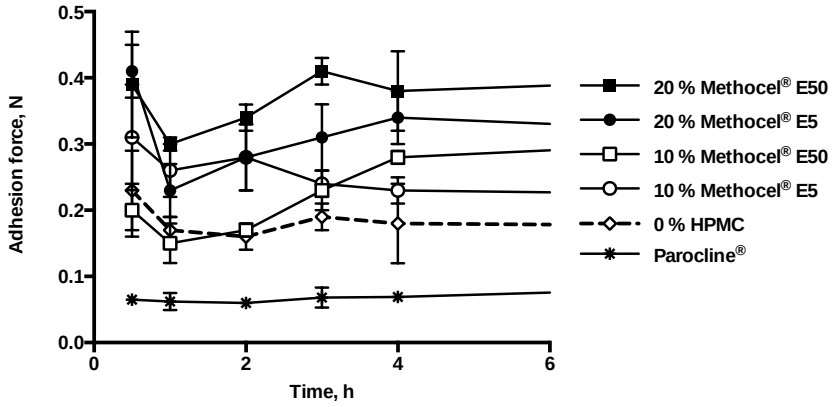
Figure 6a illustrates the impact of the addition of different *amounts* (10 % and 20 %) of Methocel® E5 and E50 on the adhesive forces of *in situ* forming implants based on PLGA RG 502 H, ATBC (10 %), loaded with 10 % doxycycline hyclate, but containing only 32 % PLGA in the liquid formulation. The change in PLGA concentration from 37 to 32 % was required to allow for the incorporation of higher amounts of HPMC, otherwise the liquid formulations became too viscous for injection. Note that this difference in PLGA concentration can be expected to impact the resulting solvent exchange kinetics (NMP leaching into the aqueous phase and water penetration into the implants). Thus, some caution should be paid when comparing these results (Figure 6) with those shown in

Figure 5. Importantly, the implants' stickiness could be further increased when increasing the HPMC content (Figure 6a): Adhesion forces as high as 0.4 N were measured with 20 % of the higher molecular weight Methocel®. This might be explained as follows: The longer the polymer chains, the easier they can create highly entangled networks. Furthermore, the higher the HPMC content, the denser and stronger are the resulting macromolecular networks. Again, the dynamic changes in the implants' stickiness at early time points can probably be explained by the dynamic changes in the systems' composition, due to solvent exchange. Note that the presence of the hydrophilic HPMC can be expected to impact the rate at which water enters the system and to impact the rate at which NMP leaches into the bulk fluid. Importantly, the adhesion forces of the implants remained high during the entire observation period.

However, the presence of significant amounts of a hydrophilic polymer might also significantly affect the plasticity/elasticity of the respective *in situ* formed implants, e.g. via altered solvent exchange kinetics (which might affect polymer precipitation and, thus, the inner implants' structure) as well as via the presence of an additional polymeric network. This is why also the mechanical properties of the implants (and potential dynamic changes thereof with time) were measured. As it can be seen in Figure 6b, the plasticity of the systems decreased, especially in the case of Methocel® E50 (the $F_{remaining}/F_{max\ deformation}$ ratio increased). This is in contrast to Figure 5b, showing systems prepared with a higher PLGA concentration (37 % versus 32 %). Thus, the difference can probably be explained by altered polymer precipitation kinetics, resulting in altered inner implant structures. In any case, the $F_{remaining}/F_{max\ deformation}$ values remained well below the reference values observed for the commercially available drug product Parocline®. Again, the observed time-dependent changes are likely to be attributable to time-dependent changes in the implants' composition, following solvent exchange.

In practice, a compromise should be made, taking into account the adhesiveness of the system as well as its deformability to optimize the resulting residence times in the patients' periodontal pockets. Of course, in addition to these key properties, also the drug release kinetics of the *in situ* formed implants is of major importance for the systems' performance. This is why the impact of the addition of different amounts and types of HPMC on doxycycline release was studied.

a)



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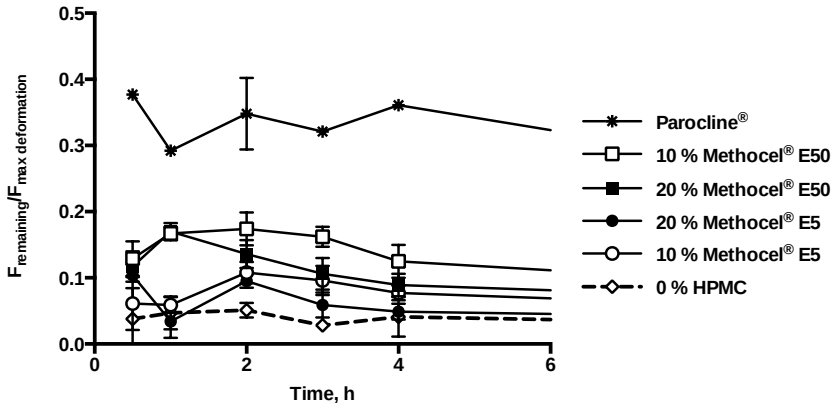


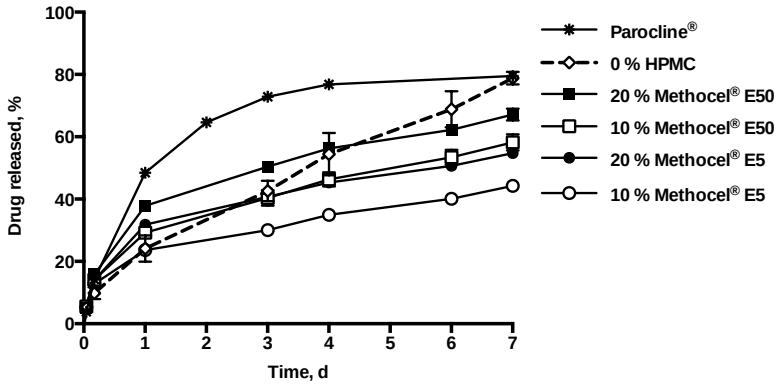
Figure 6. Effects of the addition of different types and amounts of HPMC (Methocel® E5 and E50) on the: a) adhesion force, b) mechanical properties of *in situ* forming implants, based on PLGA RG 502 H (32 %), ATBC (10 %), and loaded with 10 % doxycycline hyclate. For reasons of comparison, also Parocline® was studied.

3.4. Drug release kinetics

Figure 7 shows the drug release kinetics from *in situ* forming implants based on PLGA RG 502 H (32 %), ATBC (10 %) and 10 or 20 % Methocel® E5 or E50 (as indicated). The initial drug loading was varied from: a) 10 %, b) 5 %, to c) 2 % doxycycline hyclate. For reasons of comparison, also doxycycline release from HPMC-free systems is indicated (dotted curves). Furthermore, drug release from the commercially available product Parocline® is illustrated. However, some care should be taken when comparing the results with this commercially available product, since the drug is different: minocycline hydrochloride (in Parocline®) versus doxycycline hyclate (in the investigated PLGA implants). Very clearly, drug release is sustained during several days from all systems. Importantly, the novel *in situ* forming implants show significantly slower drug release than the commercially available product at all drug loadings (Parocline® contains 2 % drug). Furthermore, it can be seen that the addition of HPMC generally decreases the resulting drug release rate, irrespective of its molecular weight and the initial drug loading. Interestingly, the addition of shorter chain Methocel® E5 seems to retard drug release more effectively than the addition of longer chain Methocel® E50, and there is no clear tendency concerning the effect of the amount of added HPMC: 0 % versus 10 % versus 20 %. The addition of 10 % HPMC generally results in the slowest drug release patterns, irrespective of the Methocel® type and initial drug loading.

To better understand the observed effects of the addition of HPMC to the *in situ* forming implants on the resulting drug release kinetics, the dynamic changes in the systems' mass were monitored gravimetrically. These changes reflect the solvent exchange kinetics: NMP leaching into the bulk fluid and water penetration into the implants. Figure 8 shows the experimentally measured changes in the mass of the implants, drug release of which is illustrated in Figure 7 (being based on 32 % PLGA RG 502 H, 10 % ATBC, 10 % or 20 % Methocel® E5 or E50, and loaded with 2 to 10 % doxycycline hyclate).

a)



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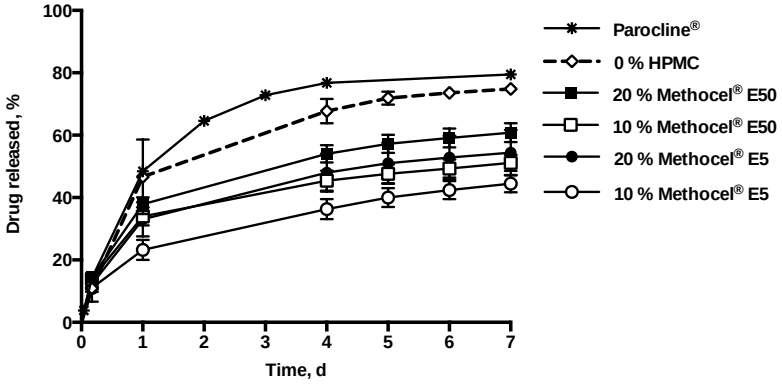

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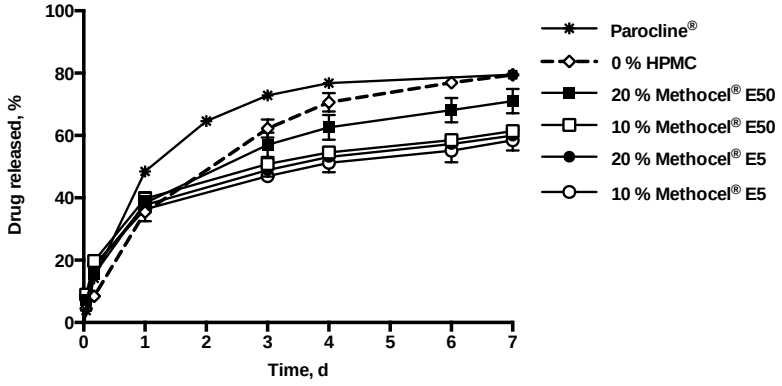
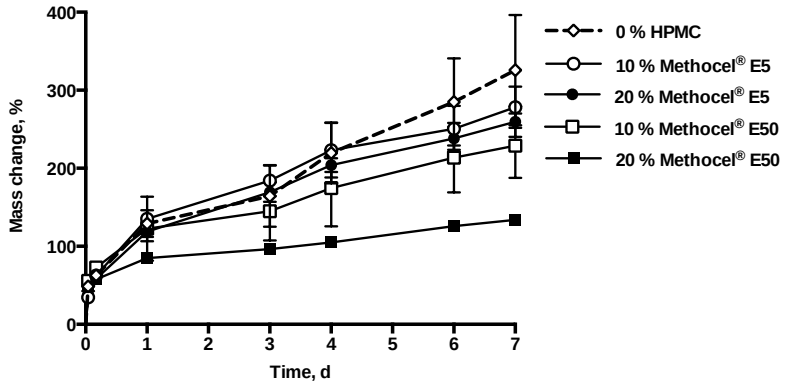


Figure 7. Impact of the addition of different types and amounts of HPMC (Methocel® E5 and E50) on drug release from *in situ* forming implants based on PLGA RG 502 H (32 %) and ATBC (10 %), loaded with: a) 10 %, b) 5 %, c) 2 % doxycycline hyclate. For reasons of comparison, also minocycline release from Parocline® is shown, but note that this is a different drug.

a)



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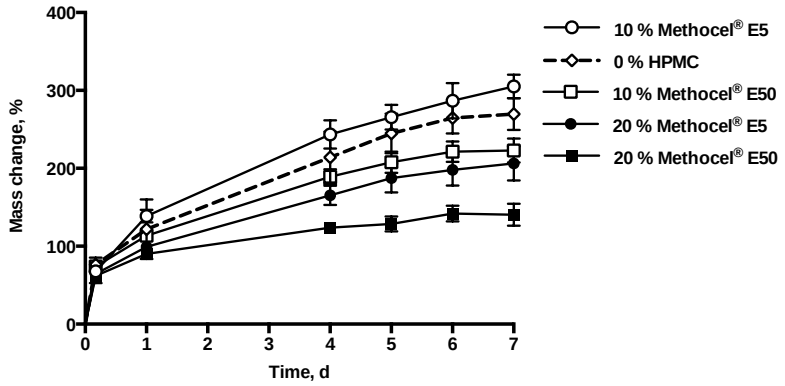

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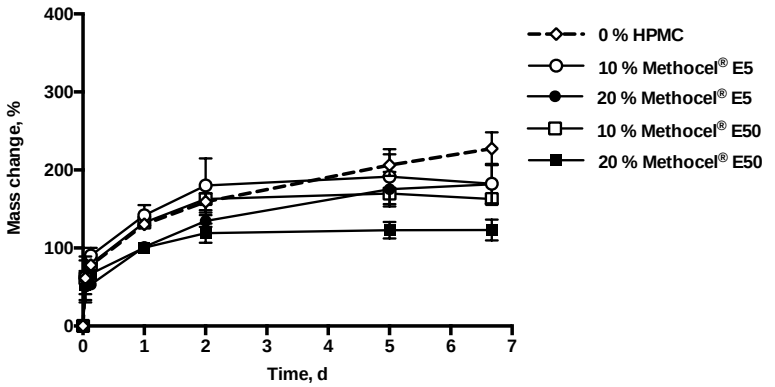


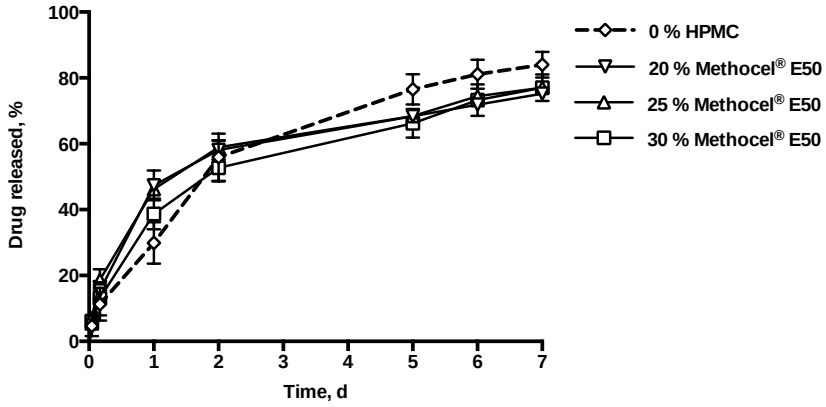
Figure 8. Impact of the addition of different types and amounts of HPMC (Methocel® E5 and E50) on the dynamic changes in the mass of *in situ* forming implants based on PLGA RG 502 H (32 %) and ATBC (10 %), loaded with: a) 10 %, b) 5 %, c) 2 % doxycycline hyclate.

As it can be seen, in all cases the implant mass increases with time during the observation period, which can be attributed to a more important water penetration into the devices than NMP leaching out of the systems. Interestingly, the addition of the hydrophilic compound HPMC generally decreases the rate and extent of this increase in mass, irrespective of the Methocel® type and initial drug loading. This might eventually be attributable to altered polymer precipitation kinetics: The presence of hydrophilic HPMC can be expected to facilitate water penetration into the system, leading to accelerated polymer precipitation and, hence, altered inner implant structures. Furthermore, it can be seen that longer chain Methocel® E50 generally more effectively hinders the mass increase at all initial drug loadings than shorter chain Methocel® E5. This correlates with generally faster drug release from these systems (Figure 7). Furthermore, there is a rough ranking order with respect to the effect of the amount of added HPMC on the rate and extent in mass increase of the implants: 0 % > 10 % > 20 %. This is in contrast to the observed drug release kinetics, where 10 % Methocel® resulted in the slowest release rates (Figure 7). These findings clearly point out: (i) that the underlying mass transport phenomena are not straightforward, and (ii) that the addition of different amounts and types of HPMC can be effectively used to fine-tune desired drug release kinetics (in addition to improving the implants' stickiness, while providing good deformability).

As a potential further tool to adjust desired drug release kinetics from the investigated *in situ* forming implants, the impact of varying the concentration of the PLGA in the liquid formulation was studied: Figure 9a shows the release of doxycycline from implants based on only 28 % (instead of 32 % as in Figure 7) PLGA RG 502 H, 10 % ATBC, and loaded with 5 % doxycycline hyclate. Comparing Figure 9a with Figure 7b (showing the same type of system, but with a higher PLGA concentration in the liquid formulation), it can be seen that the PLGA concentration indeed plays a crucial role for drug release. At 28 % PLGA content, the addition of HPMC seems to have only a very minor effect, even up to 30 % Methocel® E50 (Figure 9a). This is in contrast to the implants prepared with 32 % PLGA, for which HPMC addition impacted drug release (Figure 7b). Figure 9b shows the significant impact of adding 20 %, 25 % or 30 % Methocel® E50 to implants prepared with 28 % PLGA on their dynamic changes in mass. Interestingly, the addition of all three HPMC levels similarly strongly hindered the increase in implant mass (compared to HPMC-free systems, dotted curve). This confirms

the complex interplay between the two types of polymers (PLGA and HPMC), the plasticizer (ATBC), the drug (doxycycline hyclate) and the two solvents (NMP and water). Future studies using advanced characterization methods (such as EPR and NMR measurements) will aim at a better understanding of the involved mass transport processes.

a)



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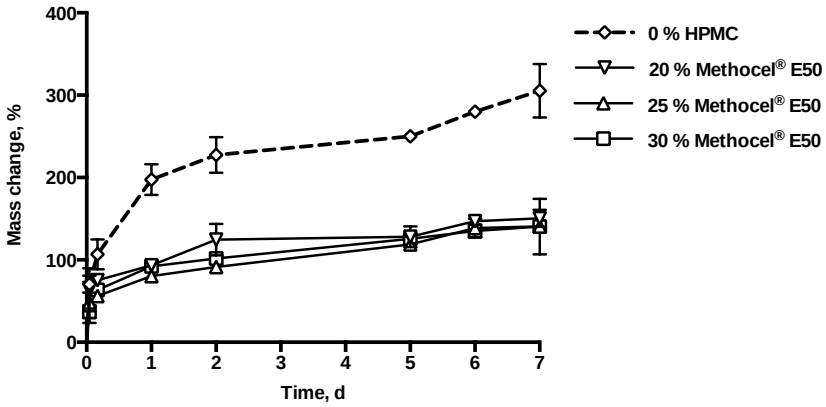

Figure 9. Effects of the addition of different types and amounts of HPMC (Methocel® E5 and E50) on the: a) drug release kinetics, b) dynamic changes in the mass from/of *in situ* forming implants based on PLGA RG 502 H (28 %) and ATBC (10 %), loaded with 5 % doxycycline hyclate.

4. Conclusion

The newly presented *in situ* forming implants show an interesting potential for the local treatment of periodontitis, since they are much more adhesive than prior art systems, while providing appropriate plasticity, the ability to control drug release during several days and show antimicrobial activity against relevant *Streptococcus* strains. In future studies the underlying mass transport mechanisms will be further elucidated and different types of drug incorporated.

Acknowledgements

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CHAPTER II

TOWARDS A BETTER UNDERSTANDING OF THE *IN SITU* FORMATION OF IMPLANTS FOR PERIODONTITIS TREATMENT

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Abstract

In situ forming implant formulations based on poly(lactic-co-glycolic acid) (PLGA), acetyltributyl citrate (ATBC), minocycline HCl, N-methyl pyrrolidone (NMP) and optionally hydroxypropyl methylcellulose (HPMC) were prepared and thoroughly characterized *in vitro*. This includes electron paramagnetic resonance (EPR), nuclear magnetic resonance (^{1}H NMR), mass change and drug release measurements under different conditions, optical microscopy, size exclusion chromatography (SEC) as well as antibacterial activity tests using gingival crevicular fluid samples from periodontal pockets of periodontitis patients. Based on these results, deeper insight into the physico-chemical phenomena involved in implant formation and the control of drug release could be gained. For instance, the effects of adding HPMC to the formulations, resulting in improved implant adherence and reduced swelling, could be explained. Importantly, the *in situ* formed implants effectively hindered the growth of bacteria present in the patients' periodontal pockets. Interestingly, the systems were more effectively hindering the growth of *pathogenic* bacterial strains (e.g., *Fusobacterium nucleatum*) than of physiological strains (e.g., *Streptococcus)*. *In vivo*, such a preferential action against the pathogenic bacteria can be expected to give a chance to the healthy flora to re-colonize the periodontal pockets.

Keywords: *In situ* forming implant; periodontitis; PLGA; antibacterial activity; EPR; NMR.

1. Introduction

Periodontitis is a highly prevalent, chronic inflammatory disease of the periodontium [1–3]. It may be defined as "a disease that affects the periodontal structures and, as a result of interactions between periodontopathogens and the host immune response, leads to the destruction of the tooth supporting tissues, periodontal ligament and alveolar bone" [1]. Briefly, microorganisms colonizing the patients' periodontal pockets are considered as a major factor, causing inflammation and tissue destruction [4]. It seems that the bacterial flora in the disease state is different from that in healthy subjects. For example, the number of gram negative anaerobic bacteria is likely to be increased, and certain clinical forms of periodontitis might be related to specific microbiota [5]. For instance, Silva-Boghossian et al. [1] reported that *Streptococcus* strains (such as *Streptococcus sanguinis*) are associated with suppuration in periodontitis subjects. However, up to date, the exact mechanisms underlying this disease are not yet fully understood. It is hypothesized that: (i) suspected periodontal pathogens produce biologically active molecules, which directly attack the host tissue, and/or that (ii) the immune response of the host organism (human body) to these pathogens results in the tissue destruction. The consequence of the tissue loss is the deepening of the periodontal pockets, and -once the mechanical anchorage of the tooth becomes insufficient -the latter is lost. Periodontitis is in fact the main cause for tooth loss in adults [6]. A recent survey estimates that 47 % of the US adults have mild, moderate or severe periodontitis [7]. The prevalence rate even increases to 64 % for adults, which are older than 65 years.

At present, the standard treatment method of periodontitis is the mechanical removal of the bacteria (in particular of bacterial biofilms). This is a procedure also called "root planing". But the geometry of the patients' pockets can be very challenging for this type of treatment: Parts of the pockets might be very difficult to access with the dentist's instruments. Thus, the removal of the bacteria might be incomplete. In theses cases, the remaining pathogenic microorganisms have a chance to re-colonize the periodontal pockets soon after the treatment. In order to reduce the risk of such pathogen re-appearance, it has been suggested to combine mechanical root planing with drug treatments [4,8,9]. However, appropriate delivery of drugs to the site of action is difficult, since many compounds do not easily partition into the periodontal pockets. In addition, the gingival crevicular fluid (GCF) flow generally rapidly eliminates the drug from its site of action [10]. For instance, it has been estimated that the

contents in a 5 mm periodontal pocket is renewed 40 times per hour [11]. Thus, using conventional administration routes, often high systemic drug levels are required, while the drug concentrations at the target site remain low. This leads to potentially severe side effects combined with limited or insufficient therapeutic efficacy, despite the availability of highly potent drugs, able to act against the pathogenic flora and inflammation. Controlled local drug delivery systems offer the possibility to overcome these crucial hurdles of limited drug accessibility to the site of action and rapid elimination, releasing the drug in a controlled manner directly in the periodontal pockets during prolonged periods of time [4,12–14]. *In situ* forming implants are particularly promising for this purpose, since these are liquid formulations, which upon injection into the periodontal pockets form *customized* solid implants: The fluids readily spread within the cavities, assuring that the entire pockets are filled with formulation and that the shape and geometry of the resulting implants are fully adapted to the characteristics of every single patient and each single pocket.

