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Ecole Doctorale Biologie-Santé

Towards a better understanding of the drug release mechanisms in PLGA microparticles

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Compréhension des mécanismes de libération de substances actives à partir des microparticules de PLGA

THESE

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Choisis un travail que tu aimes, et tu n'auras pas à travailler un seul jour de ta vie."

Confucius

GENERALITE

CHAPTER I:INTRODUCTION

Généralité

I. Introduction générale

Actuellement de nombreux traitements médicamenteux nécessitent des administrations répétées pour maintenir des concentrations plasmatiques efficaces et constantes de substance active (SA). Ceci en altérant la qualité de vie du patient peut mener à des défauts d'observance (1). Afin d'y remédier les systèmes à libération contrôlée pour l'administration parentérale constituent une bonne alternative. En effet, ils permettent de libérer la SA à une vitesse appropriée aux besoins réels in vivo pendant la durée du traitement et/ou de libérer la SA directement au niveau du site d'action, permettant ainsi d'atteindre des sites normalement non distribués, ou de limiter les effets indésirables systémiques (2). Il existe déjà sur le marché un certain nombre de formulations à libération contrôlée pour administration parentérale, parmi lesquelles nous comptons les microparticules (MP) à base de copolymère d'acides lactique et glycolique (PLGA). En effet, les avantages de ces dernières sont nombreux : une facilité d'administration par injection, une biodégradation complète, un contrôle de la libération pouvant aller de quelques jours à plusieurs mois. Ces avantages expliquent l'intérêt qui leur est porté. Cependant la compréhension des mécanismes qui contrôlent la libération de la SA à partir de ces systèmes, n'est pas toujours facile. En effet, certaines hypothèses sont décrites dans la littérature mais beaucoup de phénomènes physico-chimiques complexes impliqués restent à élucider.

II. Contexte bibliographique

II.1.Généralités

Afin de maintenir des concentrations plasmatiques efficaces et constantes, de nombreux traitements nécessitent des doses répétées de SA. Ces prises fréquentes peuvent rapidement altérer la qualité de vie du patient et mener à des défauts d'observance (1). Pour remédier à ces problèmes, de plus en plus de systèmes à libération contrôlée par voie orale ont été développés. Ces derniers permettent d'atteindre les concentrations optimales à une vitesse déterminée. Cependant, cette voie n'est pas toujours adaptée au vu des facteurs environnementaux rencontrés le long du tractus gastro-intestinal (pH, enzymes, faible capacité d'absorption, effet de premier passage hépatique …) (3). Les dispositifs à libération contrôlée pour l'administration parentérale constituent alors une bonne alternative aux problèmes rencontrés par voie orale. En effet ils en permettent de (i) libérer la SA à une vitesse appropriée aux besoins réels in vivo pendant la durée du traitement et/ou de (ii) libérer la SA directement au niveau du site d'action, permettant ainsi d'atteindre des sites normalement non distribués, ou de limiter les effets indésirables systémiques (2).

Actuellement de nombreuses formulations à libération contrôlée ont été approuvées pour l'administration parentérale telles que : les suspensions (ABILIFY MAINTENA®, aripiprazole, voie intramusculaire (4)), les liposomes (DAUNOXOME®, daunorubicine, voie intraveineuse (5)), les lipides complexes (ABELCET®, amphotéricine B, voie intraveineuse (6)), les implants (ZOLADEX®, goséréline, voie sous-cutanée (7)) et en particulier les MP comme décrit dans le Tableau 1.

La libération de la SA étant principalement contrôlée par les propriétés intrinsèques du système, les polymères (qui présentent une grande diversité de structures et de propriétés physico-chimiques) ont ainsi été largement employés dans le cadre des systèmes à libération contrôlée (3,8–12). De nombreux polymères ont largement été développés pour optimiser la conception de tels systèmes (8,10,13–16). Deux types de polymères se distinguent en fonction des conditions et de leur temps de dégradation : les polymères non biodégradables et les polymères biodégradables (8). Cependant, l'utilisation des polymères non biodégradables reste limitée car ils nécessitent d'être retirés chirurgicalement après implantation et libération de la SA. Cette limitation a conduit au développement et à l'utilisation de polymères biodégradables dont la dégradation mène à la formation de produits finaux non toxiques et préférentiellement éliminés par les voies physiologiques (10).

Tableau 1: Liste non exhaustive des spécialités commercialisées en France à base de microparticules.

Nom commercial	*Substance active*	*Voie d'administration*	*Réf.*
BYDUREON®	*Exénatide*	*Sous-cutanée*	*(17)*
DECAPEPTYL® LP	*Triptoréline (pamoate)*	*Intramusculaire*	*(18)*
ELIGARD®	*Leuproréline (acétate)*	*Sous-cutanée*	*(19)*
ENANTONE® LP	*Leuproléline*	*Sous-cutanée, intramusculaire*	*(20)*
GONAPEPTYL® LP	*Triptoréline (acétate)*	*Sous-cutanée, intramusculaire*	*(21)*
RISPERDALCONSTA® LP	*Rispéridone*	*Intramusculaire*	*(22)*
SALVACYL® LP	*Triptoréline (pamoate)*	*Intramusculaire*	*(23)*
SANDOSTATINE® LP	*Octréotide (acétate)*	*Intramusculaire*	*(24)*
SIGNIFOR®	*Pasiréotide (pamoate)*	*Intramusculaire*	*(25)*
SOMATULINE® LP	*Lanréotide (acétate)*	*Intramusculaire*	*(26)*

Les polymères biodégradables se différencient en polymères naturels et synthétiques comme illustré dans la Figure 1 (27). Les investigations sur les polymères biodégradables naturels se sont concentrées sur l'utilisation de protéines (albumine, collagène, gélatine, soie d'araignée...) et de polysaccharides (alginate, chitosan, cyclodextrine, dextran, pectine …). Cependant leurs complexités structurelles entrainent des variations d'un lot à l'autre en raison de leurs « biopréparation » via des organismes vivants (algues, plantes, bactéries ou crustacés), et leur utilisation est souvent limitée du fait de leur forte immunogénicité (8,27). Ces différents inconvénients ont mené au développement et à l'utilisation de polymères biodégradables synthétiques de composition et de fabrication plus simples (28).

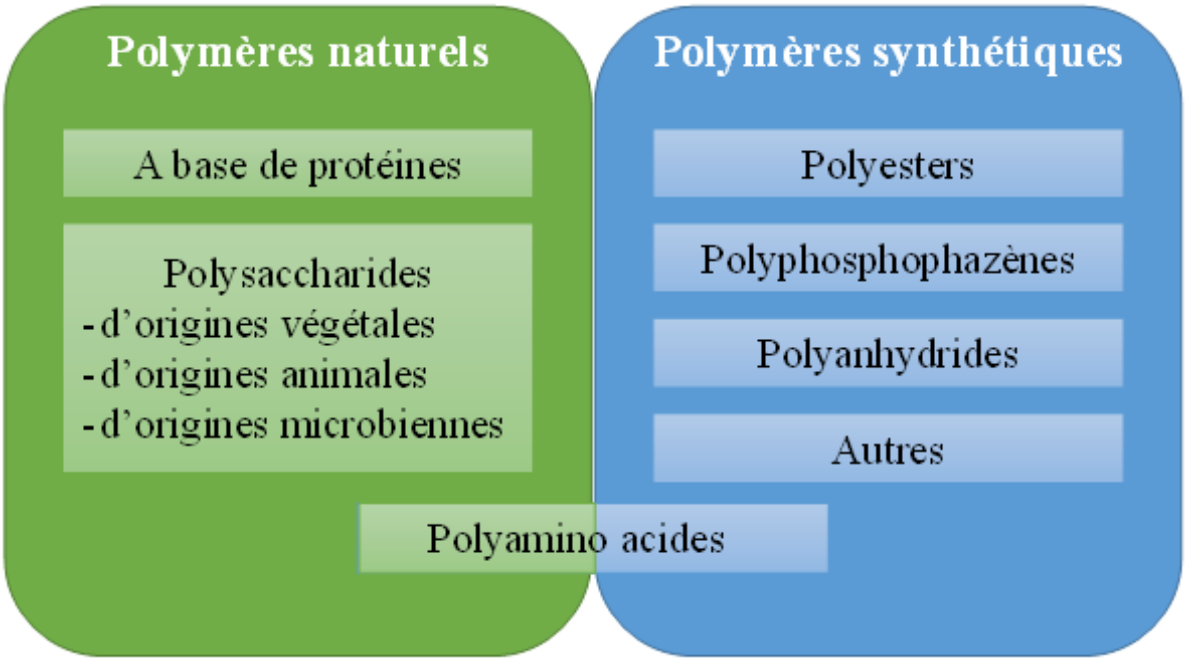

Figure 1: Vue d'ensemble des différentes familles de polymères biodégradables utilisés en libération contrôlée, classés selon leur origine (naturelle versus synthétique). Adaptée de Grund et al. 2011 (8).

Les polyesters, les polyanhydrides et les polyphosphazènes sont les familles de polymères biodégradables synthétiques les plus utilisées (8,15,28). Cependant les polyesters, et plus précisément le PLGA, sont de loin les plus largement répandus (10,29–32). Notamment du fait de leur approbation pour une utilisation humaine par l'agence réglementaire américaine : Food and Drug Administration (FDA), leur facilité de fabrication et leur biocompatibilité satisfaisante.

II.2.Copolymère d'acides lactique et glycolique

II.2.1. Formules chimiques et synthèse

Le PLGA est un copolymère linéaire qui peut être préparé avec différent ratio de monomères constitutifs : les acides lactique et glycolique (Figure 2).

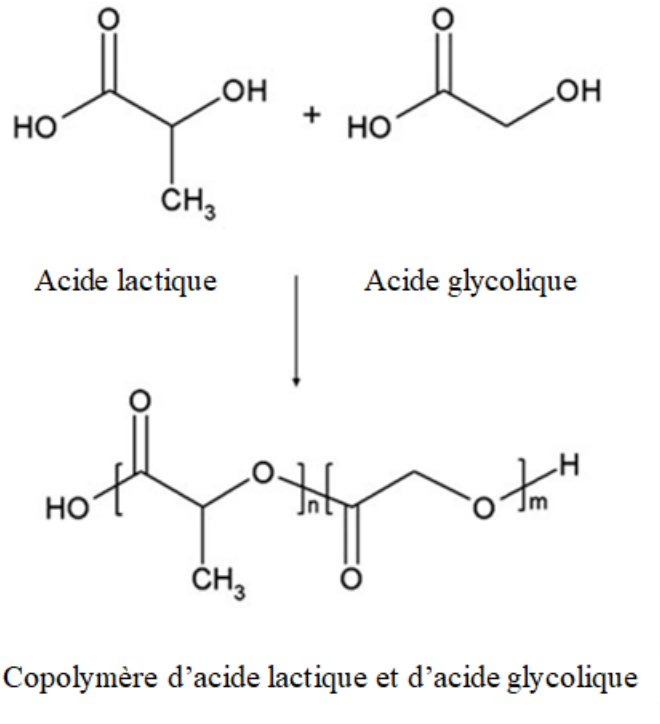

Figure 2: Structures chimiques du copolymère d'acides lactique et glycolique (n est le nombre d'unités d'acide lactique, et m le nombre d'unités d'acide glycolique) et de ses monomères.

La masse molaire (Mm) peut être contrôlée par la méthode de synthèse (13,33–35) (polycondensation ou polymérisation par ouverture de cycle) et les conditions de polymérisation (température, durée, concentration en catalyseur), alors que la nature et la quantité en monomère déterminent la composition des copolymères (13). En fonction des ratios de monomères utilisés pour la polymérisation, différents grades de PLGA peuvent être obtenus (13). Ces derniers sont généralement identifiés par le ratio de monomères utilisés.

Ces caractéristiques définissent les propriétés physico-chimiques du copolymère, influant elles-mêmes sur les vitesses de biodégradation du polymère et par conséquent, sur la libération de la SA associée à ces polymères.

II.2.2. Propriétés physico-chimiques

Contrairement aux acides polylactique et polyglycolique purs, les PLGA peuvent être dissous dans une large gamme de solvants, tels que les solvants chlorés, le tétrahydrofurane (THF), l'acétonitrile (ACN), l'acétone ou l'acétate d'éthyle (36). Ils peuvent être utilisés en tant que système à libération contrôlée de toute taille et forme, et encapsuler une large gamme de

SA (31,32). Il a été démontré que les propriétés physico-chimiques du PLGA (température de transition vitreuse (Tv), hydrophilie...) dépendent de différents facteurs, tels que le poids moléculaire, le rapport acide lactique : acide glycolique, la nature chimique de la terminaison, le temps d'exposition à l'eau et la température de stockage (37).

II.2.3. Biodégradation

Le terme dégradation correspond au clivage des chaînes polymériques en oligomères ou monomères, alors que l'érosion désigne le processus de perte de matériel (chaînes polymériques, oligomères ou monomères) à partir de la matrice polymérique (38).

La dégradation du PLGA se fait principalement, par hydrolyse au sein de la matrice polymérique. Ce mode de dégradation nécessite uniquement la présence d'eau et s'oppose à l'hydrolyse enzymatique. Dans le cas du PLGA, la pénétration de l'eau au sein de la matrice polymérique, cause une hydrolyse des liaisons ester entraînant la formation de produits acides, comme illustré dans la Figure 3. Les produits de dégradation sont principalement éliminés par voie urinaire, ou métabolisés en CO2 et en H20 dans le cycle de Krebs (28).

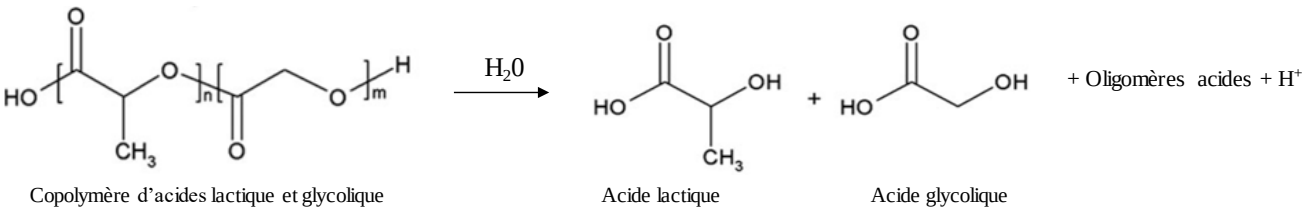

Figure 3: Réaction de dégradation du copolymère d'acides lactique et glycolique.

Les produits issus de cette dégradation, diffusent dans le milieu environnant, pouvant provoquer localement une diminution du pH (39,40). L'hydrolyse des liaisons ester étant catalysée par les protons, cette chute de pH accélère la dégradation du PLGA, c'est ce qu'on appelle l'effet autocatalytique (41). La taille du système, déterminant la longueur des chemins de diffusion des produits de dégradation, joue un rôle crucial dans l'apparition ou l'absence de l'effet autocatalytique (42).

II.3.Microparticules à base de copolymère d'acides lactique et glycolique

II.3.1. Caractéristiques générales

Les MP sont des systèmes sphériques divisés en deux catégories : les microsphères et les microcapsules, dont la taille varie de 1 à 1000 µm. Comme illustré dans la Figure 4 les premières correspondent à un système matriciel où la SA est piégée au sein d'une matrice polymérique sous forme dissoute ou dispersée (38,43). Les microcapsules, quant à elles, sont des systèmes réservoirs constitués d'un cœur de SA sous forme de solution ou de dispersion,

entouré d'une membrane polymérique de contrôle (43). Par abus de langage, dans le cas du PLGA, le terme MP désigne la plupart du temps des microsphères formées d'une matrice polymérique (44).

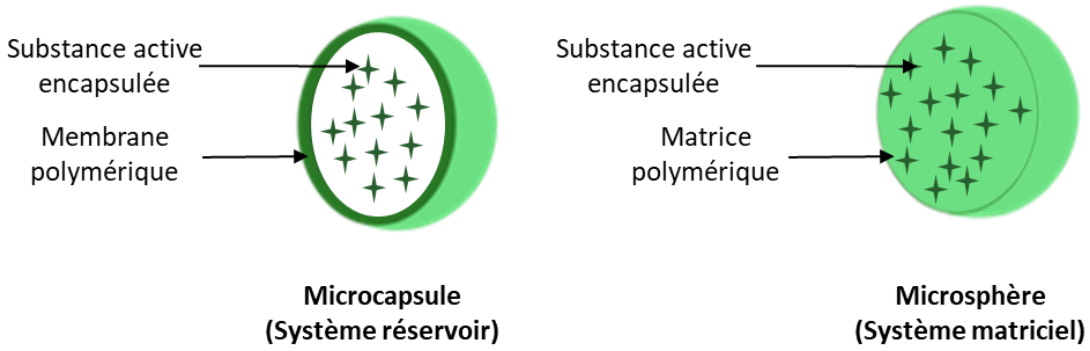

Figure 4: Représentation schématique d'une microsphère et d'une microcapsule. Adaptée de Siepmann et Siepmann 2008 (45).

Les MP sont administrées par voie parentérale, principalement intramusculaire et sous-cutanée, pour lesquelles la taille des MP désirées est comprise entre 20 et 100 µm (32). L'évolution de ces formes vers de nouvelles applications a entraîné l'investigation d'autres voies d'administration telles que : la voie intra-articulaire, intra-oculaire, parodontale, orale ou pulmonaire (46–48). Les avantages des MP de PLGA sont nombreux : une facilité d'administration par injection, une biodégradation complète, un contrôle de la libération pouvant aller de quelques jours à plusieurs mois. Ces avantages expliquent la commercialisation et le développement de nombreuses formulations, dans le but de maîtriser les profils de libération de SA.

II.3.2. Techniques de préparation

Il existe plusieurs techniques de préparation des MP. Le choix de la méthode d'encapsulation dépend de la nature du polymère, du principe actif et de l'application. Les trois méthodes les plus utilisées sont : *l'émulsion-évaporation ou extraction de solvant* (49,50), *la coacervation* (51) et *le séchage par atomisation* (9,31). Dans tous les cas la méthode choisie devra : permettre de maintenir la stabilité et l'activité de la SA, satisfaire une certaine efficacité d'encapsulation, et présenter une gamme de taille reproductible.

II.3.2.1. Emulsion-évaporation et/ou extraction de solvant

La préparation de MP par émulsion-évaporation et/ou extraction de solvant peut se faire selon différents principes. Nous détaillerons ici uniquement les méthodes d'émulsion simple de type huile dans eau (H/E) et d'émulsion double de type eau dans huile dans eau (E/H/E) car il s'agit des plus communément référencées.

- ***Emulsion simple :***

Le polymère et la SA sont dissous dans un solvant organique volatil et insoluble dans l'eau. Une émulsion H/E est formée en incorporant la solution organique dans un grand volume d'eau contenant un tensioactif (TA). L'évaporation du solvant peut être réalisée par agitation mécanique sous pression atmosphérique, ou réduite et l'extraction peut avoir lieu en incorporant l'émulsion dans un grand volume de phase aqueuse pour augmenter la vitesse de diffusion du solvant organique vers le milieu extérieur. La représentation schématique de cette méthode est illustrée dans la Figure 5.

L'interaction directe du polymère avec la phase aqueuse dans laquelle il est insoluble, induit la précipitation de celui-ci et la formation de MP solides (32,49,50). Elles sont ensuite récupérées par filtration ou centrifugation puis séchées sous vide ou par lyophilisation.

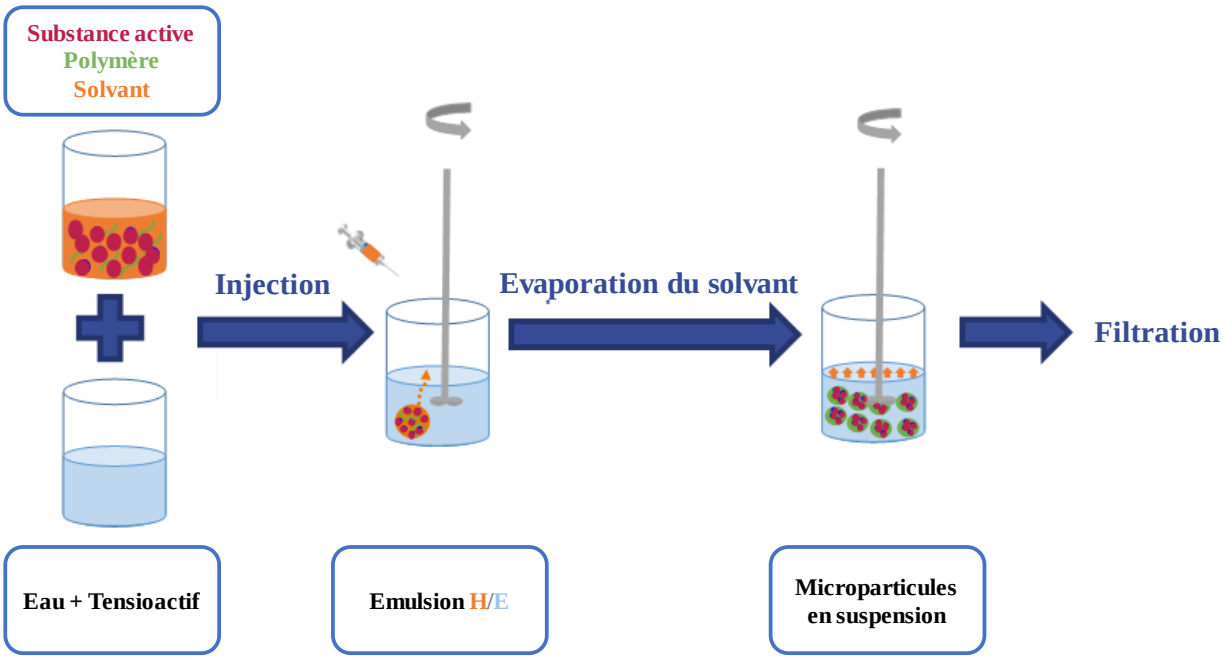

Figure 5: Représentation schématique de la méthode de préparation des microparticules par émulsion simple-évaporation de solvant.

Bien que très utilisée, cette technique s'applique essentiellement à l'incorporation de SA solubles dans le solvant organique (32). Les SA hydrophiles ont tendances à mal s'y dissoudre et à diffuser vers la phase aqueuse entrainant un faible taux d'incorporation dans les MP.

- ***Emulsion double :***

La technique d'émulsion E/H/E, qui est particulièrement adaptée à l'incorporation de SA hydrophiles, est représentée schématiquement dans la Figure 6. La solution aqueuse de SA est d'abord émulsifiée dans une solution organique dans laquelle est dissout le polymère. Cette première émulsion simple (E/H) est émulsifiée dans une phase aqueuse contenant un TA pour stabiliser la double émulsion E/H/E (36). La phase organique séparant les deux phases aqueuses agit comme une barrière empêchant le passage de la SA vers le milieu extérieur. Cette phase organique subit ensuite les mêmes étapes que pour la technique d'émulsion simple, à savoir évaporation et/ou extraction du solvant, filtration/centrifugation et séchage des MP obtenues (36).

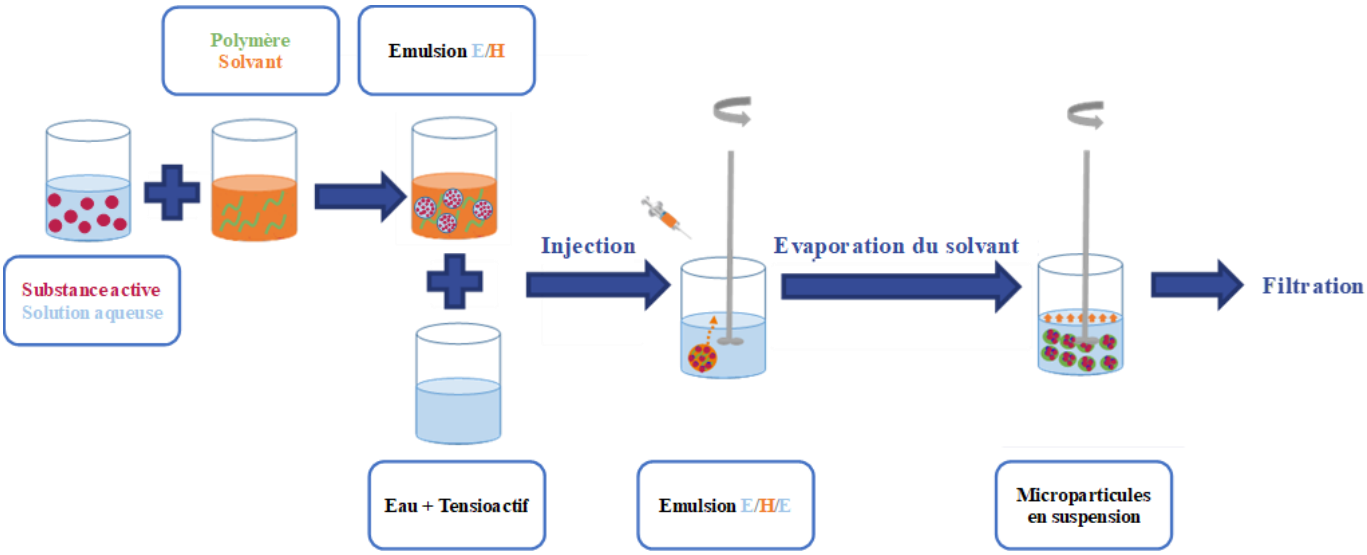

Figure 6: Représentation schématique de la méthode de préparation des microparticules par émulsion double-évaporation de solvant.

D'importants rendements d'encapsulation de SA hydrophiles peuvent être atteints par cette technique à condition que les quantités à encapsuler ne soient pas trop importantes (9). Il a été montré précédemment que le volume de la phase aqueuse interne a une influence sur la microstructure des MP. Plus le volume de la phase aqueuse interne est augmenté, plus la structure de la matrice sera poreuse (52).

II.3.2.2. Séparation de phase/coacervation

Le principe de coacervation repose sur l'abaissement de la solubilité d'un polymère en solution par ajout d'un non-solvant, d'un électrolyte ou en faisant varier la température (32). La SA est initialement dispersée dans la solution polymérique. Après ajout du non-solvant, le solvant est extrait du polymère et par diminution de la solubilité, des gouttelettes de coacervat se forment induisant une séparation de phases. Le coacervat formé est donc pauvre en solvant et riche en polymère, alors que l'inverse est retrouvé dans le surnageant. Le coacervat se solidifie en l'introduisant dans un grand volume d'un autre non-solvant, formant ainsi des MP. Cette méthode permet l'encapsulation aussi bien des SA hydrophiles que lipophiles, mais mène souvent à la formation fréquente d'agrégats (agglomération des gouttelettes de coacervat) et nécessite de grandes quantités de solvant (53).

II.3.2.3. Atomisation-séchage

Cette méthode qui permet de transformer une préparation liquide (solution, suspension ou émulsion) en MP sèches suite à son atomisation en fines gouttelettes dans un flux d'air chaud est représenté schématiquement dans la Figure 7Figure 7.

Cette technique en une seule étape, présente l'avantage d'être simple, reproductible et facile à appliquer à grande échelle. Contrairement aux autres méthodes, l'atomisation-séchage dépend beaucoup moins des paramètres de solubilité du polymère et de la SA et peut aussi bien être utilisée pour l'encapsulation de SA hydrophiles que lipophiles (32).

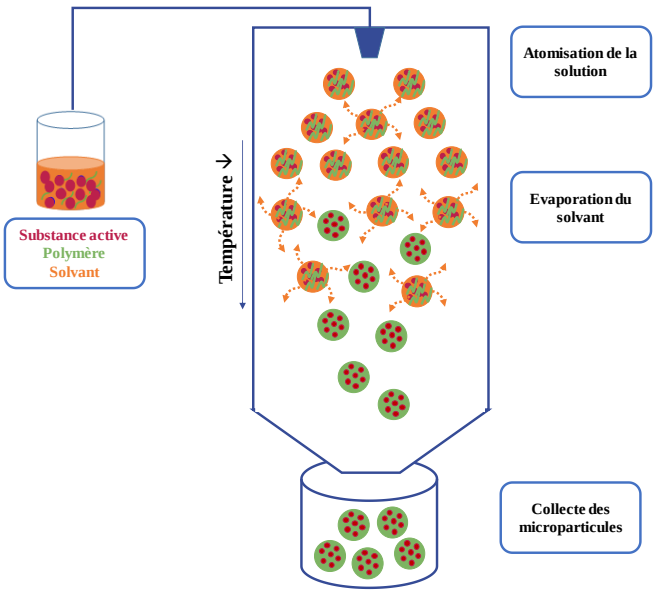

Figure 7: Représentation schématique de la méthode de préparation de microparticules par atomisation séchage.

II.3.2.4. Autres méthodes

Des méthodes de préparation de MP moins conventionnelles ont également été référencées (32). Parmi celle-ci nous comptons : les techniques d'ammonolyse du solvant, de fusion et l'utilisation des fluides supercritiques.

Après formulation, les MP doivent être caractérisées. Cette caractérisation inclut principalement une observation de la morphologie et de la structure physique des MP, ainsi que l'évaluation de la dégradation du polymère, du gonflement des MP et de la libération in vitro de la SA.

II.4.Mécanismes et profils de libération in vitro de substance active

La compréhension des mécanismes qui contrôlent la libération de la SA à partir de ces systèmes, n'est pas toujours facile. En effet, certaines hypothèses sont décrites dans la littérature mais beaucoup de phénomènes physico-chimiques complexes impliqués restent à élucider.

II.4.1. Mécanismes de libération de substance active

Le mécanisme de libération d'une SA a été défini comme pouvant être (54,55):

- La manière dont cette dernière est libérée.
- Le processus qui contrôle sa vitesse de libération.

Jusqu'ici, plusieurs processus contrôlant la vitesse de libération de la SA à partir de MP ont été référencés dans la littérature (38,41):

- Dissolution de la SA.
- Diffusion de la SA à travers des pores remplis d'eau.
- Diffusion de la SA à travers la matrice polymérique.
- Hydrolyse du polymère.
- Erosion du polymère,
- Effet osmotique.
- Absorption d'eau/Gonflement.
- Interactions SA-Polymère, SA-SA.
- Relaxation du polymère.
- Fermeture des pores.
- Dégradation hétérogène du polymère.
- Formation de fissures, déformation des MP.
- Collapse de la structure du polymère.

D’autre part Fredenberg et al. (41) ont identifié trois possibilités de libération de la SA à partir des systèmes à base de PLGA : la diffusion à travers les pores remplis d'eau, la diffusion à travers la matrice de polymère la dégradation ou érosion de la matrice polymérique, comme illustré dans la Figure 8.

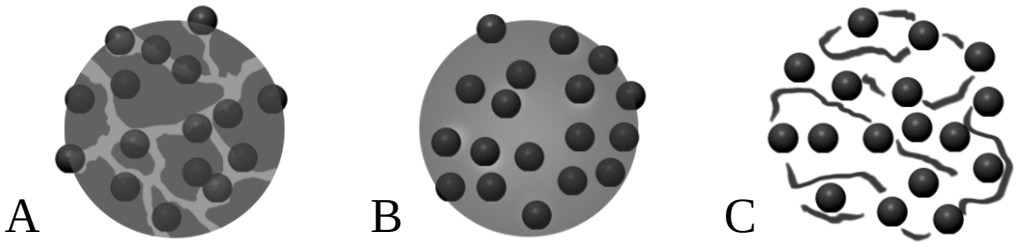

Figure 8: Représentation schématique des mécanismes de libération de SA à partir de microparticules de copolymère d’acides lactique et glycolique : (A) diffusion à travers des pores remplis d'eau, (B) diffusion à travers la matrice polymérique et (C) dégradation ou érosion de la matrice polymérique. Adaptée de Fredenberg et al. 2011 (41).

II.4.1.1. Diffusion à travers des pores remplis d’eau

Ce mécanisme permet de décrire la première étape de libération, avant le début de l’érosion du polymère. Elle est très dépendante de la structure poreuse de la MP et est donc dépendante des processus qui favorisent la formation des pores (56). Ces derniers doivent être continus et suffisamment grands pour que la SA puisse passer au travers (41). Le transport à travers les pores remplis d’eau peut se faire par diffusion, lorsque le gradient de concentration est moteur du transport et/ou par convection, dans le cas où la pression osmotique est moteur (41).

II.4.1.2. Diffusion à travers la matrice polymérique

Ce mécanisme concerne les SA hydrophobes de bas poids moléculaires (32). La vitesse de diffusion est très dépendante de l’état physique du polymère. Elle peut augmenter quand le polymère passe de l’état vitreux à l’état caoutchouteux et contrairement à la diffusion à travers les pores remplis d’eau, elle ne dépend pas de la structure poreuse (57). La diffusion est souvent plus élevée dans les polymères de bas poids moléculaire, du fait d’une plus grande mobilité des chaînes polymériques (29).

II.4.1.3. Dégradation et érosion de la matrice polymérique

La dégradation/érosion de la matrice est le processus de clivage des chaînes de polymères en oligomères et en monomères (58). Faisant et al. (29) ont répertorié deux types d’érosion : l’érosion de surface et l’érosion de masse (Figure 9).

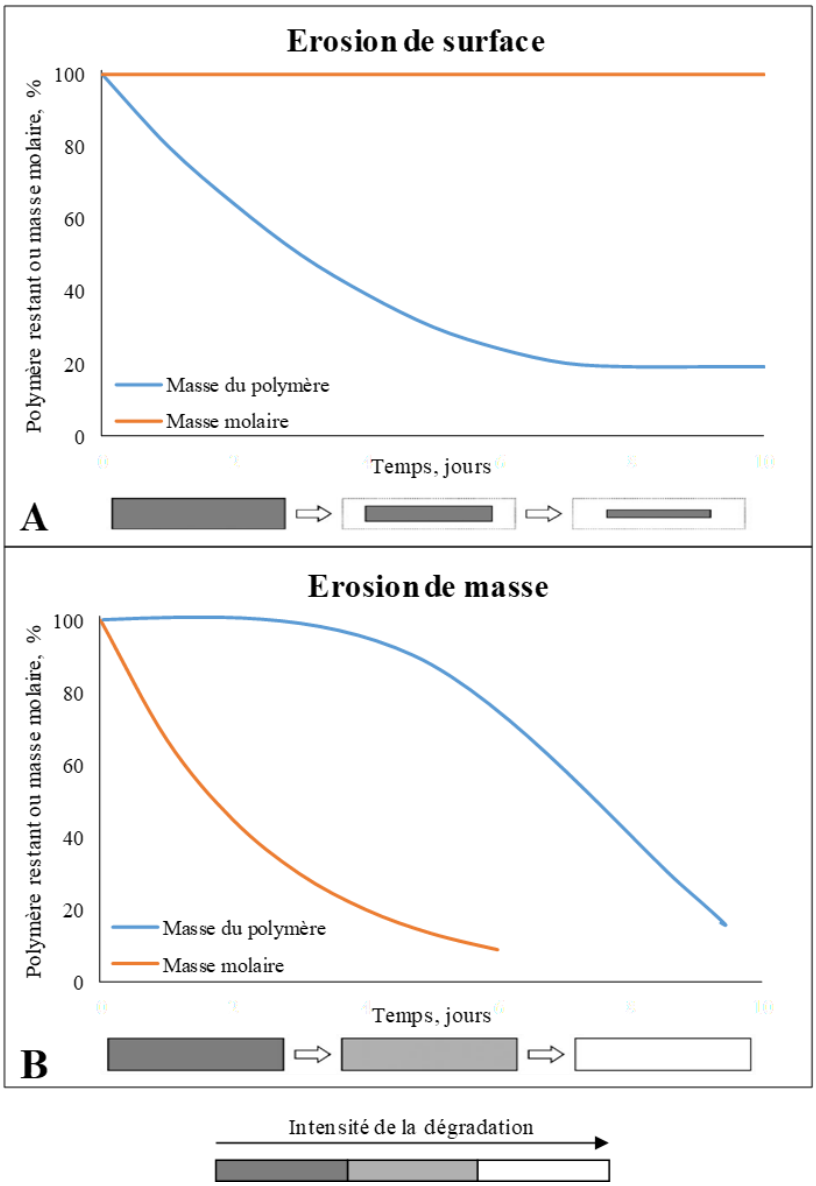

Figure 9: Variation de la masse du polymère et de la masse molaire de ce dernier dans le cas (A) de l'érosion de surface et (B) de l'érosion de masse. Adaptée de Grund et al. 2011 (8).

Dans le premier cas l'érosion se fait uniquement en surface, en raison d'une faible diffusion de l'eau dans le polymère (59). En revanche, dans le second cas, la dégradation se fait plus lentement et dans l'ensemble du système comme illustré dans la Figure 9. Les MP à base de PLGA sont généralement considérées comme des systèmes subissant une érosion de masse (8,60).

II.4.1.4. Gonflement du polymère

Le gonflement du polymère est dû aux quantités importantes d'eau pénétrant dans le système. Dès que l'eau pénètre, la mobilité des macromolécules augmente. Lorsqu'une certaine concentration d'eau critique est atteinte, les macromolécules subissent une augmentation importante de leur mobilité, ce phénomène est appelé relaxation des chaines polymériques

Le gonflement du polymère a deux conséquences principales sur la libération de SA :

- Une diminution de la vitesse de libération dû à l'augmentation des chemins de diffusion.
- Une augmentation de la vitesse de libération dû à l'augmentation de de la mobilité des molécules de SA incorporées.

Suivant l'importance relative de l'augmentation de la longueur des chemins de diffusion et de l'augmentation de la mobilité de la SA, soit la vitesse de libération de la SA augmente, soit elle diminue du fait du gonflement du polymère (45).

II.4.2. Profils de libération in vitro

Différents types de profils de libération peuvent être observés à partir de MP biodégradables. La Figure 10 décrit les trois profils de libération caractéristiques : le profil de libération monophasique, le profil biphasique et le profil triphasique.

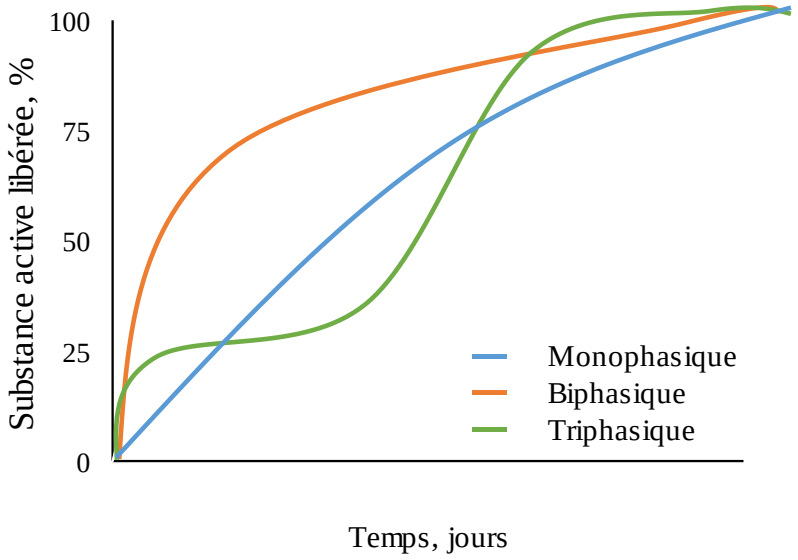

Figure 10: Représentation schématique des différents profils de libération d'une substance active à partir de microparticules.

Il n'est pas toujours évident de savoir quels sont les mécanismes de libération qui dominent lors des phases précédemment décrites (41,61). En effet, les profils de libération sont souvent dus à la combinaison de plusieurs des mécanismes décrits dans la partie *II.4.Mécanismes et profils de libération in vitro de substance active.*

II.4.2.1. Profil monophasique

Il s'agit d'une cinétique d'ordre zéro, recherchée dans la plupart des applications pour éviter l'effet « burst » pouvant être à l'origine d'effets toxiques. La libération de la SA est constante et n'est contrôlée que par un processus de diffusion. Wang et al. (62) ont montré qu'une cinétique d'ordre zéro peut être obtenue à partir de MP préparées par la technique d'émulsion simple évaporation de solvant. La libération monophasique de SA à partir de MP à base de PLGA est rare. Elle se fait principalement de façon biphasique ou triphasique (41).

II.4.2.2. Profil biphasique

Ce profil se compose de deux phases de libération : la première phase appelée effet « burst » est suivie d'un plateau (63,64).

- ***Phase I :***

Pour de nombreuses formulations, une libération rapide initiale de la SA est observée avant que la vitesse de libération n'atteigne le plateau (63). Ce phénomène est généralement appelé effet « burst ». Cela peut être définie comme la quantité de SA libérée par les MP avant le début de l'érosion du polymère (64).

