

### Développement d'une protéine à libération prolongée, mise au point du procédé d'encapsulation sans solvant halogéné et optimisation du profil de libération.

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# Thèse de Doctorat

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Mémoire présenté en vue de l'obtention du grade de Docteur de l'Université d'Angers sous le label de L'Université Nantes Angers Le Mans

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### Développement d'une protéine à libération prolongée, mise au point du procédé d'encapsulation sans solvant halogéné et optimisation du profil de libération

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### **ABREVIATIONS**

Λ
A

Α	MSC mesenchymal stem cell
AFM atomic force microscopy	MW weight average molar mass
ATP adénosine triphosphate	Ν
В	NMR nuclear magnetic resonance
BDNF brain-derived neurotrophic factor	0
BPL bonnes pratiques de laboratoire	OMS organisation mondiale de la santé
BSA bovine serum albumin	Р
С	P188 poloxamer 188
CLA chaperone-like activity	PBS phosphate buffer saline
D	PEO polyethylene oxide
DSC differential scanning calorimetry	Prx peroxiredoxin
DCM dichloromethane	PEG polyethylene glycol
DMSOdimethyl sulfoxide	PG poly(propylene) glycol
Ε	pI point isoélectrique
ELISA enzyme-linked immunosorbent assay	PLGA poly(lactic-co-glycolic acid)
Eth ethanol	PPO polypropylene oxide
F	PVA poly(vinyl alcohol)
FDA food and drug administration	S
G	SEM scanning electron microscopy
GA glycolic acid	SF silk fibroin
GF growth factor	s/o/w solid-in-oil-in-water
u	SEC size exclusion chromatography
HD henarin	Т
HSA human serum albumin	T1107 tetronic 1107
Hsp heat shock protein	Tg glass transition temperature
T	TRIS tris(hydroxymethyl)aminomethane
I IDE insulin_degrading enzyme	V
IDE insum-degrading enzyme	VLP virus-like particle
	W
	W water
MND maladie neurodegenerative	
MPA microcarrier pharmacologiquement actif	

# **INTRODUCTION GÉNÉRALE**

Ce travail de thèse a pour objectif de comprendre, d'optimiser et de permettre le transfert technologique de microsphères, formes à libération prolongée de protéines thérapeutiques. Ces microsphères s'inscrivent dans la stratégie des microcarriers pharmacologiquement actifs développés au sein du laboratoire INSERM U1066 « Micro et Nanomédecines Biomimétiques » d'Angers.

### 1 LES MICROCARRIERS PHARMACOLOGIQUEMENT ACTIFS : UN OUTIL POUR LA REPARATION TISSULAIRE

#### 1.1 Les maladies (neuro)dégénératives

Parmi les nombreux traumatismes que peut subir le corps humain, la lésion tissulaire constitue l'un des enjeux majeurs de la recherche médicale de ces dernières décennies. Certaines lésions proviennent de traumatismes ; dans la plupart des cas le corps humain est capable de s'auto-régénérer, corrélativement à son âge et à la sévérité de la lésion. D'autres sont la conséquence d'une maladie dégénérative. Dans ce cas la lésion est le résultat du dérèglement d'un ou plusieurs processus biologique(s) que le corps ne peut pas compenser. L'occurrence de ces maladies est corrélée avec l'âge : la dégénérescence a plus de chance d'apparaître chez un patient âgé. Dans le monde l'âge moyen de la population se rallongeant, l'incidence des maladies dégénératives ne cesse d'augmenter (source : Organisation Mondiale de la Santé, 2013).

Les maladies neurodégénératives (MND) en particulier constituent une priorité. Elles se caractérisent par une destruction progressive d'une population ciblée de cellules nerveuses. Elles provoquent un dysfonctionnement du système nerveux et une atrophie du tissu neuronal [1]... Plus d'un million de personnes en France et 45 millions dans le monde sont atteintes d'une MND, la moyenne d'âge des patients étant supérieure à 65 ans. La figure 1 illustre l'occurrence des maladies neurodégénératives dans le monde selon le rapport 2013 de l'OMS.



Figure 1. Répartition des patients atteints de maladie neurodégénératives. (a) Occurrence par pays (ppm). (b) Proportion du nombre de patients par tranche d'âge. Source : Organisation Mondiale de la Santé, 2013.