In this study, poly(lactic-co-glycolic acid) (PLGA) has been chosen as a matrix former for such *in situ* forming implants for periodontitis treatment, due to its biocompatibility and biodegradability. Together with the drug (here minocycline HCl) the polymer is dissolved in N-methyl pyrrolidone (NMP). Once injected, the organic solvent diffuses into the surrounding environment and aqueous biological fluids from the periodontal pocket penetrate into the liquid formulations. Since PLGA is not soluble in water, it subsequently precipitates and entraps the drug. This type of advanced local drug delivery systems for periodontitis treatment offers various important advantages, including: (1) A relatively easy administration (injection of a liquid, compared for instance with the placement of a pre-formed implant). (2) There is no need to remove empty remnants upon drug exhaust, due to complete biodegradability of the system. (3) The geometry and size of the resulting implants are adapted to the patient's dental pockets (personalized medicine). (4) The incorporated drug is locally released in a time-controlled manner through the slowly degrading polymeric system.

However, up to date major challenges remain to be addressed, namely the fact that: (i) The adherence of such *in situ* formed implants to human tissue is yet poor, resulting in premature and uncontrolled expulsion of at least parts of the implants from the dental pockets due to the non-negligible flow of gingival crevicular fluid [15]. This leads to a considerable uncertainty with respect to the amount of drug, which really reaches the target site and with

respect to the time periods during which therapeutic drug levels are provided. (ii) The elasticity/plasticity of the formed implants is generally not adapted to this type of local administration: Systems, which are difficult to deform plastically are not able to adapt their geometry to dynamic changes in the periodontal pocket' size and shape with time. Also, fully elastic implants force the periodontal pockets to keep their geometry and dimensions, which is not desirable. It has recently been proposed to add plasticizers, such as acetyltributyl citrate (ATBC) as well as a second type of polymer, such as hydroxypropyl methylcellulose (HPMC) to the liquid formulations in order to improve the adhesive and mechanical properties of the resulting implants [16]. However, yet it is unclear how these additives affect the underlying physico-chemical phenomena involved in implant formation and the control of drug release, and whether the antibacterial activity of the implants is altered.

It is well documented that the physical and chemical processes in the formation of implants based on such solvent induced phase separation are complex and that the impact of the composition of the systems on drug release is not straightforward [17–22]. For example, McHugh and co-workers reported that the addition of polyvinylpyrrolidone (PVP) accelerates the phase separation and increases the release rate of lysozyme at early time points, but does not significantly affect the water influx rate and implant morphology [23]. Increasing the polymer concentration in the formulation led to a decrease in the phase separation rate, a decreased water uptake rate and significant changes in the implants' porosity. The addition of triacetine also slowed down the phase separation rate and altered the implants' morphology, resulting in decreased drug release rates. Interestingly, the type of release medium (water versus phosphate buffer versus horse serum) did not affect the phase separation and water uptake rates as well as the implants' morphology to a noteworthy extent. In a later study, they also showed that the addition of Pluronic® led to faster phase separation and increased water uptake, but decreased lysozyme release rates [24]. Importantly, advanced physico-chemical characterization techniques, such as electron paramagnetic resonance (EPR) and nuclear magnetic resonance (^{1}H NMR) measurements can be expected to be able to provide highly valuable new insight into the underlying mass transport phenomena [25,26].

The aim of this study was to better understand the physico-chemical processes involved in the formation of PLGA-based implants and the control of drug release. EPR, ^{1}H NMR, mass change and drug release measurements under different conditions, optical

microscopy and size exclusion chromatography (SEC) were applied. Particular attention was paid to: (i) the impact of adding HPMC to the formulation, which improves the adhesive forces and mechanical properties of the implants, as well as (ii) the antibacterial activity of the systems, using gingival crevicular fluid samples, obtained from periodontal pockets of periodontitis patients.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA, Resomer® RG 504 H; Evonik, Darmstadt, Germany); acetyltributyl citrate (ATBC; Morflex, Greensboro, NC, USA); hydroxypropyl methylcellulose (HPMC, Methocel® E50; Colorcon, Dartford, UK); N-methyl pyrrolidone (NMP, 99 %), glucose and cysteine hydrochloride (Acros organics, Geel, Belgium); minocycline hydrochloride dihydrate (minocycline HCl; Fagron, Colombes, France); ascorbic acid (Cooper, Melun, France); sodium metabisulfite (Merck, Darmstadt, Germany); dimethyl sulfoxide (DMSO, 99.5 %; Gruessing, Filsum, Germany); tetrahydrofuran (THF, 99.99 %, analytical reagent grade, stabilized with 0.025 % butylhydroxytoluene), acetonitrile (HPLC grade) (Fisher Scientific, Loughborough, UK); oxalic acid (Sigma-Aldrich, Saint-Quentin Fallavier, France); ethylenediamine tetraacetic acid (EDTA; VWR, Haasrode, Belgium); 4-hydroxy-tempo benzoate (TB; Sigma-Aldrich, Seelze, Germany); agarose (GenAgarose® LE; Genaxxon BioScience, Ulm, Germany); Columbia agar base and agar (Oxoid, Basingstoke, UK); defibrinated horse blood (E&O Laboratories, Burnhouse, UK); Parocline® (2 % minocycline; Sunstar France, Levallois-Perret, France).

2.2. Preparation of the liquid formulations

PLGA (25 % w/w, based on the total liquid formulation without drug) was dissolved in NMP at 25 °C in a glass vial (30 min stirring). Optionally, the plasticizer ATBC (10 % w/w, based on the PLGA mass) and/or HPMC (10, 15, 20, 25 or 30 % w/w, based on the PLGA mass) was/were added and the mixture was vortexed for 3 min, followed by standing for 3 h at 25 °C. Subsequently, minocycline HCl (2 % w/w, based on the total liquid formulation) and ascorbic acid (0.01 % w/w, based on the total liquid formulation; minimizing drug oxidation) were added, and the mixture was vortexed for

3 min, followed by standing for 3 h at 25 °C. To eliminate air bubbles, the liquids were ultrasonicated for 10 min. The formulations were stored at -20 °C and protected from light to avoid drug degradation.

2.3. In situ implant formation and drug release measurements

Agitated vials: One hundred microliters of the respective formulation was injected at the bottom of an Eppendorf vial using a standard syringe. One and a half milliliters preheated (37 °C), degassed phosphate buffered saline pH 7.4 (Ph. Eur. 7) containing 0.01 % sodium metabisulfite (to minimize drug oxidation) was carefully added using a pipette, initiating solvent exchange and implant formation. The vials were horizontally shaken at 37 °C at 80 rpm (GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, the bulk fluid was completely withdrawn and replaced with fresh medium. The drug content in the samples was determined UV-spectrophotometrically (λ = 324 nm; UV-1650PC, Shimadzu, Champs-sur-Marne, France) (degraded and non-degraded drug) and by HPLC (non-degraded drug). The HPLC system was equipped with a ProStar 210 pump, a ProStar 410 auto-sampler, a ProStar 335 Photodiode Array Detector (Varian, Agilent Technologies, Les Ulis, France). A Kinetex column C8 (2.6 µm, 100 x 4.6 mm; Phenomenex, Le Pecq, France) was used for the separation. The mobile phase consisted of 22 % acetonitrile and 78 % of an aqueous solution (deionized water) of oxalic acid (0.02 M) and EDTA (0.0005 M), which was adjusted to pH 2.8 with aqueous sodium hydroxide solution (2 M). The operating mode was isocratic, the flow rate 1.0 mL/min, the injection volume 20 µL and the drug was detected by UV-Vis spectrophotometry at 351 nm. For reasons of comparison, also the commercially available formulation Parocline® was studied. In this case, the 100 µL of the formulation was injected using the supplied syringe.

Flow-through cells: A continuous flow-through system, as described in detail by Aubert-Pouessel et al. [27], was used. Briefly, 100 µL of the respective formulation was filled into an empty Omega column (4.6 x 50 mm; Upchurch Scientific, Oak Harbor, WA, USA) using a standard syringe. Degassed phosphate buffered saline pH 7.4 (Ph. Eur. 7) containing 0.01 % sodium metabisulfite was pumped through the column at 44 µL/h (PHD 2000 syringe pump; Harvard Apparatus, Holliston, MA, USA), simulating the continuous gingival fluid flow in patients' periodontal pockets [15]. The column was maintained at

37 °C with a water bath. The eluent was cooled to 4 °C to minimize minocycline degradation and analyzed by UV and HPLC as described above.

All tests were performed in triplicate and the results were shown as mean values ± standard deviation.

2.4. Monitoring of dynamic changes in the implants' mass

Implants were prepared and treated as described in *Section 2.3. Agitated vials*. At pre-determined time points, implants were weighed [*mass (t)*]. The *mass change in percent* was calculated as follows:

$$mass\ change\ (\%)\ (t) = 100 * [mass\ (t) - mass\ (t=0)] / mass\ (t=0) \quad (1)$$

where *mass (t=0)* is the initial weight of the formulation used for implant preparation.

2.5. Electron paramagnetic resonance (EPR) measurements

In situ forming liquid implant formulations were prepared as described in section *2.2*. The spin probe 4-hydroxy-tempo benzoate (TB) was dissolved in these liquids (1 mM). Two hundred µL of the formulations were placed into cylindrical holders, which were immerged into 3 mL phosphate buffered saline pH 7.4 (Ph. Eur. 7). As illustrated in Figure 1a, only the top circular surface of the cylindrical holder was open, the other surfaces were impermeable. The system was kept constant at 37 °C and horizontally shaken at 30 rpm (GFL 1083; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, samples (implants with holders) were withdrawn and analysed using an EPR L-band spectrometer (MagnetTech, Berlin, Germany), operating at a low microwave frequency (1 GHz). To create EPR *images*, twenty-five scans were accumulated using the following parameters: field centre = 48.9 mT; scan range = 8 mT; scan time = 40 s. For the measurement of EPR *spectra*, a scan range of 10 mT and scan time of 100 s were applied. The typical EPR parameters were calculated from the recorded EPR spectra or spectral cut of EPR images (Figures 1b and 1c).

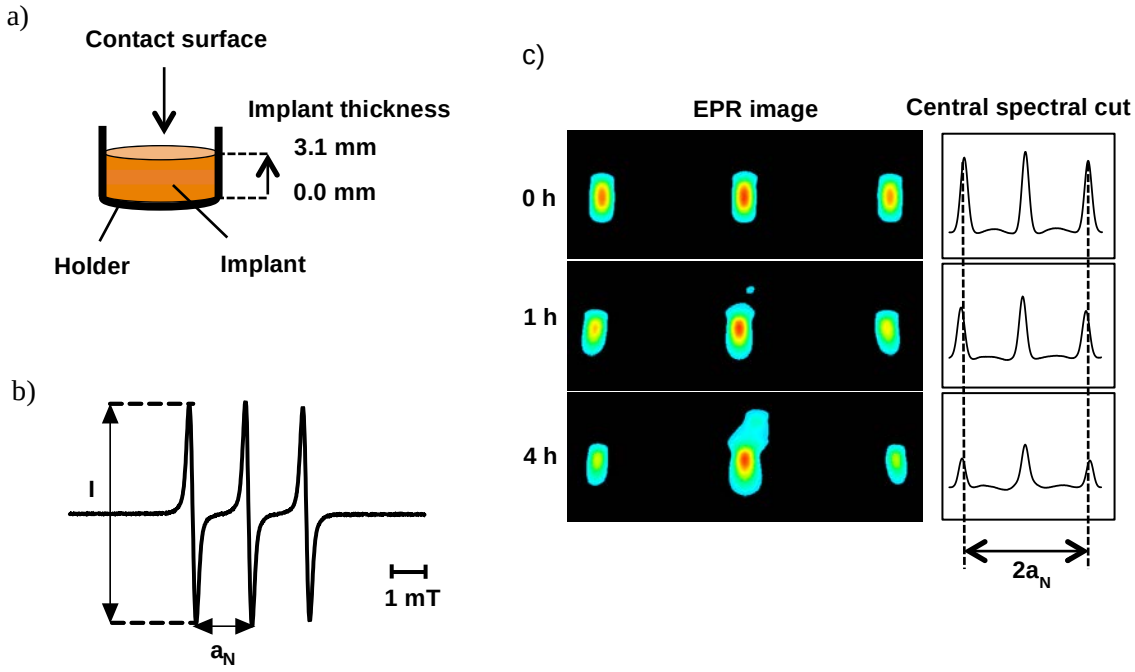

Figure 1. a) Schematic illustration of an implant with holder used for EPR measurements. Only the circular top surface of the cylindrical holder was open, all other surfaces were impermeable. b) EPR spectrum of TB dissolved in NMP (*I – signal amplitude, a_N – hyperfine splitting constant)*. c) EPR image and central spectral cut of an implant (25 % PLGA, 10 % ATBC, 20 % HPMC, 1 mM TB) recorded after 0, 1 and 4 h exposure to phosphate buffer.

2.6. ^{1}H NMR measurements

One hundred µL of the respective formulation was injected at the bottom of an Eppendorf vial filled with 1.5 mL phosphate buffered saline pH 7.4 (Ph. Eur. 7), using a standard syringe. The vials were kept constant at 37 °C and horizontally shaken at 30 rpm (GFL 1083). At predetermined time points, implants were withdrawn, carefully dried with a tissue paper and subsequently dissolved in DMSO (1:10 w/v). NMR spectra were recorded with a 400 MHz ^{1}H NMR spectrometer (Varian Gemini 2000; Varian, Agilent Technologies, Waldbronn, Germany).

2.7. Optical microscopy

Implants were prepared as described in *Section 2.3.* At predetermined time points, implants were withdrawn and freeze-dried (Epsilon 2-4 LSC; Christ, Osterode, Germany).

The process consisted of 3 phases: (i) freezing at -45 °C for 2 h; (ii) primary drying at 0.014 mbar and -9 °C shelf temperature for 10 h; (iii) secondary drying at 0.0014 mbar and 20 °C shelf temperature for 10 h. Cross-sections were obtained with a knife and analysed with a SMZ-U zoom 1:10 microscope (Nikon, Tokyo, Japan), equipped with a TV lens C-0.45x (Nikon) and a digital camera AxioCam ICc1 (Carl Zeiss, Oberkochen, Germany).

2.8. PLGA degradation

The weight average molecular weight (Mw) of PLGA was determined by size exclusion chromatography (SEC) using a Varian Prostar HPLC System (Varian, Agilent Technologies, Les Ulis, France) consisting of a Galaxie system controller, a ProStar 410 autosampler, a Prostar 230 pump and a Varian 356-LC RI detector. Freeze-dried implants were dissolved in tetrahydrofuran (0.3 % w/v) prior to the measurements. Fifty µL samples were injected into a PLGel pre-column (5 µm, 50 x 7.5 mm), which was followed by a PLGel High performance GPC column (5 µm, MIXED-D, 300 x 7.5 mm) (Polymer laboratories, Varian). The mobile phase was tetrahydrofuran, the flow rate 1.0 mL/min, the column temperature 35 °C. Polystyrene narrow molecular weight standards (Polystyrene calibration kit S-M2-10, 580 – 271,800 Da; Agilent Technologies) were used for calibration. The Mw was calculated using the Cirrus GPC software (Agilent Technologies).

2.9. Microbiological tests

Samples from periodontal pockets of periodontitis patients: Thirteen patients (4 women, 9 men; from 35 to 69 years old) were enrolled in this study (14 periodontal pockets were sampled). They were admitted at the clinical site of the Faculty of Dental Surgery, University of Lille, France. Participants did not receive any hygienic treatment at the teeth with periodontitis prior to sampling. Sterile paper points (Roeko, Coltene, Germany) were carefully inserted into each periodontal pocket (1 paper point per pocket) and left for 10 s to allow for absorption of gingival crevicular fluid (GCF). Each paper point was placed into an Eppendorf vial, filled with 1.5 mL of Ringer Cysteine. Independently, 10 paper points were weighed before and after sampling to estimate the mean amount of GCF absorbed (5.7 ± 0.6 mg) to allow for the quantification of bacteria. Further tenfold dilutions (-2 to -7) of these GCF solutions (-1) were obtained for

microbiological testing.

Susceptibility of periodontal bacteria to minocycline: Columbia agar was prepared from Columbia agar base, glucose, cysteine hydrochloride, and agar. The systems were sterilized in an autoclave (121 °C for 15 min). Prior to plating, Columbia agar was enriched with defibrinated horse blood (5 % v/v), without or with minocycline (32 mg/L) and cast into Petri dishes. After cooling to room temperature, 0.1 mL of diluted GCF solutions (from -1 to -7) was inoculated onto the agar surface (35 °C, anaerobic atmosphere; Whitley A85 workstation, Don Whitley Scientific, West Yorkshire, UK). After 5 d of incubation, the number of bacterial colonies was counted for each Petri dish, the predominant colonies were subcultured and identified to enlarge the strain collection, and the mean log CFU/g was calculated.

Antibacterial activity of the *in situ* forming implants: The in vitro efficacy of the investigated implants was assessed by their antibacterial activity against the whole periodontitis samples and against isolated bacterial strains, using the agar well diffusion method. Columbia agar was enriched with horse blood (5 % v/v) and cast into Petri dishes. After cooling to room temperature, 0.1 mL of diluted GCF (dilution -2 and -3) or isolated bacterial suspension was inoculated onto the agar surface. A cylindrical hole (diameter = 6 mm) was subsequently made at the center of the agar, and filled with 30 µL of liquid formulation using a standard syringe. Upon solvent exchange, the implants formed *in situ*. The Petri dishes were incubated for 4 d (isolated bacterial strains) or 5 d (diluted entire GCF samples) under optimum culture conditions (35 °C, anaerobic atmosphere; Whitley A85 workstation). The diameter of the observed bacteria growth inhibition zones around the center of the Petri dishes was measured using a ruler. Each experiment was conducted in triplicate, the results are presented as mean values ± standard deviation.

Minimal inhibitory concentration (MIC) of minocycline on selected strains: The MIC of minocycline was determined using the broth dilution method [28]. One hundred µL of Wilkins-West broth were pipetted into each well of a 96-well plate, except for the first column. A minocycline stock solution (64 mg/L) was prepared in the same broth, 200 µL of which were introduced into the first column of microplate wells. After thorough mixing, 100 µL of the first well was added to the second well and so on. Finally, 100 µL of bacterial suspension in Ringer Cysteine solution was introduced into each well, leading to

2-fold dilution of the drug concentration (1-fold dilution in the first well). At the end, each well row contained a dilution series of the drug from the left to the right, with progressively lower concentrations (decreasing from 32 to 0.016 mg/L). After incubation at 35 °C under anaerobic conditions (Whitley A85 workstation), the lowest concentration of drug that prevented visible growth of bacteria was determined as the MIC. According to the 2013 guideline of the *Société Française de Microbiologie* (SFM), a microorganism is called "susceptible" to minocycline if MIC ≤ 4 mg/L and "resistant" to minocycline if MIC > 8 mg/L [29]. For these tests, the bacterial strains were provided from the collection of the Laboratory of Bacteriology of the College of Pharmacy, University of Lille, France (also obtained from patients suffering from periodontitis).

3. Results and discussion

3.1. Impact of HPMC addition on the implants' key properties

Significant implant swelling with time can lead to accidental and premature expulsion of at least parts of the formulations from the patients' periodontal pockets. Hence, ideally, system swelling should remain limited with time. Figure 2 shows the experimentally measured changes in the mass of implants prepared with formulations containing 25 % PLGA, 10 % ATBC and optionally up to 30 % HPMC, upon exposure to phosphate buffer. For reasons of comparison also the behavior of the commercially available drug product Parocline® is illustrated. Very clearly, Parocline® exhibits a substantial increase in system mass with time, indicating very important water uptake. This is consistent with the visually observed significant swelling of the gel and can at least partially explain the reported premature expulsion of parts of this formulation from the patients' pockets. Very importantly, the addition of increasing amounts of HPMC to the investigated PLGA-based implants substantially decreased the increase in system mass (Figure 2), which is highly promising with respect to the *in vivo* performance of these devices.

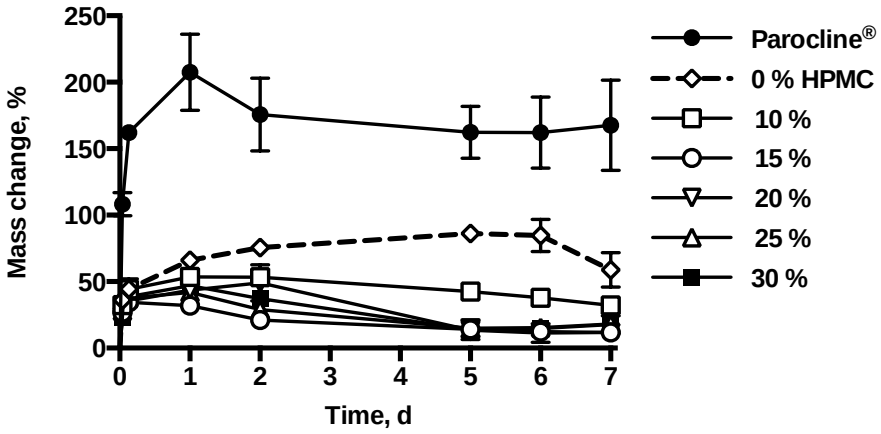

Figure 2. Effects of the addition of different amounts of HPMC (indicated in the figure) on the dynamic changes in the mass of *in situ* forming implants prepared with formulations containing 25 % PLGA and 10 % ATBC, upon exposure to phosphate buffer (agitated vial set-up).

Furthermore, it has been reported that the addition of HPMC to PLGA implants increases the latter's stickiness [16]. However, the presence of this hydrophilic polymer can also be expected to impact other implant characteristics, its drug release kinetics. The effects of adding different amounts of HPMC to liquid formulations based on PLGA (25 %) and ATBC (10 %) on the resulting minocycline release kinetics from the *in situ* formed implants are shown in Figure 3 (2 % initial drug loading). In Figure 3a, the *total* amount of drug release is illustrated, in Figure 3b the percentages of degraded drug in the withdrawn samples. Importantly, minocycline is not stable in aqueous solution. This is not a concern *in vivo*, since the living body eliminates the drug anyway, once it is released. The decisive question is whether the observed *in vitro* drug degradation occurs *within* the implants, or only once the drug is released into the bulk fluid. As it can be seen in Figure 3b, the percentage of degraded drug in the withdrawn samples strongly depended on the sampling frequency: When the sampling interval was short, the percentage of degraded drug was much lower compared to large sampling intervals. This is a good indication for the fact that a major part of minocycline degradation occurred *outside* of the implants. Within the implants, the acidic microenvironment induced by PLGA degradation may be responsible for a part of drug degradation. However, this limit can be minimized in practice considering the tinier volume of periodontal pockets (up to 1.5 µL [15]) than *in vitro* set-up (100 µL). Interestingly, the addition of HPMC to the implants led to *increasing*

release rates. This is in contrast to previously observed moderate to pronounced *decreasing* release rates when adding HPMC to PLGA-based *in situ* forming implants containing the drug doxycycline [16]. For reasons of comparison, also minocycline release from the commercial product Parocline® was measured (filled circles in Figure 3a). As it can be seen, drug release from all the investigated PLGA-HPMC implants was slower than from the commercial product. This can be expected to be advantageous in practice, combined with the improved adhesion and reduced system swelling described above. To better understand the underlying mass transport mechanisms, also EPR and NMR spectroscopy were applied to characterize the systems and dynamic changes thereof during drug release.