- ***Phase II :***

S'en suit alors une phase où la vitesse de libération de la SA est constante, appelée plateau, au cours de laquelle, le ralentissement de la libération du fait de l'augmentation des chemins de diffusion est compensé par l'érosion du polymère. En effet l'eau qui a pénétré à l'intérieur de la matrice induit l'hydrolyse des chaînes polymériques en oligomères et en monomères solubles dans l'eau (29,36,41).

II.4.2.3. Profil triphasique

Ce profil de libération présente également un effet « burst » suivi d'une deuxième phase qui correspond à une diffusion lente de la SA à travers la matrice de polymère et les pores. La troisième phase est généralement le résultat de la dégradation de la matrice polymérique qui se traduit par une libération rapide de la SA (65).

- ***Phase I :***

Comme décrit précédemment (*II.4.2.2)*, l'effet « burst » est principalement attribué à la diffusion de la SA qui est adsorbée à la surface de la particule, ou à la diffusion de la SA à travers des pores remplis d'eau en contact direct avec la surface de cette dernière (65). Des travaux précédents suggèrent que la période de « burst » se termine au moment où les pores se referment (62).

- ***Phase II :***

Cette phase, aussi appelée phase de latence, est caractérisée par une diffusion lente de la SA à travers la matrice polymérique et les quelques pores remplis d'eau (66). Pendant cette période, l'hydratation de la MP, ainsi que la dégradation du polymère ont lieu. Cette phase peut également être due à la fermeture des pores et aux interactions polymères-SA, qui pourraient limiter la libération de la SA. Il a été rapporté que plusieurs facteurs pouvaient induire la fermeture des pores, parmi eux : la dégradation du polymère, les agents plastifiants et les températures élevées (67,68). Dans certains systèmes cette phase de latence est négligeable du fait de la dégradation rapide du PLGA. La durée de cette phase dépend principalement des caractéristiques du polymère, de la taille et de la géométrie des MP (42).
Gasmi et al. (69), ont montré que cette deuxième phase de libération dépendait principalement de la teneur initiale en SA. Ils ont expliqué qu'à des teneurs élevées, une partie de la SA qui n'a pas directement accès à la surface des MP, est piégée par le PLGA et met du temps à diffuser à travers la matrice polymérique (69). Dans d'autres études, il a été montré que la SA est libérée par le gonflement des MP du fait de la diffusion de l'eau. En effet, l'eau diffuse à l'intérieur des MP provoquant la dissolution de la SA et sa diffusion dans le milieu de libération (70).

- ***Phase III :***

Cette phase est caractérisée par une libération plus rapide de la SA. La libération au cours de cette phase est principalement due à une érosion massive du polymère et au gonflement et/ou la déformation des MP (41). Le début de cette phase, se situe au moment où un réseau poreux entièrement continu se forme à l'intérieur de la particule (65). Le gonflement important observé à la fin de la deuxième phase peut résulter de la pression osmotique accumulée dans le système et débute dès que la structure polymérique atteint un poids moléculaire suffisamment faible (41,69,71).

III. Objectifs de recherche

Les MP à base de PLGA représentent un choix intéressant pour contrôler la libération de SA sur des périodes allant de quelques jours à plusieurs mois, tout en assurant une bonne biocompatibilité et une biodégradabilité complète.

Différents types de profils de libération de SA peuvent être observés à partir de MP de PLGA : mono-, bi- ou tri-phasique. Il a été reporté que différents mécanismes peuvent être impliqués dans la libération de SA à partir de MP de PLGA, tels que la dissolution de la SA, la diffusion de cette dernière à travers des pores remplis d'eau, la dégradation et le gonflement du polymère. La prédominance d'un phénomène par rapport aux autres dépend entre autres de la nature et de la teneur en SA, de la taille des MP, et de la technique de préparation des MP.

Cependant les mécanismes de transport de SA à partir de MP à base de PLGA ne sont pas complétement compris. Afin de pouvoir les élucider, ces systèmes doivent être soigneusement caractérisés avant et après l'exposition au milieu de libération. Pourtant, des ensembles de MP de tailles différentes sont généralement étudiés et il est bien connu que la dimension des MP, peut modifier l'importance des processus physico-chimiques impliqués dans la libération. Par conséquent, la cinétique de libération de SA observée à partir d'un ensemble de MP, est la somme de toutes les cinétiques de libération de SA à partir de MP individuelle.

Dans ce contexte les objectifs de ce travail ont été de :

- **(i)** Préparer et caractériser les MP de PLGA, de diamètres moyens différents encapsulant des substances actives modèles : la diprophylline et la caféine ;
- **(ii)** Etudier les cinétiques de libération *in vitro* des substance actives, de dégradation du PLGA, les modifications morphologiques et la variation de la T_v après exposition au milieu de libération, à partir d'ensembles de MP, dans différentes conditions de libération, afin de comprendre les mécanismes qui régissent la libération de la SA à partir de ces systèmes ;
- **(iii)** Etudier les cinétiques de libération *in vitro* ainsi que et le gonflement des MP de PLGA à partir de MP isolées dans le but d'approfondir la compréhension des mécanismes de libération de SA à partir de ces systèmes.

Chapter I:Introduction

I. State of the art

Drug delivery is a system concerned with the formulation and the administration of drug molecules to desired body location. Some drugs have a characteristic minimal effective concentration, below which no therapeutic effects occur, and a characteristic minimal toxic concentration, above which undesired side effects occur (Figure 11), leading to a decrease in the efficacy of the treatment of severs diseases, suggested a growing need for technologies to delivery of bioactives to targets in tissues via many routes of administration including the oral, topical, transmucosal and inhalation routes. From this, new ideas on controlling drug delivery were generated (72).

Controlled drug delivery is the use of systems to release therapeutic agent at a predictable rate. A broad range of bioactive compounds are incorporated into controlled drug delivery systems, from simple molecules to peptides and proteins, antibodies, vaccines, and gene-based drugs. These systems offer the advantages of reduction of drug side effects and reduced unwanted fluctuations in circulating drug levels. The most disadvantages of these systems include their high cost, and sometimes a decreased ability to adjust dosages (72).

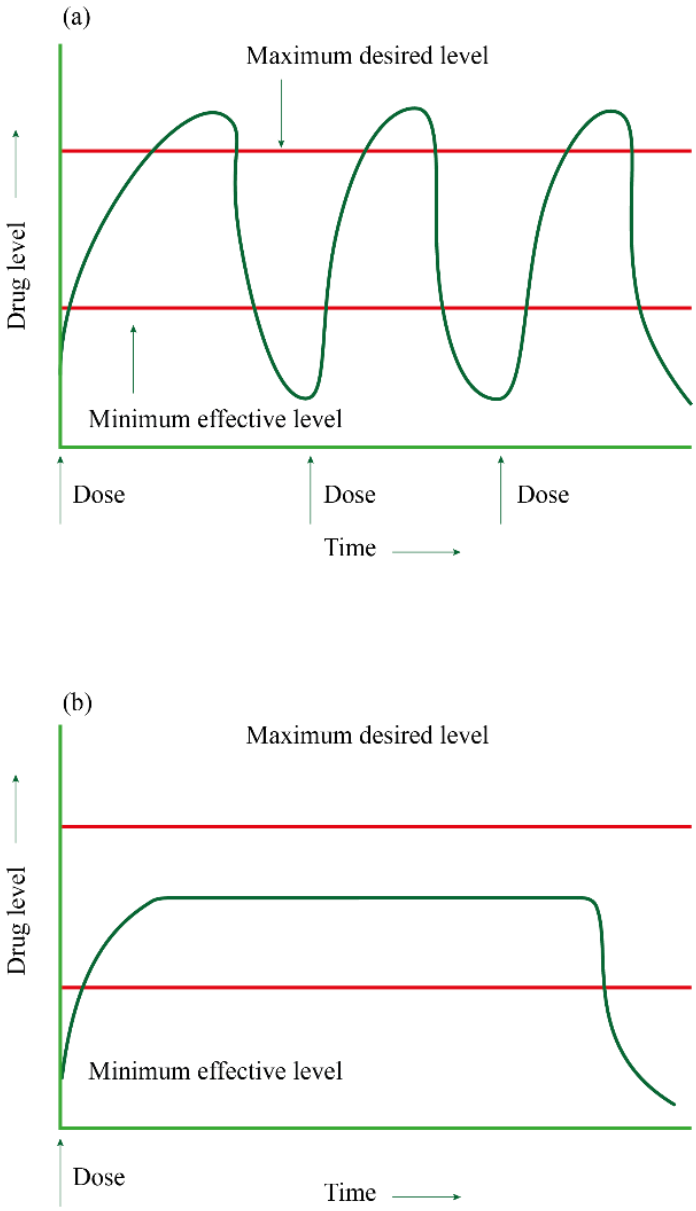

Figure 11: Drug levels in the blood with (a) immediate and (b) controlled drug delivery systems, adapted from Shaik et al. 2012 (73).

With traditional drug administration, the drug level in the blood should remain between a maximum concentration, which may represent a toxic level, and a minimum concentration, below which the drug is ineffective. In controlled drug delivery systems, the blood concentration of therapeutic agents remains constant, between the desired maximum and minimum, for a long-time. Depending on the formulation and the application, this period may be 24h (Procardia XL) to 1 month (Lupron Depot) to 5 years (Norplant). Other advantages of controlled release formulations have been identified:

- The reduction of the frequency of injections, especially those who require daily or long-term treatments.

- The targeting is possible by conjugating a molecule with affinity for a particular tissue to the controlled release systems.
- The increasing of the bioavailability or inhibiting drug resistance development by adding molecules that enhance the effect of drug.

Controlled drug delivery occurs when a natural or synthetic polymer is combined with a drug in such a way that the active agent is released from the materials in a predesigned manner. In order to be used for controlled drug delivery formulations, general requirements for polymer carriers must be respected. These requirements are (8,74):

- Chemical and biological inertia.
- Absence of immunogenicity.
- Appropriate physical structure,
- Easily processable.
- Adequate pharmacokinetics.

Any polymer selected for drug delivery is commonly characterized by its origin (natural or synthetic), chemical nature and stability (biodegradable or no) and water solubility (Figure 12). Natural polymers are abundant and often biodegradable, but their GMP-conform production needs for several purification steps because of their structural complexity. Furthermore, their use is often limited by their high immunogenicity. On the other hand, synthetic polymers are available in a wide variety of compositions with controllable properties. However, synthetic polymers are associated with inflammatory or immunogenic reactions. Several of its synthetic polymers have been particularly used for medical application to ensure controlled drug delivery. Few examples are (15,75):

- Polyesters,
- Polyanhydres,
- Polyglicolides,
- Polyorthoesters,
- Polyphosphazene.

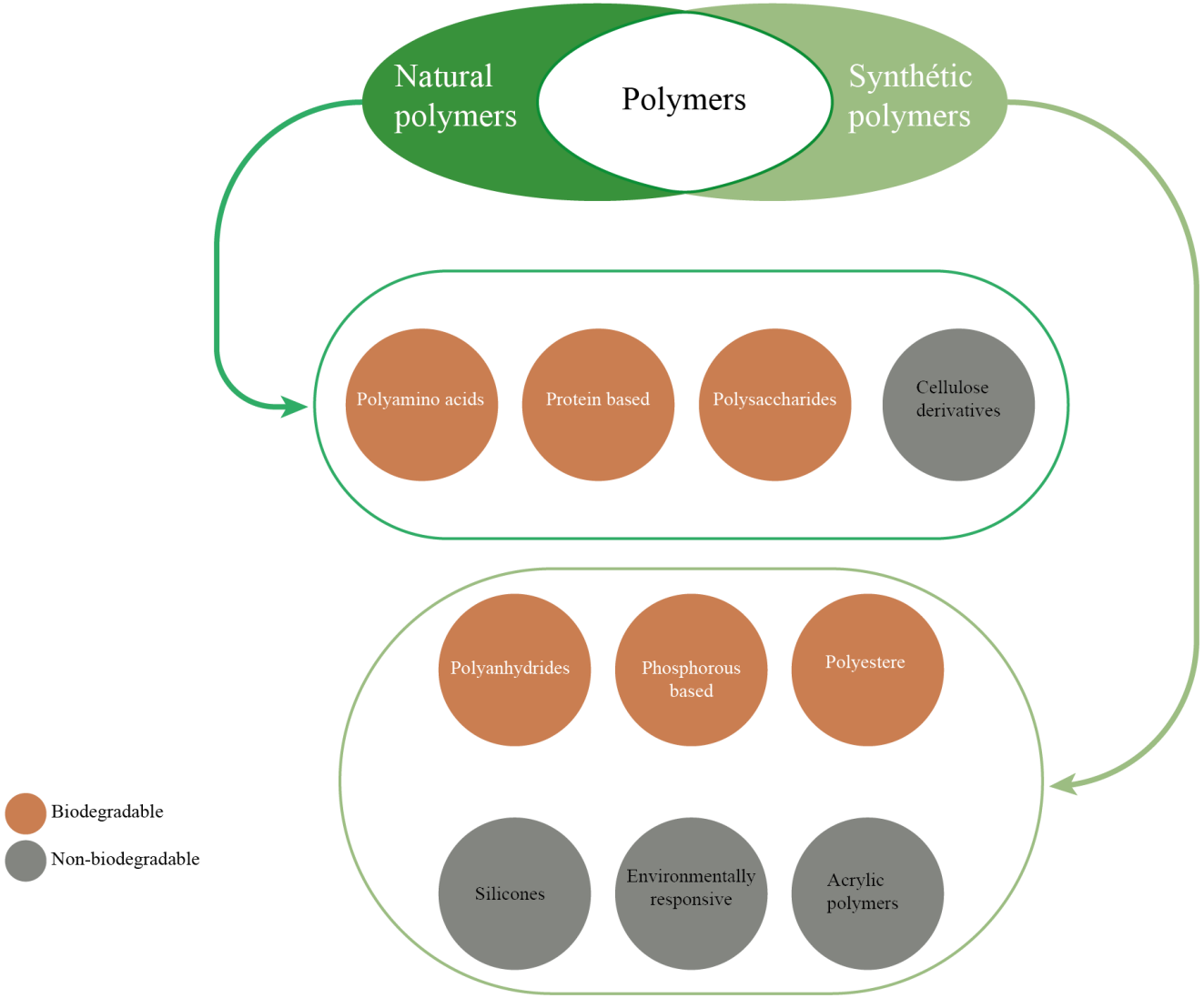

Figure 12: Classification of polymers used in drug delivery systems, based on their origin and biocompatibility, adapted from Grund et al. 2011 (8).

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

II.1.Physicochemical properties of PLGA

Poly (lactic acid-co-glycolic acid) (PLGA) is α-hydroxy acid-derived polyesters. It's a synthetic biodegradable, block copolymer obtained by random melt co-polymerization of lactide and glycolide under high vacuum in the presence of catalyst (Zinc, stannous octoate, tin, antimony…) at 160-190°C (35,76) (Figure 13). Lactides and glycolides are cyclic dimers obtained by dehydration of Lactic acid and glycolic acid. Lactic acid (LA) is a methyl-substituted glycolic acid that can be produced in D and L forms. PLGA generally contains the D- and L- lactic acid forms in equal ratio (36,76). The copolymer composition may be used to manipulate the hydrophilicity and glass transition temperature of the copolymer. Indeed, if lactic acid is used in a higher ratio than glycolic acid (GA); a more hydrophobic copolymers is formed due to the higher hydrophobicity of lactic acid (36,77). PLGA is also available with ester or acid end groups. Those with ester end group are more resistant to hydrolytic degradation (36,78).

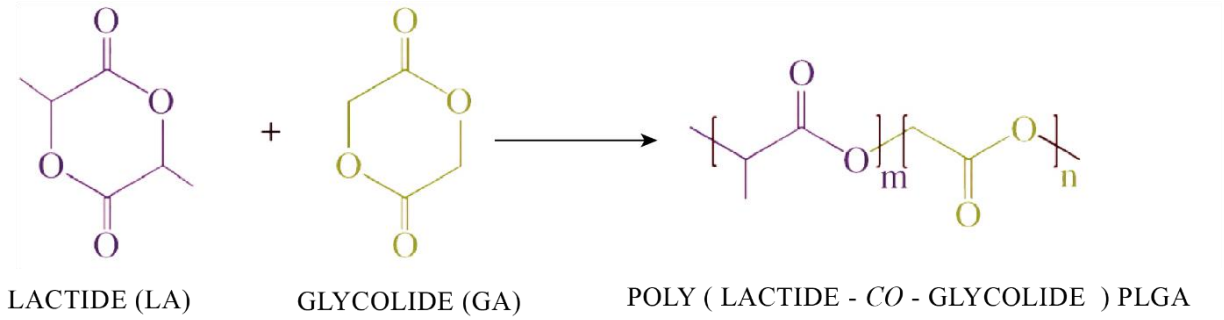

Figure 13: Copolymerization reaction of lactide and glycolide.

PLGA copolymers are amorphous with glass transition temperature (Tg) between 45 and 55°C, above the physiological temperature of 37°C and hence are glassy in nature (36). It has been reported in literature that water can act as plasticizer for PLGA. In this case, the Tg of PLGA decreases which lead to an increase of the mobility of polymer chains, influencing the release kinetics of drugs from PLGA-based drug delivery systems (DDS) (57,79,80).
PLGA copolymers are soluble in wide range of solvents such dichloromethane, tetrahydrofuran, ethyl acetate, chloroform, hexa-Fluoroisopropanol, acetone and benzyl alcohol (76,81).

II.2.Biodegradation of PLGA

Biodegradation is the chemical breakdown of materials in a physiological environment where the material is degraded by enzymes or is hydrolysed (58). Biodegradation can be distinguished in bulk and surface erosion. Since, in surface erosion the biodegradation proceeds only at the surface, the molar mass of the residual polymer remains constant, but a fast mass loss can be observed. In bulk erosion the mass loss is retarded, but the molecular weight drops very fast due to degradation throughout the whole material. The term degradation refers to bonds cleavage whereas in contrast "erosion" refers to depletion of the material and usually follows the degradation process (8,38,58,74). It is known that the degradation of polymers containing very reactive functional groups tend to be surface eroding, whereas polymers with less reactive functional group tend to be bulk eroding (29,38,82).

PLGA is classified as bulk eroding polymer. Its degradation period is between days and years and is function of the polymer's molecular weight and the ratio of lactic to glycolic acids (76). PLGA is degraded through the hydrolytic cleavage of its polyester backbone. The scission of the polymer chains leads to reduction in the molecular weight of polymer and production of water-soluble fragments. those are hydrolysed to lactic and glycolic acids that are no toxic and are eliminated from the body by normal metabolic pathways (38,76,78,83). It has been reported in the literature that the hydrolytic degradation of PLGA can also be catalysed by the acid products generated during the degradation. Those remains strapped in bulk polymer lowering microenvironment pH and causing the autocatalyze of the degradation process (38,58,76).

II.3.Factors affecting the biodegradation of PLGA

Many factors can impact the degradation of PLGA (36,84). The better understanding of these factors allows the development of drug delivery systems with desirable properties.

I.3.1. Effect of polymer composition

The chemical composition of the polymer is the most important factor affecting the biodegradation of PLGA. It directly affects the degradation rate. Indeed, the composition of the polymer determines the hydrophilicity and the glass transition temperature which in turn affect the degradation rate. Previous work provided evidences that the alteration of the chemical composition of PLGA by increasing the glycolic acid ratio increases the degradation rate (85–87). This, can be explain by the fact that glycolic acid units are more hydrophilic than lactic residues, which present an additional methyl group, and thus influences the water uptake (36,84,85,88,89).

I.3.2. Effect of molecular weight (Mw)

Molecular weight of PLGA influences scientifically the physicochemical properties of the polymer. Mw of PLGA used in controlled drug delivery applications ranges typically between 5 and 150 KDa (61). It has been provided that the molecular weight has a direct relation with the degradation rate. Polymer having higher polymer weight have longer chains, which require more time to degrade than small polymer chains (36,61,76). It can be explained by the fact that water hydration of low Mw PLGA immediately allowed the polymer to change from the glassy state to a rubbery state. This cause a rapid degradation (90).

I.3.3. Effect of drug type

The polymer degradation varies depending on the type of drug chosen. If the drug is an acid or a base, its presence might affect PLGA degradation by catalysing the cleavage of ester bonds.

I.3.4. Effect of pH

The degradation of PLGA is generally influenced by the pH of the medium. Indeed, many studies concluded that both alkaline and strongly acidic media leads to polymer degradation.

I.3.5. Effect of size and shape of the matrix

The size of the matrix impact significantly the degradation ratio of PLGA. Many studies showed that the degradation was faster for the large microspheres than for small one (60,87,91). It has been also reported that bulk degradation is faster than surface degradation for PLGA, which makes a rapid drug release from systems with higher surface area to volume.

I.3.6. Effect of enzymes

PLGA is generally degraded by hydrolytic mechanism (84,87,89). Many works have suggested the possibility that enzymes present in the physiological fluids can catalyse the degradation of the polymer (92,93). Various enzymes, such as tissue esterases, pronase and bromelain, have been investigated in order to clarify their effect on the degradation rate of PLGA (93). Conflicting results are found in literature. Some works indicated that enzymatic degradation play an important role (92,94), whereas most of others suggested that the enzymatic degradation mainly happened on the polymer surface due to the hydrophilic nature of enzymes. Indeed, a hydrophilic enzymes diffuse with difficulty into hydrophobic polymer (93,95). There is a clear difficulty in comparing and explaining the effect of enzymes due to the lack of standardization in *in vivo* studies (36,93,96).

II.4.Biocompatibility of PLGA

PLGA systems were approved by the US FDA for several therapeutic applications because of their biodegradability, biocompatibility and sustained-release properties. PLGA has been used for the first time in the 1960s as absorbable sutures and monofilament (76,78,97,98). It's generally considered as "gold standard" of biodegradable and biocompatible polymers for controlled drug delivery systems (16,78). However, some studies suggest that the majority of synthetic polymers could cause severe inflammatory or immunogenic reactions, which limits there use (8). The size, shape, chemical and physical properties of the biomaterials may be responsible for variation in the intensity and the duration of this inflammatory.

The inflammatory response is usually initiated by the implantation procedure wich in the case of microparticles involves injection of the formulation within a solvent vehicle (84). The immune response takes place in three phases (84):

- Phase one: occurs within the first two weeks following injection. It's characterized by the initiation, resolution and organisation of acute and chronic inflammatory responses. A presence of polymononuclear leucocytes, lymphocytes, plasma cells and monocytes is observed.
- Phase two: is initiated by the predominance of monocytes and macrophage. It's caracterized by the migration of monocytes into the site of injury. They differentiate into macrophages which in turn fuse to form the fibrous capsle. During this phase, the Mw of the polymer decrease to the point where the integrity of the microparticles can no longer be maintained.
- Phase three: is characterized by the breakdown of the microparticles into particles. Studies conducted by Anderson *et al*. showed that during this phase, microparticles are reduced in size to less than 10 µm (99,100). The formation of these particles initiates a tissue response. The fibrous capsle formed during the phase II becomes fibroblasts.

II. PLGA microparticles

II.1.Microparticles characteristics

Many systems can be formulated using PLGA. Its systems include: microparticles (MP), nanoparticles (NP), nano-suspensions, hydrogels, cylinders, scaffolds, foams and implants (36,41,57,61,71,79,80,88,89,101,102). They are administrated by parenteral, pulmonary, oral or nasal routes (49). PLGA-based drug delivery systems have been extensively studied. They can be applied in many fields, that is why, they represent a growing techno-economic sector.

PLGA microparticles are the most common category of PLGA-based drug delivery systems (41). In the recent literature, the term microparticles refers to micrometre-sized drug delivery systems having a size between 1 and 1000 µm. MP prepared with the conventional techniques are usually spherical, accept for those fabricated using microfabrication, wherein the particle shape and structure can be controlled (103,104). Depending on the need of the application, PLGA microparticles can be used to deliver drugs to a whole system or a target site. Application methods include (103).

- Systemic delivery: oral, intravenous and pulmonary delivery.
- Local delivery: lymph node, temporomandibular joint and cartilage delivery.

Microparticles are usually administered parenterally, mainly intramuscular and subcutaneous, for which the desired micro-articular size is between 20 and 100 µm. Smaller particles having a size less than 10 µm are necessary, if the microparticles are passively targeted to phagocytic cells. MP have been broadly studied as polymeric matrix for various therapeutics, including antigens, DNA, RNA, proteins, drugs, vaccines and cells thanks to their properties such as their degradation rate, size, shape, targeting moiety, and porosity (103).

MP are classified into two major categories as illustrated in
Figure 14 (43,45,105,106):

- Reservoir system (microcapsule): it consists of a drug depot surrounded by a release rate controlling barrier membrane (polymeric shell).
- Matrix system (microsphere): characterized by a no local separation between a drug reservoir and a release rate controlling barrier.

For both types of devices, two subclasses can be distinguished depending on the initial drug concentration (below (a) or above (b) drug solubility in the system) (43,45) on:

- ***Solution:*** when all the drug molecules are dissolved in the polymeric matrix, or the drug is rapidly and completely dissolved upon water penetration into the system.

- ***Dispersion:*** when the initial concentration of the drug exceeds drug solubility. However, dissolved and non-dissolved drug co-exist within the matrix during drug release but only partially dissolved drug diffuses out of the system upon water penetration into the matrix.

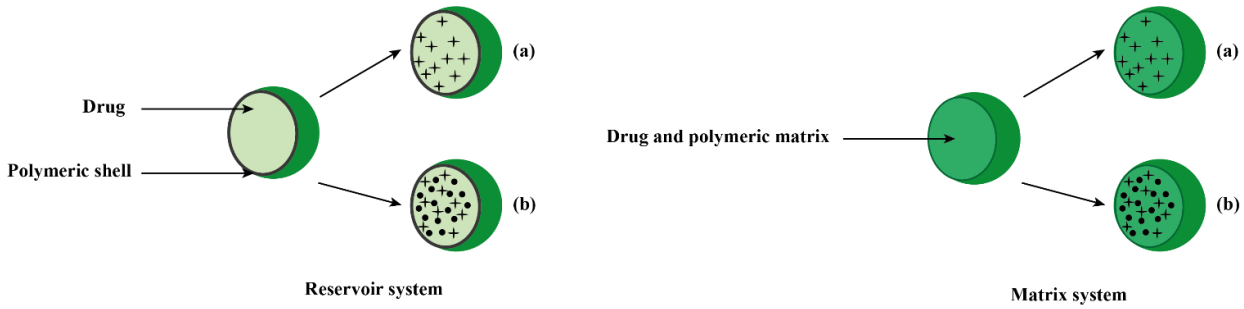

Figure 14: schematic representation of microparticles. (a) dissolved drug, (b) dispersed drug: stars represent drug molecules, black circles represent drug crystals, adapted from Siepmann et al. 2008 (45).

This pharmaceutical system offers several advantages over other forms, including (31):

- Controlled drug release rate from few days up to several months in order to reduce the frequency of administration (compared with conventional dosage forms).
- Easy administration using standard needles and syringes after redispersion in adequate medium.
- Complete biodegradability, and good biocompatibility.
- The ability to achieve certain target tissues that are normally inaccessible by some active substance.
- The ability to administer drugs directly at the site of action, reducing the administration drug concentration as well as the risk of serious side effects.

II.2.Microparticles preparation techniques

Several methods of encapsulation techniques have been used to fabricate PLGA microparticles. The preparation of MP of the desired size, surface charge, encapsulation efficiency, and release characteristics, requires precise control of synthesis parameters. Most of the methods are the modification of three conventional techniques: solvent extraction/evaporation, phase separation (coacervation) and spray-drying (31,32,49,53,107–110). The microencapsulation method employed must include the following requirements (15,31,53):

- The biological activity and chemical stability of the drug must be maintained.
- The encapsulation efficiency and the yield must be high.

- The MP must have a reasonable size range (<250 µm) facilitating the parenteral administration.
- The drug release profile should be reproducible without burst effect.
- The obtained MP must have an excellent flowability, facilitating the preparation of a homogeneous suspension.

II.3.1. Conventional preparation techniques

II.3.1.1. Solvent extraction/evaporation techniques

Solvent extraction/evaporation technique is the most studied method of fabricating microparticles (Figure 15). The single emulsion oil-in-water (O/W) is an example of Solvent extraction/evaporation techniques. In this method, PLGA is dissolved in organic solvent. The drug is then added to the polymer solution to produce the organic phase. This organic phase is then emulsified by using high speed homogenizer or ultrasound. in a large volume of water phase which contains emulsifier or surfactant. In order to harden oil droplets and obtain solid microparticles, the emulsion is subjected to solvent removal by evaporation (at reduced/atmospheric pressure and a low stirring speed) or extraction process (transferring the emulsion to a larger volume of water). The obtained particles are then washed and freeze-dried. This method is widely used for hydrophobic or water-insoluble drugs. However, for hydrophilic drugs, this method is not efficient as the drugs rapidly diffuse into the aqueous phase [51]. In this case, a double emulsion process water-in-oil-in-water (W/O/W) method is the most appropriate method. The drug is dissolved in an aqueous solution (W1) and the polymer is dissolved in an organic solvent (O). W1 is then dispersed into organic phase (O). The obtained emulsion (W1/O) is then dispersed into a second aqueous solution (W2) containing surfactant. The double emulsion is formed thanks to appropriate mixing conditions. As the Single emulsion technique, microparticles are obtained after the evaporation step. The obtained microparticles undergo the same treatment as in the case of simple emulsion (washing and freeze drying) (31,32,36,50,76,107,109–111).

In addition to the O/W and W/O/W emulsion methods, there are other emulsion methods that have been used to counteract their disadvantages. The first method, called oil-in-oil method (O/O), includes dissolving drug and polymer in an organic phase. The first organic phase is dispersed into a second organic phase. This method is used to reduce the diffusion of hydrophilic drug from oily droplets (112). The second one, solid-in-oil-in-water (S/O/W) is an emulsion method that is often used for the encapsulation of proteins to avoid problems of chemical stability. During this method, the solid drug is dispersed into the polymer in an organic

solvent that is then dispersed into a large volume of water. The protein is more stable when it is in the solid state than in the molecular state (109,113,114).

However, solvent extraction/evaporation techniques are associated with some problems. Indeed, these methods use high shear stress during homogenization which might be disadvantageous for fragile drug, such as proteins (114,115). In addition, these methods offer less precise control of size homogeneity, drug loading, reproducibility and drug release kinetics (107,110).

II.3.1.2. Coacervation technique

The coacervation technique called also liquide-liquide phase separation technique consists of deacreasing the solubility of the polymer by addition of an organic nonsolvent into organic solution that contains the polymer (dissolved) and the drug (dissolved or dispersed). The organic nonsolvent is added to the system with stirring, which extract gradually the polymer solvent. A phase separation is created and a coacervates of drug containing droplets are formed. This system is then transferred to a large quantity of another organic nonsolvent to harden droplets and form microparticles. The obtained microparticles are subsequently collected, washed and dried. This process is used for hydrophobic or hydrophilic drugs. Howerver, this method is widely used for water-soluble drugs as proteins and vaccines, because it is a method during which a non-aqueous phase is used (31,36,76,110). However, coacervation technique presents some disadvantageous. Indeed, in addition to the use of a large amounts of organic solvent during the process and the difficulty to remove residual solvent from the final product, the agglomeration is a frequent problem due to the absence of emulsifier or surfactant (31).

II.3.1.3. Spray-drying technique

Spray-drying is a rapid method with only a few processing parameters. It is used to encpsulate both hydrophilic and hydrophobic drugs. However, it has been intensively studied for protein encapsulation in order to improve the chemical and biological stability of these macromolecules. Spry-drying is based on spryaing water-in-oil (W/O) or solid-in-oil (S/O) dispersion into a stream of heated air through a noozle. This method can be used to produce reproductible uniform microparticles. Contrary to the methods cited bellow, the spray-drying process is very rapid, suitable for scalling-up, volves mild conditions, and is less dependent on the solubility parameter of the drug and the polymer. However, this method presents a serious drawbacks: the formation of fibers due to the insufficient force availible to breack up the polymer solution (31,116), the possibility of the degradation of the drug due to the high

temperature that is used during fabrication (109,117,118). But the main disavantage of this tehnique is a significant loss of the product during spray-drying caused by the agglomeration or the adhesion of the microparticles to the wall of the apparatus, which can be resolved with the use of an anti-adherent (eg. Mannitol) (31,107,119)

II.3.2. Novel preparation techniques

II.3.2.1. Microfluidic platforms

Microfluidic method is a coaxial capillary flows technique which allows for control of both the size and shape of the particles The processing parameters, such as orientation of jets, material flow rates and rates of solvent extraction can be controlled to produce uniform microparticles with simple or core-shell structure. These micorparticles are prepared using single or multiple emusions as templates (120). Additionnally, microfluidic devices can incorporate the use of electrostatic forces to control the size and morphology of particles leading to a predictable release profile (121). Two continuous and imiscible streams are infused via two separate inlets. Monodisperse droplets are generated at the junction where the two streams meet due to the high shear stress (107). By introducing the second stream, droplets may be re-encapsulated which is useful for preparing core-shell structures (111). The obtained microparticles have a size between 20 and 100 µm (122). The microfluidic method presents many advantages (109–111):

- Multiple components are generated by a single step emulsification.
- Ultra-small quantities of reagents are used.
- Drug loading, size, morphology, shell-thikness and drug release profiles are precisely controlled.

II.3.2.2. Microfabrication

Microfabrication offers a way to produce homogeneous monodisperse particles with different shape, for example, cubic particles. This method uses templates to produce monodisperse particles. The used templates can be fully removed by dissolving them in aqueous solutions once the particles are formed. The microfabrication allows to produce particles having a size between 10 nm and 200 µm. Many methods were developed:

- Particle replication in nonwetting templates (PRINT) (123).
- Microcontact hot printing (124).
- Step and flash imprint lithography (125).
- Hydrogel template (126).

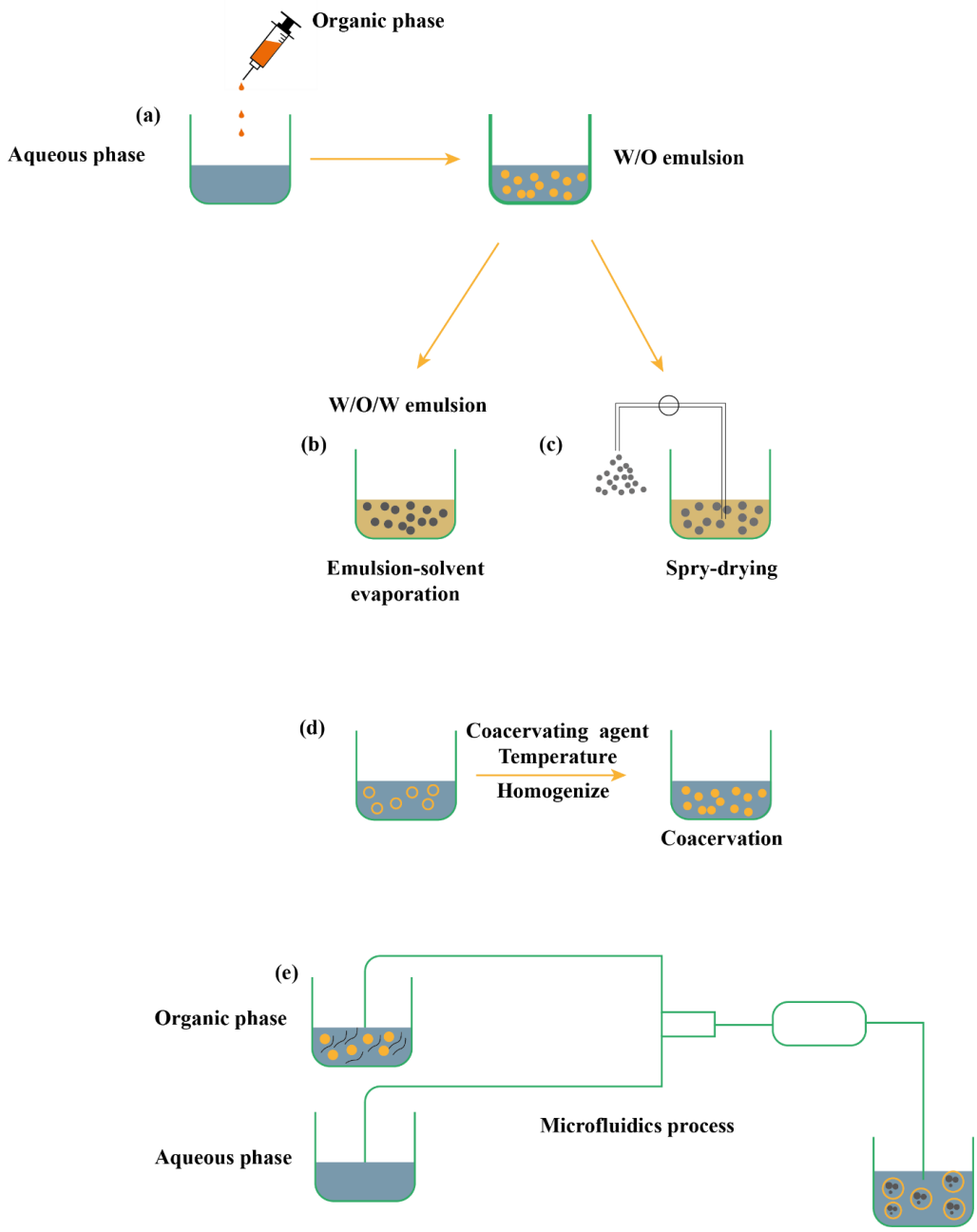

Figure 15: Schematic representation of preparation methods for PLGA MP, including (a) W/O emulsion, (b) W/O/W emulsion, (c) spray-drying technique, (d) coacervation technique and (e) microfluidic process, adapted from Ding & Zhul. 2018 (110).

II.3.Process parameters influencing the microparticles characteristics

The microparticles properties as size, shape, internal morphology, drug loading, and drug distribution can be influanced by the preparation procedure. However, some of the most important microparticles characteristics will be briefly described: *size, porosity and encapsulation efeciency*. These parameters are estimated as the key parameters in the drug release contol.

II.3.1. Process parameters affecting microparticle size

For the delivery of microparticles through injection (through a fine needle), the size of microparticles are crucial. It can be controlled by different methods, depending on the fabrication technique used. For microfabrication, the size of particles can be easily tailored by the template (107,123). For emulsificatioin solvent extraction/evaporation, the size can be controlled by adjusting (32,127,128):

- Emulsifier concentration: a higher concentration of emulsifier causes a faster saturation of droplets surface and contributes to reduce droplets coalescence and collision by increasing the aqueous phase viscosity. Thus, leading to smaller particles diameter (113,129,130). Polyvinyl alcohol (PVA) is the most commonly used emulsifier for the preparation of PLGA particles due to its excellent interaction with PLGA surfaces (131). It has been proven that an increase in the PVA concentration from 0.5% to 2% or its molecular weight leads to smaller microparticles (132–134).
- Temperature: preparing microparticles at low temperature leads to change the viscosity of both the aquouse and organic phase, and therefore the particle size. However, the solubility of the drug and the solvent evaporation can be altered (135).
- Polymer concentration: the droplets break-up is controlled by forces which are dependent on the viscosity of the polymer phase. A higher o-phase viscosity due to an increasing in the polymer concentration or polymer molecular weight result in increased particle size (128,136,137).
- Organic phase volume: When the amount of the polymer is kept constant and volume of organic phase increases, smaller particles are obtained, due to the reduction of the organic phase viscosity (129)
- Strirring speed: in order to divise the organic phase into smaller droplets, shear forces are applied to the system. By increasing the intensity of these forces, the particle size decreased (129).

The polydispersity of the obtained microparticles also depends on the fabrication method used. Spray-drying and microfabrication result in uniform particles (119). However, the emulsification methods usualy produce particles with a high polydispersity. The size and the dispersity of particles can be determined with different methods (32):

- Microscopy including light and electron microscopy.
- Coulter principle.
- Laser diffraction.
- Dynamic light scattering.
- Wet sieving of the particles through a column of sieves.

II.3.2. Process parameters affecting microparticle porosity

Process parameters during fabricating microparticles can affect the internal and external morphology of particles. Indeed, porosity is an important characteristic, which can affect and control the drug release (64,109). For the emulsification solvent extraction/evaporation method, the rate of solvent extraction, which depends on the stirring speed; the droplets size; the temperature; and the dispersed phase have an effect on the porosity. The porosity usually increases with a decrease in solvent extraction rate. The relationship between the extraction rate and particle morphology is as follows (138) (Figure 16):

- When the extraction is slow, water influx from the continuous phase into the polymer phase create water-filled channels and pores. This leads to create small and dense microparticles.
- When extraction is fast, the organic phase will instantly solidify upon contact with the aqueous phase without any change in size and morphology. Here, the solvent will be removed by quick solvent extraction into the continuous phase.