Depuis les premières descriptions de dégénérescences neuronales pathologiques par James Parkinson en 1817 et Aloïs Alzheimer en 1907 [2], de nombreuses études scientifiques sont menées afin de comprendre les mécanismes de perte neuronale et d'apporter des solutions thérapeutiques. Aujourd'hui, la médecine possède les outils cliniques et biologiques adéquats pour diagnostiquer un vieillissement normal ou pathologique des fonctions neuronales. Cependant, l'étiologie des MND demeure en grande partie inconnue. Aucun traitement médicamenteux ou chirurgical ne permet une rémission totale. Des médicaments peuvent être prescrits afin de limiter l'importance des symptômes, mais l'apparition et la progression des MND ne peuvent être empêchées.

#### **1.2 Les thérapies d'avenir**

Les thérapies classiques en échec incitent à l'exploration de thérapies alternatives et novatrices.

Parmi elles, les thérapies géniques englobent plusieurs voies de recherche par la manipulation du génome *in vivo* des cellules atteintes. Ces approches permettraient de réparer le tissu à partir de lui-même. L'une de ces approches se traduit par l'utilisation de systèmes viraux délivrant localement du matériel génétique. Dans le cadre d'un gène muté (exemple : chorée de Huntington), le matériel délivré provoque le blocage de son expression. Les systèmes viraux sont

efficaces mais restent limités par les multiples origines génétiques et environnementales des MND [3–5]. Une autre approche cible l'expression de gènes. Dans le cadre de la chute de la production de certains neuromédiateurs (exemple : la dopamine pour la maladie de Parkinson), des gènes d'intérêt sont injectés afin de remplacer ou de restaurer la fonction perdue [6–8].

Les thérapies cellulaires ont pour objectif la réparation et la reconstruction des circuits neuronaux lésés. Les premières mises en place consistaient en l'utilisation de tissus immatures différenciés provenant d'embryons humains [9]. Malgré une récupération variable des symptômes et une viabilité de la greffe [10], le recueil des tissus, la survie des cellules à transplanter, l'immunosuppression à induire ou encore les questions éthiques soulevées étaient autant de freins à l'utilisation de ces tissus. Les cellules souches adultes, pluripotentes, sont aujourd'hui préférées aux cellules souches embryonnaires. Elles sont extraites de divers tissus donc permettent une autogreffe [11] et présentent une grande capacité de différenciation et de prolifération [12,13]. Au final, les cellules souches ou néo-différenciées remplacent les cellules détruites et libèrent des facteurs neurotrophiques, restaurant le tissu endommagé [14,15].

#### **1.3 Les Microcarriers Pharmacologiquement Actifs**

Les cellules souches adultes mésenchymateuses (MSC) permettent une reconstruction neuronale. Greffées dans la lésion cérébrale, les MSC présentent une innocuité et une capacité à migrer vers toute neurodégénération [16]. Cette greffe atténue la perte neuronale et accélère la récupération des fonctions neurologiques [17,18], notamment en favorisant la neurogénèse [19].

Les MSC sont aujourd'hui étudiées dans le cadre de cette régénération tissulaire. Cependant l'utilité d'une greffe directe dans le cerveau est compromise par deux problèmes majeurs. Les cellules souches ont besoin d'un minimum de 5 semaines pour se différencier en cellules neuronales [20]. Elles nécessiteraient une primo-différenciation *in vitro* avant l'injection. De plus l'hostilité de l'environnement lésé diminue la survie, la différenciation et la prolifération des MSC [21].

L'association MSC – scaffold permet de pallier ces problèmes. Le scaffold offre un microenvironnement tridimensionnel modelable selon le type de cellules, le tissu visé, l'application envisagée. La fonctionnalisation en surface par une protéine type fibronectine fournit un mimétisme de la matrice extracellulaire. Le scaffold fonctionnalisé promeut l'adhésion et la survie, voir la différenciation et la prolifération cellulaire. Plusieurs types existent : microsphères, hydrogels, ou encore la combinaison des deux [22–25]. L'utilisation de microsphères d'une taille avoisinant les 60 µm offre une courbure de surface adéquate à l'adhésion cellulaire. Ce diamètre permet aux microsphères d'être injectables, ainsi l'administration est facilitée et peu invasive [26]. La surface et les propriétés mécaniques du matériau sont également importants. Il doit être biocompatible et

biodégradable. De nombreuses matières premières essentiellement polymériques sont utilisées pour la formulation de microsphères injectables : PLGA (poly(lactic-co-glycolic acid)), poly-ε-caprolactone, chitosan, amylose, fibroïne de soie... [27–32]

L'association des MSC avec un facteur neurotrophique type facteur de croissance leur permet d'améliorer leur survie mais aussi d'accélérer leur différenciation et leur prolifération. Certains facteurs sont connus pour induire la différenciation neuronale, tels que le NGF (Nerve Growth Factor), le BDNF (Brain-Derived Neurotrophic Factor), le NT-3 (NeuroTrophin-3) ou encore le NT-4 [33].