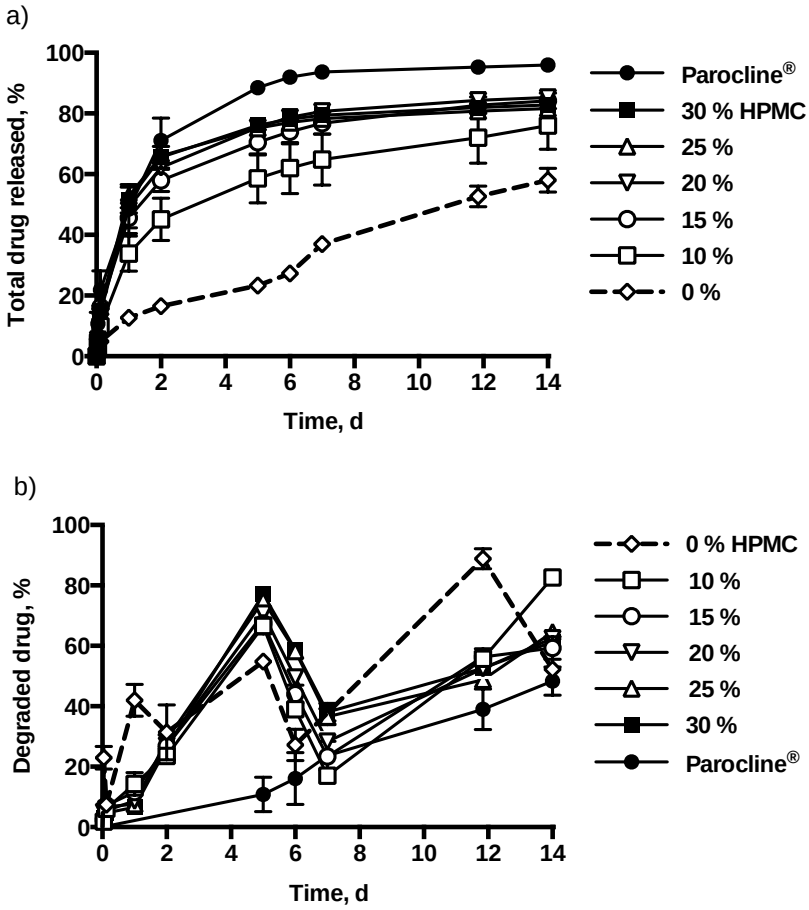

Figure 3. Drug release from *in situ* forming implants prepared from liquid formulations based on PLGA (25 %), ATBC (10 %) and 2 % minocycline HCl: a) cumulative total drug release, b) percentage of degraded drug within the withdrawn samples (agitated vial set-up).

3.2. Monitoring of the *in situ* implant formation by EPR and ^{1}H NMR

It is well known that the spectral splitting of nitroxyl radicals is sensitive to both, the molecular motion (microviscosity of the surrounding) and the polarity of their direct environment [30]. In this study, the spin probe 4-hydroxy-tempo benzoate (TB) was initially dissolved in the different liquid formulations, which formed implants *in situ*. Upon contact with phosphate buffer, solvent exchange led to substantial changes in the environment of this probe. To monitor these changes, the dependence of the hyperfine splitting ($2a_N$) on the composition of a "NMP:phosphate buffer" mixture was studied. As it can be seen in Figure 4a, the $2a_N$ values increased linearly (R^2 = 0.9886) with increasing buffer content (polarity). Based on this dependence, $2a_N$ values measured in *in situ* forming implants can be used as indicators for the solvent exchange process.

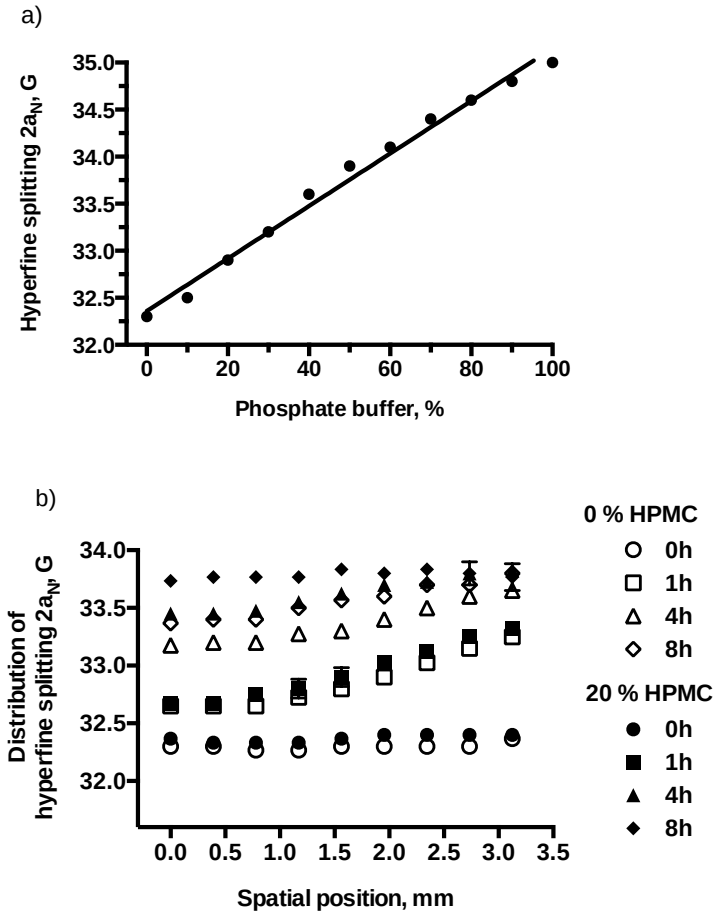

Figure 4. a) Polarity dependence of the hyperfine splitting of TB-loaded "NMP:phosphate buffer" mixtures. b) Spatially resolved profiles of TB-loaded *in situ* forming implants.

Figure 4b shows the experimentally determined hyperfine splitting of TB in *in situ* forming implants prepared with formulations based on PLGA (25 %), ATBC (10 %) and optionally HPMC (20 %). The set-up schematically illustrated in Figure 1a was used, the spatial position is plotted on the x-axis: 3.1 mm corresponds to the interface "formulation – release medium", 0 mm corresponds to the bottom of the cylindrical holder. The measurements were made before exposure to the phosphate buffer (t = 0), as well as after 1, 4 and 8 h exposure. As it can be seen, the $2a_N$ values were about position-independent and very similar at t = 0 for HPMC-free and HPMC-containing systems: around 32.3 G. This is consistent with the results shown in Figure 4a, indicating 0 % phosphate buffer for this $2a_N$ value. Importantly, after 1 h, clear spatial hyperfine splitting gradients were visible in both types of formulations: The $2a_N$ values decreased from about 33.3 to 32.7 G from the interface "formulation – release medium" to the bottom of the cylindrical holder. This indicates that water started to penetrate into the formulations and that surface near regions were much more hydrated than regions far from the surface at this time point. Interestingly, there was still no major difference between HPMC-containing and HPMC-free systems. At 4 h exposure time, much higher water contents were observed than after 1 h, and there were still spatial gradients visible between the surface and the bottom of the system, although less steep. Importantly, HPMC-containing implants showed higher water contents than HPMC-free implants at this time point (filled versus open triangles), indicating that the presence of HPMC facilitates the penetration of water into the devices. At 8 h, the water content in HPMC-containing implants is about homogeneous throughout the device (filled diamonds) and, again, higher than the water content in HPMC-free implants. The latter still exhibited a spatial concentration gradient (open diamonds). This further confirms that the presence of the hydrophilic polymer HPMC facilitates the penetration of water into the *in situ* forming implants, especially after a couple of hours, when the systems become more and more hydrophobic due to PLGA precipitation.

Figure 5a shows the ^{1}H NMR spectra of a liquid *in situ* forming implant formulation containing PLGA (25 %), ATBC (10 %), NMP, minocycline HCl (2 %), ascorbic acid (0.01 %), and HPMC (20 %) before exposure to the release medium (t = 0). Figure 5b shows the same system, but after 3 d exposure to phosphate buffer pH 7.4. Table 1 lists the peak assignments for these spectra (obtained with reference spectra of the different pure compounds). Clearly, upon exposure to the phosphate buffer, the peak

intensity of NMP decreased, whereas the peak intensity of water increased (Figure 5b versus 5a).

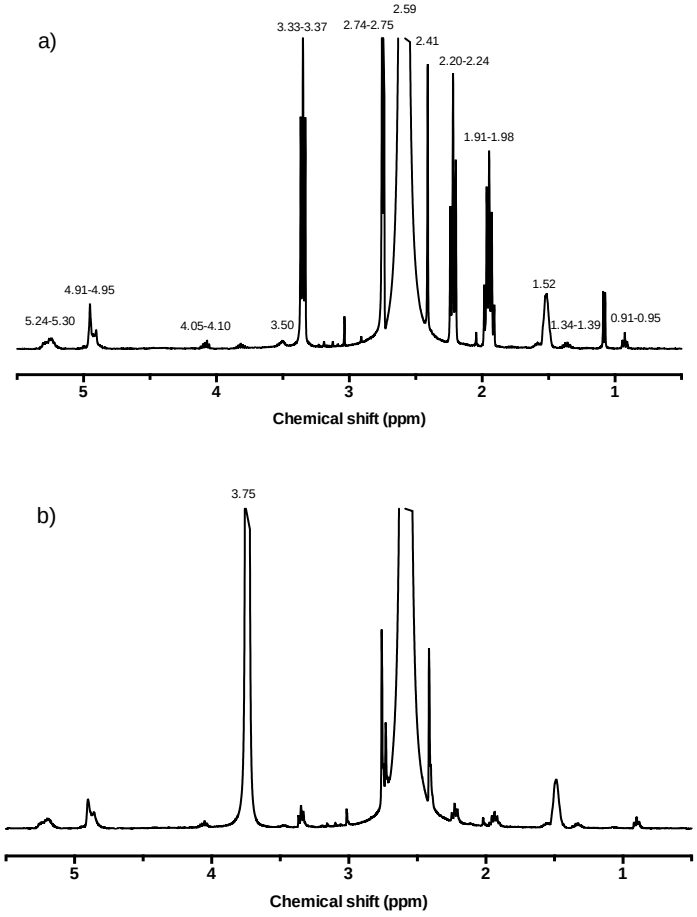

Figure 5. ^{1}H NMR spectrum of: a) a liquid *in situ* forming implant formulation containing PLGA (25 %), ATBC (10 %), NMP, minocycline HCl (2 %), ascorbic acid (0.01 %), and HPMC (20 %); (b) the system shown in a), but after 3 d exposure to phosphate buffered saline pH 7.4 (agitated vial set-up).

Chemical shift (ppm)	Assignment
0.91 - 0.95, 1.34 - 1.39, 4.05 - 4.10	ATBC
1.52, 4.91 - 4.95, 5.24 - 5.30	PLGA
1.91 - 1.98, 2.20 - 2.24, 3.33 - 3.37	NMP
2.41, 2.59, 2.74 - 2.75	DMSO
3.50	HPMC
3.75	H_20

Table 1. Assignments of the peaks observed in the ^{1}H NMR spectra of the *in situ* forming implant formulations shown in Figure 5 (the implants were dissolved in DMSO to obtain the NMR spectra).

Figure 6 shows the dynamic changes in these peak intensities for HPMC-free and HPMC-containing systems as a function of the exposure time to the release medium. Clearly, the peak intensity of NMP (and, thus, the amount of NMP remaining within the *in situ* forming implant) decreases much more rapidly in HPMC-containing devices compared to HPMC-free formulations (black versus white bars in Figure 6a). At the same time, the water penetration rate into the system is initially increased in the presence of HPMC, as it can be seen in Figure 6b at 1 d. This is consistent with the above described EPR measurements (e.g., Figure 4) and confirms that the presence of this hydrophilic polymer facilitates water uptake into the formulation. However, at much later time points (e.g., 3 d), the water content of HPMC-containing devices is lower than the water content of HPMC-free systems (Figure 6b). This might be explained by the fact that HPMC is well known to be able to form hydrogels, which can limit mass transport [31] and is in good agreement with the observed dynamic changes in implants' mass (Figure 2).

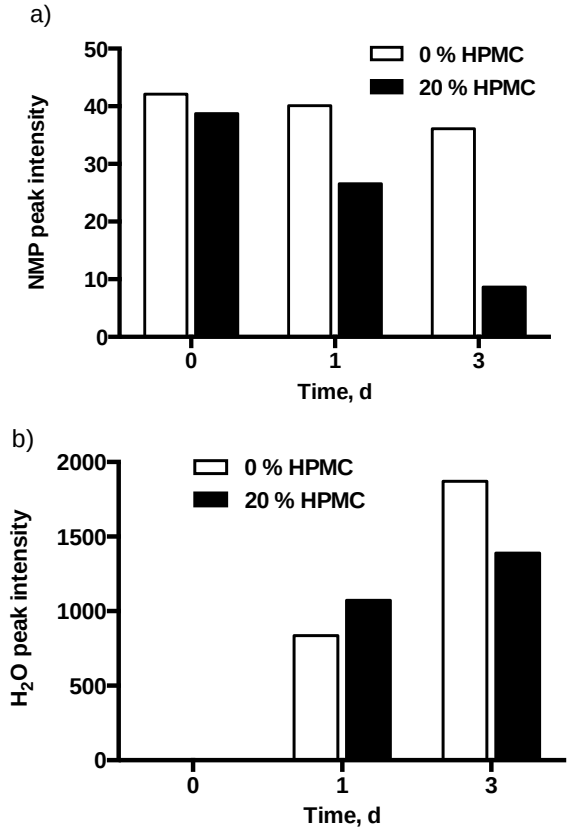

Figure 6. Dynamic changes in the peak intensity of: a) NMP and b) water observed by ^{1}H NMR spectroscopy in *in situ* forming implant formulations based on PLGA (25 %), ATBC (10 %), NMP and optional HPMC (20 %) upon exposure to phosphate buffer.

Thus, the EPR and ^{1}H NMR measurements clearly showed that the addition of HPMC significantly affected the solvent exchange kinetics in *in situ* forming implants: The leaching of NMP out of the formulations is facilitated, whereas the penetration of water into the devices is accelerated at early time points (leading to faster polymer precipitation), but slowed down at late time points. Furthermore, the building up and disappearance of water concentration gradients within the systems could be evidenced.

3.3. Optical microscopy

Figure 7 shows optical microscopy pictures of cross-sections of *in situ* formed implants after different exposure times to the release medium (as indicated). The systems were based on PLGA, ATBC, minocycline HCl and optionally contained HPMC. Note that the structures on the left hand side collapsed during sample preparation. Since the implants were prepared using the agitated vial set-up, they all have a cone-like shape (the geometry of the bottom of an Eppendorf vial). Clearly, the presence of HPMC within the formulation led to a rapid PLGA precipitation throughout the system: an about homogeneous, highly porous inner implant structure is visible on the right hand side of Figure 7. In contrast, highly heterogeneous inner structures were visible in HPMC-free implants (Figure 7, left hand side): A more dense outer shell can be distinguished from a highly porous, not even yet completely solidified inner core at early time points. These very marked differences in the implants' morphology are in good agreement with the above discussed water penetration kinetics into the systems (e.g., Figure 4b) as well as NMP diffusion kinetics out of the systems (e.g., Figure 6a), and can (at least partially) explain the observed drug release kinetics (Figure 3): In the presence of the hydrophilic polymer HPMC, water penetration into and NMP transport out of the formulation is facilitated, leading to rapid PLGA precipitation throughout the device and a highly porous system structure, resulting in high drug mobility within the implant and, thus, increased release rates. In contrast, in the absence of HPMC, the system is less hydrophilic, water penetration into the formulation and NMP transport out of the system is slowed down, leading to the formation of a denser outer system shell and a more slowly solidifying inner core. Drug transport through the denser outer PLGA shell is effectively hindered, leading to reduced drug release rates.

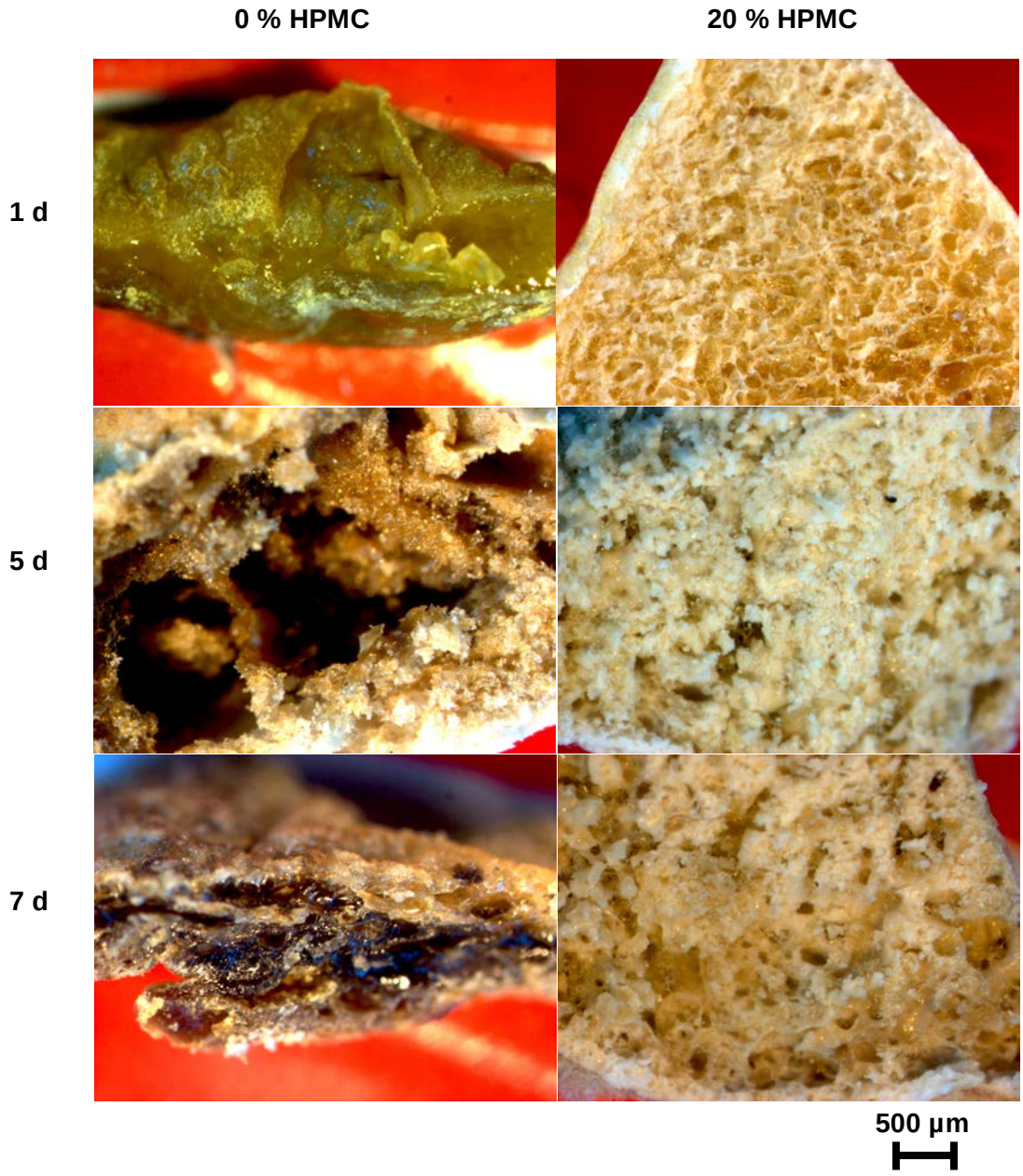

Figure 7. Optical microscopy pictures of cross-sections of *in situ* formed implants (25 % PLGA, 10 % ATBC, 2 % minocycline HCl) optionally containing 20 % HPMC after 1, 5 and 7 d exposure to phosphate buffer (agitated vial set-up).

However, care should be taken when drawing conclusions from *in vitro* experiments, especially in the case of PLGA-based systems and *in situ* forming devices, since the surrounding environment might potentially significantly affect the polymer precipitation kinetics and the overall mass transport processes [32–34]. For these reasons, the same liquid formulations were also used to prepare *in situ* forming implants in a flow-

through cell set-up [27]. Briefly, the liquids were filled into empty HPLC columns, through which the release medium was pumped at 44 µL/h. The idea was to better simulate the continuous gingival fluid flow in patients' periodontal pockets. As it can be seen in Figure 8a, the type of release set-up tremendously affected the resulting drug release kinetics: (1) In HPMC-free systems, drug release was much faster in flow-through cells than in agitated vials, whereas (2) in HPMC-containing systems drug release was very similar for both types of release set-ups. (3) The addition of HPMC significantly accelerated drug release in agitated vials and had only a moderate effect in flow-through cells. To better understand these phenomena, also the PLGA degradation kinetics in these systems as well as the latter's morphology were monitored (Figures 8b and 9).

As it can be seen in Figure 8b, PLGA degradation was much faster when using the agitated vial set-up compared to the flow-through cell set-up. Also, the addition of HPMC led to a slight slowing down of polymer degradation, irrespective of the experimental conditions. Comparing Figures 9 and 7, it becomes visible that the shape of the implants was very much dependent on the experimental set-up: the implants were "cone"-shaped in the case of agitated vials (please note the collapse in HPMC-free systems during sample preparation), and more "film"-like in the case of flow-through cells. This difference is due to the difference in the surrounding geometry during implant formation (cone-shaped bottom of an Eppendorf vial versus inner cylinder of an HPLC column, through which the release medium flows). Obviously, this difference in device geometry fundamentally affects the resulting polymer precipitation kinetics, PLGA degradation kinetics and drug release rates. In the case of flow-through cells, the film-like geometry leads to a high surface area in contact with the release medium and rapid polymer precipitation through the implants in all cases (HPMC-free and HPMC-containing systems), because the distances to be overcome by the water and NMP are short. This leads to a relatively similar, highly porous inner implant structure, irrespective of the presence of HPMC. The short pathways to be overcome and high system porosity lead to high and similar drug release rates (Figure 8a, top curves). These observations are also in good agreement with the relatively slow PLGA degradation rates observed in flow-through cells (Figure 8b), since the creation of acidic microclimates within the implants is unlikely. In contrast, in the case of agitated vials, the diffusion pathways to be overcome by acids generated upon PLGA degradation are much longer within cone-shaped implants. This renders the creation

of acidic microclimates much more likely, resulting in more pronounced autocatalytic effects [35–39] and, thus, accelerated polymer degradation, as it can be seen in Figure 8b. The longer diffusion pathways and the formation of the above discussed denser outer implant shells in the case of HPMC-free formulations are responsible for the observed slow release from these systems in agitated vials (Figure 8a).

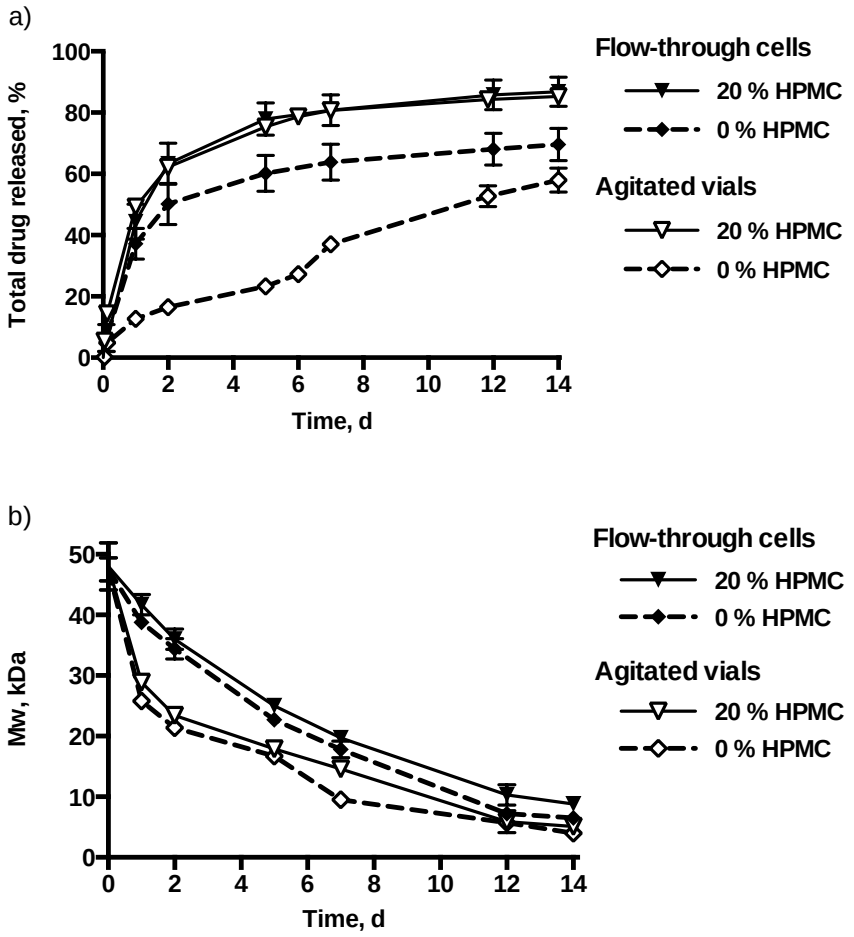

Figure 8. Impact of the experimental set-up on: a) drug release, and b) PLGA degradation from/of *in situ* forming implants (25 % PLGA, 10 % ATBC, 2 % minocycline HCl), optionally containing 20 % HPMC.