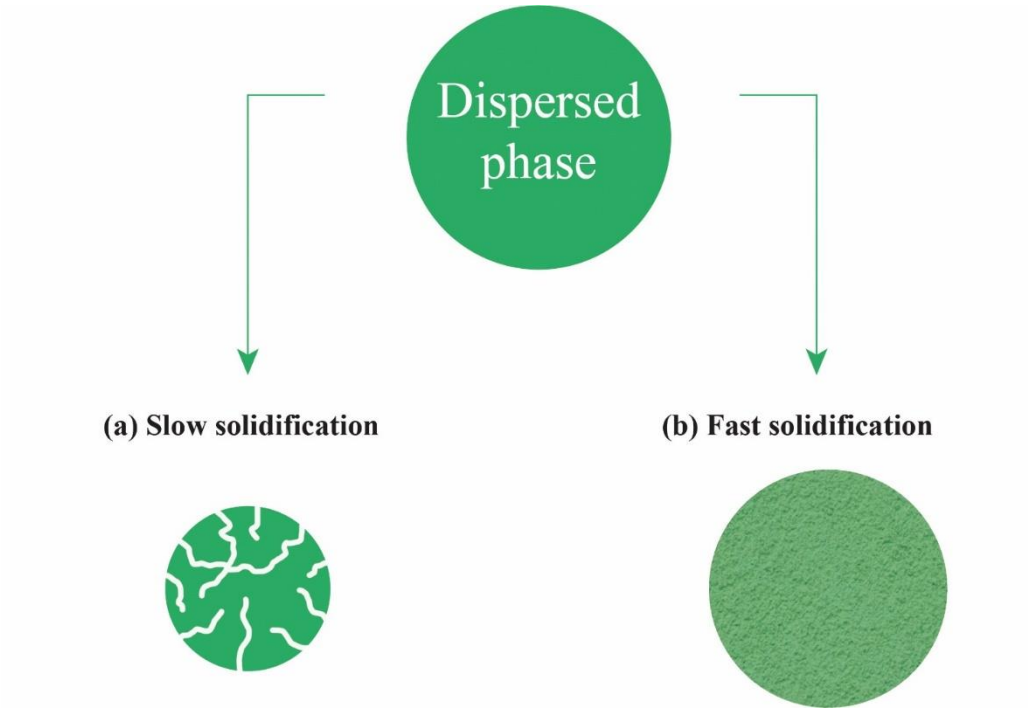

Figure 16: Schematic representation of the relation between solidification and MP morphology change, adapted from Yeo & Park, 2004 (138).

The microparticles drying process also has an impact on the internal morphology of microparticles. The drying procedure (e.g. freeze-drying) removes not only the aqueous phase and wash fluid adhering to the particles' surface but also trace of solvent and aqueous phase from the interior of the particles. Thus, the drying influences the microparticles morphology and porosity (49,60,139).

Porosity can be created as an artefact of the fabrication technique such as the loss of absorbed oil after solidification or during the freeze-drying step, or can be intentional. In the last case, porosity can be created using porogens such as gelatine and PBS or gas-forming agent such as ammonium bicarbonate. These modifications are intended to lowering the density of the particles (140) or facilitating cell attachment, proliferation and targeting (141). The internal morphology and the porosity are usually determined by scanning electron microscopy (SEM) or by confocal micrographs (64,142).

II.3.3. Parameters affecting encapsulation efficiency

A very important property should be evaluated when fabricating microparticles is the pourcentage of drug loaded and the pourcentage of encapsulation effeciency. These two parameters can be determined by the equation below:

$$Encapsulation\ efficiency\ (\%) = \frac{Practical\ drug\ loading}{Theorical\ drug\ loading}\ x\ 100$$

$$Practical\ drug\ loading\ (\%) = \frac{Weight\ of\ drug\ in\ sample}{Total\ weight\ of\ simple}\ x\ 100$$

For the emulsification method, processing parameters such as polymer nature, organic phase volume, aqueous phase volume, polymer concentration and stirring speed can alter this important characteristic (127,134,143). Microparticles with smaller size usually have a short diffusion pathway; thus, drugs can easily diffuse out of the particles during the solvent evaporation process. This, leads to a low encapsulation efficiency (144). However, Wu et al. observed a faster particle solidification due to a rapid evaporation of dichloromethane in the case of very small microparticles, which reduces the drug diffusion and consequently, increases the encapsulation efficiency (145).

For determining the encapsulation efficiency, the microparticles are first dissolved in an appropriate solvent, such as methylene chloride (146), acetonitrile (ACN), acetone, tetrahydrofuran (THF) (147) or dimethyl-sulfoxide (DMSO) (148). An additional extraction is necessary, if the drug is not dissolved in the first solvent (32,109). The obtained solution, can be then analysed by an appropriate analytical method (HPLC, UV/VIS or fluorescence spectroscopy).

III. In vitro drug release from PLGA microparticles

The term *drug release* refers to a complex phenomenon involved on the transport and the release of drugs from a dosage form (41,149). It is impossible de list all potentially involved phenomenon, but Siepmann et al., (38,45) cited a few of them:

- Wetting of the system's surface with water.
- Water penetration into the device (e.g., via pores and/or through continuous polymeric networks).
- Phase transitions of (polymeric) excipients (e.g., glassy-to rubbery-phase transitions).
- Drug and excipient dissolution.
- Drug and/or excipient degradation.
- Creation of water-filled pores.
- Pore closing due to polymer swelling.
- Creation of cracks within release rate limiting membranes.
- Creation of acidic or basic microenvironments within the dosage forms due to degradation products.
- Physical drug-excipient interactions.
- Diffusion of drugs and/or excipients out of the dosage form with potentially time- and/or position-dependent diffusion coefficients.
- Penetration of acids, bases or salts from the surrounding bulk fluid into the drug delivery system.
- Changes in the device geometry and/or dimensions.
- Creation if significant hydrostatic pressure within the delivery system.

Fredenberg et al. identified three possible ways for drug to be released from PLGA-based systems: (i) diffusion through water-filled pores, (ii) diffusion through the polymeric matrix and (iii) degradation or erosion of polymer matrix. However, The mass transport mechanisms controlling drug release from PLGA microparticles can be rather complex, including other types of Physico-chemical phenomena, such as water penetration into the system, drug dissolution (149), drug – PLGA interactions (90,101), the creation of water-soluble monomers and oligomers and the latter's diffusion into the surrounding bulk fluid, PLGA swelling, pore closure effects (150) and osmotic effects due to the presence of water-soluble compounds within the systems (151). Due to the complexity of the system, it is not always clear to know which of the process is dominating.

III.1. In vitro drug release mechanisms

III.3.1.Diffusion through water filled pores

The diffusion through water filled pores is very depends on the porous structure of the polymer and the processes that promote pore formation and closure (41). These pores must be continuous from the drug molecules to the surface of the system and sufficiently large to allow the solute to pass through (41). In many studies, the diffusion through water filled pores has been used to describe the first stage of the release period, before the onset of polymer erosion (152–154). However, other studies, mentioned that the pores are formed by erosion. Indeed, these pores are created as polymer degrades and generates small monomers and oligomers which diffuse out of the particles generating interconnected pores that provide a route of escape for drug (155,156) (Figure 17 b).

III.3.2.Diffusion through the polymer

The diffusion through the polymer is possible for hydrophobic drugs of low molecular weight (64,157). However, the drug must be dissolved in water before being released, and this process could decrease the overall release rate (43,149). The diffusion is not dependent on the porous structure of the system but the physical state of the polymer is the most important parameter. Indeed, the diffusion coefficient increases at the transition from the glassy to the rubbery state (71,158,159). It can be explained by the fact that the Tg of the original polymer is above 37°C but upon exposure to water, the plasticizing effect of water transfers the polymer into the rubbery state (57,79). The diffusivity is often higher in polymers with low Mw, because of the high flexibility of the polymer chains (29,32,79) (Figure 17 a).

III.3.3.Degradation/Erosion of polymeric matrix

The erosion is the chain scission process by which polymer chains are cleaved into oligomers and monomers (58). It is a rate controlling release mechanism during the final period of drug release and the main release mechanism for low Mw PLGA formulations (114,160,161). It has been reported that during the first time of degradation, the Mw of the polymer decreased rapidly without a significant weight loss of the observed PLGA microparticles. The Significant weight loss started only after the critical Mw of 15 KDa was reached (162–164). This critical Mw was found to be identical for all polymer investigated. However, the time-period to reach the critical Mw is dependent on the polymer composition and initial Mw (162) (Figure 17 d).

III.3.4.Swelling of polymeric matrix

The swelling is the mechanism that controlled the drug release at the end of the second phase. Indeed, Gasmi et al., have shown that substential microparticles swelling coicided the begening of the third drug release phase (rapid) from PLGA microparticles (69,71,165). This swelling might result from the osmotic pressure created within the system and generated by the accumulation of the shorter chain degradation products which are dissolved. The swelling starts as soon as the polymeric structure becomes sufficiently weak (69,71,165,166) (Figure 17 c).

According to Siepmann et al., there are two most important consequences of polymer swelling in a controlled release matrix system (43):

- The length of the diffusion pathways increases, resulting in dacreasing drug concentration and , thus potentially decreasing drug release rates.
- The mobility of the macromolecules significantly increases, resulting in increased drug mobility and, thus potentielly increasing drug release rates.

Depending on the type of polymer and type of drug delivery system, one of these effects potentieally dominates, resulting in decreasing or increasing drug release (43).

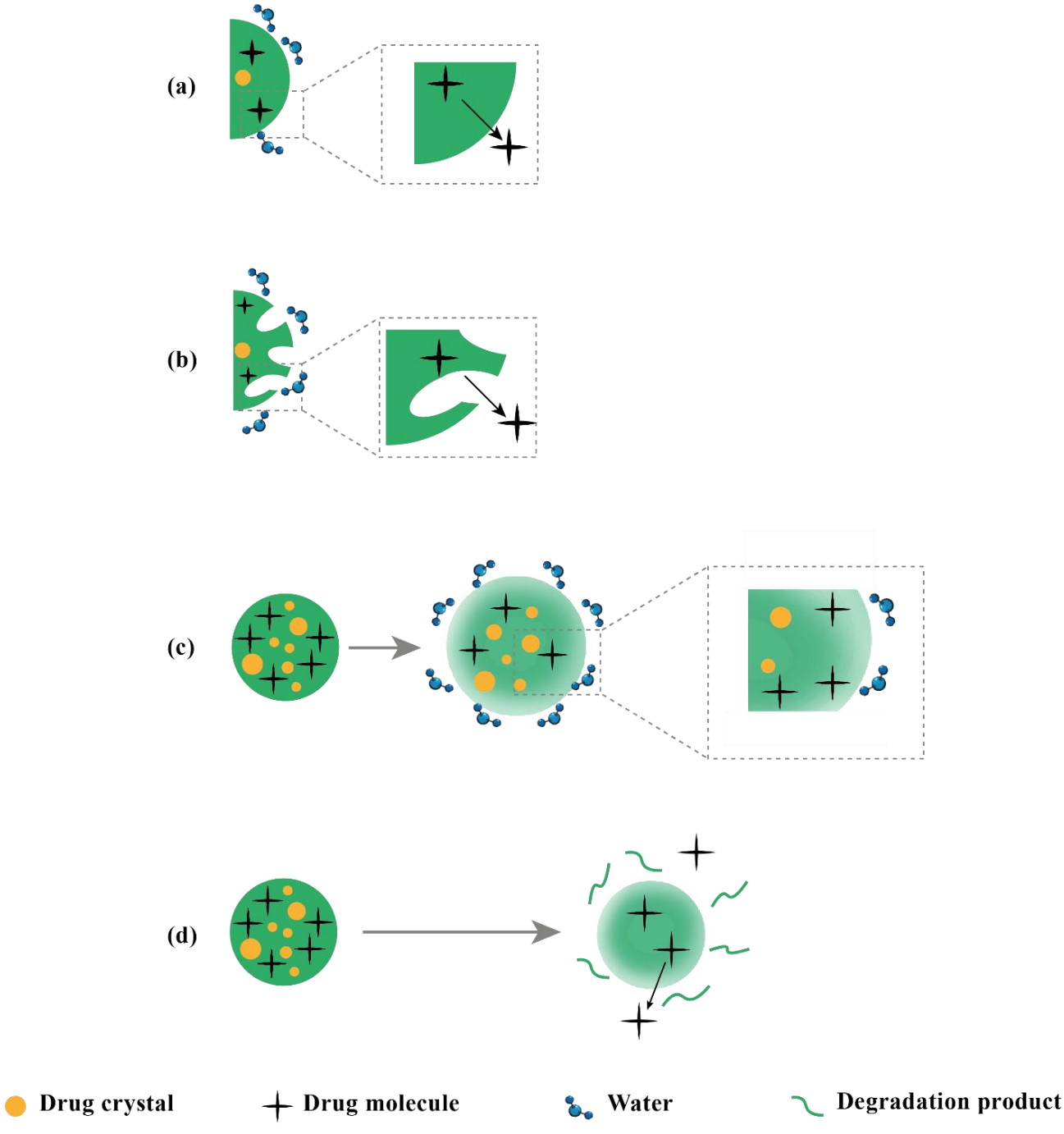

Figure 17: Schematic representation of drug release mechanisms from PLGA microparticles. (a) represent diffusion through polymer, (b) diffusion through water filled pores, (c) swelling of polymeric matrix and (d) degradation/erosion of polymeric matrix, adapted from Fredenberg et al. 2011 (41).

III.2. In vitro drug release profiles

Different types of drug release patterns can be observed from PLGA-based microparticles. Figure 18 shows the characteristics of three typical release profiles described in the literature: The monophasic profile, the biphasic profile that is characterized by the initial burst followed by a saturation, and the triphasic release profile which is composed of three phases. Phase I is often described as a burst release phase, phase II is commonly known as a period of a slower release and phase III is described as a rapid release period (41,61).

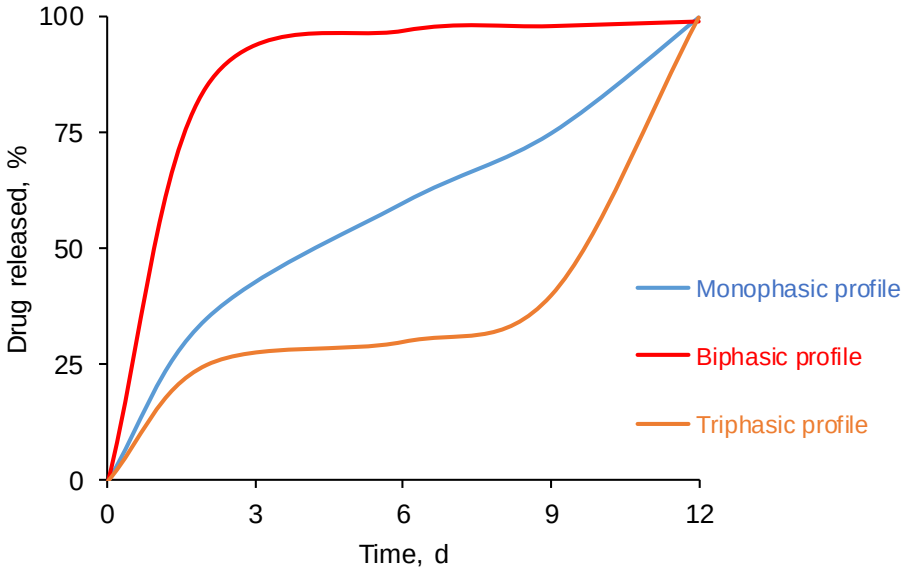

Figure 18: Schematic representation of three release profiles obtained from PLGA MP.

The monophasic profile is rare. Usually, drug release from PLGA microparticles is bi-phasic, but tri-phasic profile is the most common (41). The release of drug from PLGA microparticles obtained by extraction/evaporation solvent method is typically tri-phasic with a period of burst release, dormancy, and finally a rapid drug release (second burst) (65).

- ***The 1st Phase (Burst release):***

In many formulations, upon placement in the release medium, an initial rapid drug release is observed before the release rate reaching the plateau. This phenomenon is typically referred as "burst release" (63). It can be defined as the amount of drug that releases from microparticles prior the polymer-erosion starts (64). The burst release is mainly attributed to the diffusion of the dissolved drug which is adsorbed to the surface of the particle or the diffusion of the drug molecules through water-filled pores in direct contact with the surface of

the particle (65,69,167–173). Previous works suggested that the burst period ends once the material near the exterior of the microparticles is removed (65,69).

Recent researches have focused on the development of strategies to control the burst effect. For example, by altering the preparation method, Fu et al., obtained a homogeneous distribution of drug within the polymeric matrix due to the dissolution of both polymer and drug within the single-phase solution (174). Drug solubility in the mixed solvent system was further improved by increasing its hydrophobicity upon complexation with ionic surfactants. This modification leads to eliminate the initial burst of the drug (174). Some publications review other existing approaches, among those strategies (63,64,175–179):

- Modification of the drug (salt form of drugs may be changed, covalent modification of drugs with appropriate compounds…).
- Co-solvent system.
- Complex formation.
- The use of other excipient to modify the release of drug.
- Polymer modification.
- Surface modification.
- Surface extraction.
- Coated surfaces.

- ***The 2nd Phase:***

Also called lag-phase. It is characterized by a slow diffusion of the drug through the relatively dense polymeric matrix or the few existing water-filled pores (66). This phase may be caused by pore closure (150,180), polymer-drug interactions or drug-drug interactions (181). Many factors have been found to induce pore closure. Some of these factors are: polymer degradation, the addition of plasticizing agents or increased temperature (67,68,182). The duration of this phase is dependent mainly on polymer characteristics (e.g. Mw, lactic acid: glycolic acid ratio, etc.) and formulation size/geometry (42). In some systems, the lag-phase is negligible, due to a fast degradation of PLGA (85).

In a study on the release of octreotide acetate from PLGA-based microparticles, a slow release was observed over the first 24 h of drug release (62). This phase was correlated with the formation of a non-porous film at the surface of the microparticles following a polymer rearrangement. This polymer rearrangement was the origin of the reduction in surface porosity, the closing of pores at the surface, and the formation of a skin layer (62,77,183).

In another study, Berkland et al. hypothesized that the formation of a dense polymer skin and the closing of pores may be a result of the high concentration of rhodamine distributed toward the surface of the microparticles (184). Indeed, the hydrophilic rhodamine localized in the surface causes a rapid water uptake which leads to a rapid polymer degradation forming a porous structure near the surface. Continued polymer degradation produces a rubbery PLGA at or near the surface due to a decrease in polymer glass transition temperature, producing a dense skin covering the eroding interior (184). In an another study on the release of leuprolide acetate from PLGA-based microparticles, SEM pictures showed that the interior of the microparticles is porous while the surface remained non-porous during the second release phase (66). It is logical to assume that the low porosity at the surface was the raison of the slow release.

The co-polymer composition was also shown to be important in controlling the release rate from PLGA-based microparticles. It has been suggested that the length of the lag phase during the release profile of macromolecules from PLGA-based microparticles is dependent on the rate of polymer degradation (185). Consequently, the release profiles of macromolecules from microparticles depend on the co-polymer composition and the Mw of PLGA (42,185). Indeed, published works reported that the length of the lag phase and the duration of protein release increased with higher Mw of PLGA and with lower glycolide content. This can be explained by the fact that this polymer takes up water easier and degrades slower (157). It has been also proved that, blending a low MW-PLGA with a high Mw-PLGA could be used in order to reduce the lag phase. Indeed, the lag-phase was reduced from 1 month to approximatively 15 days by blending 25 KDa PLGA with 75 KDa PLGA (158).

Gasmi et al. provided evidence that the second drug release phase depends mainly on the initial drug loading (69). They explained the release of dexamethasone from PLGA-based microparticles prepared by an oil-in-water solvent extraction/evaporation method as follows: at very high drug loadings, a part of the drug which does not have a direct access to the surface of microparticles is effectively trapped by the PLGA and takes time to diffuse through the polymeric barrier. Importantly, a saturated drug solution is most probably provided within the system. This could be explained by the very limited amounts of water available for drug dissolution in the PLGA-based microparticles at this phase (69). These saturated drug solutions combined with sink conditions provided outside the microparticles lead to about constant drug concentration gradients (69,186,187). Consequently, a constant drug release rates ("Zero order phase") is observed (43).

- ***The 3rd phase:***

This phase is characterized by a faster release of the drug. It is sometimes called the second burst. The release during this phase is caused mainly by a massive erosion of the polymer and the swelling /deformation of the microparticles (41,188). However, the beginning of the second burst was attributed to the point at which a fully continuous porous network had formed within the particle (65,189). In other studies, it has been confirmed that, the third phase starts when the autocatalytic degradation occurs because of limited diffusion of soluble degradation products during the lag-phase. In fact, a sufficiently, acidic environment can be created in the interior of the particle to cause essentially the complete degradation of polymer in the core (91,128,156,190). Another study confirms the role of pore closing/opening in PLGA-based microspheres on drug release. Indeed, the dissolved drug and polymer degradation products cause increased osmotic pressure (90,151). This phenomenon may lead to a polymer rupture. Consequently, previous isolated pores become open and drug molecules are released (150).

III.3. Factors influencing the in vitro drug release

The drug release from PLGA microparticles depends on many factors such the microparticles structure, the physicochemical properties of the polymer and the chemical properties of the encapsulated drug. The matrix structure, particularly porosity and size, is strongly affected by the formulation parameters. Furthermore, the *in vitro* conditions used to evaluate drug release kinetics may significantly influence the resulting drug release patterns. This section presents briefly the key factors described in the literature that may alter the *in vitro* drug release profiles from PLGA microparticles.

III.3.1.Polymer properties

III.3.1.1. Influence of the copolymer composition

The chemical composition of PLGA directly affects the degradation rate, and other properties of the device. The copolymer composition determines the hydrophobicity, the crystallinity and glass transition temperature which in turns affect the degradation rate, thus the drug release rate.

- ***Effect of lactic/glycolic acid ratio:***

A faster degradation of the polymer was observed with increasing glycolic acid content. Glycolic acid units are more hydrophilic and accessible to attack by water molecules than lactic acid units, which present an additional methyl group (86,87). Indeed, Miller et al., observed a higher PLGA degradation rate when the glycolic acid content is

varied from 0% to 50% (191). The glycolic acid content controls the hydrophilicity of the matrix, which determines the degradation rate, thus the release rate of the drug.

- ***Effect of end-group:***

The PLGA end chains may present free carboxylic groups or may be capped with ester linkages. The capped polymers are more hydrophobic than the uncapped polymers. Many studies showed that the uncapped polymer degrades faster than the capped one due to higher water uptakes and to catalytic effect of the acidic end-groups (192,193). In addition, the uncapped carboxylic acid end-groups may interact with encapsulated drug by ionic interaction and thus slow down the drug release (194).

III.3.1.2. Influence of the polymer molecular weight

The initial Mw of PLGA was shown to be important in controlling the release rate from PLGA-based microparticles. It has been suggested that the degradation rate increases with decreasing polymer molecular weight. Low molecular weight polymers present a higher percentage of hydrophilic acid end-groups which leads to a higher water uptake and therefore, a rapid drug release from PLGA microparticles (36,61,76). Published work reported that the length and the duration of the lag phase during the second release phase of protein from PLGA microparticles increased with higher Mw of PLGA and with lower glycolide content (157). This phenomenon can be explained by the fact that the polymer requires more time to take up water and therefore, degrades slower (157).

III.3.2.Formulation parameters

III.3.2.1. Influence of microparticle size

A published works reported that the microparticle size is a major parameter that controls the drug release from PLGA microparticles. It was shown that that the relative and absolute drug release rate increased with deacrising system size (195). These results can be attributed to a greater surface area exposed to the medium, which facilitates the penetration of water and the diffusion of drug molecules wich are close to the surface. Generally, the drug release rate decreased with an increasing system size, which may be attributed to longer diffusion pathways (129,130). However, these effects of diffusion may be compensated by the autocatalytic effect which is more pronounced in large systems (60). In addition, the increase in microparticles size increases the initial drug loading and leads to an increase in the internal porosity and thus release rates (144).

III.3.2.2. Influence of microparticle porosity

The particle porosity significantly affects the drug release rate. Indeed, Pores and channels can exist within microparticles and can contribute significantly to drug release (64). Kang and Schwendeman showed that once PLGA microparticles are in contact with aqueous medium, pores in microspheres are open and water penetrates rapidly into the microparticle through these waters filled pores and dissolves the drug. Consequently, the drug molecules in pores will be released rapidly (150). Luan and bodmeier studied the effect of the addition of medium chain triglycerides (MCT) into microparticles structure. They have shown that the medium chain triglycerides made the surface and the inner structure more porous which affect the initial burst release and change the triphasic drug release profile into a constant profile (66).

III.3.2.3. Influence of drug loading

The drug loading play a key role in the rate and the duration of drug release. The increase of drug loading promotes the presence of drug crystals at the surface of the microparticle, and thus lead to a rapid release (137). However, Gasmi et al. provided evidence that the second drug release phase depends mainly on the initial drug loading. They explained the release of dexamethasone from PLGA-based microparticles as follows: at very high drug loadings, a part of the drug which does not have a direct access to the surface of microparticles is effectively trapped by the PLGA and takes time to diffuse through the polymeric barrier (69). Importantly, a saturated drug solution is most probably provided within the system. This could be explained by the very limited amounts of water available for drug dissolution in the PLGA-based microparticles at this phase (69). These saturated drug solutions combined with sink conditions provided outside the microparticles lead to about constant drug concentration gradients (186,187). Consequently, a constant drug release rates (“Zero order phase”) is observed (43).

III.3.2.4. Influence of the nature and the distribution of the encapsulated drug

The drug release from PLGA microparticles is greatly influanced by the nature and the distribution of encapsulated drug within the microparticle. A recent study conducted by Yang et al., confirmed that the burst release effect was mainly caused by the distribution of the drug within the microparticle. The Transition temperature microscopy (TTM) data suggested that a quantity of the drug (BSA) was present at the surface of the formulation. The BSA present near the surface would quickly dissolve and diffuse into the bulk release medium resulting in the burst release effect observed (196). However, Klose et al., hypothesized that the rapid drug release from PLGA microparticles observed during the first phase of drug release depends mainly on the nature of the encapsulated drug (194). Indeed, an attractive ionic drug-polymer

interactions can lead to unexpected decrease of the mobility of the drug within the polymeric matrix, resulting in decreased absolute and relative drug release rates (194).

III.3.3.Experimental conditions

As it was seen before, drug release from PLGA microparticles is affected by different factors as polymer and drug properties or formulation characteristics. However, this drug release may also be affected by the experimental conditions used to evaluate the *in vitro* drug release rate. As no regularly standard for *in vitro* measurements has yet been established, different experimental conditions are found to be used in practice. Therefore, several studies evaluated the potential impact of the experimental conditions on the resulting drug release profiles.

III.3.3.1. Influence of medium composition

It well known that the pH play an important role in the autocatalytic effect and thus, impact the degradation of the polymer and accelerate the drug release (42). However, the pH seems to impact the drug release in other ways. Indeed, a published work concluded that the pH impacts the inner morphology of the microparticle. They observed that at lower pH (pH 2.4), the degradation products have low solubility, crystallize and thus, leading to the formation of cracks within the microparticles. As a consequence, the polymer degradation was accelerated and drug release occurred by erosion during the second phase (197).

The drug solubility in the incubation medium also plays a crucial role in the drug release (198). The addition of any compounds able to increase drug solubility (surfactant) may accelerate the release kinetics (199–201). To commonly avoid drug saturation during the *in vitro* drug release studies, sink conditions must be provided. However, these conditions may impact significantly the drug release rate (187).

III.3.3.2. Influence of incubation temperature

The incubation temperature may also impact the drug release kinetics. The increase in the incubation temperature above the glass transition temperature of PLGA has many consequences (91,202–205):

- Higher PLGA degradation.
- Higher drug diffusivities.
- Higher water-uptake.
- Higher polymer mobility.

As a consequence, the drug release rate from PLGA microparticles is accelerated.

III.3.3.3. Influence of agitation

The agitation of bulk fluid may alter the drug release from PLGA microparticles by many ways. An unstirred medium is known to potentially decrease the drug dissolution and reduce the release due to the creation of medium layer surrounding microparticles sutured with drug (206). The agitation may also prevent aggregation by continually dispersing microparticles in the release medium, resulting in a faster release (203).

IV. Research objectives

Poly (lactic-co-glycolic) acid (PLGA)-based microparticles represent an attractive choice to sustain drug release over periods ranging from a few days up to several months, while ensuring good biocompatibility and complete biodegradability. Controlled drug delivery systems (e.g. microparticles) can be used to optimize the therapeutic effects of medical treatment, and decreasing serious side effects. Different types of drug release patterns can be obtained from PLGA microparticles, e.g. mono ,bi-, or tri-phasic drug release. Interestingly, the underlying mass transport mechanisms in PLGA microparticles are not yet fully understood, despite the great practical importance of this type of advanced drug delivery systems. This can be attributed to the complexity of the involved mass transport mechanisms.

Generally, the *in vitro* drug release is studied from *ensembles* of microparticles, differing in size, and it is well known that the system dimension can alter the importance of the involved physicochemical processes, e.g. autocatalysis. However, the observed drug release kinetics from *ensembles* of microparticles are the sum of all the individual drug release rates, which might substantially differ. For these reasons, the *in vitro* drug release was studied from an *ensemble* of PLGA-based microparticles prepared as well as from a *single* microparticles.

The aim of this work was to better understand the drug release mechanisms in PLGA microparticles by evaluating the importance of the experimental conditions on the *in vitro* drug release measurements. The key factors described in the literature such as size and temperature that may alter the *in vitro* drug release profiles from PLGA microparticles were evaluated.

The major objectives of this work were:

- **i.** To Optimize the manufacturing procedure (O/W solvent extraction/evaporation method) in order to prepare different prototype of microparticles varying in size,
- **ii.** To Physico-chemically characterize the resulting PLGA-based microparticles loaded with 5% of Diprophylline or caffeine,
- **iii.** To study the *in vitro* release kinetics of drug in various conditions,
- **iv.** To compare the *in vitro* drug release profiles obtained from single microparticles with those obtained from an ensemble of microparticles,
- **v.** To better understand the underlying delivery mechanisms, based on the physicochemical properties of the microparticles and using appropriate characterization techniques.

The research results of this Ph.D. thesis will be described in three parts:

Part I: Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Drug dispersions.

Part II: Towards a better understanding of the release mechanisms of caffeine from PLGA microparticles.

Part III: Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Impact of the temperature.

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Chapter II:Materials and methods

I. Materials

The materials used during this PhD work are:

- ***Polymer:*** Poly (D,L lactic-co-glycolic acid) (PLGA; Resomer RG 504H Evonik, Darmstadt; Germany) ; 50:50 lactic acid:glycolic acid; acid terminated, inherent viscosities of 0.1% solution in chloroform at 25°C= 0.16-0.24 and 0.45-0.60 dl.g according to the supplier.
- ***Drugs:*** Diprophylline and caffeine were offered by BASF (Ludwigshafen, Germany).
- ***Organic solvents:***
    - Acetonitrile and dichloromethane, HPLC grade were supplied by VWR (Fontenay-sous-Bois, France).
    - Tetrahydrofuran HPLC grade, anhydrous (max. 0.003% H_2O) ≥99.9% stabilized; was supplied by Fisher Scientific (Illkirch, France).
- ***Polyvinyl alcohol*** (Mowiol 4-88) provided by Sigma-Aldrich (Steinheim, Germany); Mw ~ 31 KDa.
- ***Phosphate buffer pH 7.4 (USP 42):*** was prepared using potassium dihydrogen orthophosphate and sodium hydroxide which were provided by Acros Organics (Geel, Belgium).
- ***Acetate buffer 0.01 M:*** was prepared using sodium acetate, anhydrous 99% provided by Alfa Aesar (Massachusetts, United States).

II. Methods

II.1. Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Drug dispersions

II.1.1. Microparticle preparation

Drug-loaded microparticles were prepared using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique: Appropriate amounts of diprophylline and PLGA were dispersed/dissolved (the drug was at least partially dispersed in the form of tiny particles, the polymer was dissolved) in a well-defined volume of dichloromethane (Table II- 1). "Small", "medium-sized" and "large" microparticles were prepared, adapting the formulation and processing parameters accordingly (Table II- 1). The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 or 2000 rpm, Eurostar power-b; Ika-Werke, Staufen, Germany) for 30 min. Upon solvent exchange the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25%) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40°C and 0.07 mbar for 35 h, and secondary drying at +20 °C and 0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

Table II- 1: Composition of the inner organic phase and stirring speed used for the preparation of "small", "medium-sized" and "large" PLGA microparticles loaded with diprophylline.

Microparticle size	CH_2Cl_2, mL	PLGA, mg	Drug, mg	Stirring speed, rpm
″Small″	10	900.1	204.4	2000
″Medium-sized″	6	834.3	125.0	1500
″Large″	4	909.5	101.0	1000

II.1.2. Microparticle characterization

II.1.2.1. Microparticle size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.1.2.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PTFE syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis [Thermo Fisher Scientific Ultimate 3000 Series HPLC, equipped with a LPG 3400 SD/RS pump, an auto sampler (WPS-3000 SL) and a UV-Vis detector (VWD-3400RS); Thermo Fisher Scientific, Waltham, USA]. A reversed phase column Polar C18 (Luna Omega 3 µm; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetate buffer (0.01 M, pH 4.5): acetonitrile (65:35, v:v). The detection wavelength was 274 nm and the flow rate 1 mL/min. Five µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.1.2.3. X ray powder diffraction

X ray powder diffraction analysis was performed with a Panalytical X'pert pro diffractometer (λ Cu K α = 1.54 Å) and Lindemann glass capillaries (diameter 0.7 mm) (Panalytical, Almelo, Netherland). The measurements were performed in transmission mode with an incident beam parabolic mirror and the X'celerator detector.

II.1.2.4. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: diprophylline, PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminum pans from 10 °C to 120°C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperatures (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.1.2.5. Drug release measurements from ensembles of microparticle

Ten mg microparticle samples were placed into plastic tubes (Safe-lock tubes 2.0 mL, Eppendorf, Hamburg, Germany) filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At predetermined time points, 1.5 mL samples were withdrawn (replaced with fresh medium), filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and analysed for their drug contents by HPLC analysis, as described above. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout the experiments.

II.1.2.6. Drug release measurements from single microparticles

Diprophylline release from single microparticles was monitored in 96- well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 100 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap (Simport Scientific, Beloeil, Quebec). The well microplates were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At predetermined time points, 50 µL samples were withdrawn (replaced with fresh medium) using a Hamilton syringe (Microlite #710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for their drug contents by HPLC, as described above (however, in this case the standard curve covered the range of 0.025 to 5 µg/mL).

II.1.2.7. Swelling of single microparticles

Microparticles were treated as for the drug release studies from single microparticles. At pre-determined time points, microparticles were carefully withdrawn, and pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss). The diameter of the microparticles was determined before and after exposure to the release medium (as indicated).

Furthermore, dynamic changes in the microparticles' wet mass were determined as a function of the exposure time to the release medium as follows: At predetermined time points, samples were carefully withdrawn and excess water removed using Kimtech precision wipes (Kimberly-Clark, Rouen, France). The microparticles' wet mass at time t was measured using an ultra-microbalance (XPR6U; Mettler-Toledo, Greifensee, Switzerland).

II.1.2.8. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometry (EDS)

The internal and external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan), equipped with an EDS microanalysis system (X-Max SDD detector, Aztec 3.3 software; Oxford Instruments, Oxfordshire, England). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Cross-sections were obtained after inclusion of microparticles into “OCT embedding medium” (“embedding medium” for frozen tissue specimen to ensure Optimal Cutting Temperature; VWR BDH, Chemicals, United Kingdom) and cutting with cryostat (Leica CM3050 S, Wetzlar, Germany). Microparticles were observed before and after exposure to the release medium. In the latter case, the microparticles were treated as for the drug release studies from ensembles of microparticles (described above). At predetermined time points, samples were withdrawn and freeze-dried (as described above).

II.2. Towards a better understanding of the release mechanisms of caffeine from PLGA microparticles

II.2.1. Microparticle preparation

Drug-loaded microparticles were prepared using an oil-in-water (O/W) emulsion solvent extraction/evaporation technique: Appropriate amounts of caffeine and PLGA were dissolved in a well-defined volume of dichloromethane (Table II- 2). “Small”, “medium-sized” and “large” microparticles were prepared, adapting the formulation and processing parameters accordingly (Table II- 2). The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 and 2000 rpm, Eurostar power-b; Ika-Werke, Staufen, Germany) for 30 min. Upon solvent exchange, the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25 %) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40 °C and 0.07 mbar for 35 h and secondary drying at +20 °C/0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

Table II- 2: Composition of the inner organic phase and stirring speed used for the preparation of “small”, “medium-sized” and “large” PLGA microparticles loaded with caffeine

Microparticle size	CH_2Cl_2, mL	PLGA, mg	Drug, mg	Stirring speed, rpm
″Small″	10	903.1	97.9	2000
″Medium-sized″	6	900.7	104.6	1500
″Large″	4	902.4	104.0	1000

II.2.2. Microparticle characterization

II.2.2.1. Microparticle size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.2.2.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PVDF syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis (Alliance, Separation Modules e2695, 2489, UV-Vis Detector; Waters, Milford, USA). A reversed phase column C18 (Gemini 5 µm; 110 A °; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetonitrile: water (70:30, v:v). The detection wavelength was 254 nm and the flow rate 1 mL/min. Twenty µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.2.2.3. X ray powder diffraction

X ray powder diffraction analysis was performed using a Panalytical X'pert pro diffractometer (λ Cu K α=1.54 Å) and Lindemann glass capillaries (diameter 0.7 mm) (PANalytical, Almelo, The Netherlands). The measurements were conducted in transmission mode with an incident beam parabolic mirror and the X'celerator detector.

II.2.2.4. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: caffeine and PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminium pans from 10 to 120 °C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperature (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.2.2.5. Drug release measurements from ensembles of microparticles

Ten mg of microparticles were placed into Eppendorf tubes (Safe-lock, 2.0 mL; Eppendorf, Hamburg, Germany), filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033, Gesellschaft fuer Labortechnik,

Burgwedel, Germany). At predetermined time points, 1.5 mL samples were withdrawn, filtered (PVDF syringe filters, 0.45 µm; GE Healthcare, Kent, UK) and analysed for their drug contents by HPLC analysis, as described above. To keep the volume of the release medium constant and to avoid the potential loss of microparticles due to sampling, 1.5 mL fresh release medium was injected into the Eppendorf tubes using the same syringe filters at each time point. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout the experiments.

II.2.2.6. Drug release measurements from single microparticles

Caffeine release from single microparticles was monitored in 1 mL syringes (three-part single-use syringes; HSW Henke-Ject, Tuttlingen, Germany) as follows: One microparticle was introduced into a syringe, which was filled with 200 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap [BD Luer-Lok (TM) (caps with male/female protection); Dominique Dutscher, Brumath, France]. The syringes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At predetermined time points, 50 µL samples were withdrawn (replaced with fresh medium) using Hamilton syringes (Microlite/#710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for their drug contents by HPLC, as described above (the standard curve covering the range of 0.025 to 5 µg/mL).

II.2.2.7. Swelling of single microparticles

The swelling of individual microparticles was monitored in 96-well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 200 µL phosphate buffer pH 7.4 (USP 42). The well microplates were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At pre-determined time points pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). Also, as for the drug release studies, 50 µL samples were withdrawn and replaced with fresh medium at each

sampling time point. The increase in microparticle diameter (%) at time (t) was calculated as follows:

$$Increase\ in\ diameter\ (\%)(t) = \frac{diameter\ (t)}{diameter\ (t=0)}\ x\ 100$$

where diameter (t) and diameter (t=0) represent the microparticle diameter at time t and t=0 (before exposure to the release medium), respectively.

II.2.2.8. Polymer degradation

Microparticles were treated as for the drug release studies. At predetermined time points, samples were withdrawn, freeze-dried for 3d (as described above) and the lyophilisates were dissolved in tetrahydrofuran (at a concentration for 3 mg/mL). The average polymer molecular weight (Mw) of the PLGA in the samples was determined by Gel Permeation Chromatography (GPC, Alliance, refractometer detector: 2414 RI, separation module e2695, Empower GPC software; Waters, Milford, USA), using a Phenogel 5 µm column (which was kept at 35°C, 7.8 × 300 mm; Phenomenex, Le Pecq, France). The injection volume was 50 µL. Tetrahydrofuran was the mobile phase (flow rate: 1 mL/min). Polystyrene standards with molecular weights between 1480 and 70,950 Da (Polymer Laboratories, Varian, Les Ulis, France) were used to prepare the calibration curve. All experiments were conducted in triplicate. Mean values and ± standard deviations are reported.