L'association des microsphères avec le facteur de croissance encapsulé permet à ce dernier d'être libéré de manière contrôlée et prolongée, en accord avec la différenciation cellulaire. Cette libération prolongée permet également de diminuer le nombre d'injections, ce qui représente un confort pour le patient. Le facteur libéré dans le milieu pourra également jouer un rôle sur l'environnement des cellules greffées. L'utilisation du PLGA, polymère biocompatible et biodégradable [34], fournit un outil largement étudié ces 25 dernières années.

La majorité des systèmes combinent cellules souches, scaffolds et facteurs de croissance afin de fournir un microenvironnement adéquat pour une meilleure régénération tissulaire [35–38]. La microsphère encapsule le facteur de croissance et permet l'adhésion des cellules en surface ; après injection, le facteur de croissance est progressivement libéré en un mois ce qui amène une différenciation et une prolifération des cellules. La dégradation de la microsphère « libère » les cellules qui colonisent le milieu d'implantation. Le laboratoire INSERM U1066 « Micro et nanomédecines biomimétiques » (MINT, Angers) développe les Microcarriers Pharmacologiquement Actifs (MPA) issus de l'ingénierie tissulaire [26,39,40]. La figure 2 résume les bénéfices de chaque composant principal des MPA.



Figure 2. Principaux effets recherchés des MPA pour une régénération tissulaire optimale.

Le facteur de croissance est encapsulé lors de la formulation des microsphères. Celles-ci sont ensuite recouvertes d'une couche protéique (par exemple la fibronectine ou la poly-D,L-lysine) ; cette étape est appelée « biomimétisation ». Les MSC adhèrent en surface des microsphères biomimétiques. L'ensemble constitue les MPA. La figure 3 schématise les différentes étapes conduisant à l'obtention des MPA.



Figure 3. Schématisation des étapes de production des MPA.

#### 2 LES MICROSPHERES : NECESSITE D'OPTIMISATION

#### 2.1 Protéine encapsulée : préservation de l'activité biologique

La protéine encapsulée est un facteur de croissance modélisé par le lysozyme dans cette étude. De nombreux facteurs de croissance font l'objet d'une encapsulation pour une application liée à l'ingénierie tissulaire. Sont notamment retrouvés le Platelet-Derived Growth Factor (PDGF), l'Insulin-like Growth Factor (IGF), le Glial cell-line Derived Neurotrophic Factor (GDNF), l'Epidermal Growth Factor (EGF), le Transform Growth Factor- $\beta$ 1 (TGF- $\beta$ 1), le Vascular Endothelial Growth Factor (VEGF) ou encore le NGF [41–47].

Les facteurs de croissance et le lysozyme sont des protéines globulaires. Le repliement (secondaire) des résidus fournit localement des hélices alpha et des feuillets beta. Le repliement (tertiaire) structure les repliements secondaires entre eux ; la protéine présente un cœur plutôt hydrophobe (majorité de feuillets beta) enfoui sous une enveloppe plutôt hydrophile (majorité d'hélices alpha). La structure finale de la protéine est un équilibre entre plusieurs formes réversibles

en fonction de son environnement direct. Cet environnement peut également induire des modifications irréversibles de la structure de la protéine.

Les protéines globulaires possèdent une ou plusieurs fonction(s) portée(s) par des domaines catalytiques. Ces domaines résultent de la nature et de l'enchainement des résidus d'acides aminés les composant [48,49]. La structure est très sensible à toutes formes de stress physicochimiques. Ainsi, des phénomènes de dénaturation entraînant la perte de l'activité biologique de la protéine sont observés [50]. La dénaturation peut être chimique : l'intégrité de la séquence primaire de la protéine est altérée, le plus souvent par une protéolyse. Ajouté à la perte d'activité, les produits de dégradation peuvent présenter une toxicité. La dénaturation peut être physique : la structure tertiaire de la protéine active est irréversiblement modifiée, la protéine est dite dénaturée et perd son activité biologique. La dénaturation conduit à de nombreux phénomènes tels l'agrégation des protéines, aux conséquences majoritairement cytotoxiques.