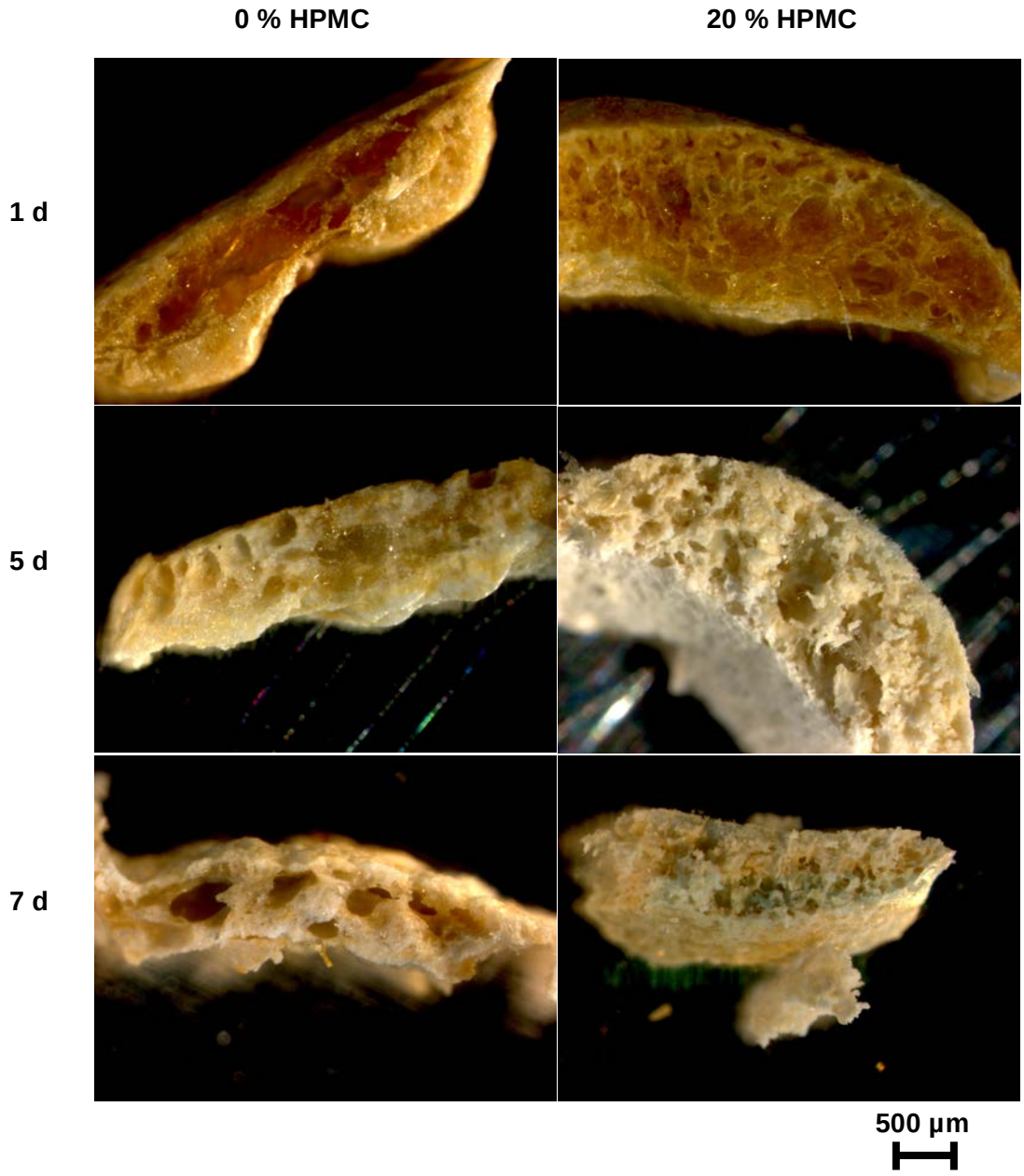

Figure 9. Optical microscopy pictures of cross-sections of *in situ* formed implants (25 % PLGA, 10 % ATBC, 2 % minocycline HCl, optionally containing 20 % HPMC) after 1, 5 and 7 d exposure to the release medium (flow-through cell set-up).

3.4. Antimicrobial activity

First, the susceptibility of the bacteria present in the periodontal pockets of patients suffering from periodontitis against minocycline was studied. For this purpose, gingival crevicular fluid (GCF) samples were incubated in the presence or absence of minocycline on Columbia agar enriched with defibrinated horse blood in Petri dishes. After 5 d, the number of bacterial colonies was counted. Table 2 shows the mean log CFU (Colony Forming Units) per gram GCF, observed with 14 samples from deep periodontal pockets of 13 patients. As it can be seen, there was no obvious relation between the pockets' depth and the CFU/g value, indicating the diversity of the bacterial levels in the different subjects. No colonies formed upon incubation of GCF samples on minocycline-loaded agar in 12 samples. Two samples showed growth of 1 colony, identified as *Candida pelliculosa* and *Candida albicans*, which are fungi. Thus, the calculated mean log CFU/g values for bacteria were ≤ 3.42 (our detection level) for all samples. On minocycline-free agar, much higher log CFU/g values were found, ranging from 5.37 to 10.07. The differences of mean log CFU/g found between the two types of agar varied from 1.94 to 6.64. It is assumed that there was less than 1 bacterium over 100 to 10,000,000 that may be resistant to minocycline. These results indicate that minocycline is suitable for the antibiotherapy of periodontitis.

In addition to these studies on bacterial cocktails, also the Minimum Inhibitory Concentration (MIC) of minocycline against ten *isolated bacterial strains* from periodontal pockets of patients suffering from periodontitis was determined. As shown in Table 3, 8 of the investigated 10 strains were susceptible to minocycline according to the *Société Française de Microbiologie* (SFM) requirements; and all strains were susceptible at the minocycline concentration used in the *in situ* forming implant formulations (2 %).

Table 2. Susceptibility of periodontal pathogenic bacteria to minocycline (n.m. = not measured).

Sample	Pocket depth (mm)	Mean log CFU/g of GCF on non selective agar	Mean log CFU/g of GCF on minocycline agar
D86	n.m.	6.88	<3.42
D87	n.m.	8.08	<3.42
D88	7	7.10	<3.42
D89	9	8.21	<3.42
D90	9	6.63	<3.42
D91	6	5.37	<3.42
D92	9	7.70	<3.42
D93	6	7.84	<3.42
D94	7	8.04	3.42
D95	7	8.68	<3.42
D96	6	8.15	<3.42
D97	8	7.72	<3.42
D98	5	10.07	<3.42
D99	6	7.97	3.42

Table 3. Minimum Inhibitory Concentration (MIC) of minocycline against specific periodontal strains.

Bacterial strain	Reference	MIC (mg/L)
Streptococcus vestibularis	D28A1	0.1
Veillonella sp.	D18B13	0.1
Fusobacterium nucleatum	JD7	0.1
Veillonella sp.	D36A19	0.2
Streptococcus mitis	D29A5	32
Streptococcus sanguinis	D28A11	0.2
Streptococcus australis	D37A12	0.1
Streptococcus salivarius	D28A9	0.1
Streptococcus cristatus	D18A2	0.1
Streptococcus sanguis	D30A3	16

Furthermore, the activity of the *in situ* forming implants against the bacteria cocktails present in the patients' periodontal pockets were studied. Figure 10 shows the inhibition zone diameters observed upon incubation of GCF samples in Columbia agar enriched with horse blood in Petri dishes. Cylindrical holes were made at the center of the agar and filled with 30 μL of liquid formulation. Upon solvent exchange, the implants formed *in situ*, and the Petri dishes were incubated for 5 d under anaerobic conditions. The biological samples were diluted either 100 times, or 1000 times, as indicated. Drug-free formulations served as negative controls. For reasons of comparison, also the activity of the commercial product Parocline® was studied with certain GCF samples. Clearly, all drug-loaded *in situ* forming implants could effectively inhibit the growth of the various bacteria present in the patients' pockets (the inhibition zone diameter varied from 1.7 to 5.3 cm). The negative controls showed negligible or only very minor growth inhibition, with 1 exception – the "D91" sample. In this case, the D91-2 and D91-3 Petri dishes recorded inhibition zone diameters of 1 and 2.9 cm, respectively. This can probably be attributed to the exceptionally low CFU concentrations present in these specific cases (9 and 88 CFU/g, respectively), leading to non-representative bacterial amounts in 0.1 mL inoculated solution. Otherwise, the bacterial loads were much higher. For instance, 1 mL of 1000 times diluted "D90" sample contained about 16,000 CFU and 1 mL of 100 times diluted "D90" sample about 160,000 CFU. As expected, the inhibition zone diameters were generally higher in the case of the 1000 fold dilutions compared to the 100 fold dilutions (since the bacterial load was 10-fold smaller). Importantly, the antibacterial efficacy of the investigated formulations forming implants *in situ* was comparable to the activity of the commercial product Parocline®.

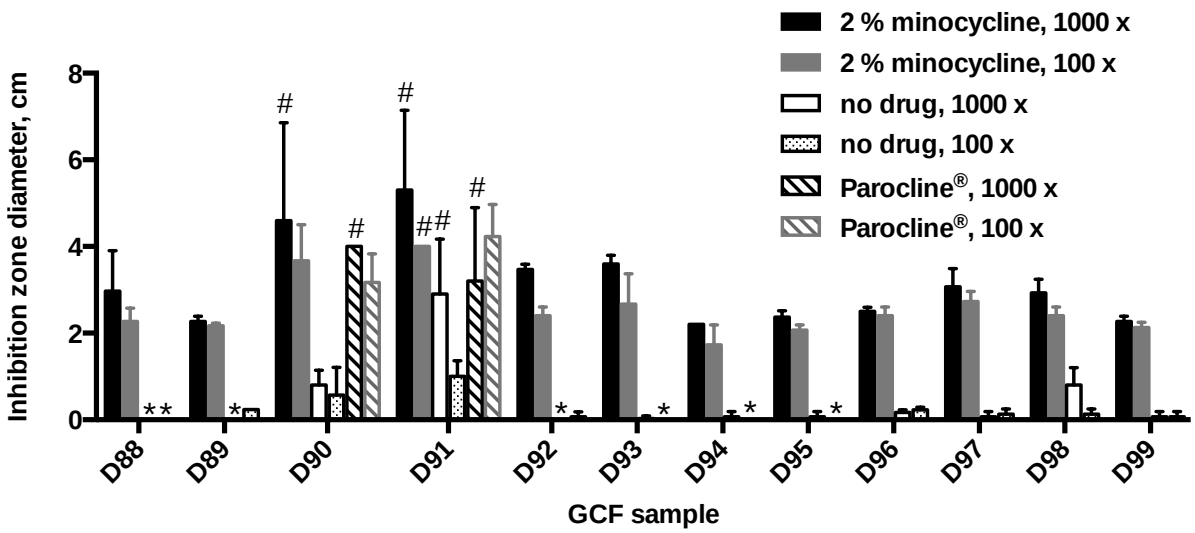

Figure 10. Antimicrobial activity of *in situ* forming implants (25 % PLGA, 10 % ATBC, 20 % HPMC, 2 % minocycline HCl) against bacterial cocktails present in the periodontal pockets of periodontitis patients: Inhibition zone diameters measured upon incubation of GCF samples in agar ("#" indicates n = 2 instead of 3; "*" indicates that no inhibition zone was detected; "100 x" indicates a sample dilution by a factor of 100; "1000 x" indicates a sample dilution by a factor of 1000). For reasons of comparison, Parocline® was also tested with certain samples.

The observed variation in the inhibition zone diameters can at least partially be attributed to the variability of the composition of the bacterial cocktails present in different GCF samples (with respect to quality and quantity). Indeed, the microflora of periodontal pockets is known to be diversified, depending for instance on the severity of the disease and on the individual subject. To get a better understanding of which specific bacterial strains might be of importance, the dominant colonies of all samples after incubation were isolated and identified. The antibacterial efficacy of the new *in situ* forming implants was also tested against these isolated bacteria strains. Out of the total 23 isolated microorganisms, there were 21 anaerobes comprising 3 gram positive, 6 gram negative obligate anaerobes and 12 gram positive facultative anaerobes. Only 2 aerobes were found, including 1 gram negative bacterium and 1 fungus. These results are in good agreement with reports in the literature, indicating a dominance of anaerobic bacteria in periodontal pockets [8,40–42]. These

microorganisms included both, “initial colonizers” (*Streptococcus spp*., *Actinomyces spp*., *Veillonella spp*.) as well as strains frequently found in more mature biofilms (*Fusobacterium spp*., *Prevotella spp*.) [42]. The microbial “orange complex” associated with periodontitis was also found, including black-pigmented *Prevotella* sp. and *Fusobacterium nucleatum* [8]. This is a good indication for the fact that the bacteria isolated in this study are likely to be representative for the microorganisms, which can be found in the periodontal pockets of patients suffering from periodontitis. As it can be seen in Figure 11, the investigated *in situ* forming implants were effectively inhibiting the growth of the various isolated bacteria strains, whereas the negative controls (formulations free of drug) showed no, or only very minor inhibitory effects, with 1 exception: *Candida pelliculosa*, a fungus that can be found in the mouth. But this microorganism is not specifically associated with periodontitis and is not susceptible to minocycline. Importantly, the antimicrobial efficacy of the *in situ* forming implants was comparable to that of the commercial product Parocline® with the investigated strains.

In addition, it has to be pointed out that the inhibition zone diameters observed with the commensal oral bacteria were generally smaller than those observed with pathogenic bacteria: The average inhibition zone diameters obtained with 7 *Streptococcus* species (commensal flora) was 2.0 cm, while those obtained with *Prevotella* sp. and *Fusobacterium nucleatum* (pathogenic strains) were 4.8 cm and 5.2 cm, respectively. This is a very interesting difference, since it indicates that minocycline is likely to rapidly destroy pathogenic bacteria, but not as quickly inhibit commensals. The commensal flora will, thus, have a chance to continue colonization and subsequently re-establish the natural microbiological balance, as in healthy subjects.

Furthermore, the impact of the amount of added HPMC on the antibacterial activity of the *in situ* forming implants was studied, using 4 selected periodontal strains. Parocline® as well as drug-free and HPMC-free systems were tested for reasons of comparison. As it can be seen in Figure 12, all drug-containing systems effectively inhibited the growth of all 4 bacterial strains, irrespective of their HPMC content. This is of importance, since HPMC addition can significantly improve key features of the implants, such as their adhesion forces,

but does not impact their antimicrobial activity to a significant extent. Also, the activity of the *in situ* forming implants was similar to the antibacterial activity of Parocline®.

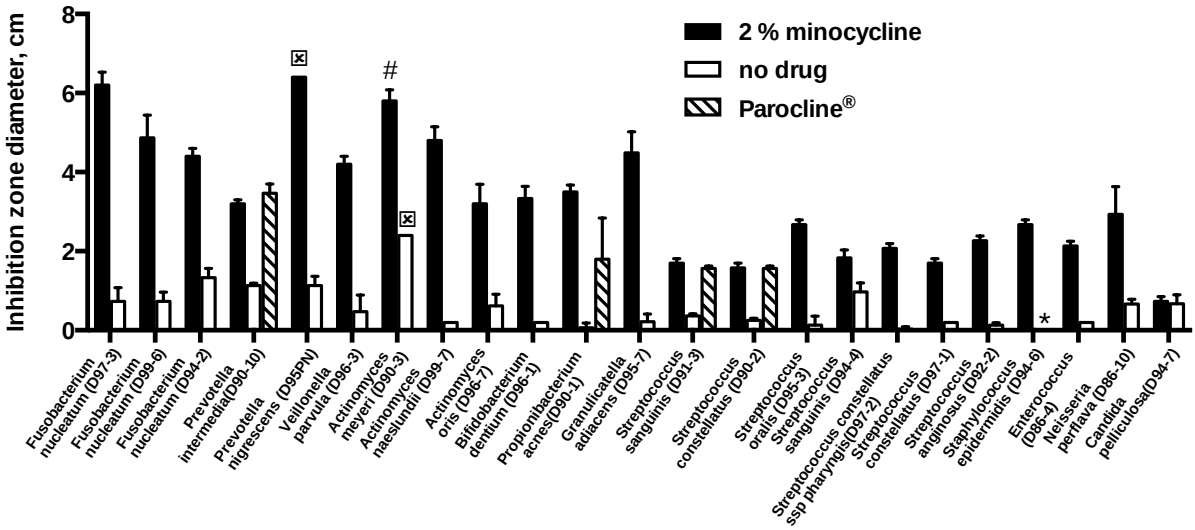

Figure 11. Antimicrobial activity of *in situ* forming implants (25 % PLGA, 10 % ATBC, 20 % HPMC, 2 % minocycline HCl) against *isolated bacteria strains* obtained from GCF samples from periodontitis patients: Inhibition zone diameters measured upon incubation in agar ("#" indicates n = 2 instead of 3; "☒" indicates n = 1 instead of 3; "*" indicates that no inhibition zone was detected). For reasons of comparison, Parocline® was also tested with certain bacteria strains.

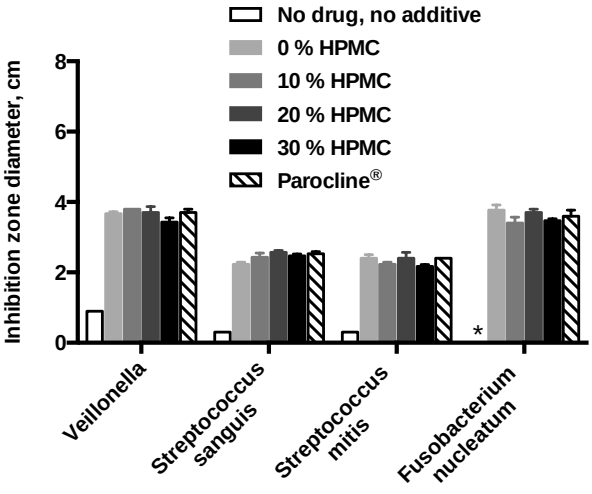

Figure 12. Effects of the addition of different amounts of HPMC to the *in situ* forming implant formulations on the antimicrobial activity of the systems (25 % PLGA, 10 % ATBC, 2 % minocycline HCl). For reasons of comparison, also Parocline® and drug-free and HPMC-free systems were studied ("*" indicates that no inhibition zone was detected).

4. Conclusion

The proposed *in situ* forming implant formulations exhibit a promising potential for improved periodontitis treatment. The addition of HPMC increases the systems' adhesive forces and limits system swelling, while the antibacterial activity remains about unaltered. The novel insight obtained by EPR and NMR measurements allows for a better understanding of the underlying mass transport mechanisms in these rather complex systems and, thus, facilitated device optimization in the future, including other applications (e.g. for different drugs or drug combinations). Interestingly, the implants more strongly inhibited the growth of pathogenic bacterial strains isolated from the periodontal pockets of patients suffering from periodontitis compared to bacteria encountered in healthy subjects. *In vivo*, such a preferential action against the pathogenic strains can be expected to give a chance to the healthy flora to re-colonize the periodontal pockets.

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CHAPTER III

IN SITU FORMING COMPOSITE IMPLANTS FOR PERIODONTITIS TREATMENT: HOW THE COMPOSITION DETERMINES SYSTEM PERFORMANCE

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Abstract

Periodontitis is the primary cause of tooth loss in adults and a very widespread disease: 47 % of the US adults have mild, moderate or severe periodontitis, and 64 % of the population older than 65 years is affected. The treatment of periodontitis is highly challenging, because drug partitioning into the periodontal pockets is not very pronounced and gingival crevicular fluid flow rapidly eliminates drugs from their site of action. Thus, using conventional administration routes high systemic drug levels are required, while the drug concentration at the target site remains low, resulting in potentially severe side effects and low or negligible treatment efficacy. Biodegradable *in situ* forming implants offer an interesting potential to overcome these hurdles: These are liquid formulations, which upon injection into the periodontal pockets form solid implants. The latter subsequently controls drug release at the site of action during pre-programmed periods of time. However, currently available systems suffer from poor adherence to the human tissue, resulting in pre-mature and uncontrolled expulsion of implant fragments from the periodontal pockets during the treatment period. This leads to unreliable drug exposure to the patient. *Composite* implants based on a *drug release rate controlling* polymer and an *adhesive* polymer can overcome this limitation. However, the processes involved in implant formation and the control of drug release are complex and the relationships between the systems' composition and the implants' performance are yet unclear. This study applies advanced characterization techniques, such as EPR analysis, to better understand *in situ* forming implants based on two different types of poly(lactic-co-glycolic acid) (PLGA), hydroxypropyl methylcellulose (HPMC) and doxycycline or metronidazole. Interestingly, HPMC addition to shorter chain PLGA slightly decreased drug release, whereas in the case of longer chain PLGA the release rate substantially increased. These tendencies could be explained based on the mass transport kinetics during implant formation and the systems' inner structures. Furthermore, the implants' antimicrobial activity against microorganisms present in the periodontal pockets of patients suffering from periodontitis is evaluated. Interestingly, the systems more effectively hinder the growth of pathogenic bacteria than of physiological microorganisms. Thus, a re-colonization of the patients' pockets with healthy flora can be expected to be favored *in vivo*.

Keywords: *in situ* forming implant; periodontitis; PLGA; EPR; doxycycline

1. Introduction

Periodontitis is a highly prevalent, chronic inflammatory disease of the periodontium. A recent survey estimates that 47 % of the US adults have mild, moderate or severe periodontitis [1]. Prevalence rates increase to 64 % for adults older than 65 years. Periodontitis is characterized by a progressive loss of the alveolar bone and periodontal ligament, leading to the formation of periodontal pockets [2–4]. If untreated, periodontitis can lead to the loosening and subsequent loss of the teeth. It is indeed the primary cause of tooth loss in adults [5]. The initiating factors of periodontitis are likely to be pathogenic bacteria and bacterial products, which form a biofilm covering the teeth' surface in the subgingival area. It seems that the microflora in the disease state is different from that in healthy subjects. For example, the number of gram negative anaerobic bacteria is likely to be increased and certain clinical forms of periodontitis might be associated with specific microbiota [6]. Recently, Silva-Boghossian et al. [2] reported that *Streptococcus* strains (such as *Streptococcus sanguinis*) are also associated with suppuration in periodontitis subjects. However, up to date, the exact mechanisms underlying this disease are not yet fully understood. It is hypothesized that: (i) the suspected periodontal pathogens produce biologically active molecules, which directly attack the host tissue, and/or that (ii) the immune response of the host organism (human body) to these pathogens results in the tissue destruction. The consequence of the tissue loss is the deepening of the periodontal pockets, and -once the mechanical anchorage of the tooth becomes insufficient -the latter is lost.