II.2.2.9. Scanning Electron Microscopy (SEM)

The internal and external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Cross-sections were obtained after inclusion of microparticles into “OCT embedding medium” (“embedding medium” for frozen tissue specimen to ensure Optimal Cutting Temperature; VWR, Lutterworth, UK) and cutting with cryostat (Leica CM3050 S, Wetzlar, Germany).

II.3. Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Impact of the temperature.

II.3.1. Microparticle preparation

Drug-loaded microparticles were prepared using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique. “Small”, “medium-sized” and “large” microparticles were prepared, adapting the formulation and processing parameters accordingly (as indicated in the following in brackets in this order). Appropriate amounts of diprophylline (204, 125 or 101 mg) and PLGA (900, 834 or 910 mg) were dispersed/dissolved (the drug was at least partially dispersed in the form of tiny particles, the polymer was dissolved) in 10, 6 or 4 mL dichloromethane. The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 or 2000 rpm, Eurostar power-b; Ika, Staufen, Germany) for 30 min. Upon solvent exchange the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25%) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40°C and 0.07 mbar for 35 h, and secondary drying at +20 °C and 0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

II.3.2. Microparticle characterization

II.3.2.1. Microparticle morphology and size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.3.2.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PTFE syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis [Thermo Fisher Scientific Ultimate 3000 Series HPLC, equipped with a LPG 3400 SD/RS pump, an auto sampler (WPS-3000 SL) and a UV-Vis detector (VWD-3400RS); Thermo Fisher Scientific, Waltham, USA]. A reversed phase column Polar C18 (Luna Omega 3 µm; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetate buffer

(0.01 M, pH 4.5): acetonitrile (65:35, v:v). The detection wavelength was 274 nm and the flow rate 1 mL/min.

Five µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.2.3. Drug release measurements from ensembles of microparticles

Ten mg microparticle samples were placed into plastic tubes (Safe-lock tubes 2.0 mL, Eppendorf, Hamburg, Germany) filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker at 37°C & 80 rpm or at 20°C & 80 rpm (GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany) or into a refrigerator at 4°C (0 rpm), as indicated. At predetermined time points, 1.5 mL samples were withdrawn (replaced with fresh medium), filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and analysed for their drug contents by HPLC analysis, as described above. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout all experiments.

In addition, the pH of the release medium was measured at pre-determined time points using a pH meter (InoLab pH Level 1; WTW, Weilheim, Germany) (n=3). Mean values ± standard deviation are reported.

II.3.2.4. Drug release measurements from single microparticles

Diprophylline release from single microparticles was monitored in 96- well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 100 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap (Simport Scientific, Beloeil, Quebec). The well microplates were placed into a horizontal shaker at 20°C & 80 rpm (GFL 3033). At pre-determined time points, 50 µL samples were withdrawn (replaced with fresh medium) using a Hamilton syringe (Microlite #710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for their drug contents by HPLC, as described above (in this case the standard curve covered the range of 0.025 to 5 µg/mL).

II.3.2.5. Swelling of single microparticles

Microparticles were treated as for the drug release studies from single microparticles. At pre-determined time points, pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss) to monitor changes in the microparticles' diameter.

Furthermore, dynamic changes in the microparticles' wet mass were determined as follows: At pre-determined time points, samples were carefully withdrawn and excess water removed using Kimtech precision wipes (Kimberly-Clark, Rouen, France). The microparticles' wet mass at time t was measured using an ultra-microbalance (XPR6U; Mettler-Toledo, Greifensee, Switzerland).

II.3.2.6. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: diprophylline, PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminum pans from 10 °C to 120°C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperatures (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.2.7. Scanning Electron Microscopy (SEM)

The external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Microparticles were observed before and after exposure to the release medium. In the latter case, the microparticles were treated as for the drug release studies from ensembles of microparticles (described above). At pre-determined time points, samples were withdrawn and freeze-dried (as described above).

II.3.2.8. Gel Permeation Chromatography (GPC)

Microparticles were treated as for the drug release studies from ensembles of microparticles. At pre-determined time points, samples were withdrawn, freeze-dried for 3d (as described above) and the lyophilisates were dissolved in tetrahydrofuran (at a concentration of 3 mg/mL). The average polymer molecular weight (Mw) of the PLGA in the samples was determined by GPC (Alliance, refractometer detector: 2414 RI, separation module e2695, Empower GPC software; Waters, Milford, USA), using a Phenogel 5 µm column (which was kept at 35°C, 7.8 × 300 mm; Phenomenex, Le Pecq, France). The injection volume was 50 µL. Tetrahydrofuran was the mobile phase (flow rate: 1 mL/min). Polystyrene standards with

molecular weights between 1480 and 70,950 Da (Polymer Laboratories, Varian, Les Ulis, France) were used to prepare the calibration curve.

II.3.2.9. Drug solubility measurements

Excess amounts of the drug (as received) were exposed to 25 mL phosphate buffer pH 7.4 in brown glass flasks and horizontally shaken at 37°C or 20 °C at 80 rpm (GFL 3033), or placed in a refrigerator at 4 °C and regularly shaken manually. At pre-determined time points, samples were withdrawn, immediately filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and diluted. The drug contents of the samples were determined by HPLC-UV, as described above. Samples were withdrawn until equilibrium was reached. Each experiment was conducted in triplicate, mean values +/- standard deviations are reported.

Chapter III:Results and Discussion

Part I

Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Drug dispersions

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Abstract

The aim of this study was to better understand the root causes for the (up to) 3 drug release phases observed with poly (lactic-co-glycolic acid) (PLGA) microparticles containing drug particles: The 1st release phase ("burst release"), 2nd release phase (with an "about constant release rate") and 3rd release phase (which is again rapid and leads to complete drug exhaust). The behavior of single microparticles was monitored upon exposure to phosphate buffer pH 7.4, in particular with respect to their drug release and swelling behaviors. Diprophylline-loaded PLGA microparticles were prepared with a solid-in-oil-in-water solvent extraction/evaporation method. Tiny drug crystals were rather homogeneously distributed throughout the polymer matrix after manufacturing. Batches with "small" (63 µm), "medium-sized" (113 µm) and "large" (296 µm) microparticles with a practical drug loading of 5-7 % were prepared. Importantly, each microparticle releases the drug "in its own way", depending on the exact distribution of the tiny drug crystals within the system. During the burst release, drug crystals with direct surface access rapidly dissolve. During the 2nd release phase tiny drug crystals (often) located in surface near regions which undergo swelling, are released. During the 3rd release phase, the entire microparticle undergoes substantial swelling. This results in high quantities of water inside the system, which becomes "gel-like". The drug crystals dissolve and dissolved drug molecules rather rapidly diffuse through the highly swollen polymer gel.

I. Introduction

Poly (lactic-co-glycolic acid) (PLGA)-based microparticles are frequently used to control drug release upon parenteral administration, because they are completely biodegradable (1), biocompatible (2) , and allow for the adjustment of desired drug release rates during rather flexible periods of time (3–7). Different types of manufacturing procedures can be used to prepare this type of advanced drug delivery systems, for example solvent extraction/evaporation techniques (8–12) and spray drying (13–15). The basic idea is to trap the drug within the polymer matrix to avoid instantaneous drug release upon injection into living tissue. The drug can be molecularly dissolved in the PLGA matrix and/or dispersed in the form of tiny (crystalline or amorphous) particles.

The resulting drug release kinetics from PLGA microparticles can be mono-phasic, bi-phasic or tri-phasic (5,16–26). The 1st release phase is more or less pronounced and also called "burst effect": The drug is rapidly released (generally during several hours or up to 1-2 days) upon contact with aqueous fluids. This phase can be followed by a 2nd release phase with an about constant drug release rate. This "zero order release phase" is variable in length (depending on the type of polymer, e.g. polymer molecular weight) and can take several days or weeks. The slope of the release curve (= the release rate) can be more or less steep. Eventually, drug release is close to negligible (e.g., this part of the release curves looks like a "plateau"). Afterwards, a 3rd release phase might be observed: In these cases, the release rate increases again at a later time point, leading to complete drug exhaust. Not all types of PLGA microparticles show drug release profiles exhibiting all 3 phases (some are only mono-phasic, other only bi-phasic). Also, the relative importance of these phases can significantly vary between different types of microparticles. Key factors influencing the shape of release profiles include the type of drug and initial drug loading, the type of PLGA (e.g. initial polymer molecular weight, type of end groups and "lactic acid: glycolic acid" ratio) as well as the manufacturing procedure (affecting the inner and outer system structure).

The mass transport mechanisms controlling drug release from PLGA microparticles can be rather complex, including different types of physico-chemical phenomena (27,28) such as water penetration into the system, drug dissolution (29), drug diffusion (30) through water-filled pores, swollen PLGA gel and/or slightly hydrated polymer networks, polyester hydrolysis (31), drug – PLGA interactions (32,33), the creation of water-soluble monomers and oligomers and the latter's diffusion into the surrounding bulk fluid, PLGA swelling, the creation of acidic micro-environments within the microparticles (especially at the center) (34–36), resulting in

accelerated PLGA degradation (autocatalysis) (31,37,38), pore closure effects (39) and osmotic effects due to the presence of water-soluble compounds within the systems (39), to mention just a few. A comprehensive review on these mechanisms has been given by the group of A. Axelsson (40).

It has to be pointed out that in practice numerous microparticles are administered at the same time and that the resulting drug release kinetics from these ensembles of microparticles are the sums of all the individual drug release profiles from the various single microparticles. For other types of multiple unit dosage forms, e.g. polymer coated controlled release pellets (41,42), it has been shown that the release behavior of the single dose units can be very different. This is not visible when only looking at the drug release kinetics observed with the ensembles of the dosage forms. The monitoring of drug release from single dose units can be very helpful to better understand how these systems work.

Recently, Gasmi et al. (43–45) reported that substantial microparticle swelling coincided with the onset of the 3rd release phase in different types of PLGA microparticles, loaded with ketoprofen, prilocaine and dexamethasone. Also, Bode et al. (46,47) reported that the onset of dexamethasone release from macroscopic, hot melt extruded PLGA implants coincided with substantial system swelling. This was true for different "lactic acid: glycolic acid ratios", as well as for poly(lactic acid) (PLA)-based implants, and was explained as follows: At early time points, the polymer chains are relatively long, thus, rather hydrophobic and highly entangled. This limits the amounts of water, which can penetrate into the system. However, some water enters and wets the entire microparticles/implants. This leads to polymer hydrolysis occurring throughout the systems ("bulk erosion") (48). With time, the polymer chains decrease in length, thus, the degree of macromolecular entanglement decreases and the network becomes mechanically less stable. In addition, ester bond hydrolysis creates additional –OH and –COOH end groups, thus, the system becomes more hydrophilic. Also, the generated monomers and short chain oligomers are water-soluble, creating a steadily increasing osmotic pressure within the system. At a certain time point, substantial amounts of water penetrate into the devices, allowing for drug dissolution and facilitated diffusion out into the surrounding bulk fluid.

However, yet the root causes for the 1st and 2nd release phases are less well understood. It is likely that surface near drug contributes to the initial burst effects, but details are often unclear and suggested potential reasons for the 2nd release phase are often not based on experimental evidence. The aim of this study was to gain further insight into the mass transport mechanisms controlling drug release from PLGA microparticles, especially during the burst release phase

and subsequent about constant drug release phase. For this reason, the behavior of single microparticles loaded with tiny diprophylline crystals was monitored upon exposure to phosphate buffer pH 7.4.

II. Materials and methods

II.1.Materials

Poly (D,L lactic-co-glycolic acid) (PLGA; Resomer RG 504H; 50:50 lactic acide:glycolic acid; Evonik, Darmstadt; Germany); diprophylline (BASF, Ludwigshafen, Germany); polyvinyl alcohol (Mowiol 4-88; Sigma-Aldrich, Steinheim, Germany); acetonitrile and dichloromethane (VWR, Fontenoy-sous-Bois, France); tetrahydrofuran (HPLC grade; Fisher Scientific, Illkirch, France).

II.2.Microparticle preparation

Drug-loaded microparticles were prepared using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique: Appropriate amounts of diprophylline and PLGA were dispersed/dissolved (the drug was at least partially dispersed in the form of tiny particles, the polymer was dissolved) in a well-defined volume of dichloromethane (Table III- 1). “Small”, “medium-sized” and “large” microparticles were prepared, adapting the formulation and processing parameters accordingly (Table III- 1). The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 or 2000 rpm, Eurostar power-b; Ika-Werke, Staufen, Germany) for 30 min. Upon solvent exchange the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25%) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40°C and 0.07 mbar for 35 h, and secondary drying at +20 °C and 0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

Table III- 1: Composition of the inner organic phase and stirring speed used for the preparation of "small", "medium-sized" and "large" PLGA microparticles loaded with diprophylline.

Microparticle size	CH_2Cl_2, mL	PLGA, mg	Drug, mg	Stirring speed, rpm
″Small″	10	900.1	204.4	2000
″Medium-sized″	6	834.3	125.0	1500
″Large″	4	909.5	101.0	1000

II.3. Microparticle characterization

II.3.1. Microparticle size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.3.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PTFE syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis [Thermo Fisher Scientific Ultimate 3000 Series HPLC, equipped with a LPG 3400 SD/RS pump, an auto sampler (WPS-3000 SL) and a UV-Vis detector (VWD-3400RS); Thermo Fisher Scientific, Waltham, USA]. A reversed phase column Polar C18 (Luna Omega 3 µm; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetate buffer (0.01 M, pH 4.5): acetonitrile (65:35, v:v). The detection wavelength was 274 nm and the flow rate 1 mL/min. Five µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.3. X ray powder diffraction

X ray powder diffraction analysis was performed with a Panalytical X'pert pro diffractometer (λ Cu K α = 1.54 Å) and Lindemann glass capillaries (diameter 0.7 mm) (Panalytical, Almelo, Netherland). The measurements were performed in transmission mode with an incident beam parabolic mirror and the X'celerator detector.

II.3.4. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: diprophylline, PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminum pans from 10 °C to 120°C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperatures (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.5. Drug release measurements from ensembles of microparticle

Ten mg microparticle samples were placed into plastic tubes (Safe-lock tubes 2.0 mL, Eppendorf, Hamburg, Germany) filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At predetermined time points, 1.5 mL samples were withdrawn (replaced with fresh medium), filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and analysed for their drug contents by HPLC analysis, as described above. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout the experiments.

II.3.6. Drug release measurements from single microparticles

Diprophylline release from single microparticles was monitored in 96- well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 100 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap (Simport Scientific, Beloeil, Quebec). The well microplates were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At predetermined time points, 50 µL samples were withdrawn (replaced with fresh medium) using a Hamilton syringe (Microlite #710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for their drug contents by HPLC, as described above (however, in this case the standard curve covered the range of 0.025 to 5 µg/mL).

II.3.7. Swelling of single microparticles

Microparticles were treated as for the drug release studies from single microparticles. At pre-determined time points, microparticles were carefully withdrawn, and pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss).

Furthermore, dynamic changes in the microparticles' wet mass were determined as follows: At predetermined time points, samples were carefully withdrawn and excess water

removed using Kimtech precision wipes (Kimberly-Clark, Rouen, France). The microparticles' wet mass at time t was measured using an ultra-microbalance (XPR6U; Mettler-Toledo, Greifensee, Switzerland).

II.3.8. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometry (EDS)

The internal and external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan), equipped with an EDS microanalysis system (X-Max SDD detector, Aztec 3.3 software; Oxford Instruments, Oxfordshire, England). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Cross-sections were obtained after inclusion of microparticles into "OCT embedding medium" ("embedding medium" for frozen tissue specimen to ensure Optimal Cutting Temperature; VWR BDH, Chemicals, United Kingdom) and cutting with cryostat (Leica CM3050 S, Wetzlar, Germany). Microparticles were observed before and after exposure to the release medium. In the latter case, the microparticles were treated as for the drug release studies from ensembles of microparticles (described above). At predetermined time points, samples were withdrawn and freeze-dried (as described above).

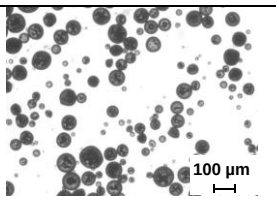
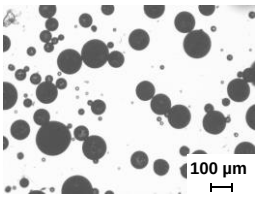
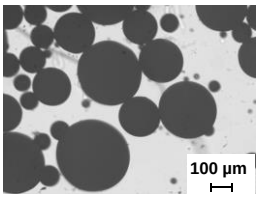
III. Results and Discussion

III.1. Ensembles of microparticles

Table III- 2 shows the practical drug loadings, mean particle sizes (+/- standard deviations), glass transition temperatures (Tgs) and optical microscopy pictures of batches of "small", "medium-sized" and "large" PLGA microparticles loaded with diprophylline. The particles were prepared with a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique: The diprophylline was at least partially dispersed in the form of tiny drug particles within an organic PLGA solution. To obtain differently sized microparticles, the stirring speed of the emulsion as well as the polymer concentration of the organic phase (determining the latter's viscosity) were varied, as indicated in Table III- 2. Higher stirring speeds and lower organic phase viscosities led to smaller organic droplets and, hence, smaller microparticles. However, also the "surface area : volume" ratio changed and, thus, the degree of drug loss into the outer aqueous phase, resulting in different practical drug loadings. With decreasing droplet size, the latter decreased (data not shown). In order to provide roughly similar practical drug loadings for the differently sized microparticles, the theoretical diprophylline loading was adjusted accordingly (Table III- 1). As it can be seen in Table III- 2, this resulted in practical drug loadings ranging from 4.8 (+/- 0.3) % to 6.7 (+/- 0.4) %. It is assumed that the variation of the above mentioned parameters and the minor differences in the drug loading do not

fundamentally alter the inner and outer microparticle structure (also, no evidence for noteworthy alterations was observed).

Table III- 2: Practical drug loadings, mean particle sizes, glass transition temperatures (Tgs) and morphology of "small", "medium-sized" and "large" PLGA microparticles loaded with diprophylline (mean values +/- standard deviations are reported).

	Practical loading, %	Mean size, µm	Tg, °C	Optical microscopy
″Small″	4.8 ± 0.3	62.9 ± 19.2	46.8 ± 0.1	
″Medium-sized″	5.8 ± 0.6	113.3 ± 40.7	46.3 ± 0.3	
″Large″	6.7 ± 0.4	295.7 ± 94.9	46.4 ± 0.4	

The obtained microparticles were spherical in shape, with mean microparticle sizes of 62.9 (+/- 19.2), 113.3 (+/- 40.7), and 295.7 (+/- 94.7) µm in the case of "small", "medium-sized" and "large" microparticles, respectively (Table III- 2). The glass transition temperatures (Tgs) were found to be about 46-47 °C (Table III- 2), irrespective of the microparticle diameter. This indicates that the PLGA is in the glassy state in the dry microparticles. However, it is well known that small amounts water relatively rapidly penetrate into PLGA-based microparticles (roughly within hours or a day) and that water acts as a plasticizer for this polymer (49,50). Consequently, the Tg of the PLGA in the investigated microparticles can be expected to be below 37 °C (and the polymer to be in the rubbery state) once the systems are wetted.

Figure III-19 shows the resulting drug release kinetics from ensembles of diprophylline-loaded PLGA microparticles in phosphate buffer pH 7.4. Batches with "small", "medium-sized" and "large" microparticles were studied (mean diameters are given). As it can be seen, all three batches exhibited tri-phasic diprophylline release patterns: A burst release (= 1st release

phase) during the first 1 day (roughly) was followed by a release phase with about constant drug release (= 2nd release phase), and a final rapid drug release phase leading to complete drug exhaust (= 3rd release phase), which started after about 1 week. The relative importance of the 3 release phases depended on the mean microparticle sizes: The batch with the lowest microparticle size (63 +/- 19 µm) showed an important burst effect (> 50 % drug release) and hardly a 3rd release phase. In contrast, the microparticle batches with "medium-sized" (113 +/- 41 µm) and "large" (296 +/- 95 µm) particles exhibited a much lower burst effect, and a much more pronounced 3rd release phase. The reasons for these differences are discussed below. Please note that, in practice, often microparticles smaller than 100 µm are used. However, for technical reasons they are difficult to study individually. The basic assumption in this study is that the internal and external structure of the systems does not fundamentally depend on their size (and no evidence was observed for such differences), so that the underlying mass transport mechanisms controlling drug release are likely the same. Larger microparticles offer the major advantage to allow for the monitoring of the behavior of single microparticles, which can be very helpful to better understand how the systems control drug release.

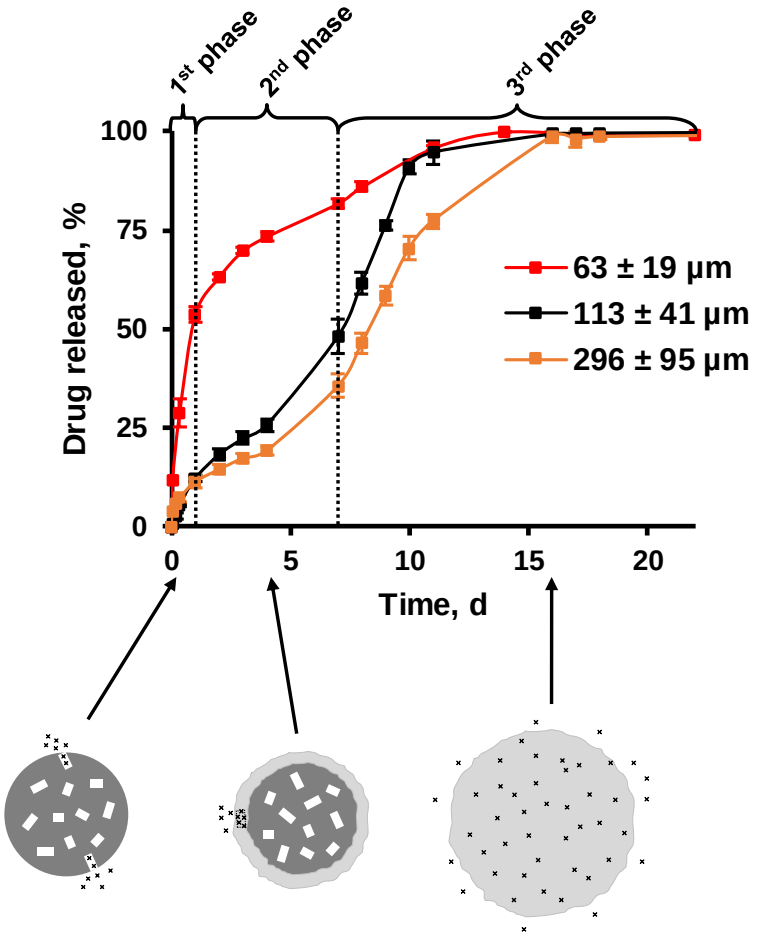


Figure III-19: Diprophylline release from ensembles of PLGA microparticles in phosphate buffer pH 7.4: Impact of the mean particle size (indicated in the diagram +/- standard deviation). The release profiles are tri-phasic: an initial burst release (= 1st phase) is followed by a period with an about constant drug release rate (= 2nd phase) and a final (again) rapid drug release phase leading to complete drug exhaust (= 3rd phase). Please note that the transition periods are not always very sharp. Also, in the case of the "small" microparticles (62 +/- 19 µm diameter), the 3rd release phase is not very pronounced, since most of the drug is already released at this time point.

Figure III-20 shows the X-ray diffraction patterns of ensembles of "small", "medium-sized", and "large" microparticles. For reasons of comparison, also the diffraction patterns of diprophylline power (as received) is illustrated. Clearly, the drug raw material was crystalline. The sharp diffraction peaks at the same angles observed with the differently sized microparticle batches indicate that diprophylline (at least partially) remained in this crystalline state. This fact can be explained by the manufacturing procedure: A suspension of tiny drug particles in a solution of PLGA in dichloromethane was emulsified into an outer aqueous phase. Upon solvent extraction/evaporation the polymer precipitated and trapped the tiny drug crystals. Thus, the latter did not change their solid state. The top rows in Figures III-3 and III-4 show representative surfaces and cross-sections of microparticles before exposure to the release medium (t = 0). They are representative for all microparticles, irrespective of their size. As it

can be seen, tiny drug crystals are randomly and rather homogeneously distributed throughout the PLGA matrix. The surface is relatively smooth and non-porous.

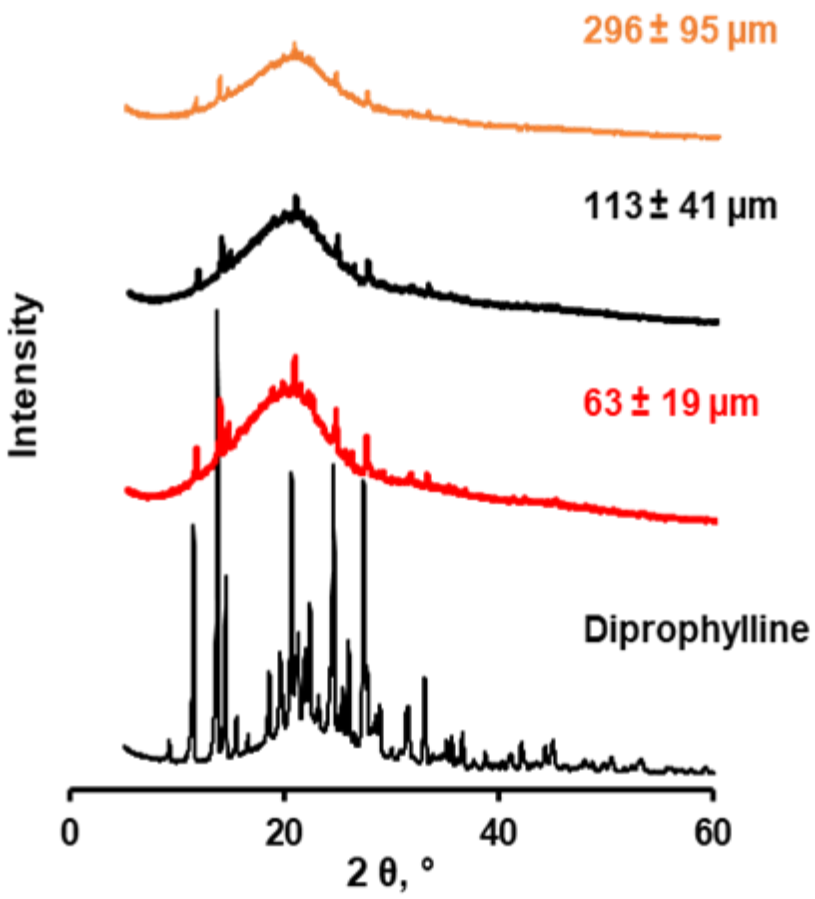


Figure III-20: X-ray diffraction patterns of ensembles of diprophylline-loaded PLGA microparticles (mean particle sizes +/- standard deviations are indicated in the diagram). For reasons of comparison, also the diffraction patterns of diprophylline powder as received) is shown.

To better understand why the different release phases were observed and why their relative importance depends on the microparticle size, the behavior of single microparticles upon exposure to the release medium was monitored.

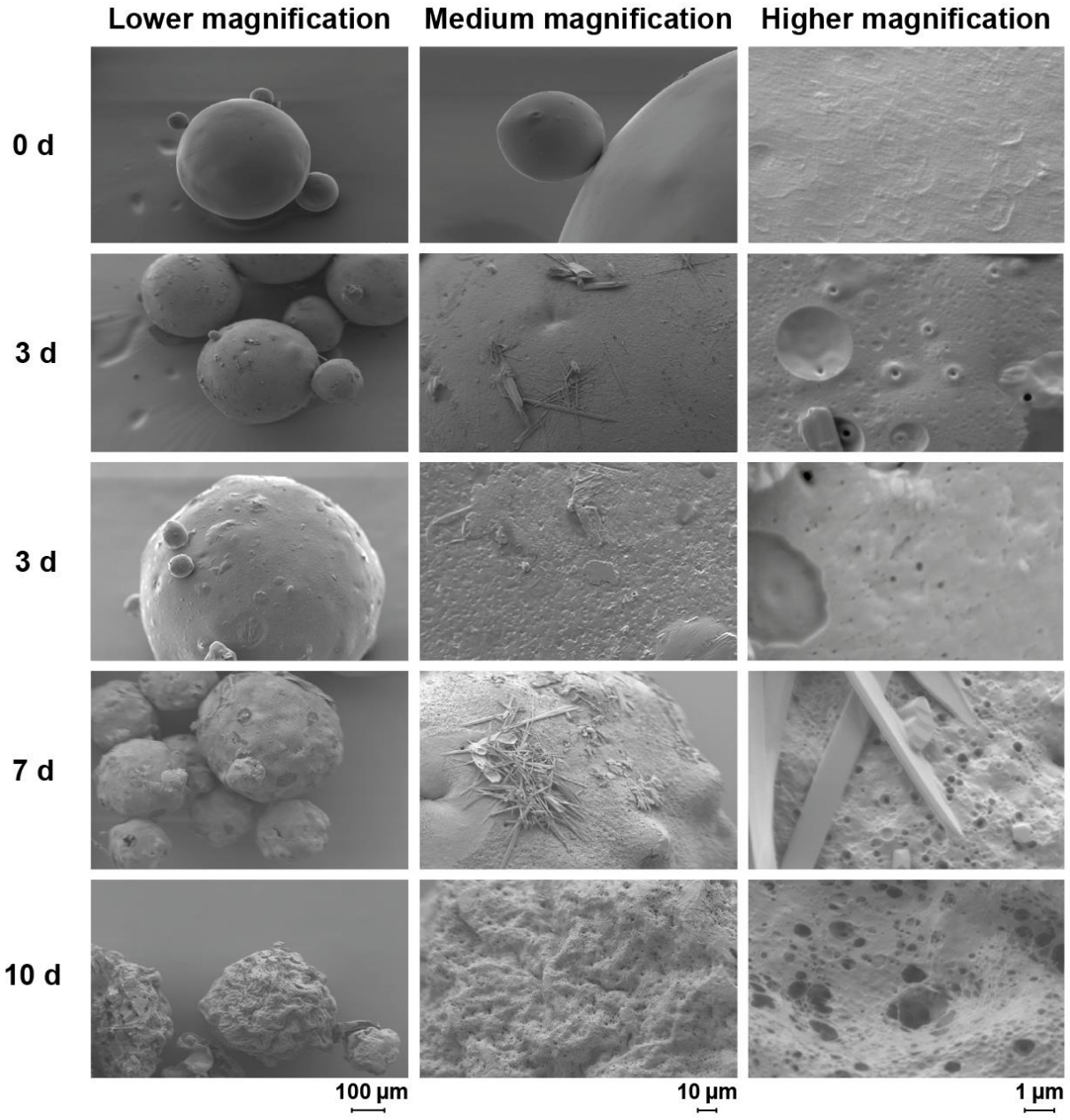


Figure III-3: SEM pictures of surfaces (lower, medium and higher magnification) of diprophylline-loaded microparticles before and after exposure to phosphate buffer pH 7.4 for different time periods (indicated on the left hand side, two examples are shown for t = 3 d). Note that the microparticles were freeze-dried after exposure to the release medium, which likely created artefacts.

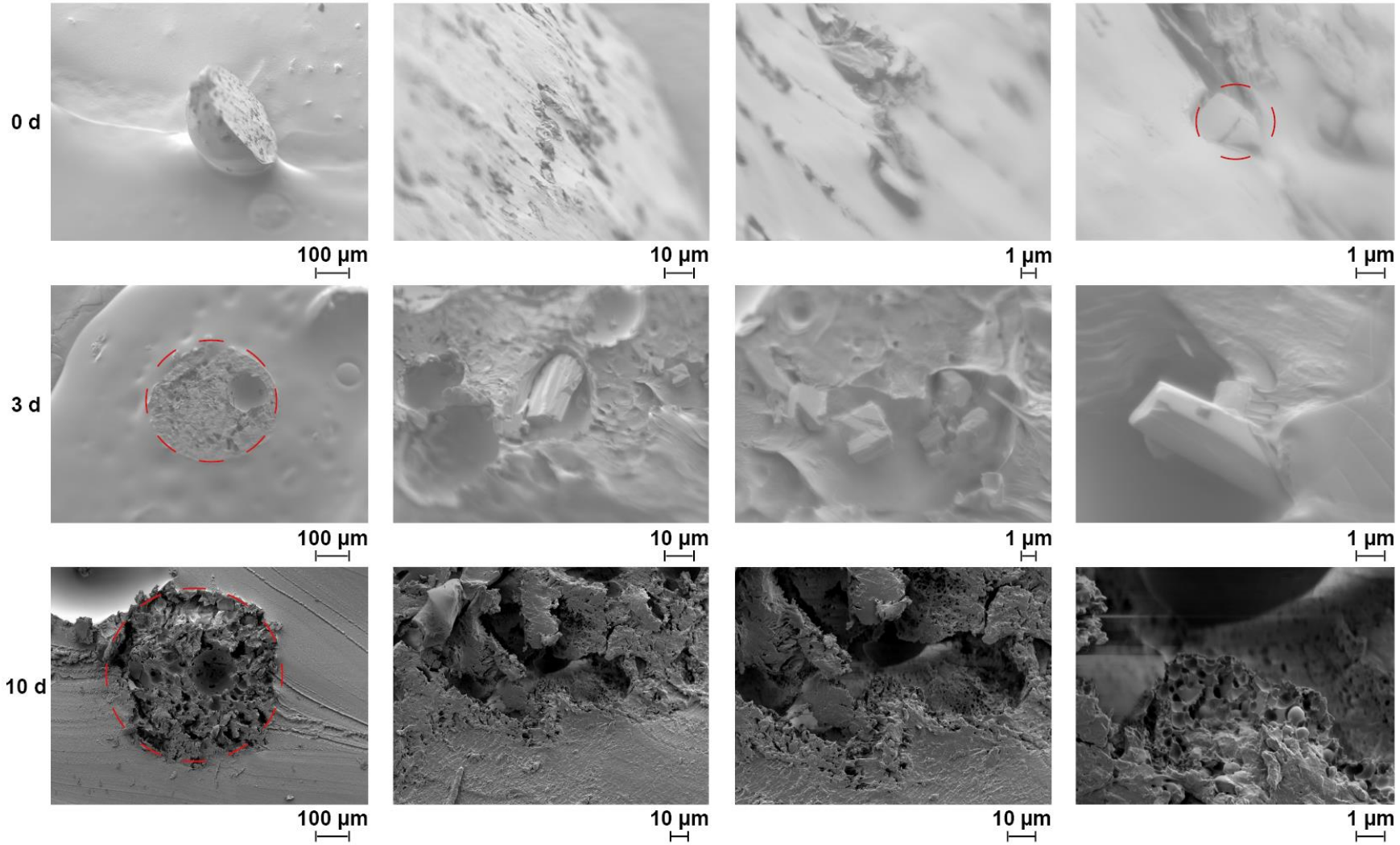


Figure III-4: SEM pictures of cross-sections at different magnifications of diprophylline-loaded microparticles before and after exposure to phosphate buffer pH 7.4 for different time periods (indicated on the left-hand side). Note that the microparticles were freeze-dried after exposure to the release medium, which likely created artefacts.

III.2. Single microparticles

Optical microscopy pictures of single microparticles, which were exposed to phosphate buffer pH 7.4 at 37 °C for different time periods are shown in Figures III-5 and III-6. Figure III-5 covers the entire relevant time period for drug release (0 to 17 d). Figure III-6 shows additional microscopic pictures of particles after 10 to 28 d exposure to the release medium, covering the phase of substantial microparticle swelling in more detail. As it can be seen, during the first few days, microparticle swelling was limited, irrespective of the system size. However, after about 1 week exposure to the release medium, substantial microparticle swelling set on. The systems became more and more transparent and "gel like". Please note that each microparticle behaved slightly differently, the swelling was not perfectly homogenous, e.g. little deformations at different locations were observed on a case by case basis. Thus, the environment of a drug crystal ("waiting to be released") varies depending on its exact location. This environment can be expected to affect the release rate of the drug crystal.

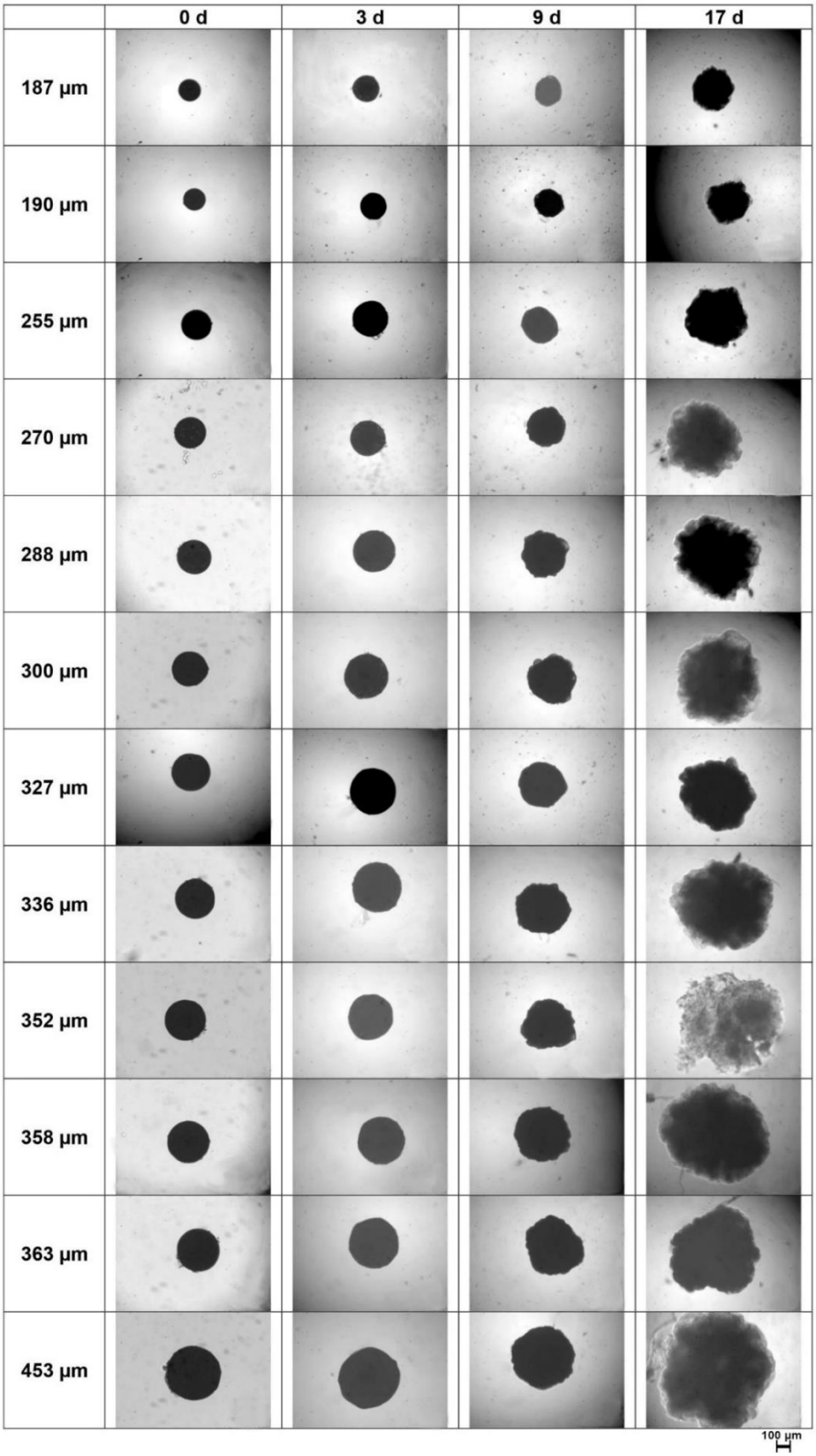


Figure III-5: Optical microscopy pictures of single diprophylline-loaded PLGA microparticles before and after exposure to phosphate buffer pH 7.4 for different time periods (indicated at the top). The initial particle size is given on the left-hand side.