Ces stress apparaissent durant les étapes de formulation et de libération à partir de la microsphère [51]. Les microsphères sont classiquement formulées par un procédé d'émulsion – extraction de solvant (cf. §2.3). Brièvement, une phase organique solvant du polymère et dispersant la protéine est émulsionnée dans une phase aqueuse. Le polymère se désolvate lors de l'extraction du solvant organique, piégeant la protéine et conduisant aux microsphères. Celles-ci sont lavées, filtrées, lyophilisées et conservées à température basse jusqu'à utilisation. La libération de la protéine encapsulée est étudiée *in vitro* et *in vivo* en parallèle d'autres contrôles des microsphères. Lors de ces contrôles la protéine est libérée et est à nouveau soumise à des stress. Le tableau 1 résume les principaux risques encourus [52–54]. On retrouve notamment de nombreux phénomènes d'adsorption : une protéine adsorbée aura sa configuration modifiée, exacerbant ses structures hydrophobes enfouies, la rendant plus sensible à la dénaturation.

Etape	Facteur	Phénomène déstabilisant	Référence
Formulation	Interface phases aqueuse - organique	Adsorption à l'interface	[55–59]
	Agitation mécanique	Augmentation des phénomènes d'adsorption aux interfaces	[60]
	Agitation ultrasonique	Production de "hot spots" (bulles gazeuses à hautes température et pression)	[61]
	Température	Déstabilisation protéique	[60]
	Origine & impuretés	Interactions avec des contaminants	[62,63]
Libération	Hydrophobie du polymère (PLGA)	Adsorption sur la matrice polymérique	[64–66]
	pH (PLGA)	Acidification par l'hydrolyse du polymère	[56,67,68]

 Tableau 1.
 Principaux facteurs dénaturant la protéine encapsulée.

Des solutions sont mises en place afin de pallier la perte d'activité : elles consistent à diminuer l'impact des stress (par le confinement de la protéine ou la modulation de l'environnement déstabilisant) [69–72], à introduire des additifs [22,55,73], à modifier la séquence des résidus d'acides aminés [74,75]... Les additifs sont le plus souvent utilisés. Le tableau 2 résume les principaux utilisés et leurs effets bénéfiques. NB : une protéine carrier est une protéine neutre. Elle est présente en grande concentration en comparaison de la protéine thérapeutique. Elle agit tel un bouclier en subissant les principaux facteurs de dénaturation [76].

Effet recherché	Type d'additif	Exemple	Référence
Anti-adsorption	Tensioactif	Poloxamère	[78,79]
		Polysorbate 20 ou 80	[71,78,80]
	Polymère	Dextran	[81,82]
		Polyéthylèneglycol-block-polyhistidine	[55]
		PEG	[71,78,81]
	Protéine	BSA, HSA	[83]
Anti-oxydation	Antioxydant	Acide ascorbique	[78,84,85]
		Ectoïne	[86,87]
		Glutathione	[78]
		Monothioglycérol	[88]
		Morine	[89]
		Polyéthylénimine	[86]
		Gallate de propyle, vitamin E	[90,91]
	Agent chélatant	Acide citrique	[90,91]
		EDTA	[85,92]
		Hexaphosphate	[78]
		Acide thioglycolique	[90,91]
pН	Tampon	Phosphate, bicarbonate, sulphate, nitrate, acétate, chlorate, pyruvate	[90,93]
	Anti-acide	Mg(OH) <sub>2</sub>	[94]
		ZnCO <sub>3</sub>	[95]
Stabilisation	Acide aminé	Alanine	[78,81]
		Arginine	[78,96,97]
		Acide aspartique	[78]
		Glycine	[78,81]
		Histidine	[98]
		Lysine	[78]
		Proline	[78,81,99]
	Sucre	Glucose	[100]
		Saccharose	[78,81,94,100–103]
		Trehalose	[78,81,101,104,105]
	Polyol	Glycerol	[78,81,106,107]
		Mannitol	[78,81,101]
		Sorbitol	[78,81,86,102]
		Cyclodextrine	[78,108–110]
	Sel	Phosphate de potassium	[78,81]
		Sulphate de sodium	[78,111,112]
	Agent chélatant	EDTA	[78,85,92]
		Hexaphosphate	[78]
	Ligand	Phénol	[113]
		Zinc	[114]
	Polymère	Dextran	[78,81,115]
		PEG	[78,81,116]
		Polyvinylpyrrolidone	[78,81,103]

Tableau 2.Principaux additifs utilisés permettant d'améliorer la conservation de<br/>l'activité biologique de la protéine encapsulée. Source : Jorgensen *et al.* [77].