The treatment of periodontitis is highly challenging, since drug partitioning into the periodontal pockets is generally not very pronounced and gingival crevicular fluid flow rapidly eliminates drugs from the site of action [7]. For example, it has been estimated that the contents in a 5 mm periodontal pocket is renewed 40 times per hour [8]. Thus, using conventional administration routes (such as oral, intravenous, intramuscular, subcutaneous etc.) often high systemic drug levels are required, while the drug concentration at the target site remains low. This leads to potentially severe side effects and limited or insufficient therapeutic efficacy, despite the availability of highly potent drugs able to act against the pathogenic flora and inflammation. Importantly, the crucial hurdles of limited accessibility of the site of action and rapid elimination can be overcome using advanced local drug

delivery systems, releasing the drug in a time-controlled manner in the periodontal pockets during prolonged periods of time [9–16]. Biodegradable *in situ* forming implants are particularly promising for this purpose [17]. These are liquid formulations, which upon injection into the periodontal pockets form solid implants. The implant formation can be induced by different mechanisms [12,18–21], for example solvent exchange: Briefly, the basic idea is to dissolve the drug and a biocompatible and biodegradable matrix former [e.g., poly(lactic-co-glycolic acid), PLGA] in an appropriate organic solvent [e.g., N-methyl pyrrolidone, NMP] [22–26]. This liquid phase can easily be injected into the periodontal cavities. Once injected, the NMP diffuses into the surrounding environment and water from the periodontal pocket penetrates into the liquid formulations. Since PLGA is not soluble in water, it subsequently precipitates and entraps the drug. Major advantages of this type of biodegradable, *in situ* forming implants include the fact that: (i) The injection of a liquid formulation is relatively easy (compared to the implantation of "pre-formed" implants). (ii) There is no need to remove empty remnants upon drug exhaust, due to complete biodegradability of the system. (iii) The geometry and size of the implants are adapted to the patient's periodontal pockets (customized systems, personalized medicine). (iv) The incorporated drug is locally released in a time-controlled manner through the slowly degrading polymer network.

However, up to date major challenges remain to be addressed, namely the fact that: (i) The adherence of *in situ* formed implants to human tissue is yet poor, resulting in premature and uncontrolled expulsion of implant fragments from the periodontal pockets due to the non-negligible flow of gingival crevicular fluid [8,27]. This leads to a considerable uncertainty with respect to the amount of drug reaching the target site and the time periods during which therapeutic drug levels are provided. (ii) The elasticity/plasticity of the formed implants is generally not adapted to this type of local administration: Systems, which are difficult to deform plastically are not able to adapt their geometry to dynamic changes in the periodontal pocket' size and shape with time. In contrast, fully elastic implants force the periodontal pockets to keep their geometry and dimensions, which is also not desirable. Recently, the addition of plasticizers, such as acetyltributyl citrate and dibutyl sebacate, as well as a second type of polymer, such as hydroxypropyl methylcellulose, has been proposed to increase the adhesiveness of the implants [28,29]. However, yet it is unclear how the systems' composition affects the key properties of the

in situ formed implants. The aim of this study was to better understand the physico-chemical phenomena involved in implant formation and the control of drug release as well as to evaluate the antimicrobial activity of doxycycline-loaded formulations.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA, Resomer® RG 502 H and 504 H; Evonik, Darmstadt, Germany); acetyltributyl citrate (ATBC; Morflex, Greensboro, NC, USA); hydroxypropyl methylcellulose (HPMC, Methocel® E5 and E50; Colorcon, Dartford, UK); N-methyl pyrrolidone (NMP, 99 %), glucose and cysteine hydrochloride (Acros organics, Geel, Belgium); doxycycline hyclate and metronidazole (Fagron, Colombes, France); 4-hydroxy-tempo benzoate (TB; Sigma-Aldrich, Seelze, Germany); agarose (GenAgarose® LE; Genaxxon BioScience, Ulm, Germany); Columbia agar base and agar (Oxoid, Basingstoke, UK); defibrinated horse blood (E&O Laboratories, Burnhouse, UK); Parocline® (2 % minocycline; Sunstar France, Levallois-Perret, France).

2.2. Preparation of the liquid formulations

PLGA (28 or 32 % w/w, based on the total liquid formulation without drug) was dissolved in NMP at 25 °C in a glass vial (30 min stirring). Optionally, the plasticizer ATBC (10 % w/w, based on the PLGA mass) and/or HPMC (10, 20, 25 or 30 % w/w, based on the PLGA mass) was/were added and the mixture was vortexed for 3 min, followed by standing for 3 h at 25 °C. Subsequently, 5 or 10 % doxycycline hyclate or 1 or 10 % metronidazole (w/w, based on the total liquid formulation without drug) was added, and the mixture was vortexed for 3 min, followed by standing for 3 h at 25 °C. To eliminate air bubbles, the liquids were ultrasonicated for 10 min. The formulations were stored at -20 °C and protected from light to avoid drug degradation.

2.3. In situ implant formation and drug release measurements

One hundred microliters of the respective formulation was injected at the bottom of an Eppendorf vial using a standard syringe. One and a half milliliters preheated (37 °C), degassed phosphate buffer pH 7.4 (USP 35) was carefully added using a pipette, initiating solvent exchange and implant formation. The vials were horizontally shaken at 37 °C at

80 rpm (GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, the bulk fluid was completely withdrawn and replaced with fresh medium. The drug content in the samples was determined UV-spectrophotometrically (λ = 325 nm for doxycycline and λ = 351 nm for metronidazole; UV-1650PC, Shimadzu, Champs-sur-Marne, France). All tests were performed in triplicate and the results were shown as mean values ± standard deviation.

2.4. Monitoring of dynamic changes in the implants' mass

Implants were prepared and treated as described in *Section 2.3. In situ implant formation and drug release measurements*. At pre-determined time points, implants were weighed [*mass (t)*]. The *mass change in percent* was calculated as follows:

$$mass\ change\ (\%)\ (t) = 100 * [mass\ (t) - mass\ (t=0)] / mass\ (t=0)$$

where *mass (t=0)* is the initial weight of the formulation used for implant preparation.

2.5. Electron paramagnetic resonance (EPR) measurements

In situ forming liquid implant formulations were prepared as described in section *2.2. Preparation of the liquid formulations*. The spin probe 4-hydroxy-tempo benzoate (TB) was dissolved in these liquids (1 mM). Two hundred µL of the formulations were placed into cylindrical holders, which were immerged into 3 mL phosphate buffer pH 7.4 (USP 35). As illustrated in Figure 1a, only the top circular surface of the cylindrical holder was open, the other surfaces were impermeable. The system was kept constant at 37 °C and horizontally shaken at 30 rpm (GFL 1083; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, samples (implants with holders) were withdrawn and analysed using an EPR L-band spectrometer (MagnetTech, Berlin, Germany), operating at a low microwave frequency (1 GHz). To create EPR *images*, twenty-five scans were accumulated using the following parameters: field centre = 48.9 mT; scan range = 8 mT; scan time = 40 s. For the measurement of EPR *spectra*, a scan range of 10 mT and scan time of 100 s were applied. The typical EPR parameters were calculated from the recorded EPR spectra (first derivative) or integrated first derivative (absorption). Figures 1b shows an EPR spectrum of TB dissolved in NMP and Figure 1c shows examples for data recorded with an implant prepared from a liquid formulation

containing 32 % PLGA RG 502 H, 10 % ATBC, 20 % HPMC and 1 mM TB before and after exposure to phosphate buffer pH 7.4.

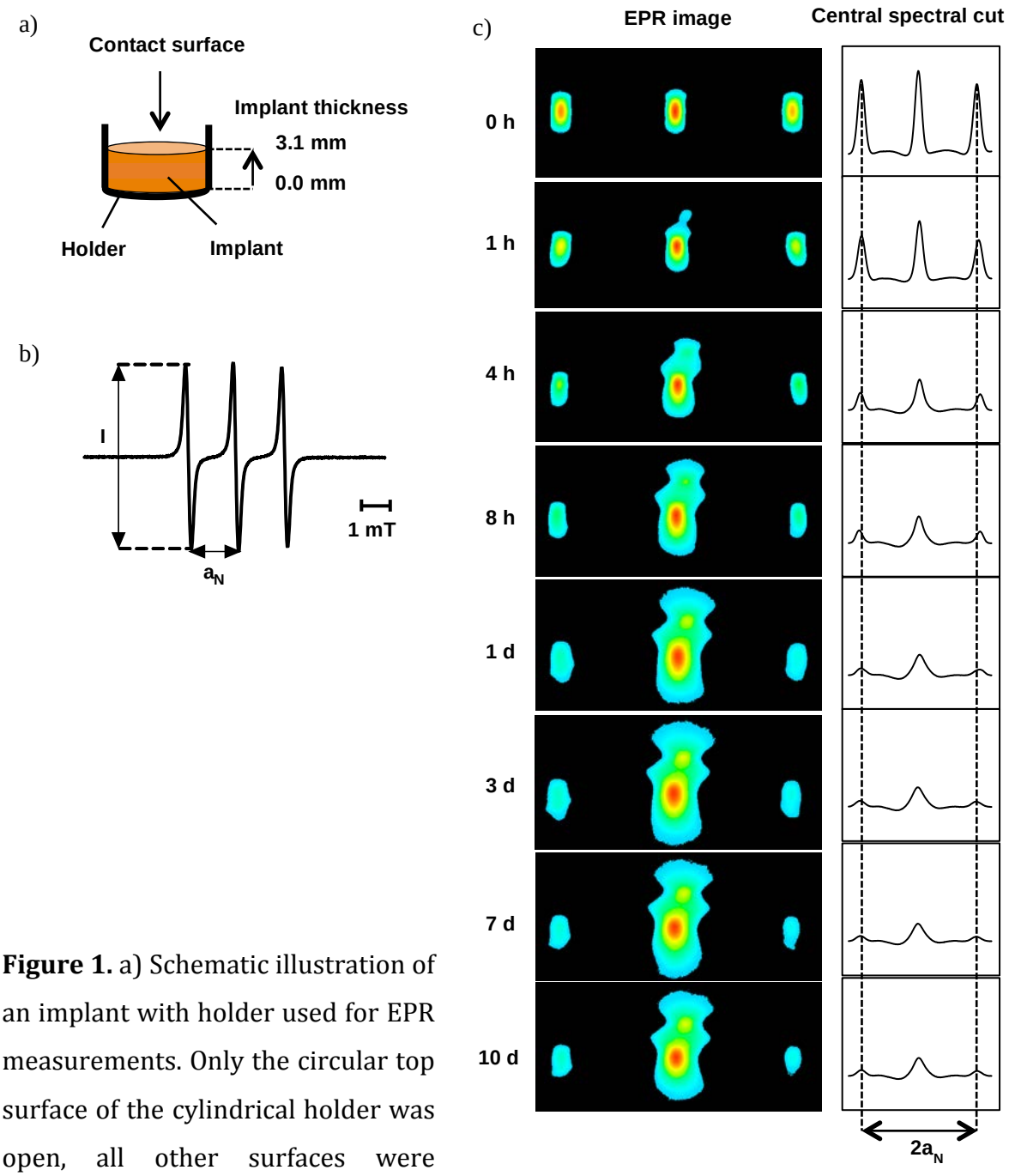


Figure 1. a) Schematic illustration of an implant with holder used for EPR measurements. Only the circular top surface of the cylindrical holder was open, all other surfaces were impermeable. b) EPR spectrum of TB dissolved in NMP (I = signal amplitude, a_N = hyperfine splitting constant). c) EPR images and central spectral cuts of an implant (32 % PLGA RG 502 H, 10 % ATBC, 20 % HPMC, 1 mM TB) recorded before and after exposure to phosphate buffer pH 7.4 for different time periods (as indicated).

2.6. Mechanical and adhesive properties

The mechanical and adhesive properties of the investigated *in situ* forming implants were determined with a texture analyzer (TA.XT.Plus; Stable Micro Systems, Surrey, UK) using the experimental set-up, which has previously been described in detail [28]. Briefly, agarose was dissolved in boiling water (0.6 % w/v), and the solutions were cast into Petri dishes (diameter = 9 cm). Upon cooling to room temperature, gels formed. At the center of the gels, cylindrical holes (diameter = 6 mm) were made and filled with 100 µL liquid formulation using a standard syringe and a drop of distilled water. Upon solvent exchange, the implants formed. At pre-determined time points, a spherical probe (diameter = 5 mm) was driven downwards (at a speed of 0.5 mm/s). Once in contact with the implant, the applied force and displacement of the probe were recorded as a function of time. When the penetration depth was 1.5 mm, this position was held for 60 s. Then, the probe was driven upwards at a speed of 10 mm/s. The maximum deformation force ($F_{max\ deformation}$) is the force measured once the probe reaches the maximum penetration depth into the implant. The force measured after the 60 s holding time is called "remaining force" ($F_{remaining}$). In this study, the ratio "$F_{remaining}/F_{max\ deformation}$" is used as a measure for the elasticity/plasticity of the implant. High values indicate high elasticity, low values indicate high plasticity. The "adhesion force" is defined as the maximum force measured with this set-up during the upward movement of the probe, accounting for the negative sign/direction of the force ($F_{adhesion}$). Each experiment was conducted in triplicate, the results are presented as mean values ± standard deviation.

2.7. Optical microscopy

Implants were prepared as described in *Section 2.3. In situ implant formation and drug release measurements.* At predetermined time points, implants were withdrawn and freeze-dried (Epsilon 2-4 LSC; Christ, Osterode, Germany). The process comprised 3 phases: (i) freezing at -45 °C for 2 h; (ii) primary drying at 0.014 mbar and -9 °C shelf temperature for 10 h; (iii) secondary drying at 0.0014 mbar and 20 °C shelf temperature for 10 h. Cross-sections were obtained with a knife and analysed with a SMZ-U zoom 1:10 microscope (Nikon, Tokyo, Japan), equipped with a TV lens C-0.45x (Nikon) and a digital camera AxioCam ICc1 (Carl Zeiss, Oberkochen, Germany).

2.8. Microbiological tests

Samples from periodontal pockets of periodontitis patients: Thirteen patients (4 women, 9 men; from 35 to 69 years old) were enrolled in this study (14 periodontal pockets were sampled). They were admitted at the clinical site of the Faculty of Dental Surgery, University of Lille, France. Participants did not receive any hygienic treatment at the teeth with periodontitis prior to sampling. Sterile paper points (Roeko, Coltene, Germany) were carefully inserted into each periodontal pocket (1 paper point per pocket) and left for 10 s to allow for absorption of gingival crevicular fluid (GCF). Each paper point was placed into an Eppendorf vial, filled with 1.5 mL of Ringer Cysteine. Independently, 10 paper points were weighed before and after sampling to estimate the mean amount of GCF absorbed (5.7 ± 0.6 mg) to allow for the quantification of bacteria. Further tenfold dilutions (-2 to -7) of these GCF solutions (-1) were obtained for microbiological testing.

Susceptibility of periodontal bacteria to doxycycline: Columbia agar was prepared from Columbia agar base, glucose, cysteine hydrochloride, and agar. The systems were sterilized in an autoclave (121 °C for 15 min). Prior to plating, Columbia agar was enriched with defibrinated horse blood (5 % v/v), without or with doxycycline hyclate (32 mg/L) and cast into Petri dishes. After cooling to room temperature, 0.1 mL of diluted GCF solutions (from -1 to -7) was inoculated onto the agar surface (35 °C, anaerobic atmosphere; Whitley A85 workstation, Don Whitley Scientific, West Yorkshire, UK). After 5 d of incubation, the number of bacterial colonies was counted for each Petri dish, the predominant colonies were subcultured and identified.

Antibacterial activity of the *in situ* forming implants: The in vitro efficacy of the investigated implants was assessed by their antibacterial activity against entire periodontitis samples from patients' periodontal pockets and against isolated bacterial strains, using the agar well diffusion method. Columbia agar was enriched with horse blood (5 % v/v) and cast into Petri dishes. After cooling to room temperature, 0.1 mL of diluted GCF (dilution -2 and -3) or isolated bacterial strains was inoculated onto the agar surface. A cylindrical hole (diameter = 6 mm) was subsequently made at the center of the agar, and filled with 30 µL of liquid formulation using a standard syringe. Upon solvent exchange, the implants formed *in situ*. The Petri dishes were incubated for 4 d (isolated

bacterial strains) or 5 d (diluted entire GCF samples) under optimum culture conditions (35 °C, anaerobic atmosphere; Whitley A85 workstation). The diameter of the observed bacteria growth inhibition zones around the center of the Petri dishes was measured using a ruler. Each experiment was conducted in triplicate, the results are presented as mean values ± standard deviation.

Minimal inhibitory concentration (MIC) of doxycycline on selected strains: The MIC of doxycycline was determined using the broth dilution method [30]. One hundred µL of Wilkins-West broth were pipetted into each well of a 96-well plate, except for the first column. A doxycycline stock solution (64 mg/L) was prepared in the same broth, 200 µL of which were introduced into the first column of microplate wells. After thorough mixing, 100 µL of the first well was added to the second well and so on. Finally, 100 µL of bacterial suspension in Ringer Cysteine solution was introduced into each well, leading to 2-fold dilution of the drug concentration (1-fold dilution in the first well). At the end, each well row contained a dilution series of the drug from the left to the right, with progressively lower concentrations (decreasing from 32 to 0.016 mg/L). After incubation at 35 °C under anaerobic conditions (Whitley A85 workstation), the lowest concentration of drug that prevented visible growth of bacteria was determined as the MIC. According to the 2013 guideline of the *Société Française de Microbiologie* (SFM), a microorganism is called “susceptible” to doxycycline if MIC ≤ 4 mg/L and “resistant” to doxycycline if MIC > 8 mg/L [31]. For these tests, the bacterial strains were provided from the collection of the Laboratory of Bacteriology of the College of Pharmacy, University of Lille, France (also obtained from patients suffering from periodontitis).

3. Results and discussion

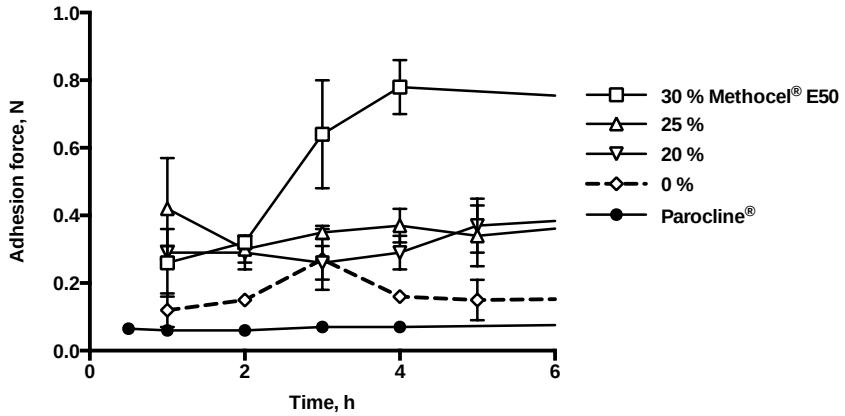
3.1. Key properties of the implants: Adhesiveness, plasticity and drug release

The therapeutic efficacy and safety of *in situ* forming implants used for periodontitis treatment strongly depends on the systems': (i) ability to remain within the periodontal pockets during the treatment period (and, thus, on their adhesiveness to the surrounding tissue), (ii) mechanical properties, in particular their capacity to adapt their size and shape to dynamic changes in the pockets' geometry, and (iii) drug release kinetics. These factors are fundamental to assure reliable drug delivery to the site of action at a pre-programmed rate during a pre-defined period of time.

Figure 2a shows the adhesion forces of composite implants formed *in situ*, measured with a texture analyzer and a spherical probe, as described in detail in the *Materials and Methods* section. Briefly, liquids containing the matrix former PLGA, the drug, a plasticizer and optionally a second polymer dissolved/dispersed in an organic, water-miscible solvent were injected into cylindrical holes at the center of agar gels in Petri dishes. The formulations contained PLGA RG 504 H (25 % w/w, referred to the total liquid), ATBC (10 % w/w, referred to the PLGA), NMP (solvent), metronidazole (1 % w/w, referred to the total liquid), and optionally up to 30 % HPMC (Methocel® E50; w/w, referred to the PLGA). Upon injection the solvent diffuses into the surrounding agarose gel and water from this gel penetrates into the liquid formulations, resulting in PLGA precipitation and drug entrapment. At different time points, a spherical probe moves downwards, penetrates into the formulation up to 1.5 mm depth, is held for 60 s and moves again upwards, while recording the forces and displacements. The maximum measured force during the upward movement of the probe is defined as the adhesion force in this study. For reasons of comparison, also the adhesiveness of the commercially available drug product Parocline® was measured (filled circles in Figure 2a). Clearly, the stickiness of the implants significantly increased with increasing HPMC contents. Importantly, the proposed new systems exhibit much higher adhesive forces than the commercial reference product. Thus, they show a very promising potential to overcome one of the fundamental bottlenecks of the current state of the art: The uncontrolled expulsion of at least parts of the

implants during the treatment period, resulting in unreliable drug exposure to the target site.

a)



b)

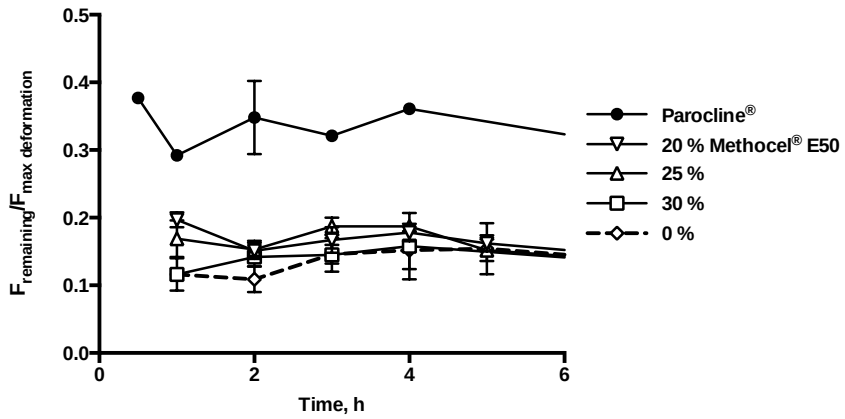


Figure 2. Importance of the composition of the liquid formulations on the resulting implants': a) adhesion force, b) mechanical properties. The formulations contained 25 % PLGA RG 504 H, 10 % ATBC, NMP, 1 % metronidazole, and optionally up to 30 % HPMC (here Methocel® E50). For reasons of comparison, also the commercial product Parocline® was studied.

Furthermore, the impact of the formulations' composition on the plasticity/elasticity of the *in situ* forming implants was quantified: The ratio of the force measured after the 60 s holding time to the maximum deformation force ("$F_{remaining}/F_{max\ deformation}$") was used for this purpose. A value of 1 indicates ideally elastic behavior, values

well below 1 indicate high plasticity. Again, the commercial product Parocline® was studied for reasons of comparison. As it can be seen in Figure 2b, the proposed new composite implants all exhibit substantially higher plasticity than the commercially available reference formulation. Thus, these composite implants can much easier adapt their size and shape to dynamic changes in the geometry of the periodontal pockets of the patients suffering from periodontitis. This key property will further contribute to a more reliable maintenance of the systems at their target site and, hence, improve the therapeutic efficacy of the treatment and the patients' safety. Interestingly, the addition of up to 30 % HPMC did not substantially affect the "$F_{remaining}/F_{max\ deformation}$" ratio.

The effects of adding up to 30 % HPMC to liquid formulations forming implants *in situ* on the resulting drug release kinetics are illustrated in Figure 3. To simulate the limited volumes of liquid the formulations are exposed to in the periodontal pockets, the experiments were conducted in Eppendorf vials. Briefly, 100 µL of a formulation was injected at the bottom of a vial, and 1.5 mL phosphate buffer pH 7.4 was added, followed by horizontal shaking at 37 °C. At pre-determined time points, the bulk fluid was completely exchanged with fresh medium. Two types of PLGA were investigated: PLGA 502 H and PLGA 504 H, differing in the average polymer molecular weight (Mw ≈ 12 and 50 kDa, respectively). Also two types of drugs were studied: metronidazole and doxycycline. The drug loadings were 1 and 5 %, respectively. Interestingly, the addition of HPMC substantially *increased* the resulting drug release rate in the case of longer chain PLGA 504 H, whereas it slightly *decreased* the release rate in the case of shorter chain PLGA 502 H. This was true for both types of drugs. This opposite impact on drug release was rather surprising, since the only difference in the formulations was the polymer molecular weight. All other compounds were identical as well as the relative amounts of all ingredients. The slight decrease in drug release upon addition of up to 30 % HPMC in the case of PLGA 502 H-based implants was further confirmed with systems containing 10 % (instead of 1 %) metronidazole, prepared with more concentrated PLGA solutions (32 instead of 28 %) and containing two types of HPMC: Methocel® E5 and E50 (differing in the average polymer molecular weight) (Figure 4a). Thus, the phenomenon was also independent of the initial drug loading, the polymer content of the formulation and the polymeric chain length of the HPMC (at least within the investigated ranges). From a practical point of view, it is very important to precisely control the resulting drug release

rate at the site of action: Too high drug concentrations lead to potentially serious side effects, whereas drug concentrations below the minimal effective concentration lead to treatment failure. To better understand why the addition of HPMC (improving the implants' adhesiveness and plasticity, as shown above) substantially increased the release rate in the case of PLGA 504 H, but decreased the release rate in the case of PLGA 502 H, the respective systems were thoroughly characterized physico-chemically during implant formation and drug release.