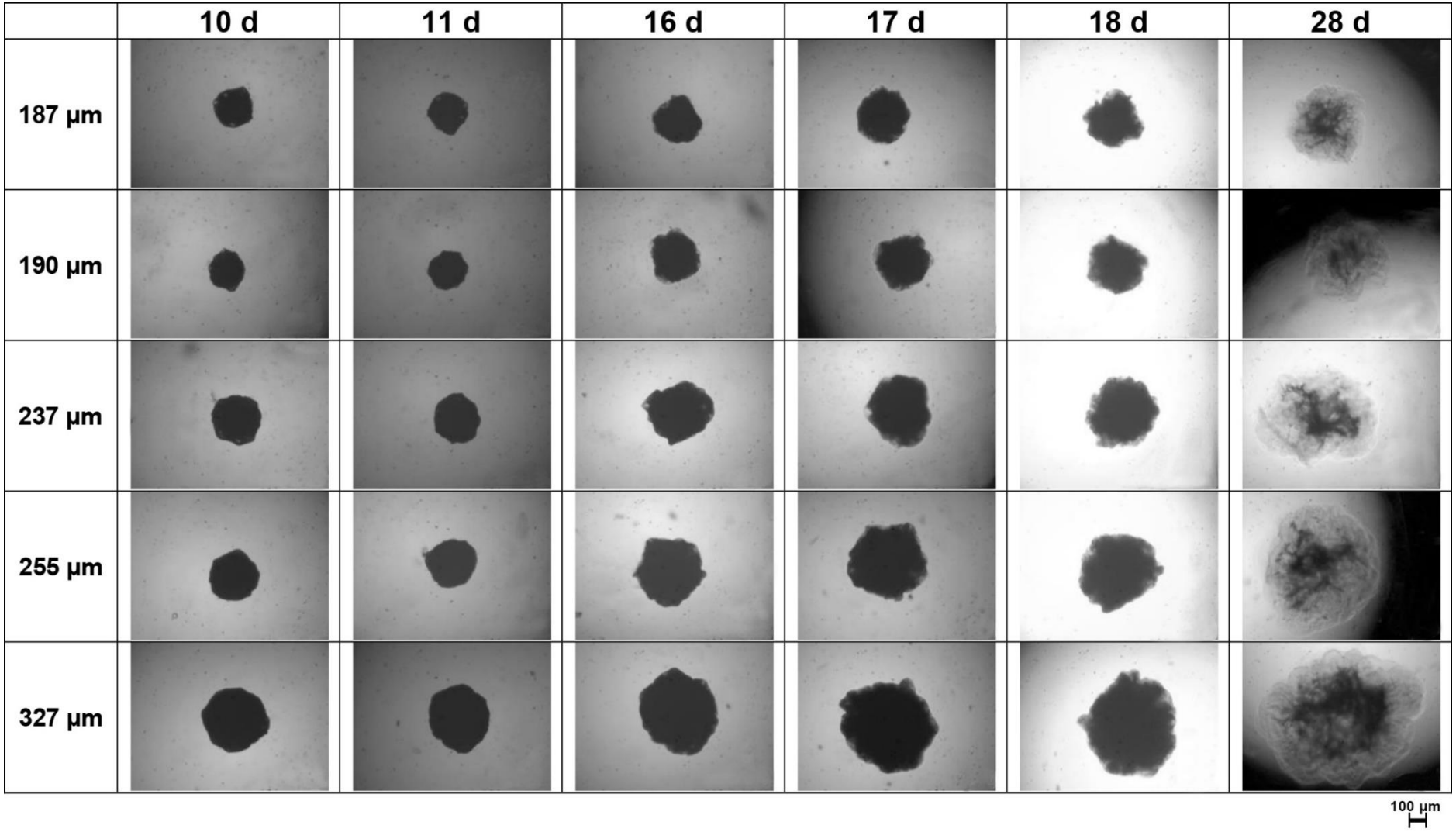


Figure III-6: Optical microscopy pictures of single diprophylline-loaded PLGA microparticles after 10-28 d exposure to phosphate buffer pH 7.4. The initial particle size is given on the left-hand side.

Figure III-7 shows the drug release profiles from single microparticles, together with their swelling kinetics. The red curves refer to the right y-axes, indicating the changes in the particle's diameter. The other (differently colored) curves refer to the left y-axes and illustrate the observed diprophylline release kinetics. The respective (initial) microparticle size is given at the top of each diagram. Interestingly, three types of behaviors can be distinguished:

(i) Certain microparticles do not release any drug prior to the onset of substantial microparticle swelling. The latter occurs after about 1 week, as it can be seen in diagram in the middle of Figure I-8, illustrating the increase in diameter of multiple single microparticles (differing in size) upon exposure to the release medium. Importantly, swelling is limited during the first couple of days, but then becomes very important. The bottom diagram in Figure III-8 shows the dynamic changes in the wet mass of single PLGA microparticles, which also indicates the fundamental swelling starting after about 1 week. This coincides with the onset of drug release from certain microparticles shown in Figure III-7, as marked by green ovals. This is also the time point at which the 3rd release phase from ensembles of microparticles sets on (final rapid release phase, Figure III-1). It has recently been reported that substantial PLGA swelling is likely the root cause for the onset of the 3rd release phase from PLGA microparticles loaded with ketoprofen (44,44), prilocaine (45) and dexamethasone (43). Also, in the case of macroscopic, hot melt extruded, cylindrical implants based on PLGA loaded with dexamethasone the onset of drug release was recently shown to coincide with substantial system swelling (46). The root cause for this type of behavior is likely as follows: At early time points, only limited amounts of water penetrate into the system, since PLGA is rather hydrophobic and the degree of polymer chain entanglement is high (the polymer molecular weight being initially elevated). However, the limited water amounts that can penetrate into the microparticles start degrading the polyester throughout the system ("bulk erosion"). Upon ester bond cleavage, new –OH and –COOH end groups are created, rendering the system more and more hydrophilic. In addition, the degree of polymer chain entanglement decreases (since the macromolecules become shorter). Also, the generated monomers and oligomers are water soluble and create a steadily increasing osmotic pressure within the microparticles. At a certain time point, the polymeric systems become sufficiently hydrophilic and "mechanically instable" to allow for the penetration of substantial amounts of water

into the microparticles: Important microparticle swelling sets on. The penetration of substantial amounts of water into the system fundamentally changes the conditions for the release of the trapped drug crystals: The latter can dissolve in the water and the dissolved drug molecules are rather mobile in the swollen "PLGA gel". Please note that non-dissolved drug cannot diffuse and that limited drug solubility effects can be of importance even in the case of freely water-soluble drugs trapped in polymeric controlled drug delivery systems, if the amounts of water available for drug dissolution are limited (51,52). In addition, the mobility of the dissolved drug molecules is much higher in a highly swollen "PLGA gel" compared to a non-swollen (only slightly hydrated) PLGA matrix. See for example the optical microscopy pictures in the columns at the right hand side versus the left hand side in Figures III-5 and III-6. This is true, even if the PLGA is in the rubbery state (please see above). Due to the fundamentally facilitated drug dissolution and increased drug mobility, the resulting drug diffusion rate increases and, thus, the release rate increases. This is likely the root cause for the onset of the 3rd drug release phase also in this study. The green region in Figure III-8 highlights the respective drug release curves (upper diagram) from single microparticles, which follow this type of behavior. Please note that in the case of the "small" microparticles, this 3rd release phase is not very much pronounced (Figure III-1), because most of the drug is already released before the onset of substantial PLGA swelling throughout the system.

It has recently been suggested to call this key role of PLGA swelling for the onset of important drug release "orchestrating role" (in the context of hot melt extruded macroscopic PLGA implants) (46,47). It is the same role that PLGA swelling likely has for the onset of the 3rd drug release phase from microparticles. Figure I-9 schematically illustrates this type of drug release behavior at the bottom: "Perfectly" trapped tiny drug crystals "have to wait for their release" until substantial system swelling sets on. Before, the amounts of water getting into contact with these crystals are too small to effectively dissolve them, and the mobility of potentially dissolved drug molecules is rather low in the only slightly hydrated PLGA matrix (even if the latter is in the rubbery state). Please note that different types of drugs likely behave differently, e.g. drugs that easily dissolve in the rubbery PLGA matrix might be able to diffuse also through the slightly hydrated polymeric system prior to the onset of substantial PLGA swelling to important extents.

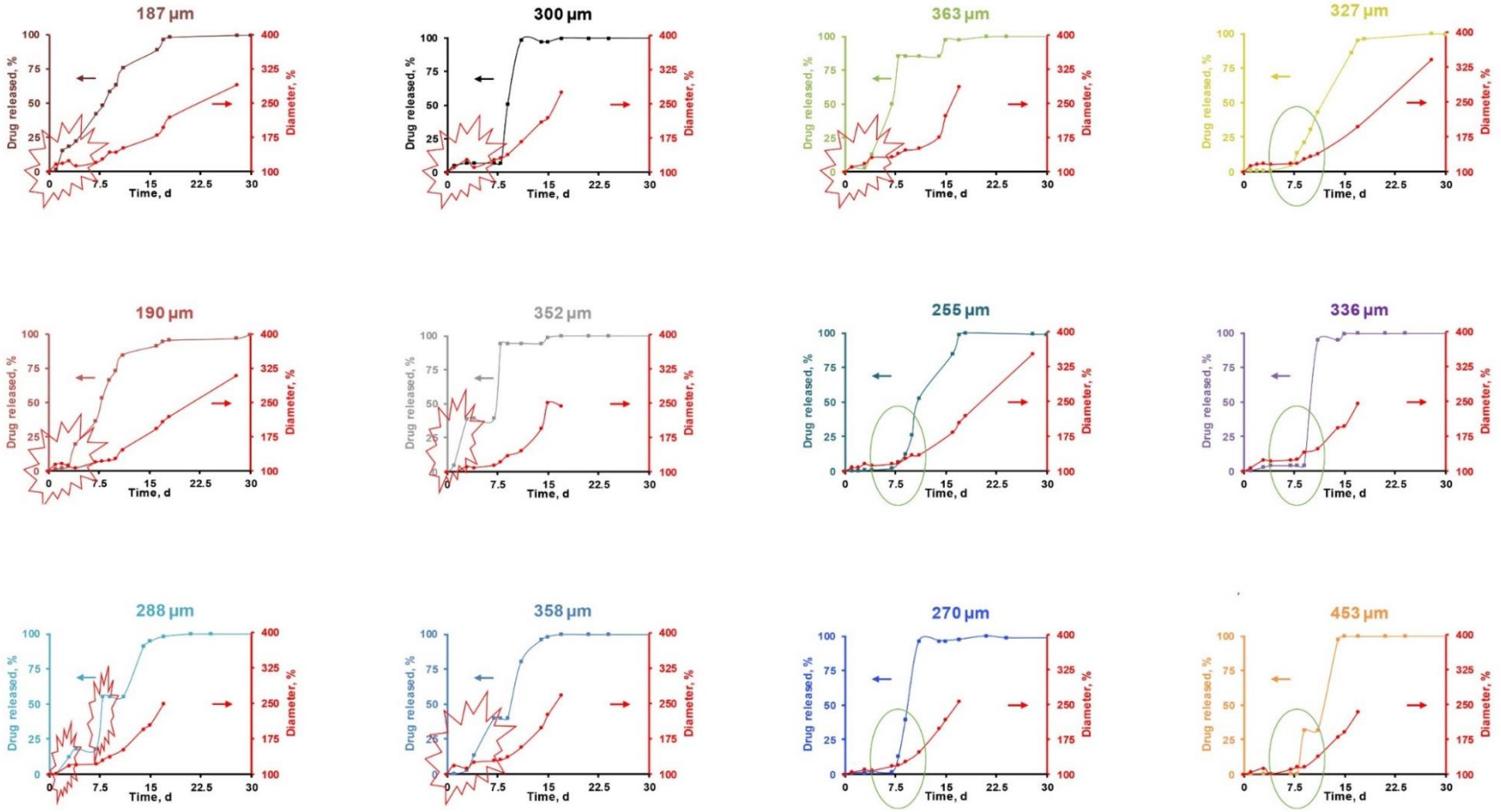


Figure III-7: Drug release and swelling of single PLGA microparticles upon exposure to phosphate buffer pH 7.4. The initial microparticle sizes are indicated at the top of each diagram. "Occasional/premature" drug release is marked in red, drug release following the onset of substantial swelling of the entire system is marked in green.

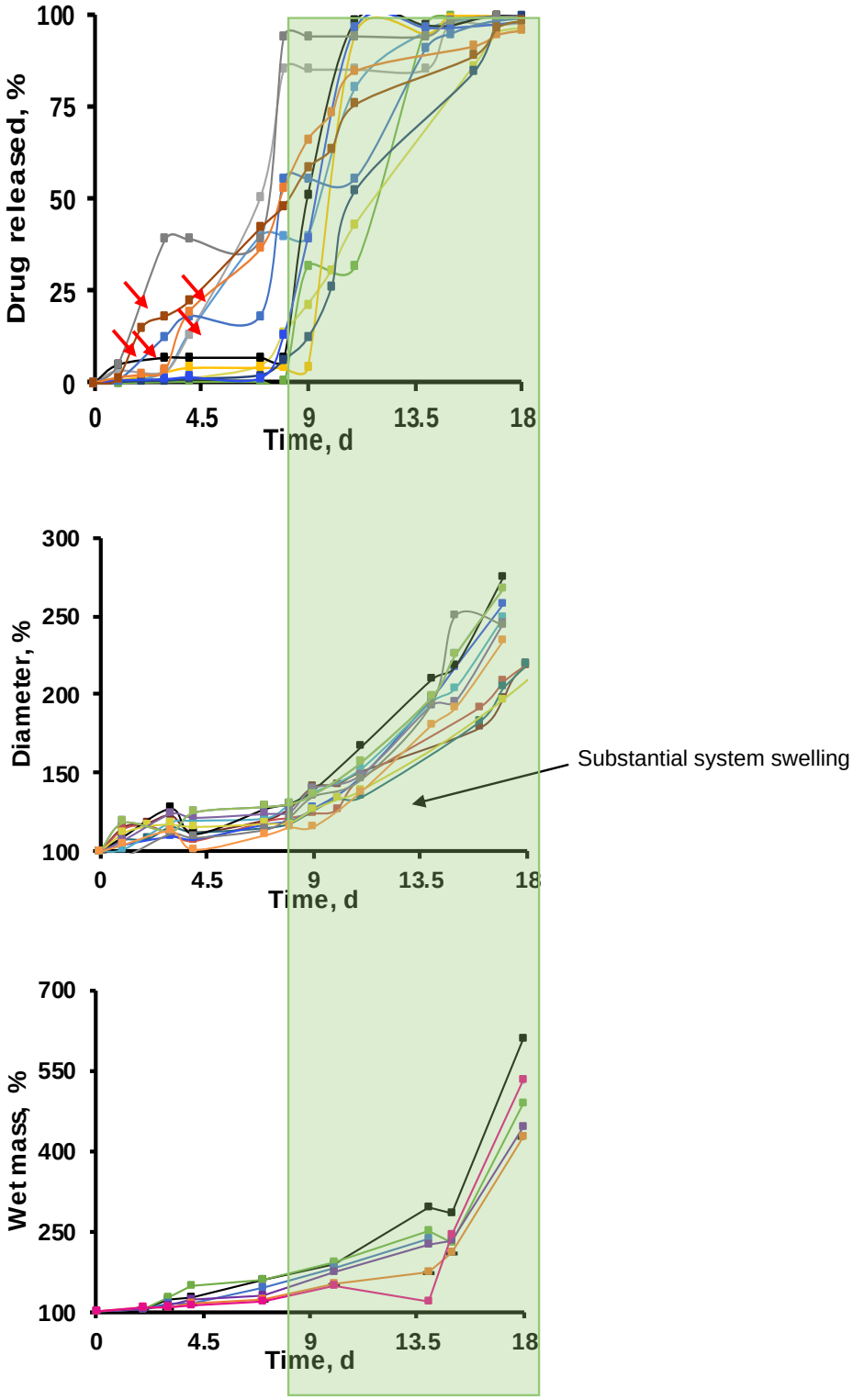


Figure III-8: Behavior of single PLGA microparticles loaded with diprophylline upon exposure to phosphate buffer pH 7.4: Drug release, dynamic changes in the diameter and dynamic changes in the wet mass. Each curve corresponds to a single microparticle.

The SEM pictures at the bottom rows in Figures I-3 and I-4 illustrate how highly swollen "PLGA gels" look like upon freeze-drying (which was required after sampling prior to the SEM measurements): Highly porous structures can be seen and no clear evidence for the presence of drug crystals (because most of the drug is already released after 10 d, Figure I-1). Please note that the exact structures that are visible in the SEM pictures are likely artefacts.

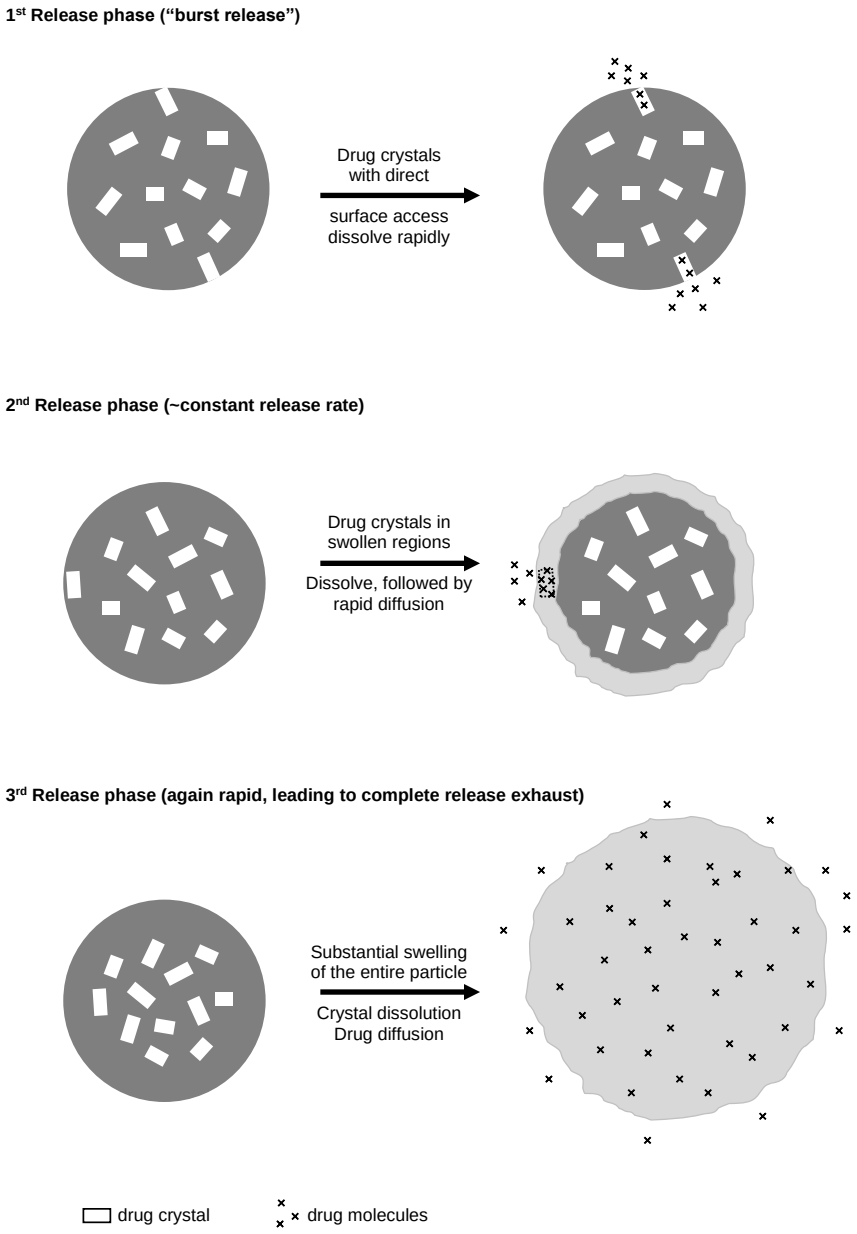


Figure III-9: Schematic illustration of the involved mass transport phenomena controlling diprophylline from the investigated PLGA microparticles during the 1st, 2nd and 3rd release phase. Non-swollen (only slightly hydrated) PLGA is marked in dark grey, swollen PLGA in light grey. Details are given in the text. Please note that the schemes are simplifications, e.g. with respect to the homogeneity of polymer swelling. Also, each microparticle has a specific, individual inner structure (e.g. location of the trapped drug crystals) and might contribute to 1 or more drug release phases.

(ii) Other microparticles release at least parts of their drug loading prior to the onset of substantial polymer swelling (before about 1 week in this case). This is marked in red in Figure III-7. In certain cases, such "premature" drug release was rather limited (e.g., in the case of the 187 µm particle in Figure III-7). In other cases, even 2 such "premature release events" were observed (e.g., in the case of the 288 µm particle in Figure III-7). This behavior can probably be explained as follows: Some of the tiny drug crystals (that are distributed throughout the PLGA microparticles) are relatively close to the systems' surface. Upon exposure to the release medium, microparticle swelling is limited during the first few days (as discussed above), but it is not completely absent. For instance, comparing the 2 columns on the left hand side in Figure III-5, showing optical microscopy pictures of microparticles at day 0 (before exposure to the release medium) and day 3, it can be seen that the particles slightly increased in diameter and that the particles' surfaces became less smooth. An example is also illustrated in Figure III-10. Thus, the outermost regions of the microparticles become deformed, indicating the swelling of these zones (at least to a certain extent). The overall extent of particle swelling is limited, for instance due to the presence of the still only slightly hydrated and mechanically rather stable inner microparticle core. As long as such a "mechanically stable" core exists, substantial swelling is hindered. Only once also the core region start to swell substantially, the microparticle can expand significantly in volume. The limited particle swelling during the first week after exposure to the release medium can also be seen in the diagrams in the middle and at the bottom of Figure I-8 (showing dynamic changes in the diameter and wet mass).

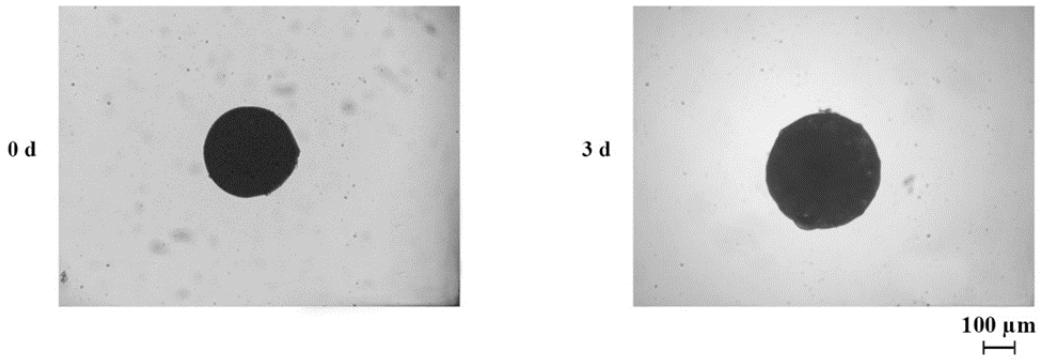


Figure III-21: Optical microscopy pictures of a single diprophylline-loaded PLGA microparticle before and after 3 d exposure to phosphate buffer pH 7.4.

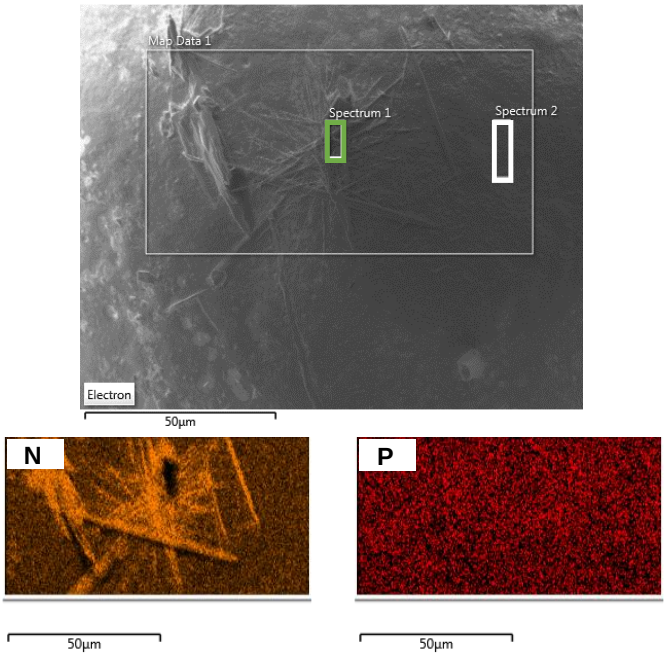
If a tiny drug crystal is located close to the microparticle's surface, at a certain time point, this zone likely swells and drug release can set on: The presence of important amounts of water in the direct vicinity of a drug crystal allows for its dissolution and the dissolved drug is rather mobile in the swollen "PLGA gel". As it can be seen in Figure III-7, this type of "occasional" drug release event (marked in red) is arbitrary and occurs only in certain microparticles, and at randomly distributed time points. This is because the tiny drug crystals are randomly distributed throughout the systems (Figure III-3) and the swelling is not perfectly homogeneous. Each microparticle has "its own" specific inner structure and releases the drug "in its own way" (Figure III-7). Adding up all the "occasional" (or "premature") drug release events can likely explain the observed 2nd drug release phase from the ensembles of microparticles (Figure III-1). Here, the term "premature" is used to express that this type of drug release occurs prior to the onset of substantial swelling of the entire microparticle (which is the root cause for the onset of the 3rd release phase, as discussed above). Several release curves in the upper diagram in Figure III-8 (left to the "green zone") exhibit such "occasional premature release events" (highlighted by small flashes). Since the surface near "swelling front" can be expected to more or less homogeneously advance towards the center of the microparticles, these "occasional"/"premature" drug release events likely occur with an about constant probability over time, explaining the about constant drug release rate in "phase 2". Please note that the decrease in surface area of the "swelling front" with time due to the spherical geometry of the system likely only plays a minor role (or is not of importance), because it lasts only about 1 week: Afterwards, substantial microparticle swelling throughout the system becomes dominant (please see above). Figure III-9 schematically illustrates this type of drug release behavior (2nd release phase).

Please note that the term "swelling front" might be misleading: In the case of macroscopic, cylindrical, hot melt extruded PLGA implants, recently swollen implants "shells" could be distinguished from only slightly hydrated, non-swollen implant cores (47). But these swollen "shells" were not very homogeneous. In the case of microparticles, it is not yet clear how sharp such "swelling fronts" might be. SEM pictures should always be seen with great caution, since system drying prior to the measurements likely creates artefacts. So, the term "swelling front" should be

viewed with great caution, it might also be a rather random swelling of certain parts of the PLGA microparticle, not necessarily a clear front that moves inwards.

Further experimental evidence for this release mechanism can be seen in Figure III-3: The second row from the top shows SEM pictures of the surfaces of microparticles after 3 d exposure to the release medium. As it can be seen, crystals are visible on the surface of some particles. Energy dispersive X-ray spectroscopy (EDS) analysis revealed that these crystals are diprophylline crystals (Figure III-11, nitrogen being present in the drug, but not in the polymer or other excipients used in this study). Please note that these drug crystals are likely artefacts created during freeze-drying (which was needed to obtain dry samples for the SEM measurements). In the wet state, during drug release, these crystals are very unlikely to exist: The drug is freely water soluble and perfect sink conditions were provided. This drug was likely dissolved either in cavities (formed upon dissolution of drug crystals) or in swollen "PLGA gel" regions ("on its way to diffuse out of the system"). Upon freeze-drying, the drug molecules precipitated and formed the needle-shaped crystals at the microparticles' surface. Importantly, not all microparticles showed this behavior. For example, the surface of the microparticle illustrated in the third row from the top in Figure III-3 was free of crystals after 3 d exposure to the release medium. This highlights the "individuality" of each PLGA microparticle. The second row from the bottom in Figure III-3 shows another example for a microparticle with clearly visible drug crystals at its surface, here after 7 d exposure to the release medium. Again, this is likely due to the precipitation of drug that was dissolved in cavities or in swollen "PLGA gel", contributing to the 2nd release phase. The middle row in Figure III-4 shows cross-sections of (freeze-dried) microparticles after 3 d exposure to the release medium. Importantly, various tiny drug crystals can be seen, the size and shape of which are much more similar to the size and shape of the diprophylline crystals distributed throughout the microparticles prior to exposure to the release medium (Figure III-4, top row). Thus, these are likely examples for "well-embedded" drug crystals, which did not come into contact with water prior to the sampling time point (here 3 d).

a)



b)

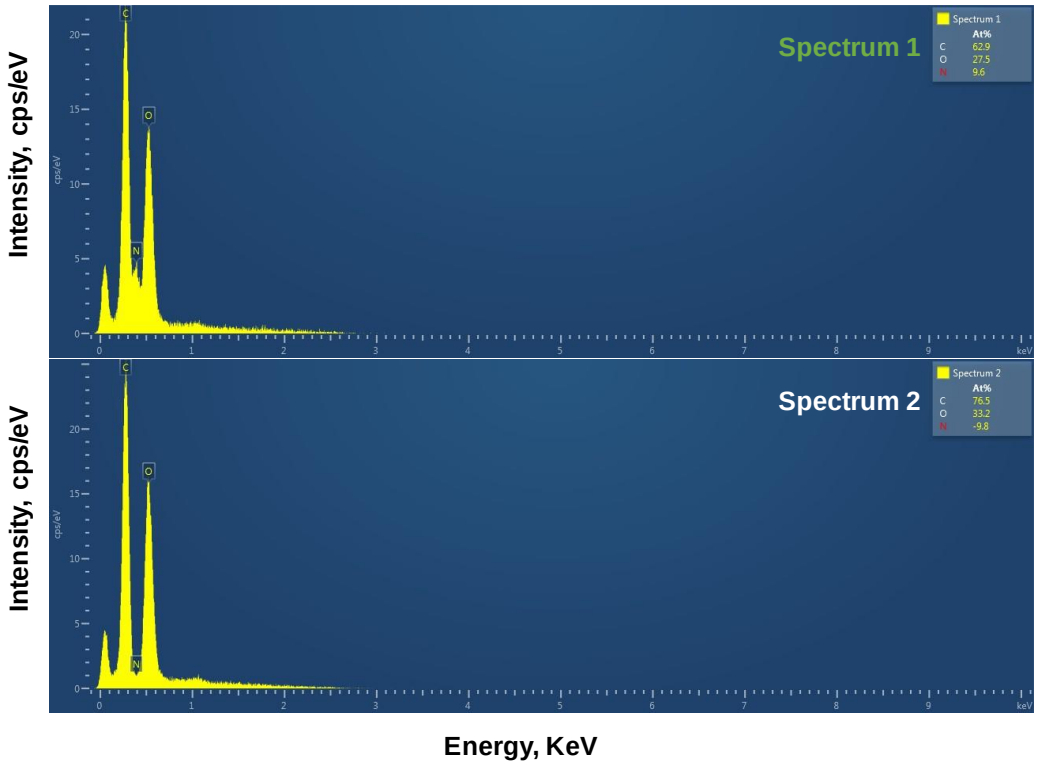


Figure III-11: : Energy dispersive X-ray spectroscopy (EDS) analysis of specific regions on the surface of diprophylline-loaded microparticles (after 3d exposure to phosphate buffer pH 7.4 and subsequent freeze-drying): a) location of the regions from which the spectra were obtained (region with crystals - spectrum 1; region without crystals - spectrum 2), b) spectrum 1 and spectrum 2.

(iii) Other microparticles contain drug crystals, which likely have direct surface access right from the beginning (or very shortly afterwards): In these cases, water can dissolve the drug crystals immediately upon exposure to the release medium, and the drug is rapidly released. The 300 µm particle and 352 µm particles in Figure III-7 are likely examples for such cases. This causes the "burst release" (= 1st release phase). Figure III-9 schematically illustrates this type of drug release behavior. If the surface area in direct contact with the release medium is limited, it can take several hours or eventually days for the entire drug crystal to be released.

As illustrated in Figure III-12a, this phenomenon is much more likely to occur in smaller microparticles than in larger microparticles (if the inner system structures are similar). The same amount of drug is located in numerous small microparticles and only a few large microparticles (the sums of the volumes of the particle populations is equal). The total number of drug crystals with direct surface access is much higher in the numerous small microparticles compared to the few large microparticles, resulting in a much more pronounced burst effect. This explains why the burst effect was much more important from the ensembles of "small" microparticles compared to the ensembles of "medium-sized" and "large" microparticles, as shown in Figure III-1. Please note that it was not possible to monitor the behavior of single microparticles much smaller than about 200 µm for technical reasons. Thus, Figure III-7 does not show any "small microparticles", which likely show many more "early drug release events", due to drug crystals with direct surface access right from the beginning (or shortly afterwards). It would be interesting to study this aspect with different techniques in the future.

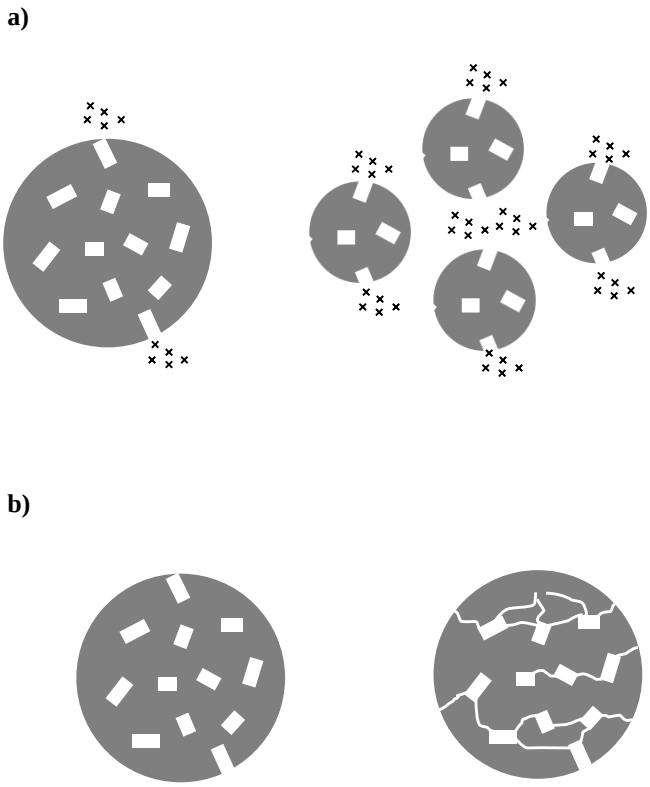


Figure II-12: a) Schematic presentation of a PLGA microparticle of a larger and several smaller microparticles: If the inner system structure is similar, the likelihood of drug crystals with direct surface access is much higher in the case of smaller microparticles, resulting in much more pronounced burst effects. b) Schematic presentation of a PLGA microparticle loaded with tiny drug crystals. The left drawing is a simplification showing only isolated drug crystals. The right drawing is likely much more realistic (at least for the investigated type of microparticles in this study), showing interconnections between some of the tiny drug crystals, forming "networks". Please note that these are 2-dimensional schemes, in reality the microparticles are spherical and interconnected networks are formed in all 3 dimensions.

Please also note that the schemes in Figure III-9 are simplifications: Drug crystals are illustrated as being individualized, without contact points to other crystals or channels connecting several drug crystals. In reality, at least some of the diprophylline crystals are either directly in contact with each other, or via "channels" (Figure III-4, top raw, please note that only 2 dimensional cross-sections are shown, the fact that the crystals are 3-dimensional and that right below the visible plane other drug crystals are located, should not be forgotten). Thus, it is likely more realistic that "interconnected networks of drug crystals" exist, as illustrated in Figure III-12b: If one of the crystals in such a "network" has "direct surface access" from the beginning (1st release phase) or its surrounding swells at a certain time point (2nd release phase), also the "connected" drug crystals will likely dissolve and be rather rapidly released afterwards. For reasons of simplicity, this fact is not shown in the other schemes of this article, but it should not be neglected.

III.3. Drug release mechanisms

In the following a short summary of the above discussed drug release mechanisms is given:

Importantly, each PLGA microparticle has its own particular inner structure, e.g. with respect to the exact locations of the tiny drug crystals distributed within the PLGA matrix. This individual structure determines whether the microparticle contributes to the 1st, 2nd and/or 3rd release phases. All options are possible, their likelihood depends among other factors on the microparticle size. The observed release kinetics from ensembles of microparticles (Figure III-1) are the sums of all the individual microparticle release behaviors in the sample. As illustrated in Figure III-9:

The 1st release phase (burst release) from the investigated PLGA microparticles can likely be attributed to the dissolution of drug crystals with direct surface access right from the beginning (or shortly afterwards) (an example is shown at the top of Figure III-13). Drug dissolution is not necessarily instantaneous, but might take up to about 1-2 d, because the drug might have to diffuse through a tiny pore.

The 2nd release phase (with an about constant drug release rate) is probably caused by the swelling of the outermost regions of the PLGA microparticles. If a tiny drug crystal is located in such a region, at a certain time point its direct environment undergoes an important change: from a slightly hydrated PLGA matrix to a swollen "PLGA gel". Once this happens, this drug crystal starts dissolving and the dissolved drug molecules are able to diffuse out through the swollen gel (an example is shown in the middle of Figure III-13). This type of "release event" occurs occasionally. Since the swelling "front" likely advances "rather" homogeneously, the probability of these events is about constant over time, resulting in about constant drug release rates.

The 3rd release phase (= final, again rapid drug release phase) is likely caused by substantial PLGA swelling throughout the system: Once the polymer chains are sufficiently hydrophilic and the network becomes "mechanically instable" and no "stable" microparticle core restricts the swelling of the entire system, the osmotic pressure created by the water-soluble degradation products attracts important amounts of water into the microparticles. Consequently, drug dissolution is very much facilitated and the mobility of the dissolved drug molecules significantly increased. Both effects lead to a substantial increase in the drug release rate and finally complete drug exhaust (an example is shown at the bottom of Figure III-13). The key

role of this substantial PLGA swelling has also been called “orchestrating role”, because the swelling determines whether the drug is able to dissolve & diffuse, or not.

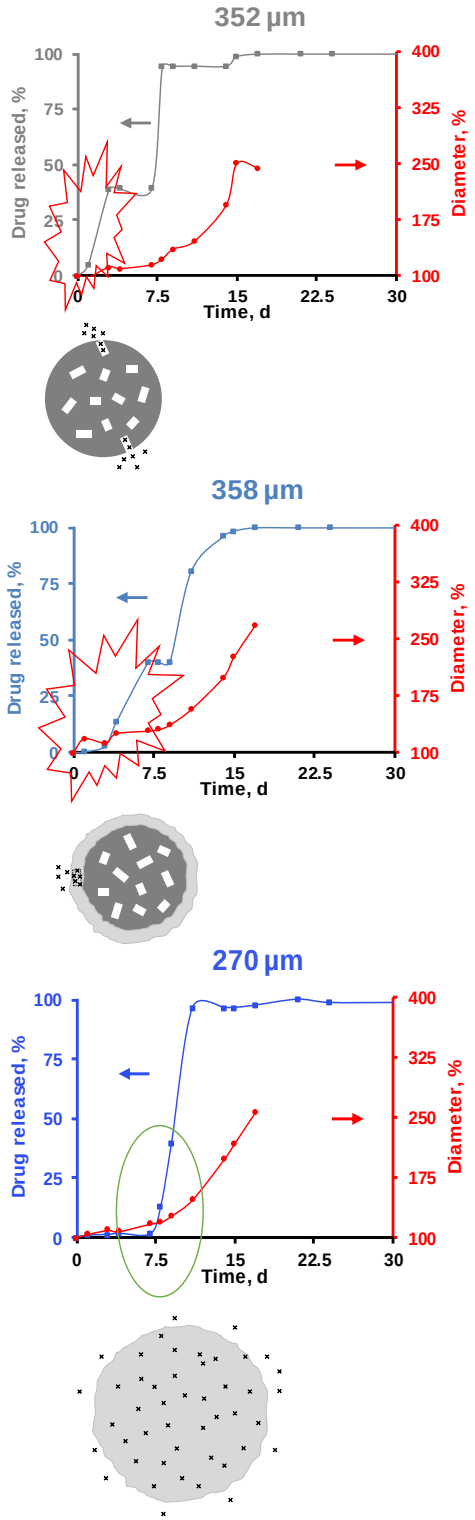


Figure III-13: Examples for single microparticles contributing to the different phases of drug release from the investigated PLGA microparticles. The experimental results show drug release from and the swelling of the systems, the schemes illustrate the likely root causes for drug release. Details are explained in the text.

IV. Conclusion

The aim of this study was to better understand the root causes for the (up to 3) drug release phases of PLGA-based microparticles loaded with drug particles (in particular of the 1st and 2nd release phase). In this case, diprophylline crystals were rather homogeneously distributed throughout the polymer matrix after manufacturing. It is suggested that every microparticle has its own, individual inner structure and drug release profile. Each microparticle contributes to one or more drug release phases. It would be interesting to study other types of microparticles in the future and to use additional experimental measurement techniques to evaluate the validity of the proposed release mechanisms also in other systems. Please note that different drugs can be expected to behave differently. For example, drugs which have a high affinity to PLGA might be able to dissolve to noteworthy extents in only slightly hydrated polymer regions and diffuse through these regions at important rates prior to the onset of substantial microparticle swelling.