La protéine est nanoprécipitée avec du poloxamère 188, puis encapsulée au sein des MPA par la technique d'émulsion – extraction de solvant. Le procédé est qualifié de s/o/w (*solid in oil in water*). Le poloxamère 188 (P188) est un polymère hydrophile biocompatible permettant de protéger la protéine contre une déstructuration irréversible. La protéine a perdu sa mobilité structurale et se retrouve ainsi protégée de l'hydrophobie du PLGA. Ces stratégies ont permis de prévenir les phénomènes de dénaturation lors de la formulation des microsphères [117]. Ainsi l'efficacité d'encapsulation est comprise entre 80 et 100%. Malgré cela seuls 50% environ de l'activité biologique de la protéine encapsulée sont retrouvés lors de la libération *in vitro* [72].

#### 2.2 Protéine encapsulée : profil de libération

La dénaturation de la protéine a donc essentiellement lieu au cours de sa libération, conduisant à un profil de libération incomplet [79,118]. La nature du polymère est directement mise en cause. Entre autres, son hydrophobie, l'acidité découlant de son hydrolyse et son profil de dégradation conditionnent la dénaturation de la protéine et son profil de libération [117]. De nombreuses études modulent la composition du polymère afin de faire varier la durée de libération de quelques heures à quelques mois [119,120]. La libération est fonction de quatre principaux mécanismes décrits dans la figure 4 [121].



Figure 4. Schématisation des mécanismes de libération de la protéine encapsulée. (a) Diffusion à travers les pores (b) Diffusion à travers le polymère (c) Gradient osmotique (d) Erosion. D'après Fredenberg *et al.* [121].

Ces quatre mécanismes sont : la diffusion à travers le réseau de pores, la diffusion à travers le polymère, le gradient osmotique et l'érosion (seul ce dernier n'implique pas le transport de la protéine) [122–125]. Ces mécanismes de libération sont fonction de l'ensemble des phénomènes physicochimiques se produisant à l'intérieur et dans l'environnement proche de la microsphère. Trois phénomènes majeurs interviennent dans un environnement aqueux : l'absorption d'eau (provoquant un « gonflement » de la microsphère), l'hydrolyse des liaisons esters entre résidus conduisant à la libération d'acides lactique et glycolique, et l'érosion [126–128]. Ces phénomènes interagissent entre eux. Les mécanismes de libération et les phénomènes physicochimiques

dépendent à leur tour des propriétés des différents composants et du milieu environnant. Citons notamment les propriétés du polymère (M<sub>w</sub>, ratio LA/GA...), les propriétés de la protéine encapsulée et des additifs, les propriétés de la microsphère (diamètre, porosité, densité...), ainsi que l'environnement *in vitro* ou *in vivo* (pH, osmolalité, température...) [129–131].

Ainsi, l'ensemble des phénomènes régulant la libération de la protéine est complexe ; seuls les mécanismes principaux sont connus. Il est difficile de connaître à l'avance le profil de libération d'une protéine encapsulée pour une composition donnée du polymère.

La composition du polymère est le facteur considéré le plus influent sur l'ensemble des phénomènes intervenant dans la microsphère [132]. Des études menées sur les MPA ont démontré l'efficacité de la modification de la composition du PLGA. Les modulations du ratio LA/GA et de la masse molaire du PLGA améliorent les profils de libération.

Le PLGA est un polymère hydrophobe. Il provoque des interactions dénaturantes pour la protéine lors de sa libération. Le copolymère PLGA-PEG-PLGA possède une hydrophobie atténuée, le segment PEG étant hydrophile. Cette hydrophilie permet de limiter les interactions dénaturantes. Les microsphères résultantes sont hydrolysées plus rapidement [39]. Plus récemment le copolymère PLGA-P188-PLGA permet de moduler finement le profil de libération de la protéine afin de mieux répondre au cahier des charges [133].