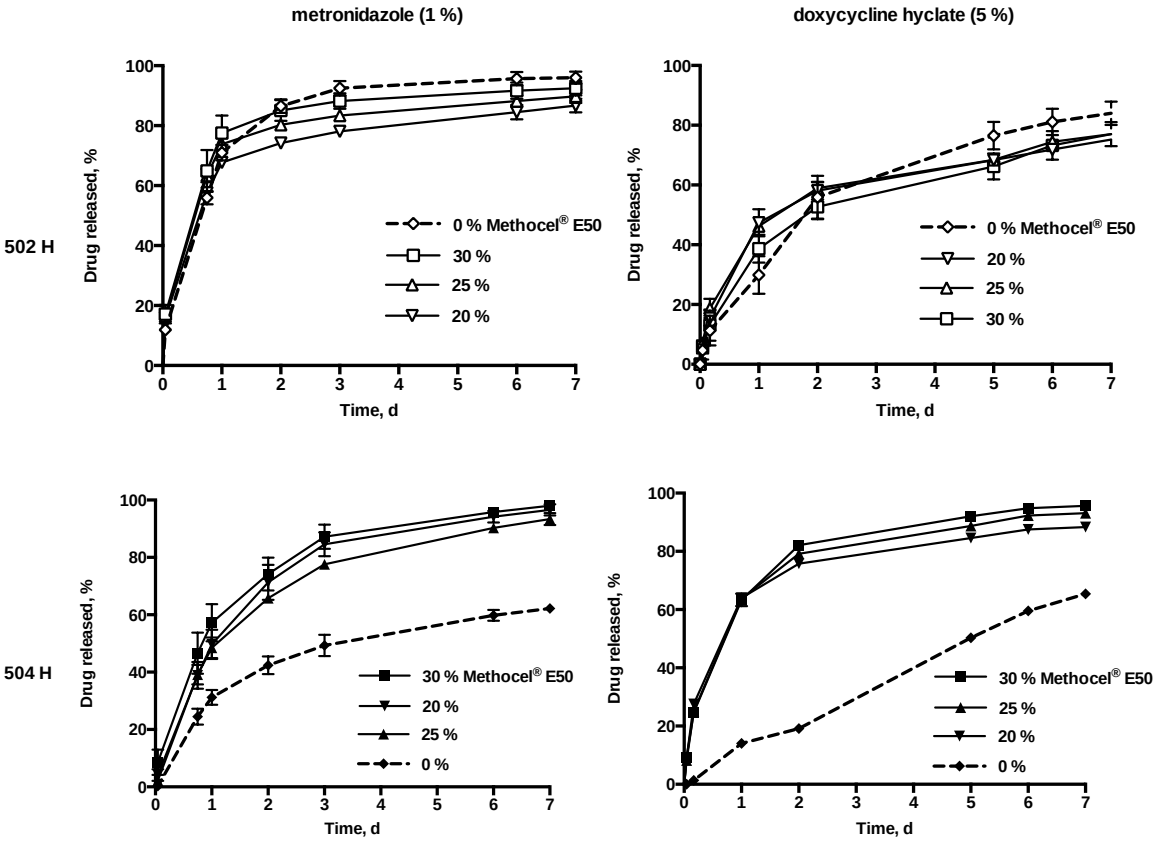


Figure 3. Impact of the addition of different amounts of HPMC (Methocel® E50) on drug release from *in situ* forming implants based on PLGA RG 502 H (top row) or PLGA RG 504 H (bottom row) (28 %, referred to the total liquid) and ATBC (10 %, referred to the PLGA). On the left hand side systems containing 1 % metronidazole are shown, on the right hand side formulations initially loaded with 5 % doxycycline hyclate.

a) b)

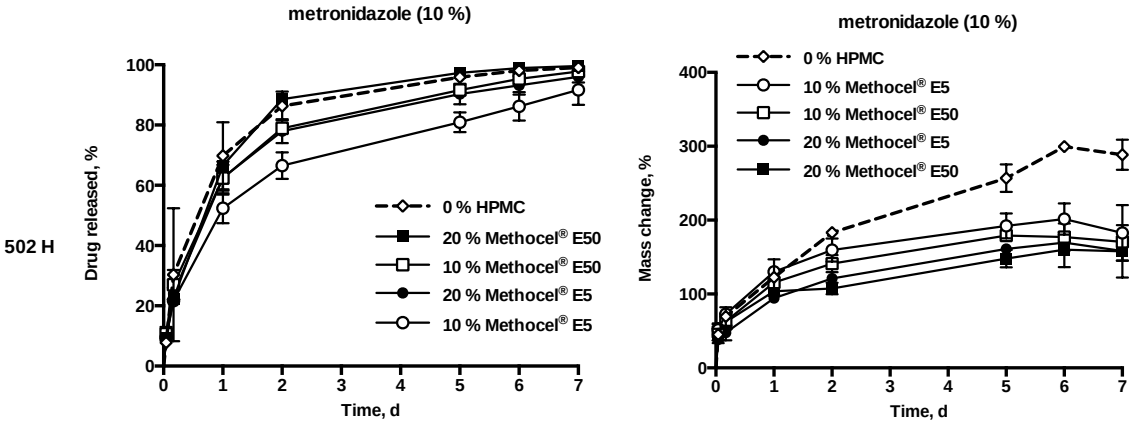


Figure 4. Effects of adding different types and amounts of HPMC (Methocel® E5 and E50) on: a) drug release, b) dynamic changes in the mass of *in situ* forming implants based on PLGA RG 502 H (32 %, referred to the total liquid) and ATBC (10 %, referred to the PLGA), loaded with 10 % metronidazole.

3.2. Underlying mass transport mechanisms

Figures 4b and 5 show the dynamic changes in the systems' mass upon exposure to the release medium. The observed mass change is essentially a consequence of the NMP diffusion into the phosphate buffer and water penetration into the formulations. As it can be seen, in all cases the mass generally increased during the observation period. This indicates that the mass gain due to water penetration is more important than the mass loss due to NMP diffusion. In practice, it is important that the mass gain is not too pronounced. Otherwise, the implants will increase too much in volume in the periodontal pockets, increasing the risk of accidental device expulsion. Importantly, the addition of HPMC led to less pronounced water uptake in all cases, further confirming the very positive impact of this compound on the maintenance of the systems within the patients' pockets. However, there is no straightforward relationship between the changes in the systems' mass and the observed release rates (Figures 3-5): HPMC addition led to faster drug release in the case of PLGA 504 H and slower drug release in the case of PLGA 502 H, whereas the increase in implant mass was reduced, irrespective of the type of PLGA. This was true for both types of drug, all investigated initial drug loadings, PLGA concentrations and HPMC polymer molecular weights.

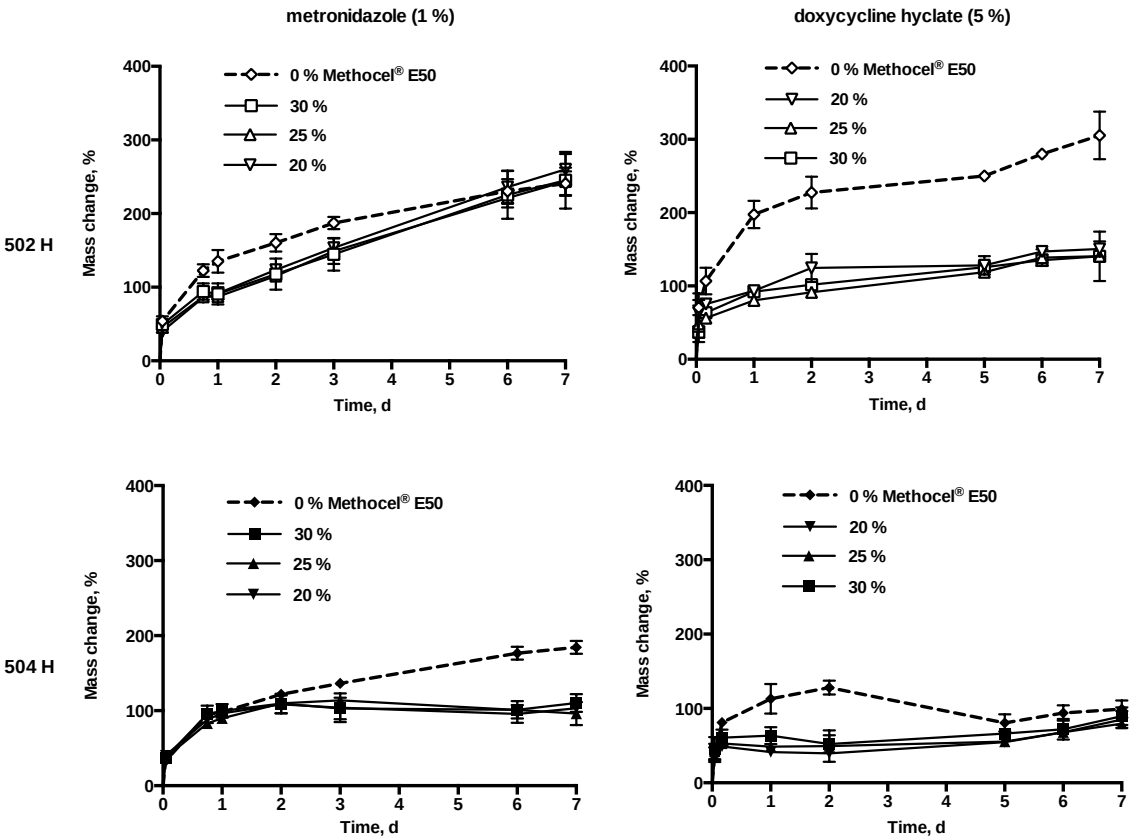


Figure 5. Impact of the addition of different amounts of HPMC (Methocel® E50) on the dynamic changes in the mass of *in situ* forming implants based on PLGA RG 502 H (top row) or PLGA RG 504 H (bottom row) (28 %, referred to the total liquid) and ATBC (10 %, referred to the PLGA). On the left hand side systems containing 1 % metronidazole are shown, on the right hand side formulations initially loaded with 5 % doxycycline hyclate.

To gain deeper insight into the rather complex phenomena involved in the *in situ* formation of the investigated implants and the control of drug release from the systems, Electron paramagnetic resonance (EPR) was applied. Briefly, an EPR spin-probe [in this study, 4-hydroxy-tempo benzoate (TB), 1 mM] was incorporated into the liquid formulations. TB is a nitroxyl radical, the spectral splitting of which is sensitive to both: the molecular motion (microviscosity of the surrounding) as well as the polarity of its environment [32]. Once the liquid formulation comes into contact with the release medium, the solvent NMP diffuses out and water penetrates into the system. This leads to PLGA precipitation and significant changes in the spin probe's environment, which can be monitored by EPR analysis. To be able to estimate the water contents of the systems at a

specific position and time point, a quantitative relationship between the measured hyperfine splitting ($2a_N$) and the phosphate buffer content in the spin probe's environment (here, NMP:phosphate buffer mixtures) was established. Figure 6a shows that a linear relationship was obtained in this case (R^2 = 0.9886). Thus, experimentally measured $2a_N$ values can be used as indicators for the changes in the systems' composition.

a) b)

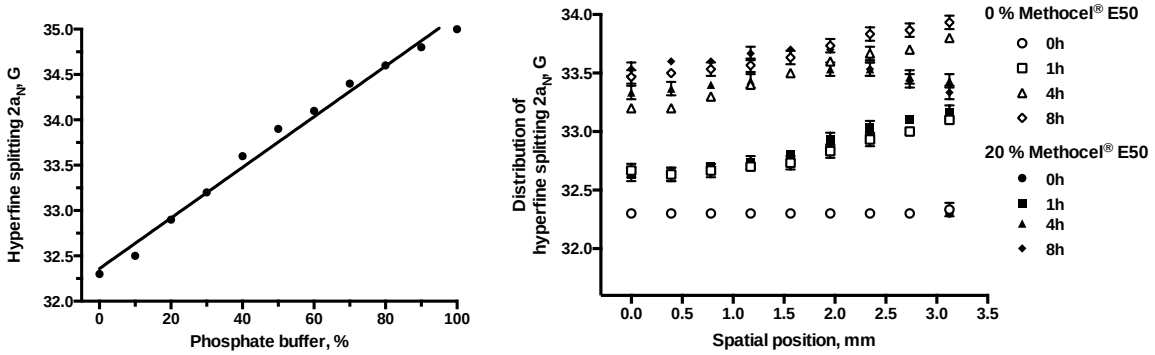


Figure 6. a) Polarity dependence of the hyperfine splitting of TB-loaded "NMP:phosphate buffer" mixtures. b) Spatially resolved profiles of TB-loaded implants based on PLGA RG 502 H (32 %), ATBC (10 %) and optionally 20 % HPMC.

Figure 6b shows the hyperfine splitting values measured at different time points in implants forming *in situ* upon exposure of NMP solutions of PLGA 502 H (32 %), ATBC (10 %) and optionally 20 % HPMC (Methocel® E50) to phosphate buffer. The spatial position is plotted on the x-axis and corresponds to the experimental set-up shown in Figure 1a: "0 mm" indicates the bottom of the holder and "3.1 mm" the interface "liquid formulation/implant – release medium". Before exposure to the release medium, the $2a_N$ values were about 32.3 G and constant within the liquid, irrespective of the presence or absence of HPMC in the formulation (open and filled circles). According to Figure 6a, 32.3 G corresponds to about 0 % phosphate buffer. This is sound, since the formulations were not yet exposed to the release medium. Importantly, the EPR measurements allowed visualizing clear water concentration gradients built up within the formulations after 1 h exposure time to the phosphate buffer (squares in Figure 6b). This indicates that surface near regions of the implants are water-rich compared to regions close to the bottom of the holder. Interestingly, the water concentration gradients in these PLGA 502 H-based

systems were very similar, irrespective of the presence or absence of 20 % HPMC (filled versus open symbols in Figure 6b). This is in contrast to previous findings with PLGA 504 H-based formulations, which showed accelerated water penetration into the systems in the presence of HPMC [29]. The difference might be explained by the different relative hydrophilicity of the two PLGA types: Both, PLGA 502 H and PLGA 504H exhibit more hydrophilic end groups at their chains (-COOH groups) and more lipophilic polymer backbones. Since the average polymer molecular weight is smaller for PLGA 502 H than for PLGA 504 H (about 12 versus 50 kDa), PLGA 502 H is more hydrophilic than PLGA 504H. This is consistent with the more pronounced water uptake of PLGA 502 H-based implants compared to PLGA 504 H-based implants shown in Figure 5 (top versus bottom row). Thus, adding hydrophilic HPMC to more hydrophobic PLGA 504 H has a more pronounced impact than when adding it to less hydrophobic PLGA 502 H. In the case of the more hydrophobic PLGA 504 H, the presence of HPMC very much facilitates water penetration into the system, whereas this effect is less pronounced in the case of the less hydrophobic PLGA 502 H. This tendency is confirmed also at later time points, namely 4 and 8 h (triangles and diamonds in Figure 6b): The water concentration profiles in PLGA 502 H-based implants are very similar in HPMC-containing and HPMC-free systems. Note that at these late time points in HPMC-containing systems, the $2a_N$ values close to the interface “implant – release medium” decrease (in contrast to HPMC-free devices). This is probably due to the fact that the formation of a HPMC gel alters the microviscosity of the environment of the spin probe and, thus, biases the measurements. In fact, increasing the viscosity of the probe’s environment has been reported to decrease the molecular tumbling rate of the nitroxyl radical [32].

Interestingly, the fact that the addition of HPMC has a major impact on the water penetration kinetics in the case of PLGA 504 H-based implant formulations (as reported in [29]) corresponds well to the major impact of HPMC addition on the resulting drug release kinetics from these systems (Figure 3). And the absence of such a major effect of HPMC addition on the water penetration kinetics in the case of PLGA 502 H-based formulations (Figure 6b) corresponds well with the absence of a major effect on the resulting drug release kinetics from these systems (Figures 3 and 4a). This clearly demonstrates that the water penetration kinetics into the *in situ* forming implants is decisive for the systems’ key properties. But yet it is unclear why accelerated water penetration into the formulations

leads to accelerated drug release. To better understand these phenomena, the morphology of the implants was studied using optical microscopy. Figures 7 and 8 show pictures of cross-sections of *in situ* formed implants based on PLGA 502 H and PLGA 504 H, respectively. The systems were exposed to phosphate buffer pH 7.4 for different time periods, as indicated (treated as for the drug release studies). The left columns show implants free of HPMC, the right columns systems containing 20 % HPMC (Methocel® E50). Very clearly, the implants solidified more rapidly in the presence of HPMC, irrespective of the PLGA type. This can probably at least partially be attributed to a faster NMP diffusion rate out of the systems, as reported in the literature [29]. Interestingly, longer chain PLGA 504 H led to a more porous inner implant structure compared to PLGA 502 H (right columns in Figures 8 versus 7) in HPMC-containing devices. This might at least partially be explained by the higher hydrophobicity of PLGA 504 H compared to 502 H, resulting in more rapid polymer precipitation upon water penetration into the systems and NMP diffusion out of the formulations. Importantly, the resulting high implant porosity can explain the significant increase in the drug release rate upon HPMC addition to these systems (Figure 3). In contrast, the much lower porosity observed in the case of PLGA 502 H corresponds very well to the substantially different effect of HPMC addition on drug release from these systems (Figures 3 and 4a). In all cases, the absence of HPMC led to slower implant solidification (Figures 7 and 8).

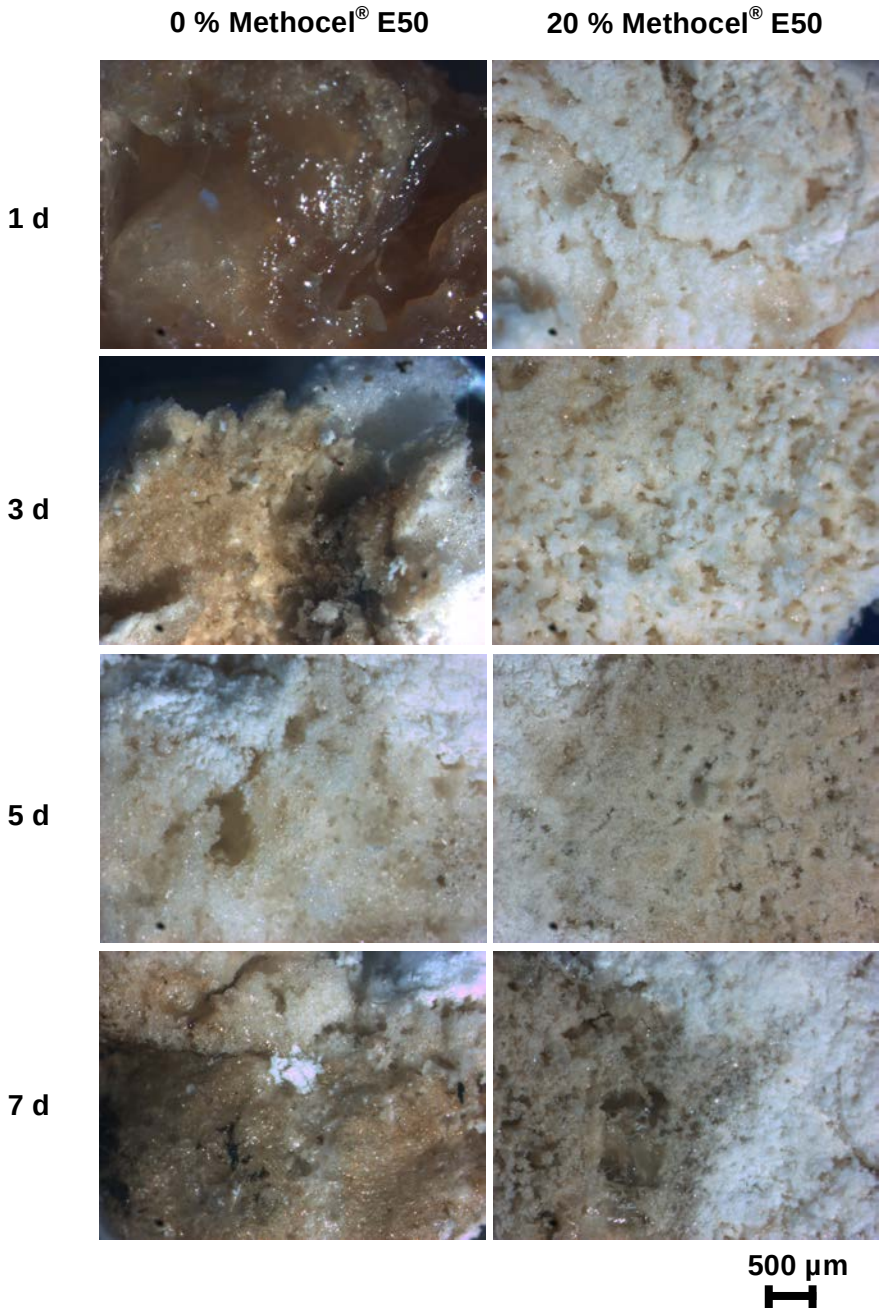


Figure 7. Optical microscopy pictures of cross-sections of *in situ* formed implants (28 % PLGA RG 502 H, 10 % ATBC, optionally 20 % HPMC (Methocel® E50), 5 % doxycycline hyclate) after 1, 3, 5 and 7 d exposure to phosphate buffer pH 7.4.