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Part II

Towards a better understanding of the release mechanisms of caffeine from PLGA microparticles

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Abstract:

Poly (lactic-co-glycolic acid) (PLGA)-based microparticles can be successfully used to control the release rate of a drug and optimize the therapeutic efficacy of a medical treatment. However, the underlying drug release mechanisms can be complex and are often not fully understood. This renders system optimization cumbersome. In this study, differently sized caffeine-loaded PLGA microparticles were prepared and the swelling and drug release behaviors of single microparticles were monitored upon exposure to phosphate buffer pH 7.4. Ensembles of microparticles were characterized by X-ray diffraction, DSC, SEM, GPC and optical microscopy. The observed tri-phasic drug release patterns could be explained as follows: The initial burst release can be attributed to the dissolution of tiny drug crystals with direct surface access. The subsequent 2nd drug release phase (with an about constant release rate) could be attributed to the release of drug crystals in regions, which undergo local swelling. The 3rd release phase (again rapid, leading to complete drug exhaust) could be explained by substantial polymer swelling throughout the systems: Once a critical polymer molecular weight is reached, the PLGA chains are sufficiently hydrophilic, insufficiently entangled and the osmotic pressure created by water soluble degradation products attracts high amounts of water into the system.

I. Introduction

Poly (lactic-co-glycolic acid) (PLGA) is frequently used as a polymeric matrix former in controlled drug delivery systems, in particular microparticles (1–6), scaffolds (7,8), nanofibers (9) and implants (10–14). This type of advanced drug products allows to pre-program the release rate of the active agent into the human body after injection or implantation. Flexible release periods can be provided, e.g. ranging from a few days up to several months (15,16). Controlling the "entry" rate into the human body allows optimizing the therapeutic efficacy and minimizing the risk of toxic side effects: Each drug has a characteristic *minimal effective concentration*, below which no therapeutic effects occur, and a characteristic *minimal toxic concentration*, above which undesired side effects occur. The aim is to achieve drug concentrations at the site of action between these two concentrations: in the so-called "therapeutic window". Unfortunately, certain drugs have narrow therapeutic windows and severe toxic side effects. Controlled drug delivery systems can be of great interest in these cases. Generally, the basic idea is to trap the drug in a polymeric matrix. The presence of the latter avoids rapid drug dissolution upon administration into the human body (e.g. by sub-cutaneous injection or implantation). The drug "has to find its way" out of the dosage form to be released. Different types of physico-chemical processes can be involved in the control of the resulting drug release rate (17), such as drug dissolution (18), drug diffusion (19), polymer degradation (20–22), polymer swelling (23–25), and osmotic effects (26) to mention just a few.

PLGA offers several major advantages as polymeric matrix former for injectable and implantable drug delivery systems, since it is biocompatible (27) and biodegradable (28). Thus, upon drug exhaust, there is no need to remove empty remnants: a major benefit for the patient. Various types of PLGA-based controlled drug delivery systems have been described in the literature (29–37). PLGA *microparticles* are often more easy to administer than PLGA *implants*, e.g. using relatively thin needles. Frequently, 3 drug release phases can be observed with PLGA microparticles (their relative importance can very much depend on the type of drug and manufacturing procedure): At early time points (e.g., during the first day), the release rate is often high. This is also called "burst effect". The 2nd release phase is generally characterized by an about constant drug release rate and can last several days or weeks. The 3rd release phase is again rapid and leads to complete drug exhaust.

Despite the great practical importance of PLGA microparticles as advanced drug delivery systems, the underlying mass transport phenomena are often not fully understood. Various types of physical and chemical processes might be involved (38–42), including for instance water penetration into the system, drug dissolution, drug diffusion through water-filled pores and/or the polymer matrix, hydrolytic polyester degradation, polymer swelling, the creation of osmotic pressure within the system due to the accumulation of water-soluble monomers and oligomers, drug – polymer interactions (e.g., plasticizing effects of certain drugs), the creation of acidic micro-environments (due to the generation of short chain acids as degradation products, especially at the center of the systems), and autocatalytic effects (since ester bond cleavage is catalyzed by protons). The relative importance of these phenomena in a particular type of PLGA microparticles likely depends on the type of drug, type of PLGA (e.g., type of end groups and average polymer molecular weight), composition of the system (e.g., presence of other excipients and drug loading) and the manufacturing procedure, which can affect the internal and external system structure (e.g. porosity). The resulting complexity makes it often difficult to reliably predict the effects of formulation and processing parameters on the resulting drug release kinetics. This renders the optimization of this type of advanced drug delivery systems cumbersome, e.g. being based on time-consuming and cost-intensive series of trial-and-error studies (with sometimes surprising tendencies).

Another particularly challenging aspect is the fact that PLGA microparticles are so called "multiple unit" dosage forms: Generally, numerous tiny microparticles (often less than 100 µm in diameter) are administered. In most cases, only such *ensembles* of microparticles are studied and characterized with respect to their drug release behavior. However, each microparticle is individual and might release the drug "in its own way", e.g. due to its unique internal structure. For this reason, it can be very helpful to monitor also the behavior of *single* microparticles. For example, the group of Anders Axelsson studied the release behavior of polymer coated "pellets" (little spherical beads, which can be filled into hard gelatin capsules to control drug release). It was shown that the release behavior of the individual beads could be very different, but the use of hundreds of these beads at the same time could provide reproducible release profiles, which were different in shape compared to the individual release profiles (43,44). For instance, if hundreds of *single* unit dosage forms release a drug in a "pulsatile manner" at randomly distributed time points,

the overall release rate of the *ensemble* of dosage forms is constant. Studying only the release of *ensembles* of dosage forms can, thus, be misleading.

The aim of this study was to prepare differently sized caffeine-loaded PLGA microparticles using an emulsion solvent extraction/evaporation method and to characterize the systems thoroughly before and after exposure to phosphate buffer pH 7.4 (a release medium, which is frequently used to simulate aqueous body fluids upon injection). X-ray diffraction, gel permeation chromatography, scanning electron microscopy, optical microscopy, differential scanning calorimetry and in vitro drug release studies were used to monitor the physical states of the drug and PLGA during drug release. Importantly, both, *single* microparticles as well as *ensembles* of microparticles were studied.

II. Materials and methods

II.1.Materials

Poly (D,L lactic-co-glycolic acid) (PLGA; Resomer RG 504H; 50:50 lactic acide:glycolic acid; Evonik, Darmstadt; Germany); caffeine (BASF, Ludwigshafen, Germany); polyvinyl alcohol (Mowiol 4-88; Sigma-Aldrich, Steinheim, Germany); acetonitrile and dichloromethane (VWR, Fontenoy-sous-Bois, France); tetrahydrofuran (HPLC grade; Fisher Scientific, Illkirch, France).

II.2.Microparticle preparation

Drug-loaded microparticles were prepared using an oil-in-water (O/W) emulsion solvent extraction/evaporation technique: Appropriate amounts of caffeine and PLGA were dissolved in a well-defined volume of dichloromethane (Table III-3). “Small”, “medium-sized” and “large” microparticles were prepared, adapting the formulation and processing parameters accordingly (Table III-3). The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 and 2000 rpm, Eurostar power-b; Ika-Werke, Staufen, Germany) for 30 min. Upon solvent exchange, the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25 %) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40 °C and 0.07 mbar for 35 h and secondary drying at +20 °C/0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

Table III-3: Composition of the inner organic phase and stirring speed used for the preparation of "small", "medium-sized" and "large" PLGA microparticles loaded with caffeine.

Microparticle size	CH_2Cl_2, mL	PLGA, mg	Drug, mg	Stirring speed, rpm
″Small″	10	903.1	97.9	2000
″Medium-sized″	6	900.7	104.6	1500
″Large″	4	902.4	104.0	1000

II.3.Microparticle characterization

II.3.1. Microparticle size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.3.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PVDF syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis (Alliance, Separation Modules e2695, 2489, UV-Vis Detector; Waters, Milford, USA). A reversed phase column C18 (Gemini 5 µm; 110 A °; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetonitrile: water (70:30, v:v). The detection wavelength was 254 nm and the flow rate 1 mL/min. Twenty µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.3. X ray powder diffraction

X ray powder diffraction analysis was performed using a Panalytical X'pert pro diffractometer (λ Cu K α=1.54 Å) and Lindemann glass capillaries (diameter 0.7 mm) (PANalytical, Almelo, The Netherlands). The measurements were conducted in transmission mode with an incident beam parabolic mirror and the X'celerator detector.

II.3.4. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: caffeine and PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminium pans from 10 to 120 °C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperature (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.5. Drug release measurements from ensembles of microparticles

Ten mg of microparticles were placed into Eppendorf tubes (Safe-lock, 2.0 mL; Eppendorf, Hamburg, Germany), filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033, Gesellschaft fuer Labortechnik, Burgwedel, Germany). At predetermined time points, 1.5 mL samples were withdrawn (replaced with fresh medium), filtered (PVDF syringe filters, 0.45 µm; GE Healthcare, Kent, UK) and analysed for their drug contents by HPLC analysis, as described above. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout the experiments.

II.3.6. Drug release measurements from single microparticles

Caffeine release from single microparticles was monitored in 1 mL syringes (three-part single-use syringes; HSW Henke-Ject, Tuttlingen, Germany) as follows: One microparticle was introduced into a syringe, which was filled with 200 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap [BD Luer-Lok (TM) (caps with male/female protection); Dominique Dutscher, Brumath, France]. The syringes were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At predetermined time points, 50 µL samples were withdrawn (replaced with fresh medium) using Hamilton syringes (Microlite/#710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for

their drug contents by HPLC, as described above (the standard curve covering the range of 0.025 to 5 µg/mL).

II.3.7. Swelling of single microparticles

The swelling of individual microparticles was monitored in 96-well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 200 µL phosphate buffer pH 7.4 (USP 42). The well microplates were placed into a horizontal shaker (37°C, 80 rpm, GFL 3033). At pre-determined time points, microparticle samples were carefully withdrawn, and pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). Also, as for the drug release studies, 50 µL samples were withdrawn and replaced with fresh medium at each sampling time point.

II.3.8. Polymer degradation

Microparticles were treated as for the drug release studies. At predetermined time points, samples were withdrawn, freeze-dried for 3d (as described above) and the lyophilisates were dissolved in tetrahydrofuran (at a concentration for 3 mg/mL). The average polymer molecular weight (Mw) of the PLGA in the samples was determined by Gel Permeation Chromatography (GPC, Alliance, refractometer detector: 2414 RI, separation module e2695, Empower GPC software; Waters, Milford, USA), using a Phenogel 5 µm column (which was kept at 35°C, 7.8 × 300 mm; Phenomenex, Le Pecq, France). The injection volume was 50 µL. Tetrahydrofuran was the mobile phase (flow rate: 1 mL/min). Polystyrene standards with molecular weights between 1480 and 70,950 Da (Polymer Laboratories, Varian, Les Ulis, France) were used to prepare the calibration curve. All experiments were conducted in triplicate. Mean values and ± standard deviations are reported.

II.3.9. Scanning Electron Microscopy (SEM)

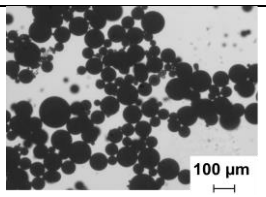
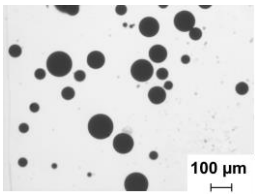
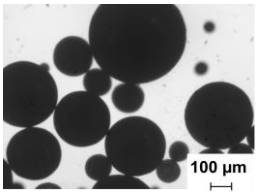
The internal and external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Cross-sections were obtained after inclusion of microparticles into “OCT embedding medium” (“embedding medium” for frozen tissue specimen to ensure Optimal Cutting Temperature; VWR, Lutterworth, UK) and cutting with cryostat (Leica CM3050 S, Wetzlar, Germany).

III. Results and Discussion

III.1. Ensembles of microparticles

To obtain differently sized PLGA microparticles loaded with caffeine using an emulsion oil-in-water (O/W) solvent extraction/evaporation method, the stirring speed and polymer concentration of the organic phase were varied, as shown in Table III-4. A higher stirring speed and lower polymer concentration (resulting in a lower viscosity of this phase) led to smaller organic phase droplets and, hence, smaller microparticles. Under the given conditions, the mean particles sizes (+/- standard deviations) of "small", "medium-sized" and "large" microparticles were equal to 62 (+/- 19), 94 (+/- 31) and 287 (+/- 159) µm, respectively. In practice, most often microparticles with a diameter of less than 100 µm are used. However, they are difficult to study individually, for technical reasons. In this study, also larger microparticles were prepared and their behavior upon exposure to the release medium was monitored individually: This can provide very interesting information on the underlying drug release mechanisms, especially in the case of multiple unit dosage forms (as PLGA microparticles): Generally, only drug release from the *ensembles* of numerous microparticles are measured. However, these are only the sums of all the individual microparticle release profiles, which might substantially vary from particle to particle. The basic underlying assumption of this study is that the inner and outer structures of the prepared smaller and larger microparticles are similar. If this would not be the case, the underlying drug release mechanisms might be different. No evidence was observed in this study for any relevant differences in the internal or external structures of the investigated microparticles. There was a minor difference in the practical drug loadings, which varied from 6 to 7 % (Table III-4): The smaller particles had a slightly lower practical drug content. This can be explained by the smaller size of the droplets of the organic phase formed during microparticle preparation, resulting in higher drug loss into the outer aqueous phase (due to shorter diffusion pathways). We believe that these differences have no major impact on the resulting drug release mechanisms.

Table III-4: Practical drug loading, mean practical size, glass transition temperature (Tgs) and morphology of "small", "medium-sized" and "large" PLGA microparticles loaded with caffeine.

	Practical loading, %	Mean size, µm	Tg, °C	Optical microscopy
″Small″	5.9 ± 0.5	61.8 ± 19.4	44.6 ± 0.2	
″Medium-sized″	5.5 ± 0.2	94.1 ± 31.5	44.3 ± 0.1	
″Large″	7.1 ± 1.0	286.9 ± 158.9	43.5 ± 0.4	

The glass transition temperatures (Tgs, determined by DSC analysis) were equal to about 44 °C in all cases (Table III-4). Please note that this indicates that the PLGA is in the glassy state at 37 °C body temperature. However, it is well known that upon contact with aqueous fluids, limited amounts of water rather rapidly penetrate into the entire system (often within hours or up to 1 d). Although these amounts are low, they effectively decrease the Tg of the PLGA by about 10 °C (45,46) (and start polyester hydrolysis throughout the system: "bulk erosion"). Thus, it can be expected that the polymer undergoes a transition from the glassy to the rubbery state rather rapidly upon administration into the human body.

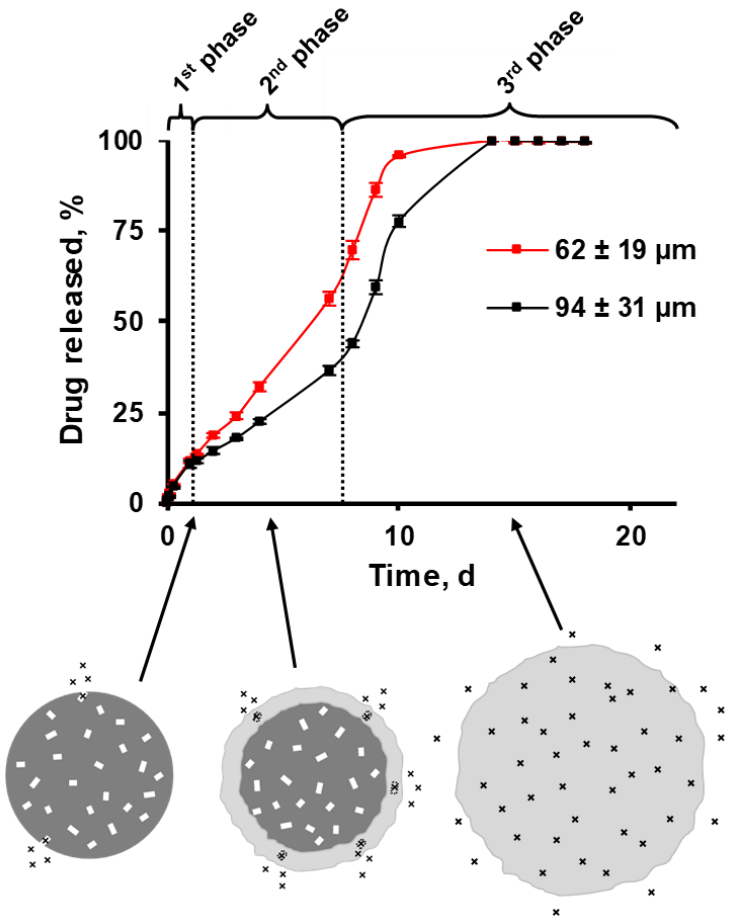


Figure III-14: Caffeine release from ensembles of PLGA microparticles in phosphate buffer pH 7.4: Impact of the mean particle size (indicated in the diagram +/- standard deviation). The release profiles are tri-phasic (although the first phase is not very pronounced): an initial (limited) burst release (= 1st phase) is followed by a period with an about constant drug release rate (= 2nd phase) and a final (again) rapid drug release phase leading to complete drug exhaust (= 3rd phase). The cartoons at the bottom indicate the hypothesized drug release mechanisms (details are given in the text).

Figure III-14 shows the experimentally measured caffeine release kinetics from *ensembles* of PLGA microparticles, differing in size: The mean particle diameters (+/- standard deviations) are indicated in the diagram. As it can be seen, classical "tri-phasic" drug release profiles were observed (although the 1st release phase was not very pronounced), irrespective of the microparticle size:

(i) At early time points (during the first day), the drug release rate was high. This is also called the "burst effect".

(ii) Then, the release rate remains about constant during several days. This is generally called the "2nd release phase". Please note that the slope of the release curve was higher for the smaller microparticles in this phase.

(iii) At a later time point (here, after about 1 week), a final rapid drug release phase set on, leading to complete drug exhaust. This phase is often referred to as the "3rd release phase".

The optical microscopy pictures in Table III-4 and the SEM pictures at the top of Figure III-15 show that the microparticles were spherical in shape and exhibited a rather smooth, non-porous surface before exposure to the release medium. The SEM pictures at the bottom of Figure III-5 show cross-sections of the differently sized microparticles. As it can be seen, small pores were distributed throughout the systems, irrespective of the microparticle size. Importantly, very small crystals (1 µm or less in size) were visible in the different cross-sections at higher magnification (bottom row in Figure III-15). These crystals are likely caffeine crystals, since X-Ray diffraction revealed sharp Bragg peaks in the different microparticle batches at the same angles as observed with the caffeine raw material (as received) (Figure III-16). This is important information for the underlying drug release mechanisms. The investigated microparticles are dispersions of very small drug crystals in a PLGA matrix. Please note that the caffeine was *dissolved* in the organic phase during microparticle preparation. However, at least parts of the drug recrystallized upon solvent evaporation. This is consistent with the fact that the glass transition temperature (Tg) of the PLGA raw material (as received) was equal to 47 +/- 0.2 °C, as compared to Tg values around 44 °C in the case of the PLGA microparticles loaded with 6-7 % caffeine (Table III-4). The slight decrease in Tg (by about 3 °C) can serve as an indication that parts of the drug are likely *dissolved* in the PLGA and act as a plasticizer for this polymer. But the solubility of caffeine in PLGA is likely limited: The decrease in Tg is limited and crystals are visible in cross-sections of particles loaded with 6-7 % drug.

To better understand why the different release phases were observed from the investigated caffeine-loaded PLGA microparticles (and why there was a moderate difference in the release rate during the 2nd release phase), the behavior of *single* microparticles was studied, in particular their swelling and drug release kinetics upon exposure to an aqueous phase simulating body fluids at the administration site.

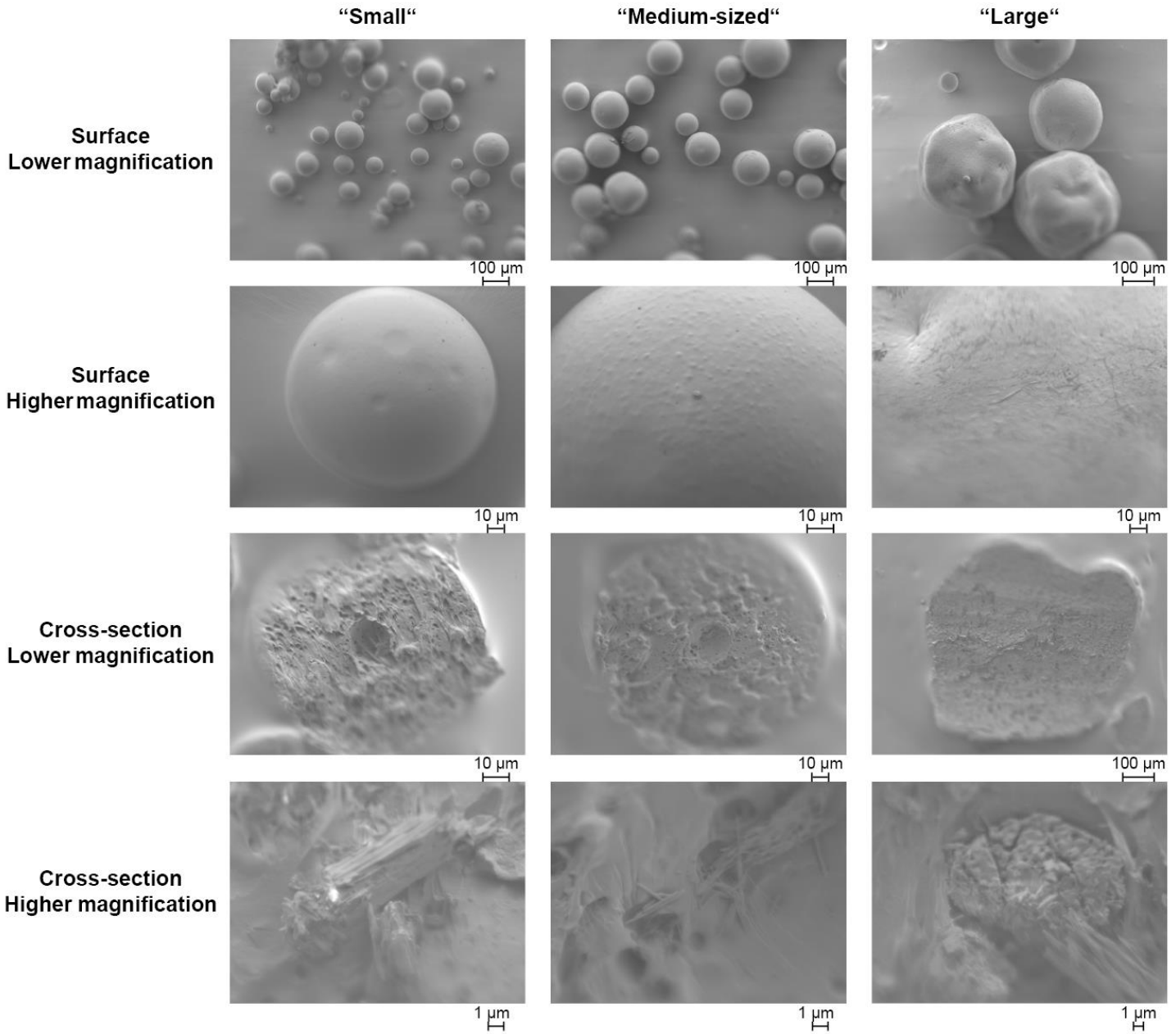


Figure II-15: SEM pictures of surfaces (lower and higher magnification) and cross-sections (lower and higher magnification) of caffeine-loaded microparticles before exposure to the release medium.

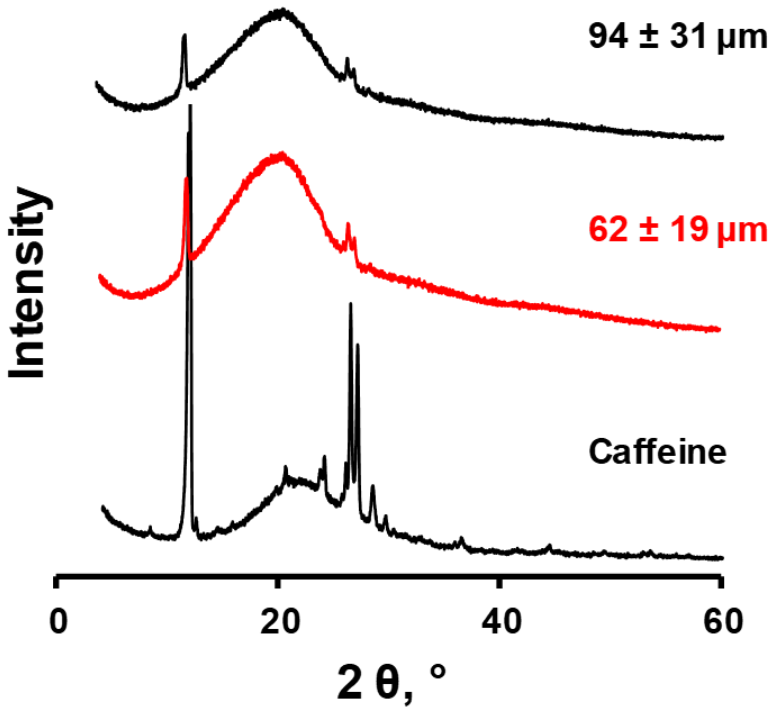


Figure III-16: X-ray diffraction patterns of ensembles of caffeine-loaded PLGA microparticles (mean diameters +/- standard deviations are indicated), and of caffeine raw material (as received) for reasons of comparison.

III.1. Single microparticles

Figure III-17 shows optical microscopy pictures of differently sized PLGA microparticles loaded with caffeine upon exposure to phosphate buffer pH 7.4 at 37 °C. As it can be seen, the size of the particles remained about constant during the first few days, but after about 1-week substantial microparticle swelling set on. The dynamic changes in the diameters of the *single* microparticles are plotted as a function of exposure time to the release medium in Figure III-18. The initial particle sizes are indicated at the top of the diagrams. A superposition of the different curves is shown in the diagram at the bottom of Figure III-18 on the right-hand side. Clearly, microparticle swelling was very much limited during the first week, but then substantial swelling set on. This phenomenon has recently been explained as followed, in the context of macroscopic cylindrical PLGA implants (47): Initially, the PLGA chains are rather hydrophobic and intensively entangled. This effectively limits the amounts of water, which can penetrate into the system upon contact with aqueous fluids. However, the limited amounts of water that enter the microparticles start cleaving the ester bonds of the PLGA throughout the system ("bulk erosion") (48).

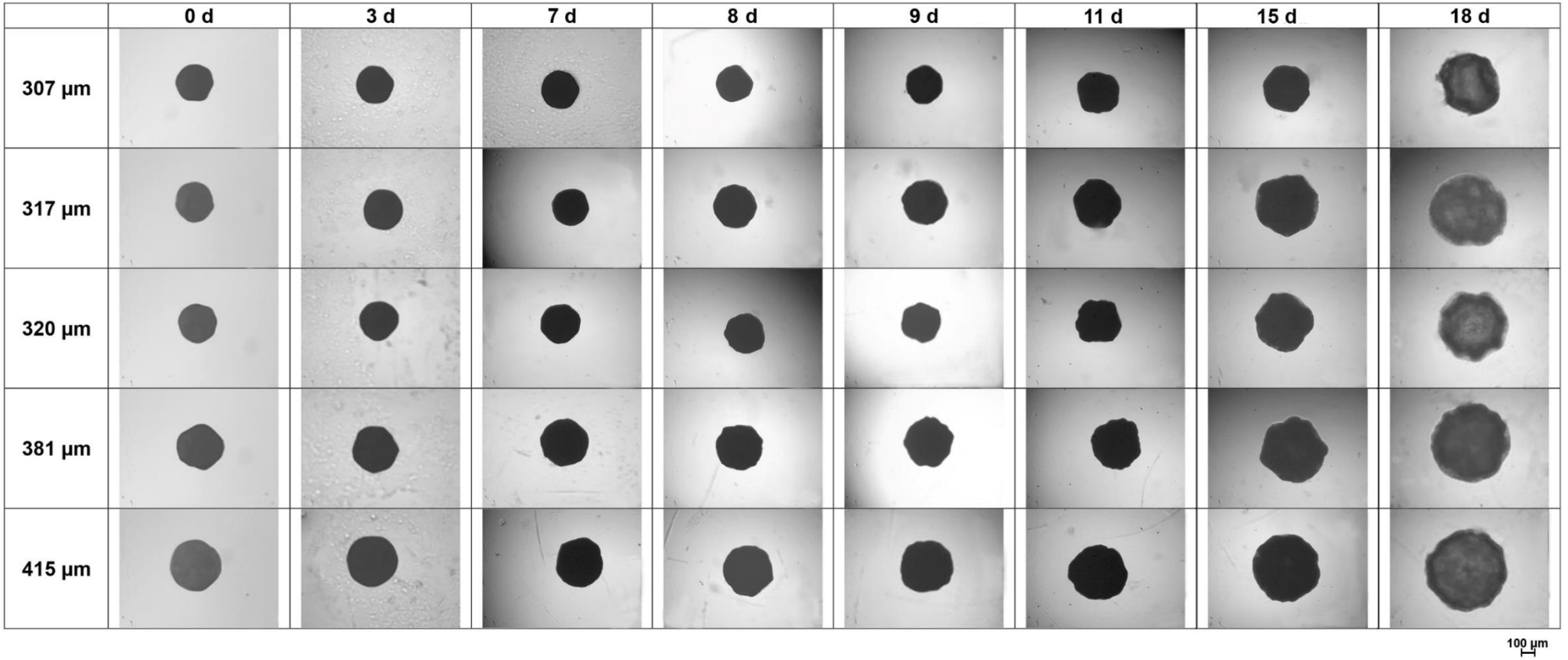


Figure I1I-17: Optical microscopy pictures of single caffeine-loaded PLGA microparticles before and after exposure to phosphate buffer pH 7.4 for different time periods (indicated at the top). The initial particle size is given on the left-hand side.

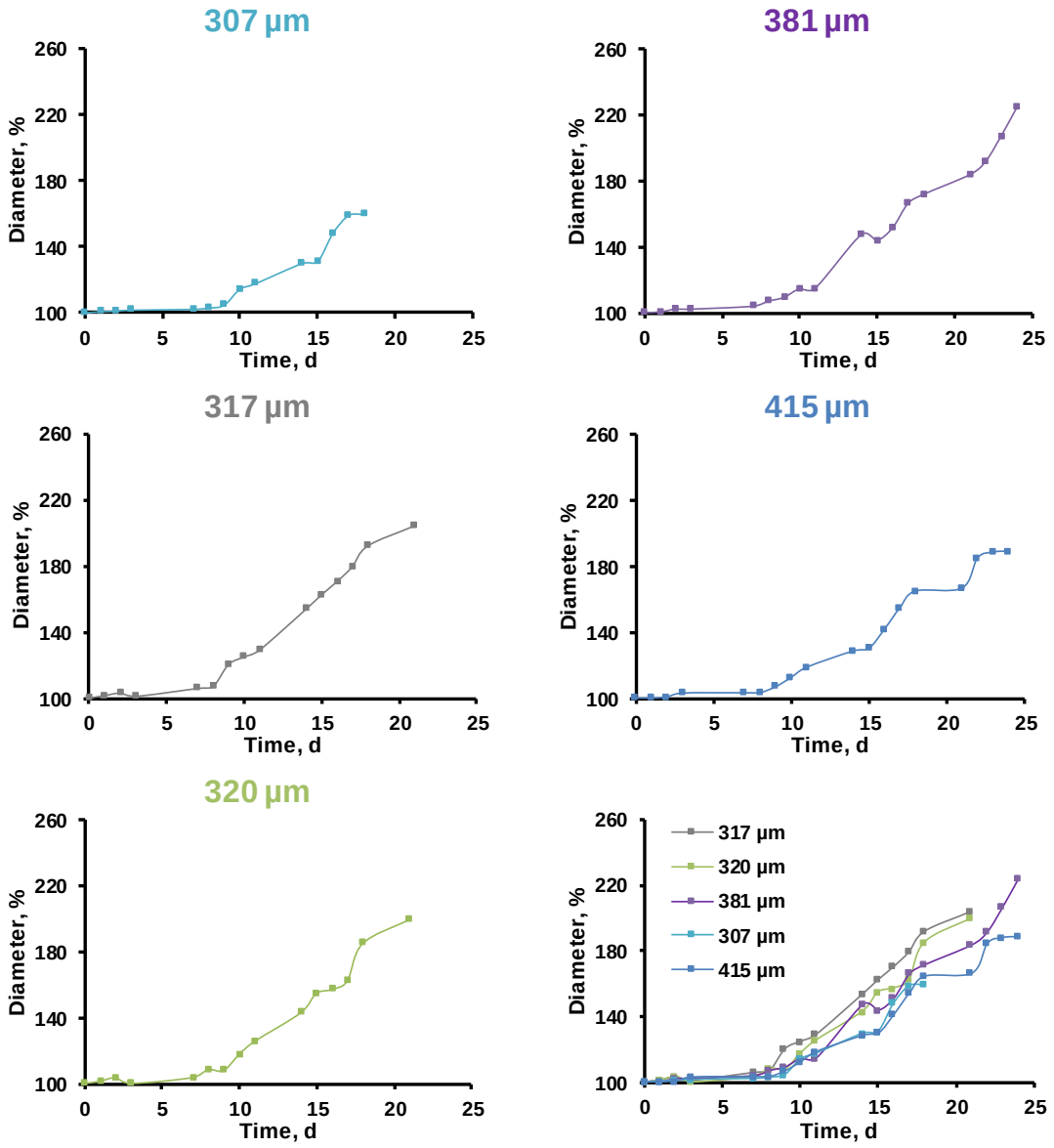


Figure III-18: Swelling kinetics of single PLGA microparticles upon exposure to phosphate buffer pH 7.4 (monitored by optical microscopy). The initial microparticle sizes are indicated at the top of each diagram. The diagram at the right-hand side at the bottom shows the superposition of all individual curves.

This has at least 3 major consequences: (i) The polymer chains become more and more hydrophilic, since new –OH and –COOH end groups are created upon ester bond hydrolysis. (ii) The polymer chains become less entangled, because their molecular weights decrease. This affects the "mechanical stability" of the polymeric matrix. (iii) Water soluble monomers and oligomers are generated, creating a steadily increasing osmotic pressure within the system. As

soon as a certain, critical threshold value is reached, the polymer matrix is sufficiently hydrophilic and mechanically instable, so that high amounts of water are effectively attracted by the osmotic pressure built up within the microparticles: Substantial swelling of the entire system sets on. The presence of high amounts of water within the microparticles allows for the complete dissolution of the caffeine crystals and results in relatively high mobilities of the dissolved drug molecules in the PLGA gels. Both effects lead to an increase in the resulting drug release rate: The final, rapid drug release phase (= 3rd release phase) starts.

This type of drug release mechanism is likely also of importance in the investigated caffeine-loaded PLGA microparticles: As it can be seen in Figure III-14, after about 1 week the final rapid drug release phase set on, irrespective of the microparticle size. Also, the drug release profiles observed with *single* microparticles confirm this theory: Figure III-19 shows the release of caffeine from individual PLGA microparticles in phosphate buffer pH 7.4 at 37 °C (body temperature). The diagram on the right-hand side at the bottom of Figure III-19 shows the superposition of the different curves. As it can be seen, a final rapid drug release phase was observed in all cases (marked in green). Please note that there is some variability in the onset time of this 3rd release phase. Often, the onset is slightly delayed with respect to the onset of the substantial swelling of the entire microparticles (Figure III-18). This might be due to inter-particle variability (e.g., only a few microparticles have been studied, *ensembles* of microparticles consist of numerous *single* particles), and/or it might take some time for the drug to diffuse out upon polymer swelling.

Interestingly, the onset of substantial microparticle swelling was observed after about 1 week in this study, which corresponds to a polymer molecular weight of about 20 kDa: Figure III-20 shows the decrease in the average polymer molecular weight (Mw) of the PLGA in the investigated microparticles as a function of the exposure time to the release medium at 37 °C. The degradation kinetics were similar for the differently sized microparticles. A threshold value of about 20 kDa was also observed by Gasmi et al.,(23,24) studying dexamethasone- as well as prilocaine-loaded PLGA microparticles. In contrast, a threshold value of about only 8 kDa was reported to coincide with the onset of substantial PLGA *implant* swelling by Bode et al.(47) Those implants were based on Resomer RG 502H, which is a shorter chain polymer compared to the one used in this study. But the most likely reason for the difference in the threshold value might be the difference in the dimensions of the systems: microparticles versus macroscopic implants: One pre-

requisite for substantial swelling of the entire drug delivery system is the absence of a stable core. As long as such a stable core exists, it mechanically restricts the swelling of the other regions. Once also the core of the device starts to swell, the entire system can rather easily expand. In the case of macroscopic implants this takes more time than in much smaller microparticles. But this is only a hypothesis, and it would be interesting to study this aspect in more detail in the future.

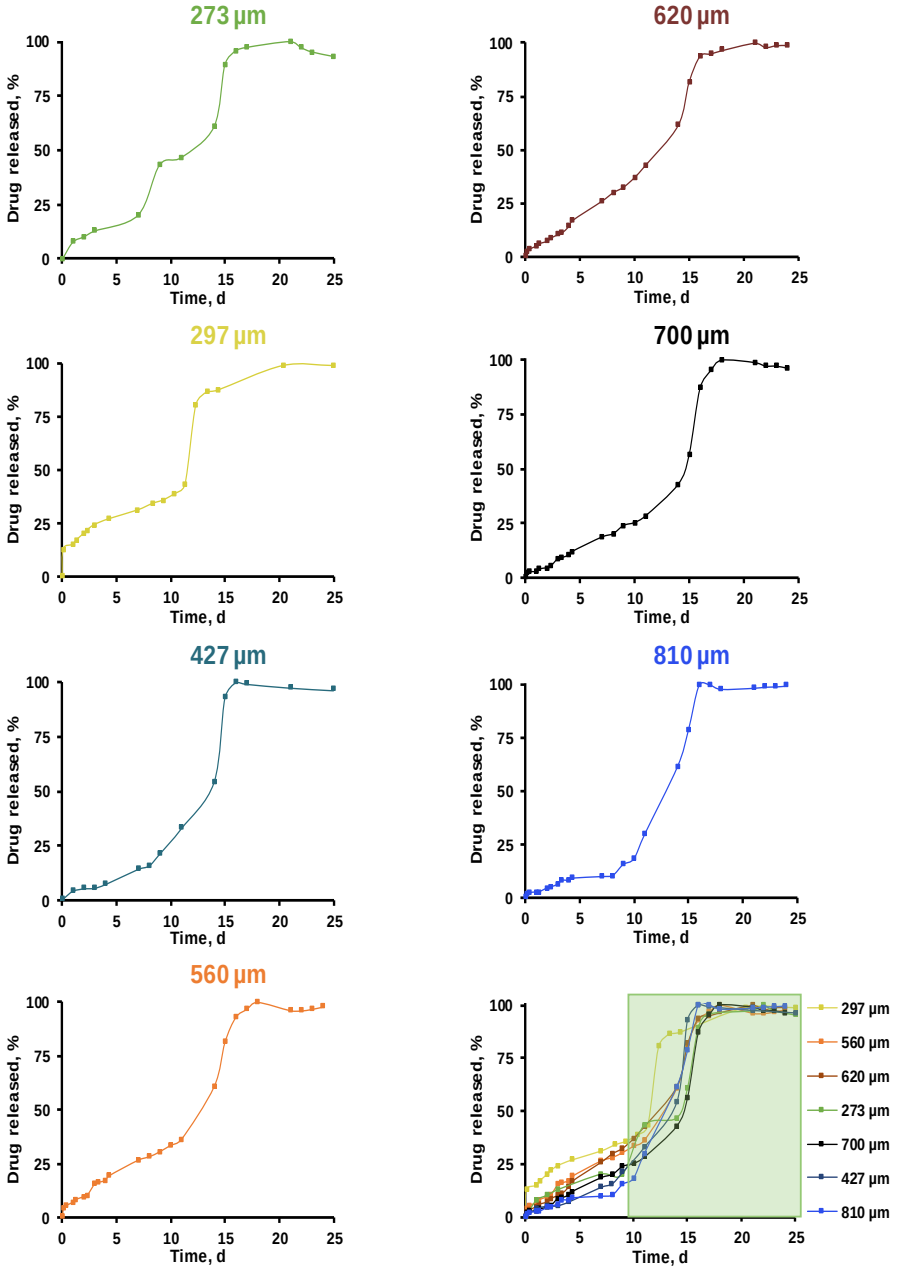


Figure III-19: Caffeine release from single PLGA microparticles in phosphate buffer pH 7.4. The initial microparticle size is indicated at the top of each diagram. The diagram on the right hand side at the bottom shows the superposition of all individual curves. The green region indicates the 3rd release phase (= final, rapid drug release phase).