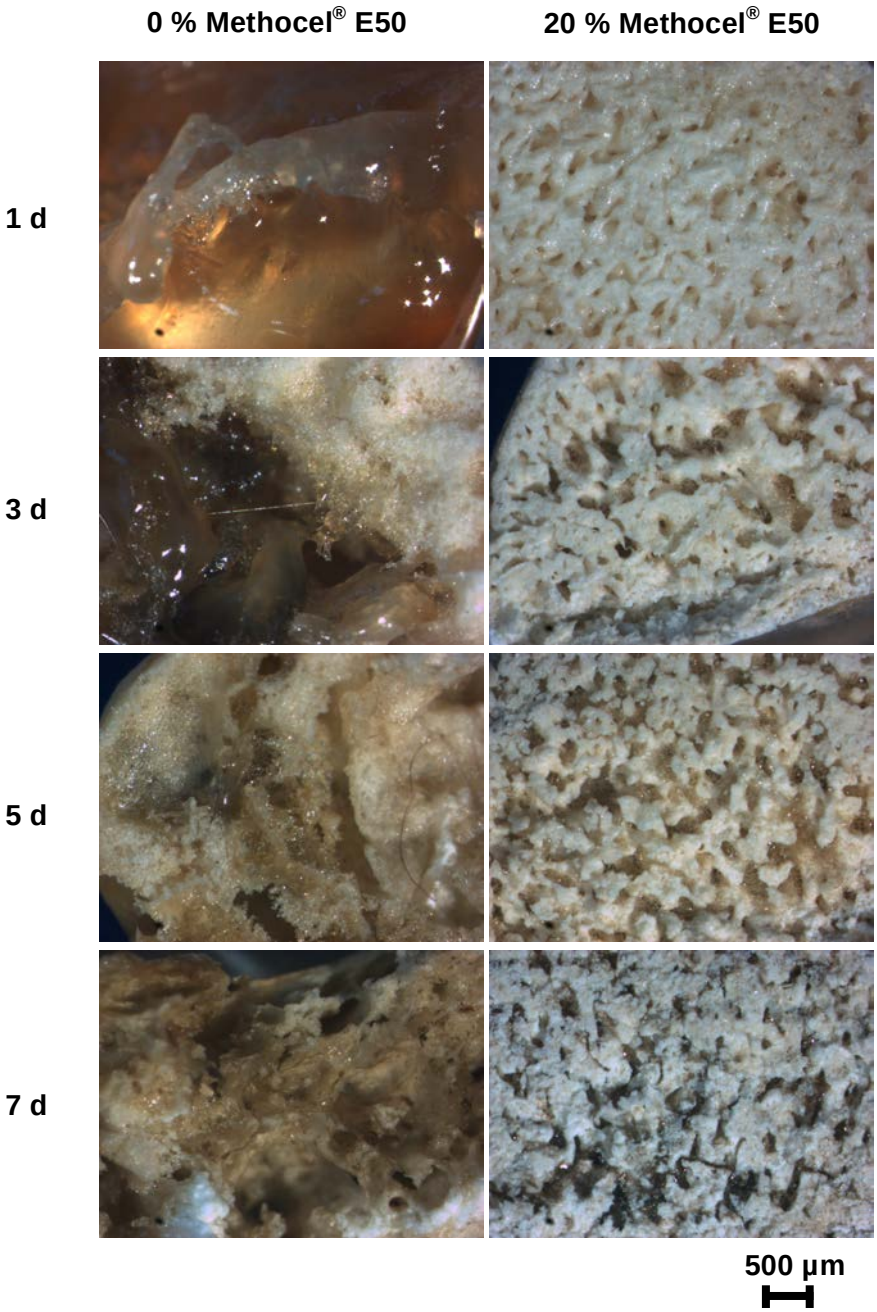


Figure 8. Optical microscopy pictures of cross-sections of *in situ* formed implants (28 % PLGA RG 504 H, 10 % ATBC, optionally 20 % Methocel® E50, 5 % doxycycline hyclate) after 1, 3, 5 and 7 d exposure to phosphate buffer pH 7.4.

The EPR measurements could also be used to quantify the release of the spin probe TB: The “normalized AUC” calculated from the EPR spectra can be used as a measure for the amount of TB still remaining within the system. Figure 9 illustrates how these values decreased with time upon exposure of formulations based on PLGA 502 H to the release medium. Open symbols correspond to HPMC-free formulations, closed symbols to HPMC-containing formulations. The triangles, squares and circles correspond to three different samples. As it can be seen, the “normalized AUC” values decreased more rapidly in HPMC-free systems than in HPMC-containing devices. This indicates faster TB release from HPMC-free implants and is consistent with the generally faster drug release observed with HPMC-free formulations based on PLGA 502 H compared to HPMC-containing systems (Figures 3 and 4). Eventually, the presence of HPMC leads to gel formation in the tiny implant pores and, thus, increased resistance for drug and spin probe diffusion [33].

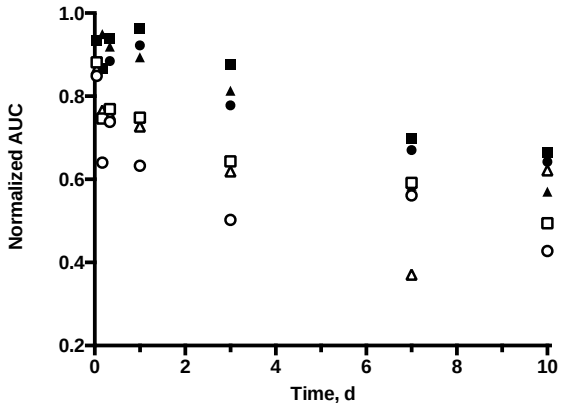


Figure 9. Release of the EPR spin probe TB (expressed as the decrease in the normalized AUC) from implants based on PLGA RG 502 H (32 %), ATBC (10 %), being free of Methocel® E50 (open symbols) or containing 20 % Methocel® E50 (closed symbols) upon exposure to phosphate buffer pH 7.4, determined via double integration of EPR spectra. The triangles, squares and circles correspond to three different samples.

3.3. Antimicrobial implant activity

For the efficacy of the periodontitis treatment the sensitivity of the bacteria in the patients’ pockets against the drug is decisive: In case of resistance, the treatment fails, even if the drug reaches its target site. For this reason it was important to verify whether or not

the bacteria, which are present in the periodontal pockets of patients suffering from periodontitis, are sensitive to the drug (here, we selected doxycycline). To be able to do so, gingival crevicular fluid (GCF) samples from 13 patients (4 women, 9 men; from 35 to 69 years old) were incubated on Columbia agar (containing or not doxycycline), enriched with defibrinated horse blood in Petri dishes. Upon 5 days of incubation, the number of Colony Forming Units (CFU) (per gram GCF) was counted. Table 1 shows the mean log (CFU)/g GCF values for 14 pocket samples. Importantly, no colonies formed in 13 out of the 14 samples in the presence of drug; and the only growth observed (in sample D99) was identified as *Candida albicans*, which is a fungus. Thus, the calculated mean log CFU/g for bacteria was ≤ 3.42 (our detection level) for all the samples. In contrast, on doxycycline-free agar, much higher log CFU/g values were found, ranging from 5.37 to 10.07. The differences of mean log CFU/g found between the two types of agar varied from 1.94 to 6.64. It is assumed that there was less than 1 bacterium over 100 to 10,000,000 that may be resistant to doxycycline. This result indicates that doxycycline is suitable for the antibiotherapy of periodontitis. Furthermore, Table 1 shows that there was no clear relationship between the pockets' depth and number of Colony Forming Units, indicating the diversity of the bacterial levels in the enrolled subjects. In addition to these studies on *bacterial cocktails*, also the activity of doxycycline against *isolated bacterial strains*, which were obtained from periodontal pockets of periodontitis patients was determined. For this purpose, the Minimum Inhibitory Concentration (MIC) of drug for specific strains was measured. Briefly, different dilutions were prepared and the concentration identified above which bacterial growth was inhibited. The results are shown in Table 2 and indicate that 8 out of the 10 strains are susceptible to doxycycline according to the Société Française de Microbiologie (SFM) requirements; and all strains were susceptible at the doxycycline concentration used in the investigated *in situ* forming implant formulations (5 or 10 %).

Table 1. Susceptibility of periodontal bacteria from periodontitis patients to doxycycline (n.m. = not measured).

Sample	Pocket depth (mm)	Mean log CFU/g of GCF on non selective agar	Mean log CFU/g of GCF on doxycycline agar
D86	n.m.	6.88	< 3.42
D87	n.m.	8.08	< 3.42
D88	7	7.10	< 3.42
D89	9	8.21	< 3.42
D90	9	6.63	< 3.42
D91	6	5.37	< 3.42
D92	9	7.70	< 3.42
D93	6	7.84	< 3.42
D94	7	8.04	< 3.42
D95	7	8.68	< 3.42
D96	6	8.15	< 3.42
D97	8	7.72	< 3.42
D98	5	10.07	< 3.42
D99	6	7.97	3.42

Table 2. Minimum inhibitory concentration (MIC) of doxycycline against periodontal strains.

Bacterial strain	Reference	MIC (mg/L)
Streptococcus vestibularis	D28A1	0.5
Veillonella sp.	D18B13	0.1
Fusobacterium nucleatum	JD7	0.03
Veillonella sp.	D36A19	1.0
Streptococcus mitis	D29A5	32
Streptococcus sanguinis	D28A11	1.0
Streptococcus australis	D37A12	0.2
Streptococcus salivarius	D28A9	0.2
Streptococcus cristatus	D18A2	0.5
Streptococcus sanguis	D30A3	32

The activity of the *in situ* forming implants against the bacteria cocktails present in the patients' periodontal pockets was measured as follows: 30 µL of the liquid formulations (containing 32 % PLGA RG 502 H, 10 % ATBC, 20 % Methocel® E50, 10 % doxycycline hyclate) were injected into cylindrical holes at the center of Columbia agar plates, enriched with horse blood and inoculated with 100- or 1000-times diluted gingival crevicular fluid (GCF) samples from patients. Upon contact with the agar gel, NMP diffused out of the formulations and water into the systems, resulting in PLGA precipitation and drug entrapment. The Petri dishes were incubated for 5 days under optimum culture conditions (35 °C, anaerobic atmosphere). In case of bacterial growth inhibition, an inhibition zone around the *in situ* formed implant was visible. The diameter of this zone was measured with a ruler. For reasons of comparison, also drug-free formulations were studied (as negative controls) and the commercially available drug product Parocline® (as reference, with two of the GCF samples: D90 and D91). Figure 10 shows the observed inhibition zone diameters. As it can be seen, the microorganisms present in all samples were susceptible to the *in situ* forming implants loaded with doxycycline as well as to Parocline® (note that this product contains a different drug: minocycline). The inhibition zone diameters varied from 1.9 to 5.7 cm, depending on the specific GCF sample, with 1 exception: For the sample D91-3, the inhibition zone of doxycycline-loaded implants was at least as large as the Petri dish. The negative controls (*in situ* forming implants free of drug) did not inhibit bacterial growth (*) or exhibited small inhibition zones (diameter ≤ 0.7 cm), with 1 exception: For the samples D91-2 and D91-3, the recorded inhibition zone diameter of the negative controls was 2.4 and 5.1 cm, respectively. This exception can be attributed to the low bacterial concentration present in these specific samples, namely: 88 and 9 CFU/g, respectively. Such a low contamination leads to non-representative numbers of Colony Forming Units resulting from incubation of 0.1 mL solution. The bacterial loads in the other samples were much higher: For instance, 1 mL D90-2 solution contained 160,000 CFU. As expected, the inhibition zone diameters were generally smaller in the case of 100-fold dilutions compared to 1000-fold dilutions (since the bacterial concentration was 10-fold higher).

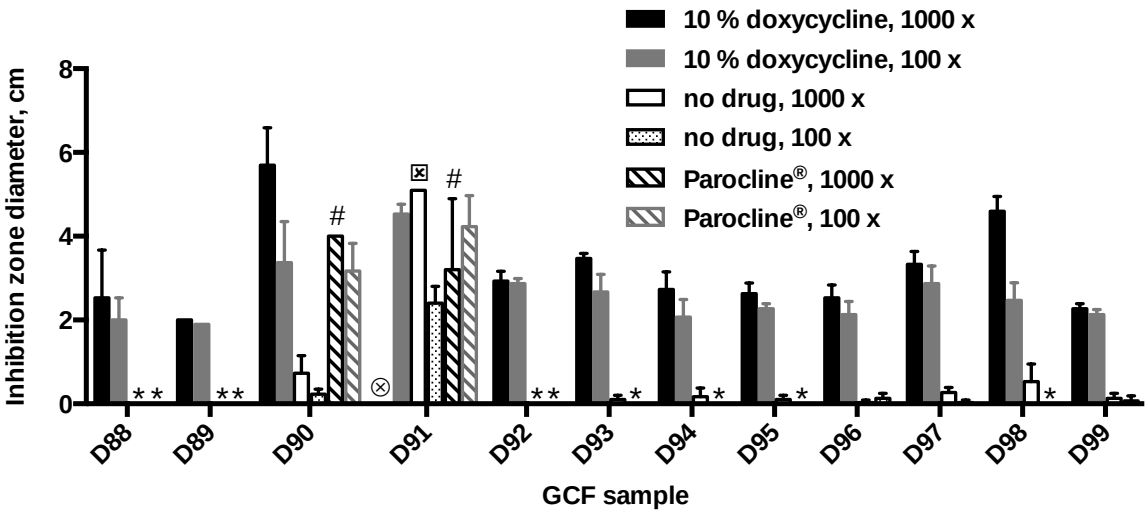


Figure 10. Antimicrobial activity of *in situ* forming implants (32 % PLGA RG 502 H, 10 % ATBC, 20 % Methocel® E50, 10 % doxycycline hyclate) against bacterial cocktails sampled from periodontal pockets of periodontist patients: Inhibition zone diameters measured upon incubation of gingival crevicular fluid samples in agar ("*" indicates that no inhibition zone was detected; "⊗"indicates that inhibition zone was at least as large as the Petri dish; "100 x" indicates a 100-fold sample dilution; "1000 x" indicates a 1000-fold sample dilution; "#" indicates n = 2 instead of 3; "☒" indicates n = 1 instead of 3). For reasons of comparison, drug-free formulations were studied as negative controls and Parocline® as a reference (with some samples).

The observed variation in the inhibition zones can be explained by the different compositions of the contents of the periodontal pockets of the patients: It is well known that this microflora is highly diverse and the quality and quantity of bacteria present depends on many factors, including the severity of disease state. To better understand which bacterial strains are likely to be of importance, the dominant colonies in all the samples from periodontitis patients were isolated and identified. In this study, 23 microorganisms were isolated: 21 anaerobes (3 gram positive, 6 gram negative obligate anaerobes and 12 gram positive facultative anaerobes) and only 2 aerobes (1 gram negative bacterium and 1 fungus). These results are in good agreement with data reported in the literature, showing the dominance of anaerobic bacteria in periodontal pocket [34–37]. The isolated microorganisms included both, initial colonizers (*Streptococcus spp*., *Actinomyces spp*., *Veillonella spp*.) as well as strains frequently present in more mature biofilms

(*Fusobacterium spp*., *Prevotella spp*.) [37]. The so-called microbial "orange complex" associated with periodontitis was also found, including black-pigmented *Prevotella* sp. and *Fusobacterium nucleatum* [34]. This indicates that the bacteria identified in this study are likely to be representative for the principal microorganisms present in periodontal pockets of periodontitis patients.

Once having identified these microorganisms, it was interesting to determine how active the proposed novel *in situ* forming implants were against them. For this reason, the same experimental set-up was used as described above for the entire fluid samples from the periodontal pockets, but inoculating only the isolated bacteria. Figure 11 shows the measured inhibition zone diameters observed with *in situ* forming implants prepared from formulations containing 32 % PLGA 502 H, 10 % ATBC, 20 % HPMC (Methocel® E50) and 10 % doxycycline hyclate. For reasons of comparison, also drug-free systems were studied (as negative controls) and the commercially available Parocline® (as a reference, with some of the bacterial strains). Clearly, efficient growth inhibition was observed with all drug-containing systems in 22 out of 23 cases. The only exception was *Candida pelliculosa*, but this is a fungus (and not a bacterium), and it can be found in the physiological mouth flora. It is not surprising that doxycycline is not active against it. Depending on the type of microorganism, the inhibition zone diameter varied from 1.5 to 6.5 cm (Figure 11). This variation is due to the different susceptibility of the respective bacteria to the drug. Importantly, the corresponding inhibition zone diameters observed with the placebo-formulations were generally much smaller, ranging from 0.0 to 3.4 cm. The 3.4 cm value was measured upon incubation of *Prevotella nigrescens*. Although this value is about 2-fold smaller than the corresponding value obtained with doxycycline-loaded formulations (6.5 cm), it indicates a possible susceptibility of this bacterium to the drug-free implant systems, eventually because of the decrease in pH upon PLGA degradation. Furthermore, it can be seen in Figure 11 that the activity of the novel *in situ* forming implants was similar to the activity of Parocline® (in the case of the investigated strains).

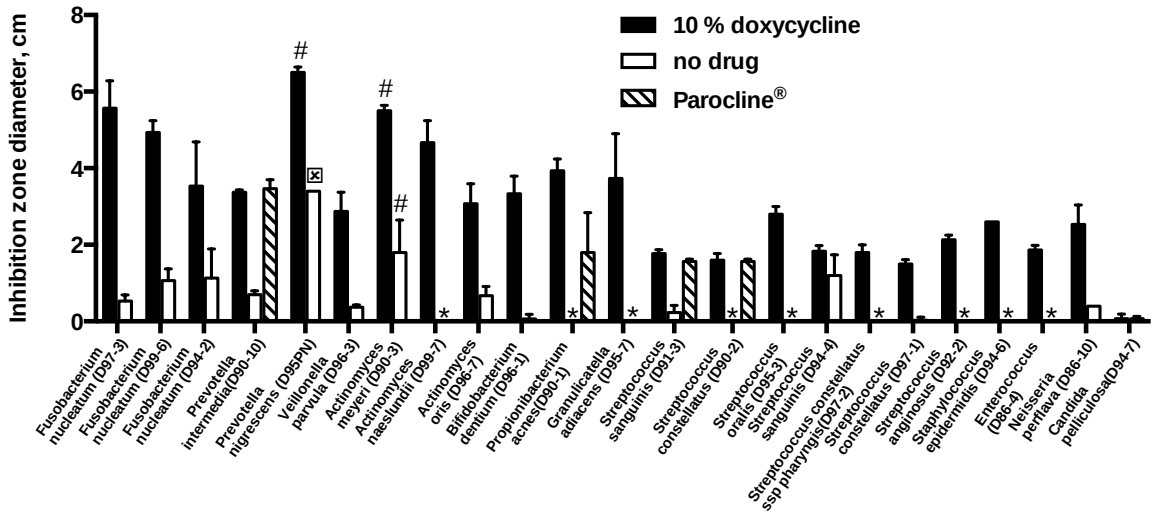


Figure 11. Antimicrobial activity of *in situ* forming implants (32 % PLGA RG 502 H, 10 % ATBC, 20 % Methocel® E50, 10 % doxycycline hyclate) against *isolated bacteria strains* obtained from gingival crevicular fluid samples of periodontitis patients: Inhibition zone diameters measured upon incubation in agar ("*" indicates that no inhibition zone was detected; "#" indicates n = 2 instead of 3; "⊠" indicates n = 1 instead of 3). For reasons of comparison, drug-free formulations were studied as negative controls and Parocline® as a reference (with some bacterial strains).

In addition, it has to be pointed out that the inhibition zone diameters of the commensal oral bacteria were smaller than the ones of pathogenic bacteria. For example, the average inhibition zone diameter observed with 7 *Streptococcus* strains (commensal flora) was 1.9 cm, while the one from *Prevotella* sp. and *Fusobacterium nucleatum* (pathogenic strains) were 4.9 and 4.7 cm, respectively. This difference indicates that the investigated implants are likely to rapidly destroy pathogens and not as quickly inhibit commensals. Thus, the commensal flora will have an opportunity to continue to colonize the periodontal pockets of the patients, re-establishing the natural microbiological balance as in healthy subjects. This is a very important aspect in practice, since the current standard treatment "root planing" suffers from a high risk of re-colonization of the pockets with pathogenic bacteria.

4. Conclusion

The novel composite *in situ* forming implants show a very promising potential as innovative local and controlled drug delivery systems for the treatment of periodontitis: They are likely able to overcome crucial current bottlenecks in this field, including unreliable residence times at the site of action (due to accidental system expulsion) and re-colonization with pathogenic bacteria. Future studies should address the *in vivo* activity of these novel types of advanced drug delivery systems.

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CONCLUSION

Conclusion

Periodontitis is a highly prevalent disease worldwide. Its treatment is challenging due to the complexity of its etiology and common recurrence post-treatment. *In situ* forming implant was reported to be an effective local periodontitis treatment, even though the lack of bioadhesiveness is still a limitation unsecuring the therapy efficacy. In this thesis, we developed biodegradable and bioadhesive *in situ* forming implants loaded with antibiotics for the treatment of periodontitis. By means of characterization techniques, the properties and antimicrobial activity of drug devices were evaluated.

In the Introduction, general definition, cause and treatment methods of periodontitis were reviewed. Short summary about the antibiotherapy was shown including various systemic or local antibiotic delivery systems and their reported clinical results. Among that, *in situ* forming implants are particularly promising for the treatment of periodontitis. The formulation composition and their impact on the controlled drug release capacity of these drug devices were briefly introduced.

Chapter 1 presented new *in situ* forming implants based on PLGA in NMP for the local treatment of periodontitis, with good mechanical properties by means of addition of a plasticizer ATBC and a second polymer HPMC. Indeed, these systems perform much higher adhesiveness than prior art systems and provide appropriate plasticity, which favor their residence times in the patients' periodontal pockets. The ability to control drug release during more than 7 days and good antimicrobial activity against relevant *Streptococcus* strains makes these *in situ* forming implants very promising for improved periodontitis treatment.

Chapter 2 focused on the controlled drug release capacity of the *in situ* forming implants and its mechanism. It was demonstrated that the presence of HPMC in the systems was a key parameter to increase the systems' adhesive forces and limit system swelling. The role of HPMC in modifying drug release kinetics was exhibited by advanced characterization techniques such as EPR and NMR measurements. In the PLGA RG 504 H matrices, the presence of HPMC increased significantly the solvent exchange rates in *in situ* forming implants, leading to rapid PLGA precipitation throughout the device and a highly porous system structure. These phenomena resulted in high drug mobility within the implant and, thus, increased release rates.

The addition of HPMC is an interesting tool to achieve desired drug release kinetics of PLGA-based *in situ* forming implants.

Chapter 3 investigates how the formulation composition determines system performance. In contrast to the substantial effect of HPMC in higher molecular weight PLGA (RG 504 H), the impact of HPMC is negligible in lower molecular weight PLGA (RG 502 H), irrespective of the type of drug. Interestingly, the increase in implant mass was reduced, irrespective of PLGA type. This was true for all investigated initial drug loadings and drug type, PLGA concentrations and HPMC polymer molecular weights. This insight can be very helpful for device optimization in the future, including other applications (e.g. for different drugs or drug combinations).

Chapter 2 and Chapter 3 showed that the proposed *in situ* forming implant formulations (loaded with 10 % doxycycline hyclate or 2 % minocycline hydrochloride) exhibited good antimicrobial activity against all the tested complex or isolated bacteria from periodontitis patients. Interestingly, the implants more strongly inhibited the growth of pathogenic bacterial strains isolated from the periodontal pockets of patients suffering from periodontitis compared to bacteria encountered in healthy subjects. This preferential activity is highly expected to favor the re-establishing of healthy microorganisms after treatment period.

In the future, the *in vivo* activity of these novel types of advanced drug delivery systems should be addressed.

RESUME

Résumé

Les maladies parodontales sont des infections diverses des tissus parodontaux, y compris la gingivite et la parodontite. Ces maladies sont causées par un biofilm bactérien résidant sur les dents adjacentes à la gencive, conduisant à une inflammation des gencives. Alors que la gingivite est la forme la plus douce, qui ne nuit pas aux structures de soutien sous-jacent des dents et est réversible, la parodontite quant à elle mène à la perte de tissus conjonctifs et de soutien de l'os.

Selon la classification de l'Académie américaine de parodontologie, la parodontite peut être divisée en plusieurs catégories: la parodontite chronique, la parodontite agressive, la parodontite associée à la maladie systémique et la parodontite nécrosante. Elles sont généralement caractérisées par une destruction du ligament alvéolo-dentaire, une résorption de l'os alvéolaire et une migration de la jonction épithéliale le long de la surface de la dent, menant à la formation de poches parodontales.

La cause principale de ces maladies est la prolifération de bactéries pathogènes bouleversant l'équilibre naturel de défense de l'hôte et de la flore commensale. Ces organismes se développent sur les surfaces dentaires d'abord en tant que micro-colonies, puis s'attachent les uns aux autres et forment des biofilms. La gingivite est souvent causée par une hygiène buccale insuffisante, provoquant la formation de la plaque dentaire. Les autres facteurs qui peuvent contribuer à la cause de cette maladie sont la génétique, le tabac, la consommation d'alcool, les carences nutritionnelles, l'infection au VIH, l'ostéoporose, le diabète, le stress, la réponse de l'hôte altérée et certains médicaments. Les lésions gingivales non traitées peuvent évoluer en parodontite, dans laquelle la plaque s'élargit et remonte sous la gencive, créant encore un meilleur environnement pour les colonies bactériennes, notamment les bactéries à gram négatif et anaérobies.