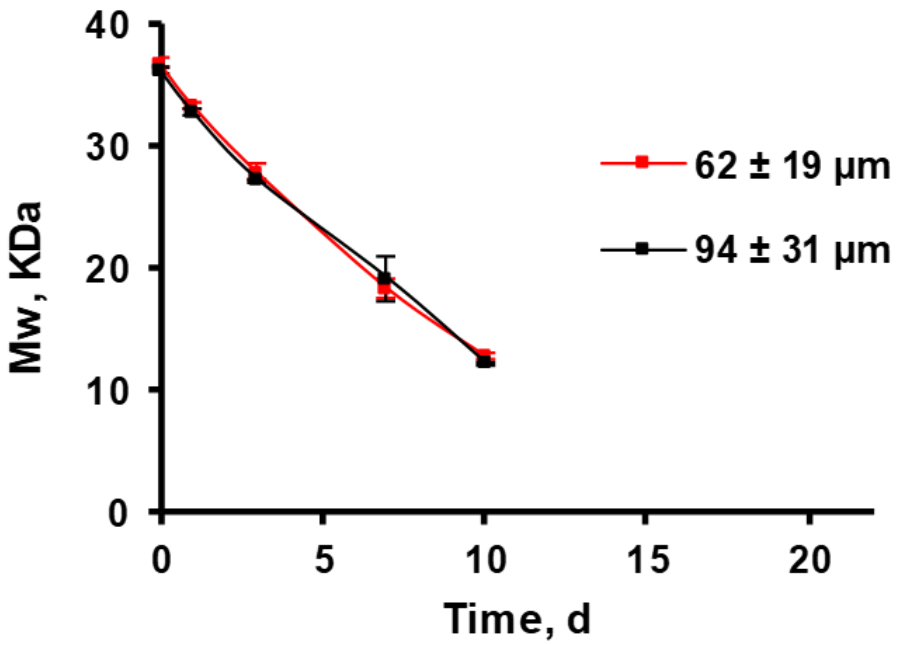


Figure IiI-20: Polymer degradation kinetics upon exposure to phosphate buffer pH 7.4 for differently sized microparticle batches (the mean diameters +/- standard deviations are indicated in the diagram). The polymer molecular weight (Mw) was determined by GPC analysis

The proposed drug release mechanism for the 3rd drug release phase is also illustrated in the scheme at the bottom of Figure III-14 The rectangles represent caffeine *crystals* (which cannot diffuse), the crosses represent caffeine *molecules*, which can diffuse. Prior to the onset of substantial microparticle swelling, the amounts of water in the systems are limited and insufficient to dissolve major portions of the drug. However, once substantial microparticle swelling starts, the drug crystals can dissolve and the dissolved caffeine molecules are rather mobile in the swollen PLGA gel.

The burst release (= 1st release phase) from the investigated PLGA microparticles can probably be explained by the presence of caffeine crystals, which are located close to or at the surface of the systems, with immediate direct access to the surrounding bulk fluid (or obtaining such access shortly after exposure to the release medium). As illustrated in the scheme at the top of Figure III-9, water can immediately dissolve these drug crystals. If the drug has to diffuse through a tiny pore to be released, this process might take some time. However, this type of "early drug release" is very much limited in the investigated microparticles (Figure III-14). This is consistent with the drug release profiles observed with *single* microparticles, shown in Figure II-6. The particle with an initial size of 297 µm exhibits a burst release of about 10 % of its loading,

but the other particles show much less caffeine release within the first day. This is in contrast to recently reported PLGA microparticles loaded with *diprophylline* crystals, which exhibited burst releases of up to more than 50 % (*Chapter III, Part 1*). Importantly, in that study, the drug crystals were much larger than in the present case. If a large drug crystal rapidly dissolves during the first day, the impact on the relative drug release rate is much higher than if a small drug crystal dissolves (containing much less drug).

The scheme in the middle of Figure III-9 illustrates the root cause for the 2nd drug release phase, which has recently been proposed for diprophylline-loaded PLGA microparticles. Upon contact with aqueous media, the hydrophobicity and mechanical stability of the systems initially limit the amounts of water that can penetrate into the microparticles. As discussed above, a certain lag time (here about 1 week) is observed prior to substantial swelling of the entire systems. However, already during the first few days, the microparticles become less spherical and the surfaces of the systems becomes more and more (locally) deformed. This can serve as an indication for the fact that locally, especially in surface near regions, parts of the system start swelling. Some kind of "swelling front" might be observed, as illustrated in the scheme in the middle of Figure III-17 but caution should be paid: In reality, no clear "swelling front" might exist, it might be a more or less random swelling of certain microparticle regions (with a higher likelihood of swelling in surface near regions). If a drug crystal is located in such a region, it will get into contact with important amounts of water, dissolve and the dissolved caffeine molecules will subsequently rather rapidly diffuse out through the swollen PLGA.

In the case of the recently reported diprophylline-loaded microparticles (*Chapter III, Part1*), this led to "step-like" release profiles from single microparticles, such as observed in this study with the 273 µm particle shown in Figure III-19: After about 7 d, within a short period of time about 25 % of the drug was released. This likely corresponds to a high number of caffeine crystals in this case, which might be interconnected via tiny pores or be in direct contact with each other. Importantly, such "steep drug release steps" were not observed with the other *single* microparticles in this study (Figure III-19). This is consistent with the very small drug crystal size (probably less than 1 µm, please see above). Once such a small caffeine crystal dissolves and is released, only a "small step" can be observed, and the 2nd phases of the release profiles in Figure III-19 can be attributed to the release of various small caffeine crystals at random time points. The fact that the "swelling front" more or less homogeneously moves inwards can likely

explain that the release rate remains about constant in this phase (changes in the surface area are likely of minor importance, since substantial swelling of the entire system sets on after about 1 week).

Please note that the slope of the release curve in the 2nd release phase of *ensembles* of microparticles was higher for the smaller systems (red versus black curve in Figure III-14). This is consistent with the hypothesized release mechanism: If the inner microparticle structure is similar, the number of surfaces near crystals is higher in an *ensemble* of smaller microparticles compared to an *ensemble* of larger microparticles.

III.2. Drug release mechanisms

Based on the above described experimental findings and discussion, the following drug release mechanisms are suggested for the control of caffeine release from the investigated microparticles (as illustrated in Figure III-9):

The burst release (= 1st release phase) is caused by the rapid dissolution of caffeine crystals with immediate direct surface access. This phenomenon is very much limited in the present study, e.g. due to the very small size of the drug crystals.

The 2nd drug release phase with an about constant release rate is caused by the local swelling of certain PLGA regions (e.g., visible as deformations of the spheres' surfaces during the first few days): Drug crystals located in these regions dissolve and the dissolved drug molecules rather rapidly diffuse through the swollen PLGA gel.

The 3rd drug release phase (= final, again rapid drug release phase) can be attributed to substantial swelling of the *entire* microparticles, which starts as soon as the polymer chains are sufficiently hydrophilic and less intense entangled, driven by the osmotic pressure generated by the water soluble PLGA degradation products. The presence of high amounts of water dissolves the drug crystals throughout the system, and the dissolved drug molecules rather rapidly diffuse through the swollen PLGA gel. This leads to complete drug exhaust.

Please note that a certain portion of the caffeine is likely also *dissolved* in the PLGA matrix: For instance, the glass transition temperature of the PLGA decreased from about 47 to 44 °C. This might indicate that some of the drug might have a possibility to diffuse also through the non-swollen PLGA matrix (which is likely in the rubbery state, as discussed above). However, the importance of such a contribution is difficult to estimate. The observed release profiles from *single* microparticles (Figure III-19) suggest that it might not be of major impact: Otherwise the shape of

the release curves prior to the onset of substantial swelling of the entire system should be different: the release rate would be expected to monotonically *de*crease with time, due to the increasing length of the diffusion pathways. It would be interesting to study this aspect in more detail in the future, especially also for other types of drugs, which can dissolve to important extents in PLGA and act as efficient plasticizers for this polymer.

IV. Conclusion

The obtained new knowledge of the underlying drug release mechanisms in caffeine-loaded PLGA microparticles can probably be helpful to understand also the drug release mechanisms in other types of PLGA microparticles and even macroscopic implants. This type of advanced drug delivery systems offers many interesting advantages and is of increasing practical importance, but device optimization is often challenging, due to the complexity of the involved mass transport mechanisms. Rather surprising effects can be observed when varying formulation and processing parameters. A better understanding of how these systems work can facilitate their optimization. Also, “worst case scenarios” can be considered in a more realistic manner, rendering the respective medical treatments safer.

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Part III

Mechanistic explanation of the (up to) 3 release phases of PLGA microparticles: Impact of the temperature

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Abstract:

The aim of this study was to better understand the underlying drug release mechanisms in poly(lactic-co-glycolic acid) (PLGA)–based microparticles. Differently sized diprophylline-loaded microparticles were prepared using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique. The microparticles were thoroughly characterized before and after exposure to phosphate buffer pH 7.4 at different temperatures: 37, 20 and 4 °C. In vitro drug release was measured from ensembles and single microparticles. GPC, DSC, SEM, drug solubility measurements and optical microscopy were used to elucidate the importance of polymer swelling and degradation, drug dissolution and diffusion as well as pore closure effects. The drug was initially homogeneously distributed in the form of tiny crystals throughout the microparticles. The burst release (1st phase) is likely attributable to the dissolution of drug crystals with direct surface access (eventually via tiny pores). The 2nd release phase (with an about constant release rate) can probably be explained by the dissolution of drug crystals in surface near regions undergoing local swelling. The 3rd (again rapid) drug release phase seems to result from substantial PLGA swelling throughout the entire microparticles. This phase starts as soon as a critical polymer molecular weight of about 20 kDa is reached: Significant amounts of water penetrate into the systems, dissolving the remaining drug crystals. Importantly, the dissolved drug molecules are rather mobile in the highly swollen polymeric matrix.

I. Introduction

Poly(lactic-co-glycolic acid) (PLGA)–based microparticles offer an interesting potential for parenteral controlled drug delivery, because they are: (i) biodegradable (avoiding the removal of empty remnants upon drug exhaust) (1–4), (ii) biocompatible (5), allow the control of drug release during flexible periods of time (ranging from a few days up to several months) (6–8), and can be rather easily injected compared to macroscopic implants. Since decades various types of PLGA microparticles are commercially available, in particular for the treatment of cancer. A variety of manufacturing methods can be used to prepare this type of advanced drug delivery systems, including emulsion solvent extraction/evaporation techniques, hot melt extrusion and grinding, spray-draying (9–11). Numerous types of PLGA-based microparticles have been reported in the literature, exhibiting a broad range of drug release kinetics (12–14). However, in general the observed drug release patterns are either mono-, bi- or tri-phasic. In the latter case an initial rapid drug release phase (also called "burst effect" = 1st release phase) is followed by a period with an about constant drug release rate (= 2nd release phase) and a final (again rapid) 3rd release phase, which leads to complete drug exhaust. It can be hypothesized that certain PLGA microparticles only exhibit mono- or bi-phasic drug release patterns, because all drug is released before the 2nd or 3rd release phase sets on.

However, despite their steadily increasing practical importance, the underlying mass transport mechanisms controlling drug release from PLGA microparticles are often not fully understood and product optimization is generally based on time-consuming and cost-intensive series of trial-and-error experiments (15–20). This can at least partially be explained by the potential complexity of the involved chemical and physical phenomena (1,21–26). A variety of phenomena can be involved, such as water diffusion, drug dissolution, drug diffusion, polymer degradation and erosion, drug – polymer interactions (e.g., plasticizing effects), polymer swelling and autocatalytic effects. The latter can occur, because the degradation products of PLGA are acids, which are generated throughout the microparticles. Importantly, the rate at which they are generated can be higher than the rate at which they diffuse out or are at which they are neutralized (27–31). Consequently, the pH within PLGA microparticles can significantly drop. Since ester hydrolysis is catalyzed by protons, this can lead to autocatalysis. The importance of this effect has been reported to depend on the microparticle size and porosity (32,33).

Based on drug release and swelling measurements from/of single PLGA microparticles (instead of from/of ensembles of numerous microparticles), it has recently been hypothesized that the role of PLGA swelling is often underestimated (34–38). This is also true for macroscopic PLGA implants (39,40). In brief, it has been suggested that PLGA swelling is not negligible, but instead plays an "orchestrating role" for the control of drug release from PLGA-based drug delivery systems exhibiting 3-phasic drug release patterns. The rational for this theory is as follows: Upon contact with aqueous body fluids (e.g., upon s.c. or i.m. injection), water penetrates rather rapidly into the entire PLGA microparticles. However, at this stage, the polymer is rather hydrophobic and the macromolecules are highly entangled. This limits the amounts of water that can enter the microparticles. Nevertheless, the entire microparticles are wetted (often within less than 1 d) and polyester bond cleavage starts throughout the system: The particles undergo "bulk erosion" (41). Consequently, the polymer chains become shorter with time and new –OH and –COOH groups are created throughout the system. This renders the polymeric matrix more and more hydrophilic. Also, the degree of polymer chain entanglement decreases (as the chains become shorter). In addition, low molecular weight degradation products are water soluble (e.g. lactic acid and glycolic acid). Thus, a steadily increasing osmotic pressure is built up in the microparticles, attracting water into the system. At a certain time point (e.g., when a certain critical polymer molecular weight is reached), the system becomes sufficiently hydrophilic to allow for the penetration of significant amounts of water and the mechanical stability of the polymer network becomes insufficient to withstand the generated osmotic pressure: Substantial system swelling sets on, fundamentally altering the conditions for drug release.

Recently, Tamani et al. (2019a, b) proposed that in the case of diprophylline-loaded PLGA microparticles (in which the drug is dispersed homogeneously in the form of tiny crystals throughout the matrix) the initial burst release and the 2nd release phase (with an about constant release rate) can be explained by the occasional release of parts of the drug loading in individual microparticles. In other words, every microparticle has its own structure and its "own way" to release the drug. If a drug crystal has direct surface access (eventually via pores/channels), it will rapidly dissolve and the dissolved drug molecules will rapidly be released. The sum of such events constitutes the burst release. With time certain PLGA regions (especially surface near regions) undergo local swelling (prior to the onset of substantial polymer swelling throughout the entire system). If a drug crystal is located in such a region, it gets into contact with significant amounts

of water, dissolves and the dissolved drug molecules rather rapidly diffuse through the swollen PLGA. The about constant release rate from ensembles of microparticles is the sum of numerous randomly occurring individual release events of this type. The aim of the present study was to get further insight into the underlying dug release mechanisms from this type of PLGA microparticles, altering the temperature of the release medium: The systems were also exposed to phosphate buffer pH 7.4 at 20 and 4 °C. The idea was to be able to slow down key phenomena, such as polymer degradation, drug diffusion and alter the drug's solubility in the bulk fluid. It was expected that the resulting release kinetics substantially change. These changes were to be explained based on a thorough characterization of the microparticles before and upon exposure to the release medium at the different temperatures. In particular, the release and swelling behavior of single microparticles as well as the degradation and release kinetics of ensembles of microparticles were to be monitored. Differently sized microparticle batches were prepared, with mean particle diameters of 63 +/- 19 µm, 113 +/- 41 µm and 296 +/- 95 µm. Please note that in this study it was technically not possible to monitor small single microparticles. The assumption is that the underlying drug release mechanisms do not fundamentally depend on the system size, given the fact that there were no visible signs for differences in their inner structure and composition. This does not mean that the relative importance of specific phenomena does not depend on the system size (as discussed in detail in this article).

II. Materials and methods

II.1.Materials

Poly (D,L lactic-co-glycolic acid) (PLGA; Resomer RG 504H; 50:50 lactic acid:glycolic acid; Evonik, Darmstadt; Germany); diprophylline (BASF, Ludwigshafen, Germany); polyvinyl alcohol (Mowiol 4-88; Sigma-Aldrich, Steinheim, Germany); acetonitrile and dichloromethane (VWR, Fontenay-sous-Bois, France); tetrahydrofuran (HPLC grade; Fisher Scientific, Illkirch, France).

II.2.Microparticle preparation

Drug-loaded microparticles were prepared using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique. "Small", "medium-sized" and "large" microparticles were prepared, adapting the formulation and processing parameters accordingly (as indicated in the following in brackets in this order). Appropriate amounts of diprophylline (204, 125 or 101 mg) and PLGA (900, 834 or 910 mg) were dispersed/dissolved (the drug was at least partially dispersed

in the form of tiny particles, the polymer was dissolved) in 10, 6 or 4 mL dichloromethane. The organic phase was emulsified into 2.5 L of an outer aqueous polyvinyl alcohol solution (0.25% w/w) under stirring (1000, 1500 or 2000 rpm, Eurostar power-b; Ika, Staufen, Germany) for 30 min. Upon solvent exchange the PLGA precipitated, trapping the drug. The formed microparticles were hardened by adding 2.5 L of the same outer aqueous polyvinyl alcohol solution (0.25%) and further stirring at 700 rpm (Eurostar power-b) for 4 h. The microparticles were separated by filtration (Nylon filter, 0.45 µm, 13 mm; GE Healthcare Life Sciences Whatman, Kent, UK), washed with de-mineralized water and subsequently freeze-dried (freezing at -45°C for 1 h 45 min, primary drying at -40°C and 0.07 mbar for 35 h, and secondary drying at +20 °C and 0.0014 mbar for 35 h) (Christ Epsilon 2-4 LSC+; Martin Christ, Osterode, Germany).

II.3.Microparticle characterization

II.3.1. Microparticle morphology and size

Microparticle sizes were determined by optical microscopy: Microscopic pictures were taken using an Axiovision Zeiss Scope-A1 microscope, equipped with an AxioCam ICc1 camera and the Axiovision Zeiss Software (Carl Zeiss, Jena, Germany). For ensembles of microparticles, each measurement included 200 particles. Mean values +/- standard deviations are reported.

II.3.2. Practical drug loading

The practical drug loading was determined by dissolving approximately 5 mg microparticles in 5 mL acetonitrile, followed by filtration (PTFE syringe filters, 0.45 µm; GE Healthcare, Kent, UK). The drug content was determined by HPLC analysis [Thermo Fisher Scientific Ultimate 3000 Series HPLC, equipped with a LPG 3400 SD/RS pump, an auto sampler (WPS-3000 SL) and a UV-Vis detector (VWD-3400RS); Thermo Fisher Scientific, Waltham, USA]. A reversed phase column Polar C18 (Luna Omega 3 µm; 150 x 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was a mixture of acetate buffer (0.01 M, pH 4.5): acetonitrile (65:35, v:v). The detection wavelength was 274 nm and the flow rate 1 mL/min. Five µL samples were injected. The standard curve covered the range of 0.1 to 50 µg/mL. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.3. Drug release measurements from ensembles of microparticles

Ten mg microparticle samples were placed into plastic tubes (Safe-lock tubes 2.0 mL, Eppendorf, Hamburg, Germany) filled with 2 mL phosphate buffer pH 7.4 (USP 42). The tubes were placed into a horizontal shaker at 37°C & 80 rpm or at 20°C & 80 rpm (GFL 3033;

Gesellschaft fuer Labortechnik, Burgwedel, Germany) or into a refrigerator at 4°C (0 rpm), as indicated. At predetermined time points, 1.5 mL samples were withdrawn (replaced with fresh medium), filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and analysed for their drug contents by HPLC analysis, as described above. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported. Sink conditions were provided throughout all experiments.

In addition, the pH of the release medium was measured at pre-determined time points using a pH meter (InoLab pH Level 1; WTW, Weilheim, Germany) (n=3). Mean values ± standard deviation are reported.

II.3.4. Drug release measurements from single microparticles

Diprophylline release from single microparticles was monitored in 96- well standard microplates (Tissue culture plate 96 well; Carl Roth, Karlsruhe, Germany) as follows: One microparticle was introduced into each well, which was filled with 100 µL phosphate buffer pH 7.4 (USP 42) and closed with a cap (Simport Scientific, Beloeil, Quebec). The well microplates were placed into a horizontal shaker at 20°C & 80 rpm (GFL 3033). At pre-determined time points, 50 µL samples were withdrawn (replaced with fresh medium) using a Hamilton syringe (Microlite #710, 100 µL; Hamilton, Bonaduz, Switzerland) and analysed for their drug contents by HPLC, as described above (in this case the standard curve covered the range of 0.025 to 5 µg/mL).

II.3.5. Swelling of single microparticles

Microparticles were treated as for the drug release studies from single microparticles. At pre-determined time points, pictures were taken using an Axiovision Zeiss Scope-A1 microscope and the Axiovision Zeiss Software (Carl Zeiss) to monitor changes in the microparticles' diameter.

Furthermore, dynamic changes in the microparticles' wet mass were determined as follows: At pre-determined time points, samples were carefully withdrawn and excess water removed using Kimtech precision wipes (Kimberly-Clark, Rouen, France). The microparticles' wet mass at time t was measured using an ultra-microbalance (XPR6U; Mettler-Toledo, Greifensee, Switzerland).

II.3.6. Differential scanning calorimetry (DSC)

DSC thermograms of raw materials (as received: diprophylline, PLGA) and of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland).

Approximately 5 mg samples were heated in sealed aluminum pans from 10 °C to 120°C, cooled to -70 °C and reheated to 120 °C at a rate of 10 °C/min. The indicated glass temperatures (Tgs) were obtained from the second heating cycles. Each experiment was conducted in triplicate. Mean values +/- standard deviations are reported.

II.3.7. Scanning Electron Microscopy (SEM)

The external morphology of microparticles was studied using a JEOL Field Emission Scanning Electron Microscope (JSM-7800F, Tokyo, Japan). Samples were fixed with a ribbon carbon double-sided adhesive and covered with a fine chrome layer. Microparticles were observed before and after exposure to the release medium. In the latter case, the microparticles were treated as for the drug release studies from ensembles of microparticles (described above). At pre-determined time points, samples were withdrawn and freeze-dried (as described above).

II.3.8. Gel Permeation Chromatography (GPC)

Microparticles were treated as for the drug release studies from ensembles of microparticles. At pre-determined time points, samples were withdrawn, freeze-dried for 3d (as described above) and the lyophilisates were dissolved in tetrahydrofuran (at a concentration of 3 mg/mL). The average polymer molecular weight (Mw) of the PLGA in the samples was determined by GPC (Alliance, refractometer detector: 2414 RI, separation module e2695, Empower GPC software; Waters, Milford, USA), using a Phenogel 5 µm column (which was kept at 35°C, 7.8 × 300 mm; Phenomenex, Le Pecq, France). The injection volume was 50 µL. Tetrahydrofuran was the mobile phase (flow rate: 1 mL/min). Polystyrene standards with molecular weights between 1480 and 70,950 Da (Polymer Laboratories, Varian, Les Ulis, France) were used to prepare the calibration curve.

II.3.9. Drug solubility measurements

Excess amounts of the drug (as received) were exposed to 25 mL phosphate buffer pH 7.4 in brown glass flasks and horizontally shaken at 37°C or 20 °C at 80 rpm (GFL 3033), or placed in a refrigerator at 4 °C and regularly shaken manually. At pre-determined time points, samples were withdrawn, immediately filtered (PTFE syringe filters, 0.45 µm; GE Healthcare) and diluted. The drug contents of the samples were determined by HPLC-UV, as described above. Samples were withdrawn until equilibrium was reached. Each experiment was conducted in triplicate, mean values +/- standard deviations are reported.

III. Results and Discussion

In all cases, spherical particles were obtained, with mean sizes of 63 +/- 19 µm, 113 +/- 41 µm and 296 +/- 95 µm for “small”, “medium-sized” and “large” microparticle batches, respectively. SEM pictures revealed no signs for noteworthy external porosity prior to exposure to the release medium (top row in Figure III-1). The practical drug loadings were about 5-7 % [4.8 +/- 0.3, 5.8 +/- 0.6 and 6.7 +/- 0.4 %] and the glass transition temperatures (Tgs) about 46-47 °C [46.8 +/- 0.1, 46.3 +/- 0.3 and 46.4 +/- 0.4 °C]. It has recently been reported that the Tg of the polymer raw material (as received) was 47.0 +/- 0.2 °C (Tamani et al., 2019a) (37). Hence, diprophylline is not acting as a plasticizer for PLGA. Furthermore, X-ray diffraction and SEM pictures of cross-sections of these microparticles revealed numerous tiny drug crystals distributed throughout the systems. Hence, probably only minor amounts of the hydrophilic diprophylline are dissolved within the much more hydrophobic PLGA (37).

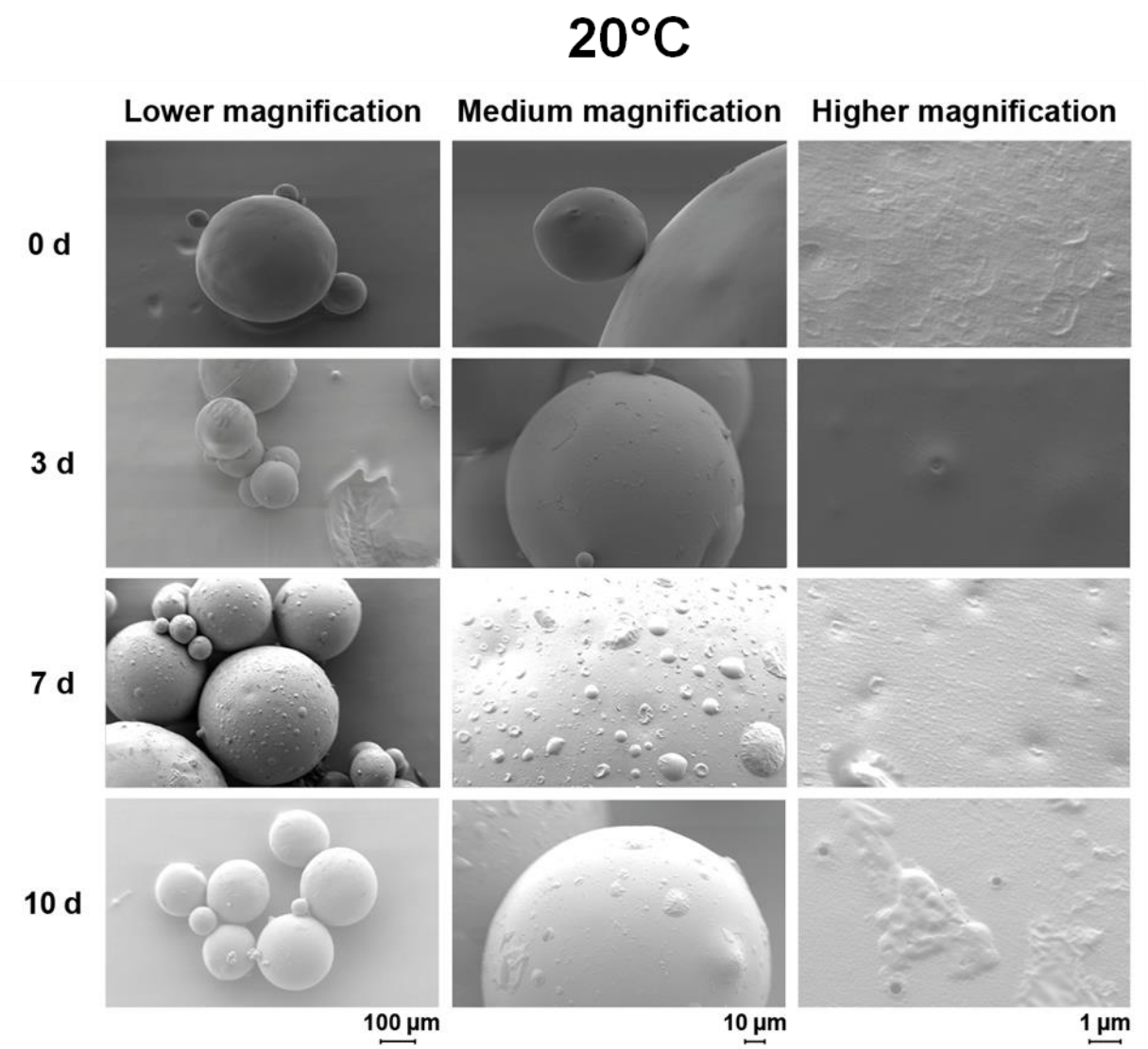

Figure III-23: SEM pictures of surfaces (lower, medium and higher magnification) of diprophylline-loaded PLGA microparticles before and after exposure to phosphate buffer pH 7.4 (treated as for drug release studies from ensembles of microparticles) at 20 °C (80 rpm). The exposure times are indicated on the left hand side. Note that the microparticles were freeze-dried after exposure to the release medium.

III.1. Drug release from ensembles of microparticles

Figure III-22 shows the resulting diprophylline release kinetics from the investigated PLGA microparticles in phosphate buffer pH 7.4 at 37 °C (top), 20 °C (middle) and 4 °C (bottom). Please note that the systems were agitated (80 rpm) at 37 and 20 °C, but not at 4 °C. However, it has been demonstrated that the impact of agitation (0 vs. 80 rpm) on drug release from these systems is limited at 37 °C (Data not shown). The mean microparticle sizes (+/- SD) are indicated in the diagrams. "Small", "medium-sized" and "large" microparticle batches are marked in red, black and orange, respectively. At 37 C, also a zoom on the first 20 d is shown.

Interestingly, the following observations were made:

- The drug release rate increased with increasing temperature, irrespective of the microparticle size.
- At all temperatures, the release rate increased with decreasing microparticle size.
- At 37 °C, 3-phasic drug release was observed, irrespective of the microparticle size (although the importance of the 3rd release phase was only minor in the case of the "small" microparticles): An initial rapid drug release phase ("burst") was followed by a 2nd release phase with an about constant release rate, and a final (again rapid) 3rd release phase (leading to complete drug exhaust).
- At 20 and 4 °C, the drug release patterns were only bi-phasic in the observation period (100 d), irrespective of the microparticle size: A "burst" release was followed by a 2nd release phase with an about constant release rate. Please note that at the end of the observation period, diprophylline was far from being complete at these temperatures, especially at 4 °C (e.g., less than 30 % diprophylline was released at this time point). It can be hypothesized that at later time points, also a 3rd release phase might be observed at these temperatures. Actually, looking at the orange curve in the middle of Figure III-22 (20 °C, "large" microparticles), such a final rapid release phase might just begin (after 90 – 100 d).
- The slopes of the 2nd release phases (with about constant release rates) strongly increase with increasing temperature.
- The importance of the burst release ("1st phase") increases with increasing temperature.

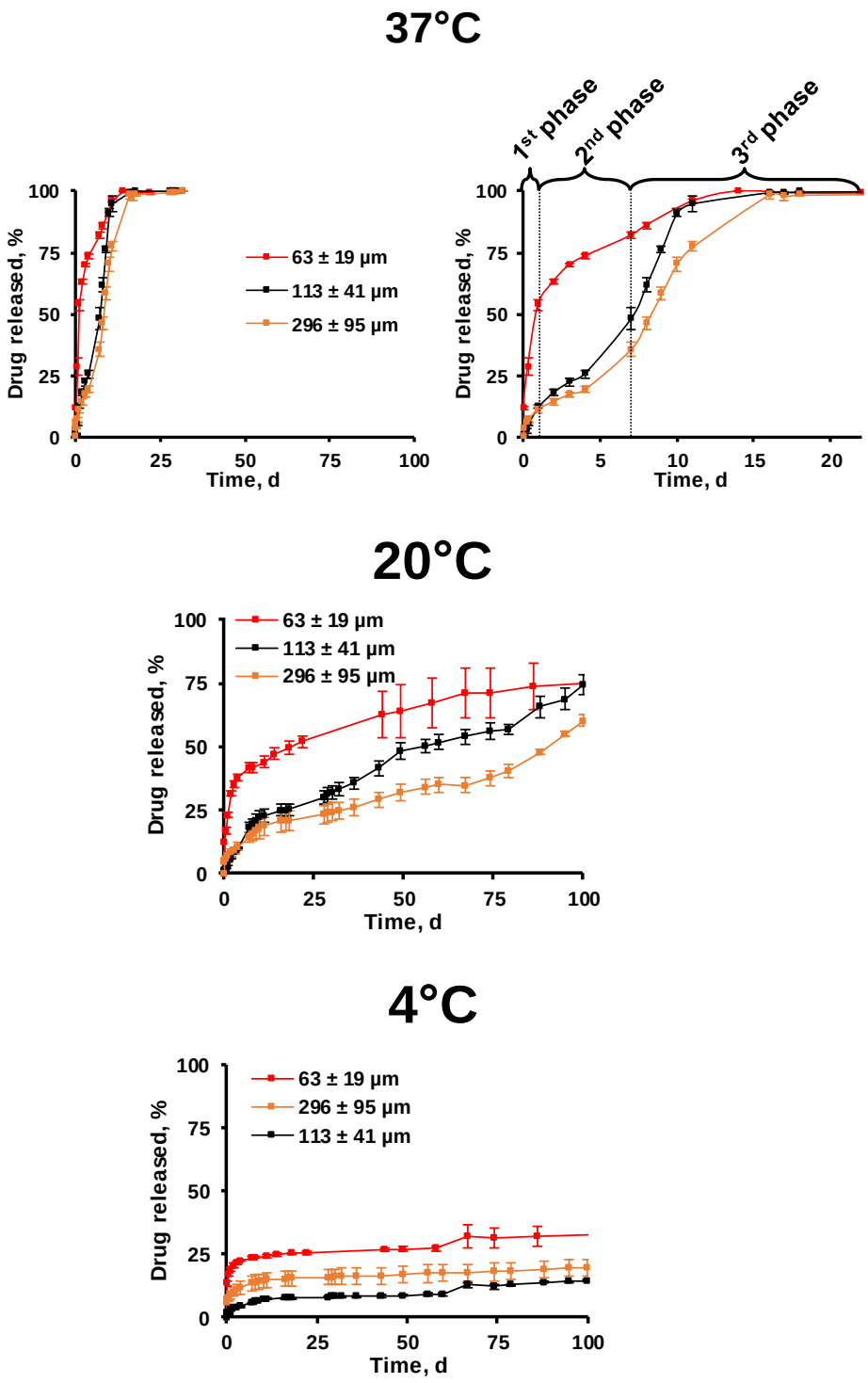

Figure III-24: Impact of the temperature on diprophylline release from ensembles of PLGA microparticles in phosphate buffer pH 7.4. Note that the systems were agitated at 20 and 37 °C, but not at 4 °C. Three microparticle batches with different mean particle sizes (indicated in the diagram +/- SD) were studied. The results obtained at 37 °C are reproduced from F.Tamani et al (37) with permission. Mean values +/- SD are indicated (n = 3).

To better understand these phenomena and tendencies, the degradation of the polymer upon exposure to the release medium as well as potential changes of the external porosity and pH of the surrounding bulk fluids were monitored. Also, the swelling and drug release kinetics of single microparticles were measured at the different temperatures.

III.2. Polymer degradation, bulk fluid pH and outer microparticle morphology

Figure III-23 shows the decrease in polymer molecular weight (Mw) of the diprophylline-loaded PLGA microparticles as a function of the exposure time to phosphate buffer pH 7.4 at the different temperatures. The systems were treated as for the in vitro drug release studies described above. Clearly, the polymer degradation rate substantially increased with increasing temperature: At 37 °C, the polymer molecular weight decreased from about 48 kDa to only about 8 kDa in the first 3 weeks. At 20 °C, the Mw decreased to only about 41 kDa in the same time period, and at 4 °C there was virtually no change in the polymer molecular weight in this observation period. This can be explained by the fact that chemical reactions (including hydrolytic ester bond cleavage) can strongly depend on the temperature.

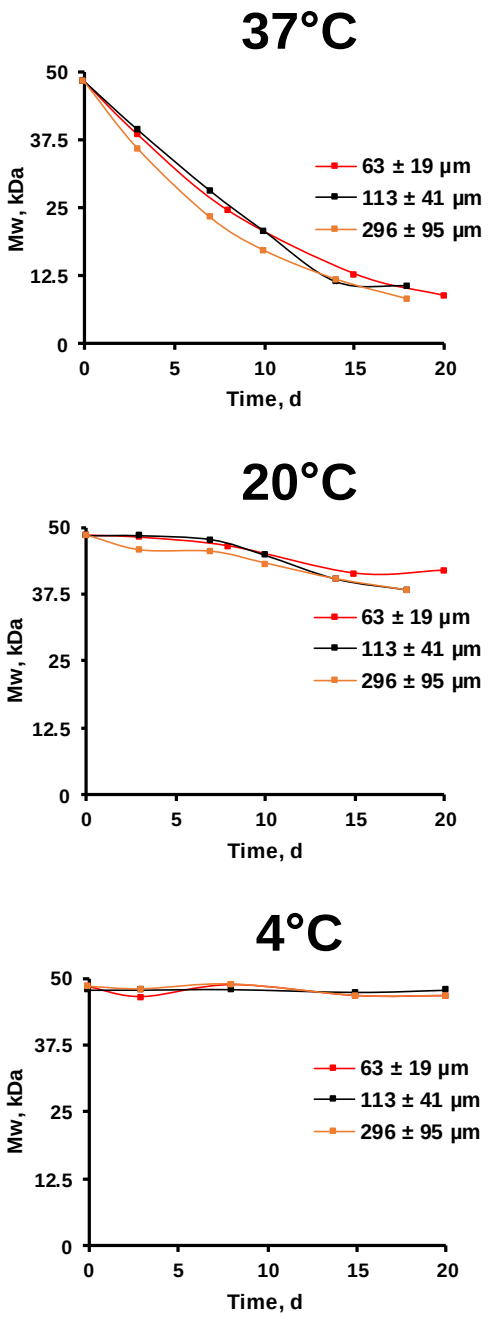

Figure III-23: Impact of the temperature on PLGA degradation upon exposure of ensembles of microparticles to phosphate buffer pH 7.4. Note that the systems were agitated at 20 and 37 °C, but not at 4 °C. Mean values +/- SD are indicated (n = 3). Three microparticle batches with different mean particle sizes (indicated in the diagram +/- SD) were studied.

The dynamic changes in the pH of the release medium upon exposure of ensembles of diprophylline-loaded PLGA microparticles to phosphate buffer pH 7.4 are illustrated in Figure III-4. The systems were treated as for the in vitro drug release studies described above. Clearly, the pH of the surrounding bulk fluid remained about constant during the observation periods in all cases: at all temperatures and for all microparticle sizes. Thus, under the given conditions, potential acidifications of the release medium due to the leaching of short chain acids (PLGA degradation products) from the microparticles into the bulk fluid does not seem to play a noteworthy role.

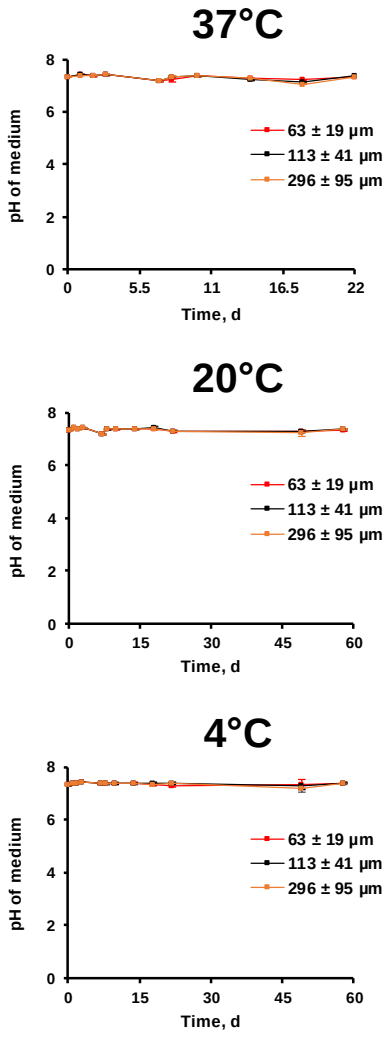

Figure III-24: Dynamic changes in the pH of the release medium upon exposure of ensembles of diprophylline-loaded PLGA microparticles to phosphate buffer pH 7.4 at 37, 20 and 4 °C. Note that the systems were agitated at 20 and 37 °C, but not at 4 °C. Mean values +/- are indicated (n = 3). Three microparticle batches with different mean particle sizes (indicated in the diagrams +/- SD) were studied.