La microflore orale normale (10^8 bactéries/mL de salive) contient principalement des aérobies à gram positif ainsi que plusieurs espèces pathogènes à faible virulence. Les espèces pathogènes associées à la parodontite se composent principalement d'anaérobies à gram négatif. Chaque type de parodontite présente une flore sous-gingivale spécifique avec ses micro-organismes propres. Le changement de composition bactérienne associé à l'apparition des souches bactériennes spécifiques dans les canaux radiculaires infectés pourrait être un facteur déterminant dans la progression de la maladie. Le premier complexe bactérien associé à la parodontite est appelé «complexe orange» et se compose

des bacilles gram négatif anaérobies strictes comme *Prevotella intermedia* et *Fusobacterium nucleatum*. La forme la plus sévère s'accompagne du «complexe rouge» formé par l'association de 3 bactéries: *Porphyromonas gingivalis, Tannerella forsythia* et *Treponema denticola*. Le gram négatif facultatif tel que *Actinobacillus* (maintenant *Aggregatibacterium actinomycetemcomitans)* est aussi communément associé à cette maladie, en particulier chez les jeunes adultes.

Le traitement des maladies parodontales vise à rétablir la santé parodontale en interrompant la progression de la maladie, en prévenant la récidive et en maintenant des dents en bonne santé. Cet objectif peut être atteint par diverses thérapies chirurgicales et non chirurgicales, en fonction de la maladie et de sa gravité. La première possibilité pour le traitement des maladies parodontales consiste en l'élimination de la plaque, qui est effectuée par le soin d'hygiène bucco-dentaire et le traitement professionnel appelé le détartrage et le surfaçage radiculaire. Ces 2 techniques doivent être réalisées régulièrement pour maintenir l'hygiène buccale et stabiliser la flore buccale normale, interrompant l'inflammation gingivale. Cette thérapie non chirurgicale peut avoir une bonne efficacité dans la parodontite initiale comme la baisse de l'inflammation des tissus et l'amélioration du « clinical periodontal attachment". Toutefois, dans les cas graves, ce traitement mécanique ne suffit pas pour atteindre les résultats cliniques souhaités. Par exemple, la recolonisation des espèces pathogènes associées aux maladies et la récurrence de la parodontite sont très fréquentes.

Afin de renforcer le traitement non chirurgical de la parodontite, une thérapie antimicrobienne est souvent utilisée comme complément au détartrage et au surfaçage radiculaire. L'antibiothérapie systémique est également utilisée pour le traitement de la parodontite sévère. Cependant, cette voie d'administration présente des désavantages en raison de leurs effets secondaires, à savoir l'hypersensibilité et l'intolérance gastro-intestinale. Par ailleurs, la concentration du principe actif au niveau du site d'action (le parodonte) est assez faible et ne suffit pas pour un traitement antimicrobien efficace. Ces limites pourraient être améliorées par l'administration locale d'agents antimicrobiens. La mise en place d'un système à libération contrôlée contenant un principe actif dans la poche parodontale pourrait améliorer de manière significative sa concentration locale. En contrôlant la libération de ces systèmes, les effets secondaires pourraient également être réduits.

Cette forme galénique doit être facilement administrée et libérer l'antibiotique au sein de la poche parodontale à un taux optimum sur une longue période. Le premier système commercialisé sous-gingival est Actisite®: il est constitué de fibres de poly(éthylvinylacétate) (EVA) chargées en chlorhydrate de tétracyclines. Malgré une libération prolongée pendant 9 jours *in vitro* et une efficacité clinique démontrée, les cliniciens rapportent des difficultés avec la technique de placement d'Actisite®. Chez les patients, les principaux inconvénients sont l'anesthésie nécessaire pour le placement, l'inconfort pendant le traitement et des effets indésirables significatifs (rougeur gingivale, pigmentation de la langue). En outre, ce système doit être fixé par un adhésif cyanoacrylate à cause du manque de bioadhésivité. Le produit commercialisé Elyzol® contenant 25% de métronidazole peut être facilement placé dans la poche parodontale par une seringue fournie. Néanmoins, suite aux études cliniques, l'efficacité de ce gel utilisé en combinaison avec le détartrage et le surfaçage radiculaire est controversée. Ceci est probablement dû à la mauvaise rétention de gel Elyzol® à l'intérieur de la poche parodontale. De même, l'efficacité clinique de Periochip®, un insert biodégradable composé de gluconate de chlorhexidine à la gélatine hydrolysée n'a pas été confirmée. Cet insert adhésif peut maintenir la libération du principe actif pendant 7 jours. Toutefois, suite à une étude clinique systématique, les résultats cliniques et microbiologiques de Periochip® en combinaison avec le détartrage et le surfaçage radiculaire sont limités et controversés. Par ailleurs, le gel de minocycline à 2 % qui a été commercialisé sous plusieurs marques (Dentomycin®, Periocline®, Parocline®) semble être meilleur au niveau de la thérapie clinique. Il a été rapporté que le Dentomycin® fournissait une réduction importante du « probing depth » et du « clinical attachment gain" ainsi que de meilleurs résultats pour le « bleeding on probing". Toutefois, ces gels manquent encore de biodégradabilité, d'où la nécessité de retrait du dispositif vide après le traitement. Un autre système injectable biodégradable largement étudié est Atridox®. Ce système est constitué d'un polymère biodégradable, l'acide polylactique (PLA) dissous dans le solvant biocompatible N-méthyl-2-pyrrolidone (NMP) chargé à 10 % hyclate de doxycycline. Il s'agit d'un système de formation *in situ* en raison de son changement d'état de liquide à solide après l'injection dans la poche parodontale. Cet implant peut maintenir la libération de la doxycycline sur 7 jours. Dans deux grandes études cliniques (n = 411), Atridox® a démontré une efficacité clinique supérieure à l'hygiène bucco-dentaire et au véhicule seul. Cependant, l'Atridox®

tend à sortir prématurément de la poche parodontale (mécaniquement), l'addition de colles parodontales a pour effet de minimiser cet inconvénient.

Les implants se formant *in situ* (ISFI) sont des formulations galéniques liquides parentérales qui se transforment en dépôt (semi-) solide après injection par une seringue. L'ISFI a d'abord été étudié au début des années 1980 avec pour objectif de développer des formulations injectables antimicrobiennes pour le traitement local des maladies parodontales. Jusqu'à présent, l'ISFI retient encore beaucoup l'attention des chercheurs en raison de son avantage par rapport aux autres dispositifs d'administration de médicaments par voie parentérale tels que les liquides, les liposomes, les émulsions, les microsphères et les microparticules. Les principaux avantages des ISFI sont leur plus faible coût de production et un procédé de fabrication simple.

Les ISFI peuvent être classés en 3 groupes principaux, en fonction du mécanisme de formation de l'implant: (i) les systèmes réticulés, (ii) les organogels solidifiant et (iii) les systèmes de séparation de phases. Parmi ceux-ci, le système de séparation de phase par échange de solvant est très attractif en raison de son grand potentiel commercial. En effet, cette thèse se concentre sur les implants se formant *in situ* à base d'échange de solvant, l'abréviation ISFI sera utilisée pour désigner les systèmes de séparation de phase par l'échange de solvant. La formulation de ces systèmes ISFI est généralement constituée d'un solvant, d'un polymère et d'un principe-actif. Des quantités relativement élevées de solvant sont utilisées dans la formulation d'ISFI pour dissoudre le polymère, formant une solution polymérique. Cette dernière est ensuite injectée dans le corps et se diffuse dans les tissus environnants, le solvant utilisé doit donc répondre à certaines exigences. Il doit être non toxique et biocompatible, par conséquent, il ne provoquera aucune irritation sévère des tissus ni de nécrose au site d'injection. Par ailleurs, le solvant doit être miscible à l'eau pour diffuser rapidement dans le fluide corporel et permettre à l'eau de diffuser dans la solution polymérique, conduisant à la précipitation du polymère. Les solvants préférés comprennent la N-méthyl-2-pyrrolidone, la 2-pyrrolidone, l'acétone, le diméthylsulfoxyde, en raison de leur capacité de solvatation et de leur biocompatibilité. Il existe un grand choix de polymères biodégradables qui peuvent être utilisés dans l'ISFI. Les polymères à faible degré de cristallinité et grande hydrophobicité sont préférables en raison de leur grande solubilité dans les solvants organiques. Des exemples de tels polymères sont les polylactides, les polycaprolactones et les acides poly(lactique-co-glycolique). Ils

présentent des régions plus amorphes pour améliorer la solubilité. Ces polymères sont également largement étudiés en raison de leur sécurité approuvée par la FDA et une longue histoire d'utilisation clinique. L'acide poly(lactique-co-glycolique) (PLGA) est un copolymère de l'acide polylactique (PLA) et de l'acide polyglycolique (PGA). La gamme des produits de PLGA est large en raison de la capacité de copolymérisation des deux polymères PLLA et PDLLA avec différents ratios de monomères. Le PLGA se dégrade dans l'eau par hydrolyse de ses liaisons esters. Pour les systèmes à libération contrôlée, le choix d'un PLGA avec une cinétique de dégradation appropriée est important pour obtenir une cinétique de libération souhaitée. Le PLGA est à ce jour considéré comme le polymère le mieux défini disponible pour les systèmes à libération contrôlée à l'égard de la conception et de la performance. Le choix du principe actif dépend de l'application de l'ISFI. Pour le traitement de la parodontite, les substances choisies sont des antiseptiques ou des antibiotiques à large spectre antibactérien approprié. Pour le traitement du cancer de la prostate, le peptide agoniste des récepteurs hormonaux, l'acétate de leuprolide a été choisi comme principe actif. Par ailleurs, de nombreux dispositifs d'ISFI ont été étudiés en utilisant de petites molécules comme le diclofénac sodique et l'aspirine ainsi que de grandes molécules de protéines comme l'albumine de sérum bovin et l'hormone de croissance humaine. Les propriétés de la substance active (le poids moléculaire, la solubilité, l'affinité pour le solvant) et sa teneur dans la formulation peuvent affecter son profil de libération à partir des systèmes d'ISFI.

La libération du principe actif à partir de l'ISFI de PLGA est le résultat d'un processus physico-chimique complexe se produisant dans la matrice de PLGA, de l'injection de la solution polymérique jusqu'à la fin de la dégradation de la matrice. Ce processus commence par l'échange de solvant provoquant la précipitation du polymère, aboutissant à la formation d'un dépôt solide. Au sein de la matrice de PLGA, la présence d'eau provoque l'hydrolyse du PLGA, coupant les liaisons ester et augmentant la mobilité des chaînes polymériques. La diminution de la masse moléculaire du polymère conduit finalement à l'érosion de la matrice polymérique, ce qui pourrait affecter la libération du principe actif. En bref, le mécanisme sous-jacent de libération de la substance active peut se résumer à deux procédés principaux: la diffusion et l'érosion. Ces mécanismes peuvent se produire simultanément et sont influencés par des paramètres de formulation et par l'environnement du site d'injection. La diffusion a été décrite comme l'un des mécanismes

de libération principal contrôlant la libération du principe actif à partir de systèmes à base de PLGA. Elle est directement liée à la porosité de la matrice polymérique, et par conséquent au processus de formation de pores. Dans le cas de l'ISFI, l'échange de solvant se produit lors du contact de la solution polymérique avec l'environnement aqueux conduisant à une séparation de phases liquide-liquide et à la formation des pores. Le coefficient de diffusion du principe actif à partir de l'ISFI dépend du coefficient de diffusion dans les pores remplis par le liquide, sa porosité et sa tortuosité. Par conséquent, ce paramètre n'est pas constant mais il change en fonction du temps car l'altération de la structure du dépôt est induite par la dégradation du polymère. L'érosion commence quand le poids moléculaire du polymère passe en dessous d'un seuil de 15 kDa. Dans un premier temps, l'érosion augmente la formation des pores et augmente ainsi la vitesse de diffusion. Par ailleurs, l'érosion peut être considérée comme un vrai mécanisme de libération, induisant une libération directe de principe actif en même temps qu'une perte de masse du polymère. D'autres mécanismes de libération de la substance active à partir des systèmes à base de PLGA comprennent la diffusion au travers du réseau polymérique et le pompage osmotique. Dans le cas de l'ISFI, l'absorption de l'eau conduit à un gonflement du polymère. Ce phénomène peut alors compenser la pression osmotique à l'intérieur des systèmes. Par conséquent, la diffusion à travers des pores remplis d'eau est généralement le mécanisme de libération dominant.

De nombreux facteurs peuvent influencer la libération du principe actif des ISFI de PLGA, y compris les paramètres de formulation et les propriétés de l'environnement du site d'injection. Le solvant organique a un impact significatif sur la formation de la matrice polymérique et la libération de principe actif sous-jacente. En fonction de la force du solvant (la miscibilité dans l'eau), la matrice polymérique peut être poreuse ou presque non-poreuse. Les solvants ayant une grande miscibilité dans l'eau (NMP, DMSO...) favorisent une rapide séparation de phase liquide-liquide et par conséquent la formation de la structure poreuse, ce qui peut augmenter la libération du principe actif. En revanche, la structure plus uniforme créée par les solvants les plus faibles (la triacétine, l'acétate d'éthyle, le benzoate d'éthyle ...) conduit à une libération du principe actif plus lente. Les propriétés physico-chimiques du PLGA ont également un impact significatif sur la cinétique de libération du principe actif à partir de l'ISFI de PLGA. Tout d'abord, la masse moléculaire (Mw) est une propriété importante du PLGA, celle-ci est proportionnelle à la

longueur de la chaîne du polymère, et par conséquent proportionnelle à sa viscosité intrinsèque. Selon Ahmed et al. (2012), une Mw faible conduit à un polymère moins hydrophobe avec une augmentation du taux d'absorption de l'eau et de la dégradation de la matrice, ce qui entraîne une vitesse de libération plus rapide par rapport aux Mw élevées. Deuxièmement, une concentration en polymère plus élevée conduit à une augmentation de la viscosité et de l'hydrophobicité de la solution polymérique. Par conséquent, le taux d'afflux de l'eau, le taux de séparation des phases et la diffusion du principe actif dans le milieu de libération sont limités, conduisant à des taux de libération plus lents. Ensuite, le groupement situé à l'extrémité du PLGA peut être un acide carboxylique ou un ester, entraînant un changement de ses propriétés chimiques. Des polymères avec un groupement carboxylique sont plus hydrophiles, donc augmentent la vitesse d'absorption de l'eau, de l'hydrolyse et de l'érosion. Par conséquent, le groupe d'extrémité a un impact considérable sur le taux de libération du principe actif. Enfin, le PLGA peut se composer de n'importe quel rapport acide lactique/acide glycolique (L:G). Cette proportion influence la cristallinité du polymère et donc son absorption d'eau et sa vitesse de dégradation. Grâce aux propriétés plus hydrophile du PGA par rapport au PLA, le PLGA avec un L:G plus faible est moins hydrophobe que celui avec un rapport L:G plus haut. Par conséquent, il absorbe plus d'eau et se dégrade plus rapidement. Dans l'ISFI, le principe actif peut être dissous ou dispersé dans la solution polymérique, en fonction de sa solubilité dans le solvant organique. Etant donné que la diffusion est considérée comme le mécanisme principal de libération, la solubilité du principe actif dans le milieu de libération est un paramètre important. D'une part, la nature du principe actif peut modifier l'échange de solvant de la solution polymérique et ainsi modifier la vitesse de libération. Généralement, une substance active hydrophile conduit à un taux de diffusion et de dégradation plus élevé que les substances hydrophobes. Certains principes actifs acides (le N-acétyl cystéine, l'aspirine) ont montré qu'ils facilitaient la dégradation de la matrice de PLGA et donc amélioraient leurs libérations à partir de l'ISFI de PLGA. D'autre part, la libération du principe actif à partir de la matrice de PLGA dépend aussi de ses propriétés chimiques. Les substances basiques peuvent créer une interaction ionique forte avec le polymère, en les gardant dissoutes dans la matrice. Cette interaction protège les groupes carboxyliques terminaux du polymère, ce qui entraîne une érosion plus lente de la matrice et réduit la diffusion à travers la matrice. En revanche, en raison de leur faible interaction avec le

PLGA, les substances acides et neutres précipitent rapidement sous la forme de cristaux dans la matrice pendant la libération. Par conséquent, la solubilité de ces principes actifs dans la matrice hydratée devient un paramètre dominant affectant sa diffusion. Plusieurs additifs ont été ajoutés dans la formulation des ISFI de PLGA pour modifier ses propriétés de libération. Les polymères hydrophiles comme la polyvinylpyrrolidone (PVP) ou les Pluronics ont démontré un impact sur la vitesse de libération à partir des ISFI. L'addition d'un co-solvant, d'un plastifiant ou d'un excipient qui peut interagir avec le principe actif peut également affecter le profil de libération de l'ISFI.

L'objectif de ces travaux était de développer de nouveaux implants biodégradables se formant *in situ* pour le traitement des maladies parodontales, les infections les plus fréquentes au monde. Ces implants permettront de délivrer localement le principe actif et de contrôler sa libération. L'avantage des implants se formant *in situ* est la possibilité d'épouser parfaitement la poche parodontale. Ces implants sont basés sur un polymère biodégradable et biocompatible, l'acide polylactique (PLA) ou l'acide poly(lactique-co-glycolique) (PLGA). Il est aussi important de souligner que l'un des pré-requis pour ces nouveaux systèmes est de présenter une bonne bioadhésion et des propriétés mécaniques permettant d'éviter une expulsion prématurée hors de la poche parodontale. Les cinétiques de libération résultantes seront contrôlées sur au moins 7 jours avec une dégradation simultanée de la matrice polymérique. Les principaux objectifs de cette thèse incluaient: (i) la préparation et la caractérisation physico-chimique d'implants se formant *in situ*, (ii) l'étude de l'effet des paramètres de formulation et de procédé (ex: différents types et teneurs en polymère, agent bioadhésif et plastifiant) sur les cinétiques de libération résultantes, (iii) l'élucidation des mécanismes de libération sous-jacents en se basant sur les propriétés physico-chimiques des implants caractérisés par microscopie optique, suivi des cinétiques de libération, chromatographie à perméation de gel (GPC), résonance paramagnétique électronique (EPR) et (iv) l'évaluation de l'activité antimicrobienne des implants développés par des tests microbiologiques sur la flore complexe et sur des bactéries isolées du fluide gingival de patients atteints de parodontite.

Dans un premier temps les travaux menés dans le cadre de cette thèse se sont attachés à développer de nouveaux implants se formant *in situ* avec un potentiel prometteur pour surmonter l'un des inconvénients majeurs liés au traitement local de la

parodontite: l’adhérence limitée aux tissus environnants, entraînant l'expulsion accidentelle d'au moins une partie des implants de la poche parodontale. Cela conduit à de fortes incertitudes quant au temps de résidence des systèmes au site d'action et ainsi au temps d'exposition au principe actif. Dans cette étude, l'addition de diverses concentrations de différents types de plastifiants (l’acetyltributyl citrate, ATBC et le dibutyl sebacate, DBS) et de polymères adhésifs (dérivés cellulosiques tels que l'hydroxypropyl méthylcellulose, HPMC) ont permis d’obtenir une augmentation significative de l’adhésion des implants à base de l’acide poly(lactique-co-glycolique) (PLGA). Ces systèmes sont formés *in situ* à partir des formulations liquides de N-méthyl-2-pyrrolidone (NMP). Il est important de noter que, dans le même temps, une bonne aptitude à la déformation plastique des implants a été obtenue et les cinétiques de libération du principe actif souhaitées ont pu être affinées à l'aide de plusieurs outils de formulation. L'activité antimicrobienne de ce nouveau type d'implants se formant *in situ*, chargés à l’hyclate de doxycycline, a été démontrée en utilisant la méthode de diffusion en gélose sur plusieurs souches de Streptococcus isolées à partir de la microflore buccale des patients souffrant de parodontite.

L’objectif dans un deuxième temps visait à une meilleure compréhension des mécanismes de formation *in situ* des implants. Des implants se formant *in situ* à base de PLGA, d’ATBC, de chlorhydrate de minocycline, de NMP et d’HPMC, ont été préparés et caractérisés en détail *in vitro*. Pour cela différentes techniques ont été utilisées: la résonance paramagnétique électronique (EPR), la résonance magnétique nucléaire (^{1}H NMR), le suivi de l’évolution de la masse et la cinétique de libération du principe actif dans différentes conditions, la microscopie optique, la chromatographie d'exclusion stérique (SEC), ainsi que des tests d'activité antibactériens utilisant des échantillons de fluide gingival des poches parodontales des patients atteints de parodontite. En se basant sur ces résultats, une vision approfondie sur les phénomènes physico-chimiques impliqués dans la formation de l'implant et sur le contrôle de la libération du principe actif a pu être acquise. Par exemple, les effets de l'ajout d’HPMC dans la formulation, qui améliore l'adhérence de l'implant et réduit le gonflement, ont pu être expliqués. De manière importante, les implants se formant *in situ* ont efficacement empêché la croissance bactérienne dans les poches parodontales des patients. Il est intéressant de noter que ces systèmes ont été plus efficaces sur la croissance des souches bactériennes pathogènes (par exemple le *Fusobacterium nucleatum*) que sur des souches physiologiques (par exemple

les *Streptococcus*). *In vivo*, on peut donc s'attendre à une action préférentielle contre les bactéries pathogènes permettant ainsi à la flore saine de recoloniser les poches parodontales.

Enfin dans un dernier temps, l'impact de la composition des implants sur la performance des systèmes a été étudié. Les processus impliqués dans la formation de l'implant et dans le contrôle de la libération du principe actif sont complexes et les relations entre la composition des implants et ses performances sont encore obscures. Afin d'élucider ces relations, des techniques de caractérisation de pointe, telles que l'analyse EPR ont été utilisées pour mieux comprendre les implants se formant *in situ* basés sur deux différents types de PLGA, d'HPMC et de doxycycline ou de métronidazole. Il est intéressant de noter que l'ajout d'HPMC et de PLGA de plus faible poids moléculaire a légèrement diminué la libération du principe actif, alors que dans le cas de PLGA de poids moléculaire plus élevé, la vitesse de libération a substantiellement augmenté. Ces tendances peuvent être expliquées en se basant sur la cinétique du transport de masse au cours de la formation de l'implant et des structures internes des systèmes. En outre, l'activité antimicrobienne des implants contre les micro-organismes présents dans les poches parodontales de patients atteints de parodontite a été évaluée. Il est intéressant de noter que ces systèmes gênent plus efficacement la croissance des bactéries pathogènes que celle des micro-organismes physiologiques. Ainsi, une recolonisation de la flore saine dans les poches des patients peut être envisagée *in vivo*.

Pour conclure, cette thèse présente des implants se formant *in situ* à base de PLGA qui se dissolvent dans le NMP, sont bioadhésifs et chargés en antibiotique (la minocycline, la doxycycline ou le métronidazole) pour le traitement des parodontites. L'ajout de diverses concentrations de plastifiants (l'ATBC et le DBS) et de polymères adhésifs (dérivés cellulosiques tels que l'HPMC) a apporté une bonne aptitude à la déformation plastique des implants. Parallèlement, les cinétiques de libération de substance active souhaitées ont pu être affinées à l'aide de plusieurs outils de formulation. En utilisant plusieurs techniques de caractérisation *in vitro*, une vision approfondie des phénomènes physico-chimiques impliqués dans la formation de l'implant et du contrôle de la libération du principe actif a pu être acquise. Par exemple, les effets de l'ajout d'HPMC dans la formulation, qui améliore l'adhérence de l'implant et réduit le gonflement, ont pu être

expliqués. En outre, l'activité antimicrobienne des implants contre les micro-organismes présents dans les poches parodontales de patients atteints de parodontite a été évaluée. Il est intéressant de noter que ces systèmes sont plus efficaces contre la croissance des souches bactériennes pathogènes que contre celle des souches physiologiques. Ces résultats préliminaires ouvrent des perspectives pour évaluer plus en détail ces formulations *in vivo.*