The SEM pictures in Figure III-21 show surfaces of the microparticles after 0, 3, 7 and 10 d exposure to the release medium at 20 °C. Please note that after exposure to the phosphate buffer the microparticles first had to be dried (in this study they were freeze-dried) prior to analysis. Thus, artefact creation cannot be excluded. As it can be seen, no signs for pore formation were observed. The same is true for microparticles which were exposed to the release medium at 4 °C for up to 10 d (Data not shown). In contrast, when the microparticles were exposed to phosphate buffer pH 7.4 for 10 d, surface pores became visible (Tamani et al., 2019a) (37), reflecting substantial PLGA degradation (please see above).

III.3. Drug release from and swelling of single microparticles

Figure III-25 shows optical microscopy pictures of the investigated microparticles upon exposure to phosphate buffer pH 7.4 at 20 °C for up to 74 d. The initial microparticle diameters are indicated on the left hand side. Importantly, no noteworthy changes were observed in this time period. This is in contrast to substantial microparticle swelling that was reported upon exposure of the same microparticles to the same release medium, but at 37 °C (Tamani et al., 2019a) (37): At body temperature, substantial microparticle swelling set on after about 1 week, irrespective of the microparticle size. This can be explained by the difference in the PLGA degradation rates (Figure III-23) and has important consequences for drug release. Figure III-26 shows the dynamic changes in the diameters of individual microparticles and in their wet mass as a function of the exposure time to phosphate buffer at 20 °C. The results are consistent with the optical microscopy pictures in Figure III-25 and indicate the absence of important microparticle swelling under the given conditions. Please note that at late time points, eventually the beginning of some kind of microparticle swelling might be observed, when looking at the changes in the systems' diameters (top diagram in Figure III-26). This would coincide with the potential onset of a 3rd drug release phase observed for the "larger" microparticles after 90-100 days (orange curve in Figure III-22). At 4 °C, no moteworthy microparticle swelling was observed during the first 100 d (Data not shown).

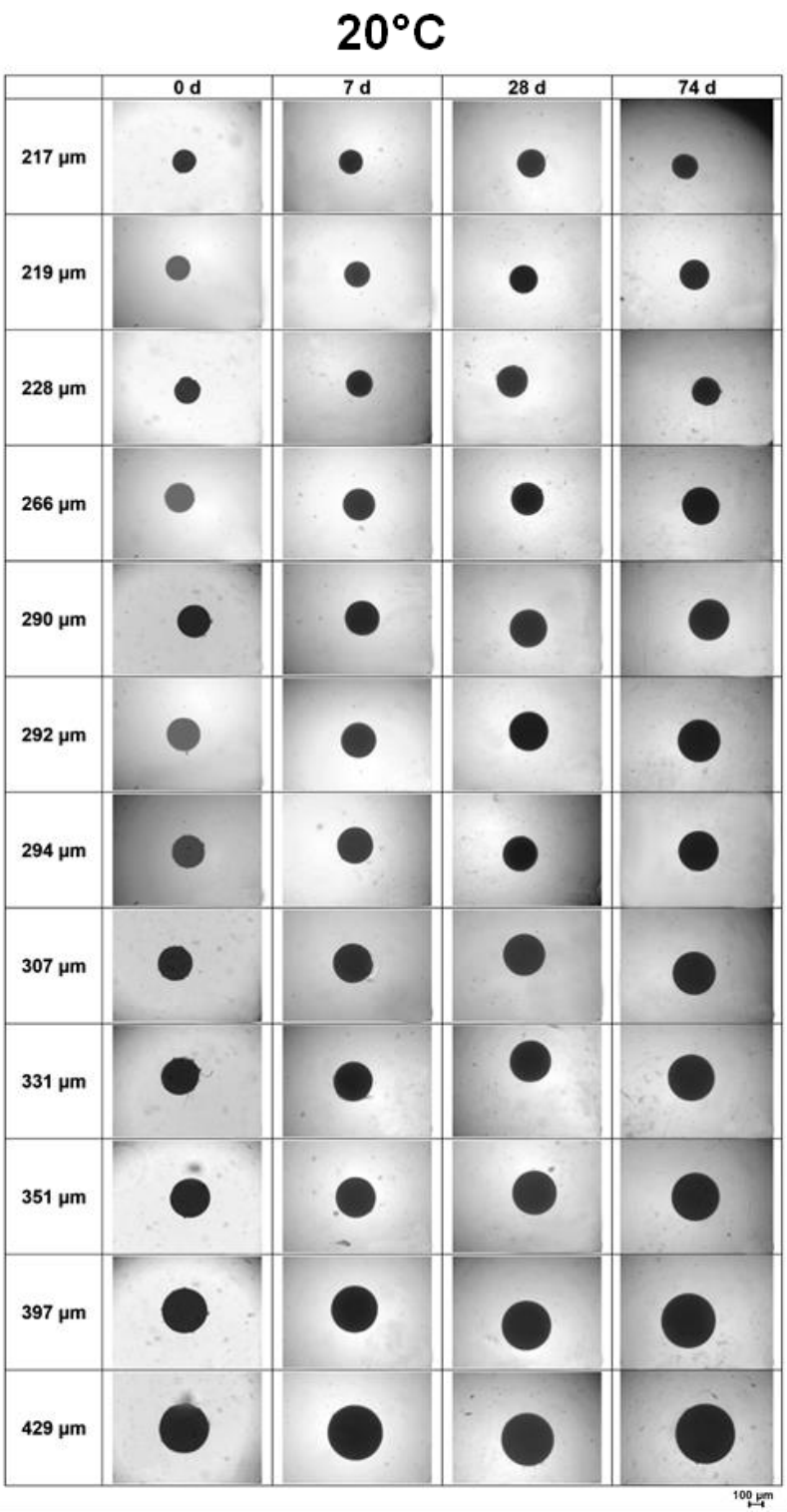

Figure III-25: Optical microscopy pictures of single diprophylline-loaded PLGA microparticles before and after exposure to phosphate buffer pH 7.4 at 20°C (80 rpm) for different time periods (indicated at the top). The initial particle sizes are given on the left han left hand side.

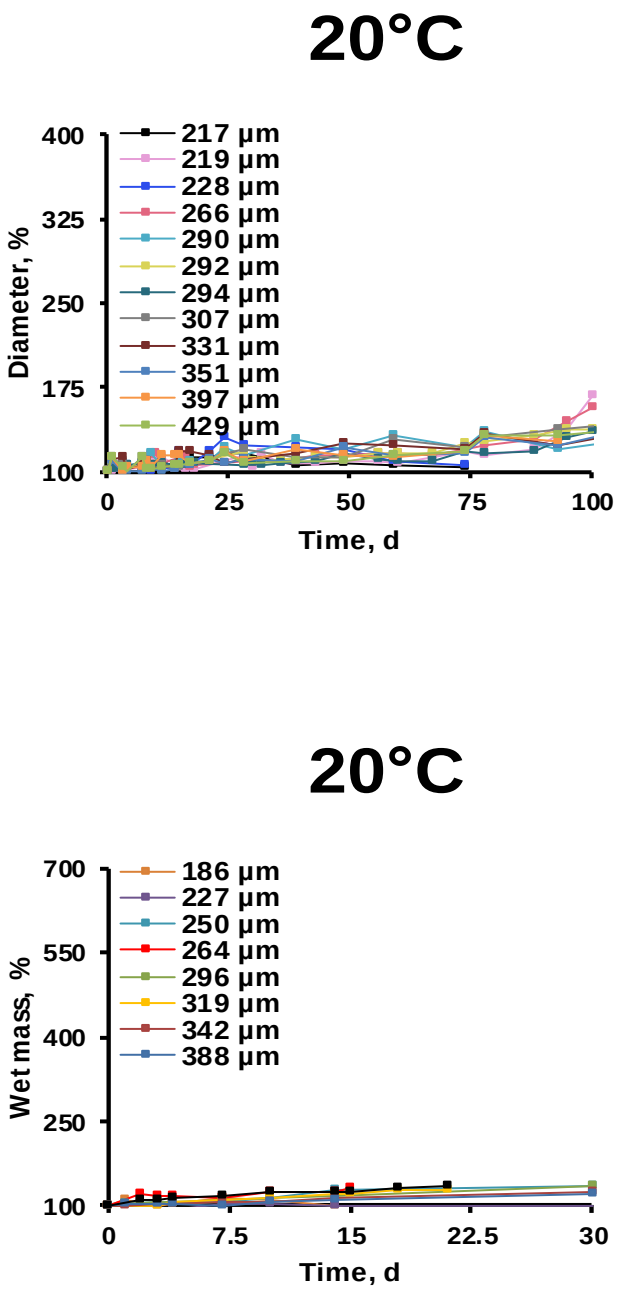

Figure III-26: Swelling kinetics of single diprophylline-loaded PLGA microparticles upon exposure to phosphate buffer pH 7.4 at 20 °C (80 rpm): Dynamic changes in the microparticles' diameter (top) and wet mass (bottom). The initial microparticle diameters are indicated in the diagrams.

Figure III-27 shows the swelling kinetics of the single microparticles together with their drug release in the same diagrams upon exposure to phosphate buffer pH 7.4 at 20 °C. The left y-axes refer to drug release, the right y-awes to changes in the systems' diameters. The initial particle size (before exposure to the release medium) is indicated at the top of each diagram. As it can be seen, each microparticle behaved differently, "released the drug "in its own way". In some cases, especially at lower initial microparticle sizes, more or less important portions of the drug were released in rather short time periods (marked by red stars). Figure III-28 shows an all the individual microparticle release profiles at 20 °C in one diagram. As it can be seen, many microparticles do not release any diprophylline to a noteworthy extent during the observation period, whereas others release parts of their drug load rather arbitrarily at different time points to different extents.

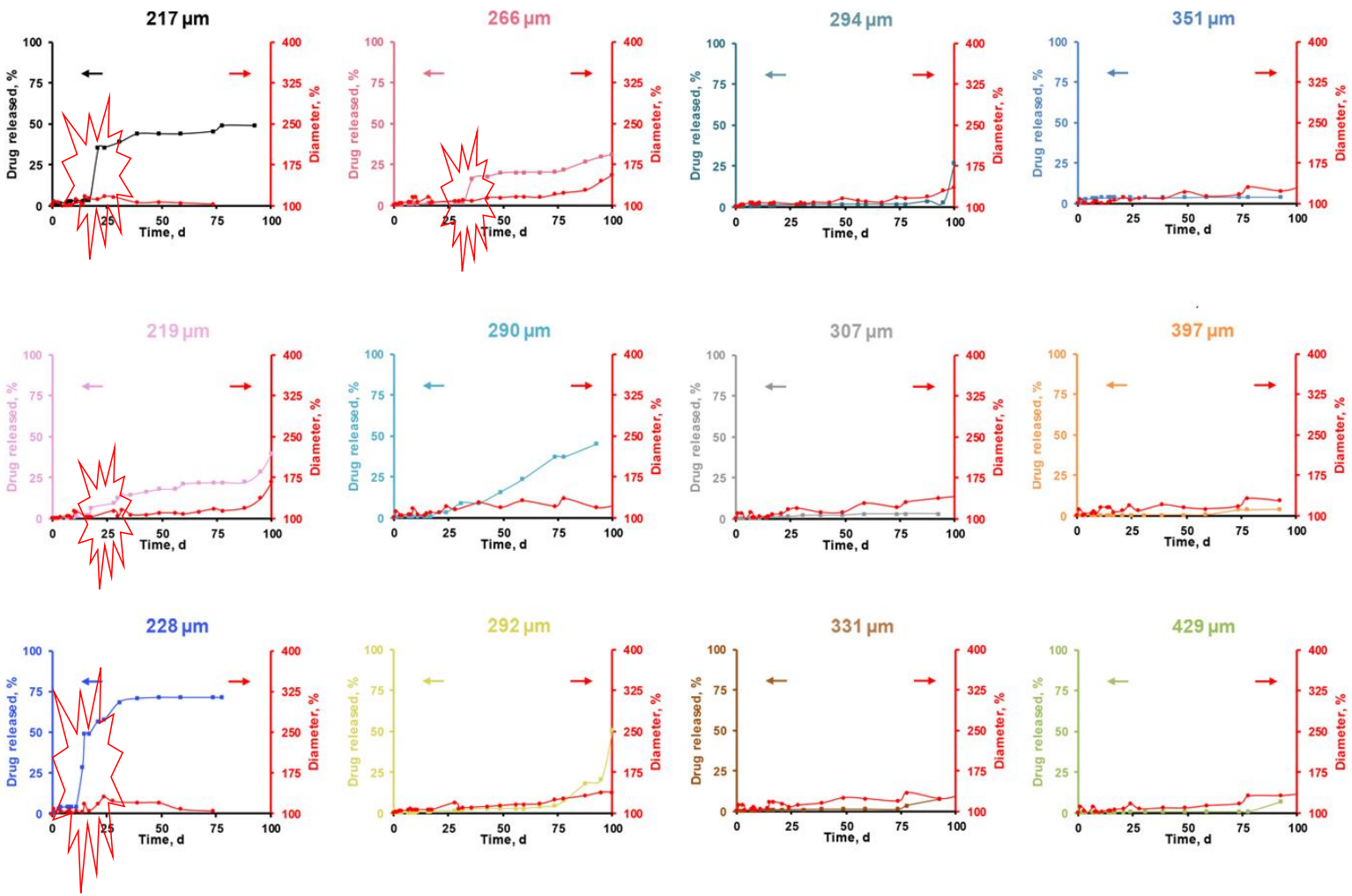

Figure III-27: Drug release and swelling of single diprophylline-loaded PLGA microparticles upon exposure to phosphate buffer pH 7.4 at 20 °C (80 rpm). The initial microparticle diameters are indicated at the top of each diagram.

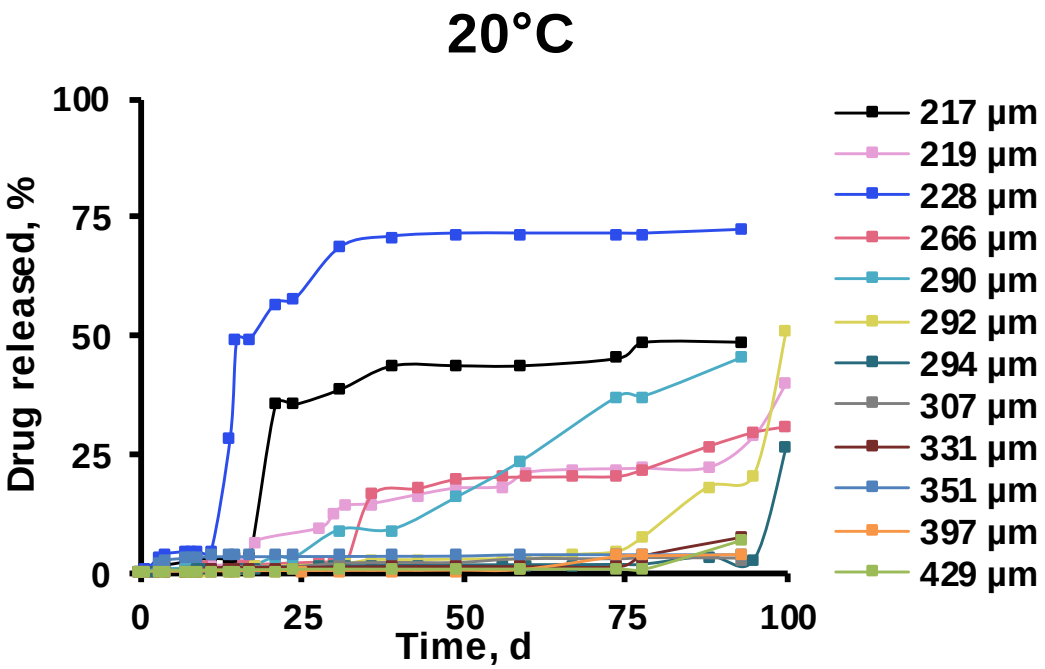

Figure III-28: Diprophylline release from single PLGA microparticle in phosphate buffer pH 7.4.at 20 °C (80 rpm). The initial microparticle diameters are indicated in the diagram.

III.4. Hypothesized drug release mechanisms

Based on the above described experimental observations, the following drug release mechanisms are hypothesized for the different release phases:

The initial "burst" release (= 1st phase), is caused by the release of drug crystals with direct surface access from the beginning. This does not mean that a part of the respective drug crystal is necessarily directly located at the system's surface: Also an access via a tiny pore can allow for rapid drug crystal dissolution and subsequent diffusion through the pore. The schema at the top of Figure III-29 illustrates this release mechanism. Please note that such pores might be very small and not visible on SEM pictures (e.g., due to the sputtering of a gold layer in this study). Previously, *single* microparticle release studies evidenced that some of the microparticles release parts of their drug loading right from the beginning upon exposure to phosphate buffer pH 7.4 *at 37 C* (Tamani et al., 2019a) (37). In the present study, none of the investigated *single* microparticles showed such a release behavior at *20 C* (Figure III-27). This is consistent with the observed drug release kinetics from *ensembles* of microparticles at 20 °C (diagram in the middle of Figure III-22): As it can be seen, the "burst effect" was very much limited in the case of "medium-sized" and "large" microparticles (black and orange curves). Only in the case of "small" microparticles (red curve), an important burst release was observed

at this temperature. But the release from “small” microparticles could not be monitored for technical reasons. The fact that the “initial burst release” phase is much more important in the case of small microparticles compared to larger microparticles can be explained as follows: The total surface area of numerous small microparticles is much higher than the surface area of much fewer, larger microparticles (the sum of the volumes of the two populations being equal). Consequently, the probability that a drug crystal has “direct surface access” (eventually via a tiny pore) is much higher in the case of smaller microparticles. This principle is schematically illustrated in Figure III-30.

Interestingly, the importance of the initial burst release phase also strongly depends on the temperature: As it can be seen in Figure III-1, the burst release was much more pronounced at 37 °C compared to 20 °C and 4 °C. This cannot solely be explained by differences in the drug’s diffusivity or solubility at the investigated temperatures: Such differences can explain a decrease in the release *rate* during this time period, but not a difference in the *extent* (here the “height” of the “plateau phase”). The fact that the extent of the initial burst release from the microparticles strongly depends on the temperature likely indicates that pore closure effects as described by the group of Steven Schwendeman (42,43) are of importance: Upon contact with aqueous media, limited amounts of water rapidly penetrate into the entire systems and polymer degradation starts throughout the system (“bulk erosion”). These limited amounts of water can be expected to also lead to limited PLGA swelling, closing the tiny pores, which gave some of the drug crystals “direct surface access” at early time points. Once the pores are closed, this type of “burst release” ends. As long as the pores are “open”, drug crystals in contact with such pores can (at least partially) dissolve, and the dissolved drug molecules can rather rapidly be released. At 37 °C, the diffusivity of the drug in the water-filled channels can be expected to be higher than at 20 and 4 °C. In addition, at 37 °C, the solubility of diprophylline is higher solubility in the release medium than at 20 and 4 °C: 169 +/- 8 mg/mL compared to 128 +/- 11 and 79 +/- 6 mg/mL, respectively. The higher mobility of the drug and the higher drug solubility (leading to higher concentration gradients: the driving forces for diffusion) result in higher drug release rates during the “burst release phase”. In brief, at 37 °C the drug can more easily dissolve and more rapidly diffuse through the “open” pores compared to 20 and 4 °C. Once the pores are “closed” by (limited) PLGA swelling, the burst release phase is terminated.

The 2nd release phase can probably be attributed by the occasional release of tiny drug crystals, which are located in “surface near” regions. As illustrated in the cartoon in the middle of Figure III-29, surface near regions polymer can be expected to undergo more substantial

swelling before the entire system starts to substantially swell. Experimental evidence (optical microscopy pictures) for this phenomenon were recently reported for the investigated microparticles upon exposure to phosphate buffer pH 7.4 at 37 °C (Tamani et al., 2019a). At 20 °C this effect occurs less rapidly, because PLGA degradation is much slower (Figure III-23). This surface near polymer swelling can be attributed to the high water amounts the surface is in contact with: The high concentrations of water likely *locally* accelerate ester bond cleavage as well as water penetration into these regions. In the cartoon in the middle of Figure III-29, such swollen PLGA regions are marked in light grey, the only slightly hydrated PLGA regions are marked in dark grey. As long as a drug crystal is surrounded by essentially non-swollen polymer, the amount of water available for dissolution is very much limited and the mobility of dissolved drug molecules in the PLGA is low. However, once the neighborhood of a drug crystal undergoes substantial swelling, much higher amounts of water get into contact with the crystal and dissolved drug molecules can much more easily diffuse out. Such an event can be expected to lead to rather rapid release of the concerned drug crystal (or network of crystals, if they are interconnected by pores or channels). This type of event was observed with some of the microparticles shown in Figure III-27: The red stars highlight "sudden" partial drug release events. The size of the drug crystal (or network of interconnected drug crystals) can be expected to determine the "height of the release step". Importantly, the time points of these events are randomly distributed. Thus, in a population of numerous microparticles shows an about constant drug release rate. This is consistent with the "2nd release phases" from *ensembles* of microparticles shown in Figure III-22. The rather random distribution of the time points of sudden partial drug release events throughout the duration of the 2nd drug release phase can be attributed to the fact that the drug crystals are homogeneously distributed throughout the microparticles (as evidenced by SEM pictures of cross-sections, Tamani et al., 2019a) and an about constant rate at which the surface near swelling zones growths with time. Please note that this theory is also consistent with the experimentally observed slight and about constant increase in the microparticles' diameter with time during the 2nd drug release phase (Figure III-26, 20 °C).

Importantly, the slope of the 2nd drug release phase strongly depended on the temperature (Figure III-22): The release rate clearly increased when increasing the temperature from 4 to 20 to 37 °C. This can be explained by the more rapid local swelling of the PLGA, as evidenced for instance by optical microscopy, comparing the results obtained in this study at 20 °C (Figure III-25) with the results obtained by Tamani et al. (2019a) at 37 °C.

The 3rd release phase can be explained by the onset of substantial polymer swelling throughout the entire microparticles once a critical polymer molecular weight is reached. Initially, the PLGA chains are rather hydrophobic and highly entangled. This limits the water penetration at early time points. With time the polymer chains are cleaved into shorter fragments throughout the system ("bulk erosion"). The polymer molecular weight decreases (Figure III-3), new –OH and –COOH groups are created, rendering the system more and more hydrophilic. Also, the degree of polymer chain entanglement decreases. In addition, water-soluble degradation products create a continuously increasing osmotic pressure within the microparticles. As soon as a certain, critical PLGA Mw threshold is reached, substantial amounts of water are driven into the system, allowing for the dissolution of the drug crystals. This is illustrated in the cartoon at the bottom of Figure III-29. Importantly, the mobility of the dissolved drug molecules in the substantially swollen PLGA is rather high, resulting in the onset of the 3rd (again rapid) drug release phase, leading to complete drug exhaust.

As it can be seen at the top of Figure III-21 on the right-hand side, this 3rd drug release phase set on after about 1 week exposure to the release medium at 37 °C. This corresponds to a PLGA polymer molecular weight of about 25 kDa (top of Figure III-23). It has recently been reported that the investigated microparticles undergo substantial swelling once a polymer molecular weight of about 20 kDa was reached in PLGA microparticles loaded with caffeine (Tamani et al., 2019b), dexamethasone (Gasmi et al., 2015) and prilocaine (Gasmi et al., 2015). The difference in these threshold values is not fully understood. At 20 and 4 °C the PLGA degradation is much slower and during the observation periods the critical Mw threshold value was not reached. Consequently, substantial microparticle swelling and the 3rd release phase did not yet set on. However, please note that after 90-100 d exposure to the release medium at 20 °C, certain signs for the eventual onset of the 3rd release phase also at this temperature might be visible, e.g. in Figure III-28, III-26 and III-22.

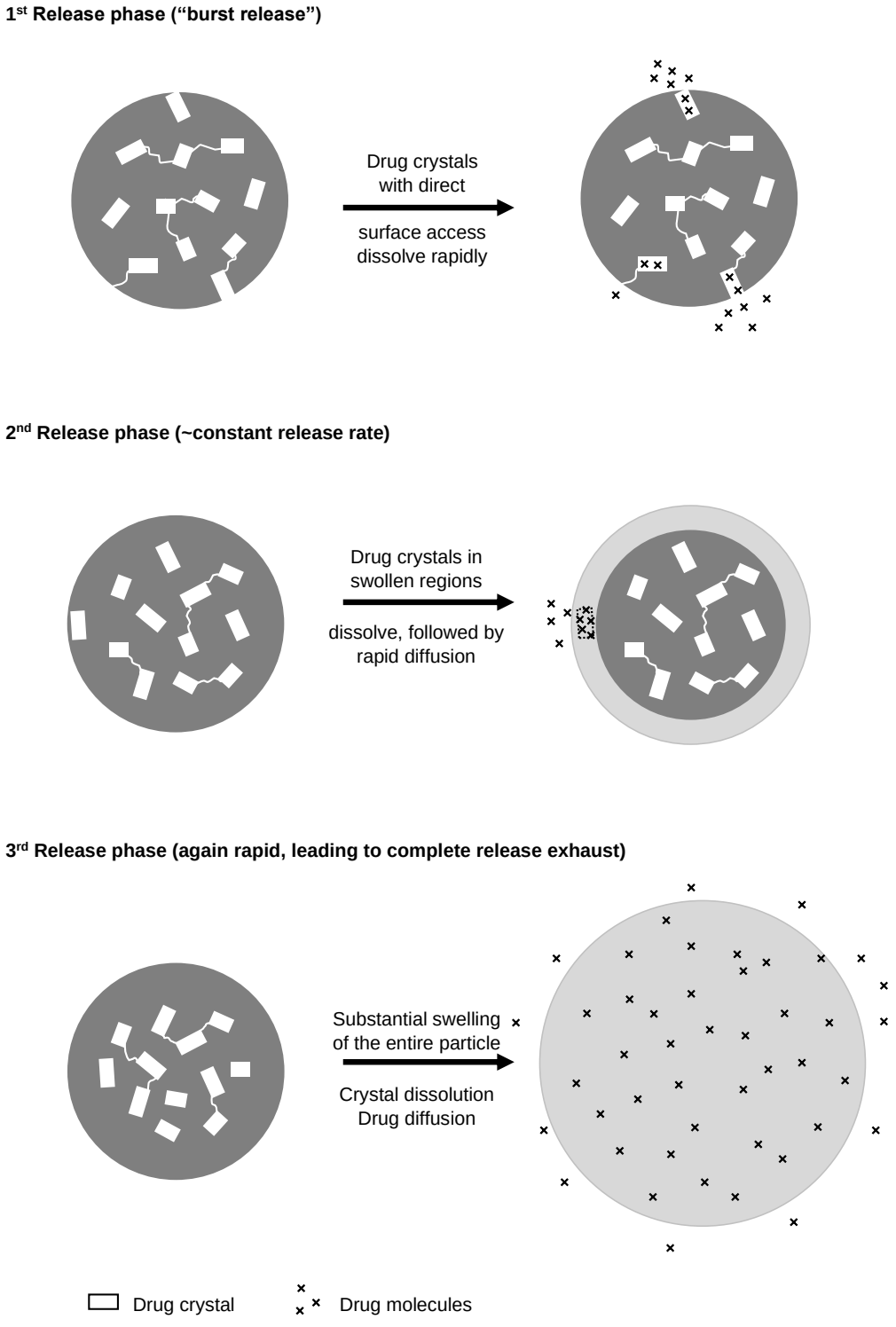

Figure III-29: Schematic presentation of the hypothesized drug release mechanisms from the investigated diprophylline-loaded PLGA microparticles. Please note that the cartoons are simplifications, e.g., with respect to the homogeneity of polymer swelling. Details are explained in the text.

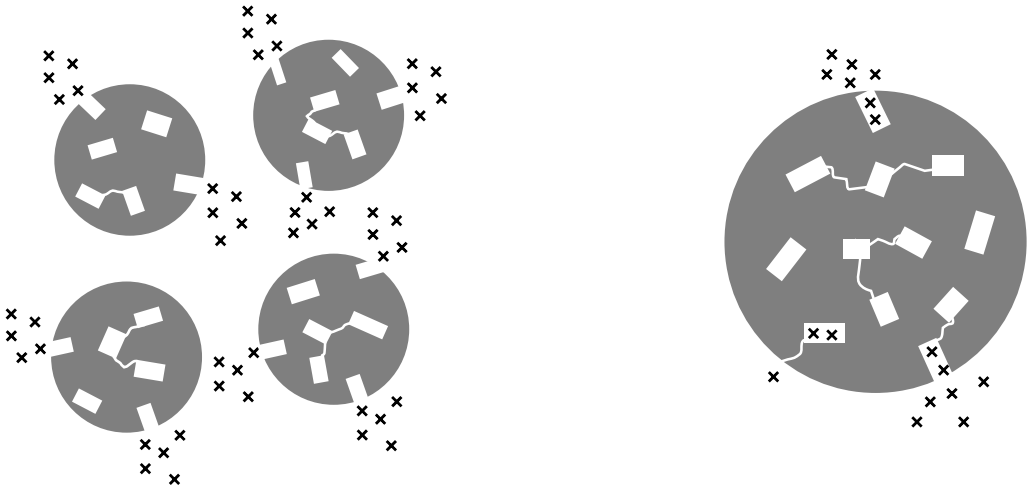

Figure III-25: Cartoon illustrating the root cause for the higher burst release observed with smaller microparticles compared to larger microparticles. The total surface area of many small particles is much higher than the total surface area of fewer, larger particles, resulting in a higher probably of drug crystals having direct surface access. Details are explained in the text.

IV. Conclusion

The results obtained in this study at 20 and 4 °C confirm the hypothesized drug release mechanisms from PLGA-based microparticles containing dispersed drug particles (here crystals): The burst release can likely be attributed to the release of drug crystals with direct surface access (eventually through tiny pores). The 2nd release phase results from the random dissolution of (eventually interconnected) drug crystals located in surface near regions undergoing local swelling. The 3rd release phase is due to substantial PLGA swelling throughout the microparticles, resulting in significant amounts of water available for drug crystal dissolution and elevated mobility of the dissolved drug molecules in the highly swollen polymer.

It will be interesting to study other type of PLGA microparticles in the future, e.g. loaded with drugs which can dissolve in the polymer to important extents. In these cases, eventually also drug transport through essentially non-swollen PLGA might be of importance, especially in the case of drugs acting as plasticizers for PLGA.

A thorough mechanistic understanding of how this type of advanced drug delivery systems work can be helpful facilitating device optimization.

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General conclusion

Poly (lactic-co-glycolic acid) (PLGA)–based microparticles are an interesting parenteral controlled drug delivery system, because their biodegradability and biocompatibility. Since decades various manufacturing methods were used to prepare this type of advanced drug delivery systems, including emulsion solvent extraction/evaporation techniques, hot melt extrusion and grinding, spray-draying. Numerous types of PLGA-based microparticles have been reported in the literature, exhibiting a broad range of drug release kinetics. However, in general the observed drug release patterns are either mono-, bi- or tri-phasic.

PLGA microparticles are called "multiple unit" dosage forms: Generally, numerous tiny microparticles are administered. In most cases, only such *ensembles* of microparticles are studied and characterized with respect to their drug release behavior. However, each microparticle is individual and might release the drug "in its own way", e.g. due to its unique internal structure. For instance, if hundreds of *single* unit dosage forms release a drug in a "pulsatile manner" at randomly distributed time points, the overall release rate of the *ensemble* of dosage forms is constant. Studying only the release of *ensembles* of dosage forms can, thus, be misleading (Chapter I).

The aim of this study was to better understand the root causes for the (up to 3) drug release phases of PLGA-based microparticles loaded with drug particles (in particular of the 1st and 2nd release phase). In this case, drug crystals were rather homogeneously distributed throughout the polymer matrix after manufacturing. It is suggested that every microparticle has its own, individual inner structure and drug release profile. Each microparticle contributes to one or more drug release phases.

Chapter II summarized the materials and methods used during this work. PLGA-based microparticles were prepared by simple emulsion solvent extraction/evaporation method. Diprophylline and caffeine were selected as a model drugs at 5% of drug loading. In all cases, the release medium was phosphate buffer pH 7.4. Particle size analysis, thermal analysis, morphology, swelling and polymer degradation were evaluated to better understand the observed phenomena.

Chapter III presents all the experimental finding obtained. It is organized in three parts.

In part I and II, the following drug release mechanisms are suggested for the control of the release from the investigated microparticles:

- The 1st release phase can be attributed to the rapid dissolution of drug crystals with immediate direct surface access. This phase might take up 1-2 days because the drug has to diffuse through a tiny pore before dissolution.
- The 2nd release phase is caused by the local swelling of certain PLGA regions. In this case, drug crystals located in these regions dissolve and diffuse rapidly through the swollen PLGA gel.
- The 3rd release phase is likely caused by the substantial swelling of the entire microparticles once the polymer chains are sufficiently hydrophilic. During this phase, the water-soluble degradation products attracts important amounts of water into the microparticles. Consequently, drug dissolution and diffusion are very much facilitated.

In part III, further insight into the underlying dug release mechanisms from this type of PLGA microparticles were obtained, by altering the temperature of the release medium. The idea was to be able to slow down key phenomena, such as polymer degradation, drug diffusion and alter the drug's solubility in the bulk fluid.

The results obtained at 20 and 4 °C confirm the results obtained in the first part of the work:

- The burst release can be attributed to the release of drug crystals with direct surface access.
- The 2nd release phase results from the random dissolution of drug crystals located in surface near regions undergoing local swelling.
- The 3rd release phase is due to substantial PLGA swelling throughout the microparticles.

As for the continuation of this work, it could be interesting:

- To study other type of PLGA microparticles, loaded with drugs with a high affinity to the polymer or drugs acting as plasticizers for PLGA. In these cases, eventually also drug transport through essentially non-swollen PLGA might be of importance.
- To realize a mechanistic understanding of how this type of advanced drug delivery systems work can be helpful facilitating device optimization.

Abstract

Poly (lactic-co-glycolic) acid (PLGA)-based microparticles represent an attractive choice to control drug release over periods ranging from a few days up to several months, while ensuring good biocompatibility and complete biodegradability. Different types of mass transport phenomena might be involved in the control of drug release from PLGA-microparticles, including for instance water diffusion, drug dissolution, drug diffusion, polymer degradation, autocatalysis and polymer swelling. The relative importance of these phenomena can strongly depend on the composition, size and preparation technique of the systems. However, generally ensembles of microparticles are studied, differing in size and behavior.

In order to better understand the drug release mechanisms from PLGA microparticles, the behavior of single microparticles after exposure to the release medium was studied.

On the one hand, the main objective of this work was to better understand the root causes for the (up to) 3 drug release phases observed with poly (lactic-co-glycolic acid) (PLGA) microparticles containing drug particles: The 1st release phase ("burst release"), 2nd release phase (with an "about constant release rate") and 3rd release phase (which is again rapid and leads to complete drug exhaust). The behavior of single microparticles was monitored upon exposure to phosphate buffer pH 7.4, in particular with respect to their drug release and swelling behaviors. In this study, PLGA-based microparticles were prepared by simple emulsion solvent extraction/evaporation method. Diprophylline and caffeine were selected as a model drugs at 5% of drug loading. In all cases, the release medium was phosphate buffer pH 7.4. Particle size analysis, thermal analysis, morphology, swelling and polymer degradation were evaluated to better understand the observed phenomena. Importantly, each microparticle releases the drug "in its own way", depending on the exact distribution of the tiny drug crystals within the system. During the burst release, drug crystals with direct surface access rapidly dissolve. During the 2nd release phase tiny drug crystals (often) located in surface near regions which undergo swelling, are released. During the 3rd release phase, the entire microparticle undergoes substantial swelling. This results in high quantities of water inside the system, which becomes "gel-like". The drug crystals dissolve and dissolved drug molecules rather rapidly diffuse through the highly swollen polymer gel.

On the other hand, the importance of the experimental conditions on the in vitro drug release measurements was evaluated. The key factors described in the literature such as size, and temperature that may alter the in vitro drug release profiles from PLGA microparticles were evaluated. PLGA-based microparticles were prepared by simple emulsion solvent

extraction/evaporation method. Diprophylline was selected as a model drug at 5% of drug loading. The studies were carried out both from ensembles of microparticles (in vitro release kinetics, PLGA degradation kinetics, morphology of microparticles after exposure to the medium) and from single microparticles (in vitro release, swelling kinetics and wet mass). All studies were performed under different release conditions (37°C/80 rpm, 20°C/80 rpm, 4°C/0 rpm) in order to identify which mechanisms, control the release of diprophylline. The obtained results show that the experimental conditions can impact the release kinetics in a significant or negligible way. These differences are due to the complicity of the mechanisms involved in the release of drug from PLGA microparticles.

Keywords: Poly (lactic-co-glycolic) acid (PLGA), single microparticles, swelling, controlled release, mechanisms,

Résumé

Les microparticules à base de poly (acide lactique-co-glycolique) (PLGA) représentent un choix attrayant pour le contrôle de la libération de substance active sur des périodes allant de quelques jours à plusieurs mois, tout en assurant une bonne biocompatibilité et une biodégradabilité complète. Différents types de mécanismes peuvent être impliqués dans le contrôle de la libération de substance active à partir de microparticules de PLGA, par exemple la diffusion de l'eau, la dissolution de substance actives, la diffusion de substance actives, la dégradation des polymères, l'autocatalyse et le gonflement des polymères. L'importance relative de ces phénomènes peut dépendre fortement de la composition, de la taille et de la technique de préparation des systèmes. Toutefois, on étudie généralement des ensembles de microparticules dont la taille et le comportement diffèrent. Afin de mieux comprendre les mécanismes impliqués dans le contrôle de la libération de substances actives à partir des microparticules de PLGA, le comportement des microparticules isolées après exposition au milieu de libération a été étudié.

D'une part, l'objectif principal de ce travail était d'étudier et de comprendre les différentes phases constituant le profil triphasique de libération d'une substance active à partir de microparticules de PLGA : phase 1 (acide lactique-co-glycolique) (PLGA) : (« libération rapide »), phase 2 (avec un « taux de libération constant ») et phase 3 (qui est rapide et mène à la libération complète de la substance active). Le comportement des microparticules individuelles a été suivie après exposition au tampon phosphaté pH 7,4. Dans cette étude, les microparticules à base de PLGA ont été préparées par la méthode d'émulsion simple, extraction/évaporation de solvant. La diprophylline a été choisies comme substance active modèle. Dans tous les cas, le tampon phosphate pH 7,4 était utilisé comme milieu de libération. L'étude de la taille, l'analyse thermique, la morphologie, le gonflement et la dégradation des polymères ont été évalués afin de mieux comprendre les phénomènes observés.

Il a été observé que chaque microparticule se comporte de manière individuelle. La libération de la substance active dépond particulièrement de la distribution des cristaux de cette dernière dans le système. En effet, au cours de la 1ére phase, les cristaux de substances actives qui ont un accès direct à la surface se dissolvent et sont rapidement libérés. Au cours de la deuxième phase de libération, des cristaux de substances actives de plus petite tille (souvent) situés en surface à proximité des régions qui subissent un gonflement, sont libérés. Pendant la troisième phase de libération, l'ensemble des microparticules subissent un gonflement important. Il en

résulte une diffusion importante d'eau à l'intérieur du système. Les cristaux de substance actives se dissolvent et diffusent rapidement à travers le gel polymérique.

D'autre part, l'impact des conditions expérimentales sur les essais de libération in vitro de substances actives à partir des microparticules de PLGA a été évalué. Les principaux facteurs décrits dans la littérature, comme la taille du système, le processus de séchage, la vitesse d'agitation et la température du milieu ont été étudiés. Les microparticules à base de PLGA ont été préparées par une méthode d'émulsion simple extraction/évaporation de solvant. La diprophylline a été choisie comme substance active modèle à une teneur de 5 %. Les études ont été réalisées à partir d'un mélange de microparticules (cinétique de libération in vitro, cinétique de dégradation du PLGA, morphologie des microparticules après exposition au milieu) et à partir de microparticules isolées (libération in vitro, cinétique de gonflement et prise en eau). Toutes les études ont été réalisées dans des conditions de température différentes (37 °C/80 tr/min, 20 °C/80 tr/min, 4 °C/0 tr/min) afin de déterminer quels mécanismes contrôlent la libération de la diprophylline à partir des microparticules de PLGA. Les résultats obtenus montrent que les conditions expérimentales peuvent impacter les cinétiques de libération d'une manière significative ou négligeable. Ces différences sont dû à la complicité des mécanismes impliqués dans la libération des substances actives à partir des microparticules de PLGA.

Mots-clés : acide poly lactique-co-glycolique (PLGA), microparticules isolées, gonflement, libération contrôlée, mécanismes.