Cependant le profil de libération idéal n'est pas atteint. Pour permettre une différenciation cellulaire optimale, le facteur de croissance doit être libéré de manière continue sur un mois. De nouvelles stratégies doivent donc être mises en place.

#### 2.3 Procédés de formulation

Du fait de l'étape de nanoprécipitation de la protéine, le procédé de formulation s/o/w n'est plus un facteur de déstabilisation de la protéine.

Durant une formulation s/o/w, les nanoprécipités protéiques sont dispersés dans la phase organique. Cette phase est un mélange de solvants organiques (dichlorométhane (DCM) et acétone), solvants du copolymère. Cette phase organique est dispersée dans une phase aqueuse sous agitation contenant l'alcool polyvinylique (PVA). L'ajout important de phase aqueuse permet l'extraction des solvants organiques. Les microsphères sont récupérés par filtration, lavées puis lyophilisées afin d'enlever toute trace d'eau.

Les solvants halogénés tels le DCM sont considérés comme toxiques (classe 2) selon la Pharmacopée Européenne. Formuler des microsphères sans DCM ou autre solvant toxique serait un atout pour le transfert vers la clinique. De plus, la polydispersité des diamètres des microsphères

rend les mécanismes de libération de la protéine plus délicats à maîtriser. S'intéresser à une méthode parallèle de formulation permettrait de s'affranchir de ces problèmes. Le tableau 3 présente les principales méthodes de formulation de microsphères pour la libération prolongée de protéines.

Type de formulation	Procédé	DDS : protéine encapsulée	Référence
Emulsion	Water in oil in water	GDNF	[134–136]
		Insuline	[137–141]
		VEGF	[142]
		FGF-2, TGF	[143]
	Solid in oil in water	GDNF	[134,144,145]
		VEGF	[146,147]
		HGF, IGF-1	[148]
		TGF-β3	[133,149]
		Insuline	[137,150]
		α-chymotrypsine	[151]
		Erythropoïétine	[152,153]
	Water in oil in oil	Ovalbumine	[154]
		α-cobrotoxine	[155]
	Solid in oil in oil	Insuline	[156]
		LHRH	[157]
		Péroxidase	[158]
Atomisation	Spray drying	Insuline	[159]
		BMP-2	[160]
	Ultrasonic atomization	BSA	[161]
Microfluidique	Microcanaux	BSA	[162]
	Electrostatic droplet generation	BSA	[163]
		Insuline, hémoglobine	[164]
	Jet excitation	Subtilisine	[165]
		Lysozyme	[166]
	Flow focusing	Insuline	[141]
		GFP	[167]
		BSA	[162]

Tableau 3. Procédés de fabrication de microsphères de PLGA contenant des protéines.

Les procédés de formulation par émulsion listés dans le tableau précédent sont les premiers à être mis au point et aujourd'hui les plus couramment utilisés. Un de leurs principaux défauts est la production de microsphères polydisperses.

D'autres méthodes sont développées depuis quelques années. Ces nouvelles techniques mettent en avant une formulation rapide (une seule étape) et continue, pour une maîtrise des caractéristiques des microsphères. On retrouve notamment deux méthodes de formulation par atomisation. Lors d'une formulation par *spray drying*, la phase organique est pulvérisée dans un

courant d'air chaud. Le solvant organique est évaporé, la gouttelette se solidifie et conduit à la formation des microsphères [168]. La température nécessaire à l'évaporation du solvant organique peut entraîner une dénaturation de la protéine [169]. Lors d'une formulation par atomisation, la phase organique est pulvérisée par vibration [170]. Ces méthodes de formulation rapide conduisent à la production de microsphères polydisperses au diamètre moyen peu contrôlables [52].

Les méthodes de formulation par microfluidique, notamment les membranes microporeuses et les microcanaux, produisent des microsphères par injection de la phase organique (contenant le polymère et la protéine dissouts) dans une phase aqueuse via un système de micro-orifices. Ces orifices déterminent le diamètre des microsphères [171,172]. Bien que les microsphères obtenues soient monodisperses, ces méthodes sont notamment limitées par les faibles quantités produites [173].

D'autres méthodes microfluidiques dites *jet break-up* conduisent à l'obtention de microsphères à partir d'un flux laminaire de la phase organique. Le flux est interrompu à intervalle régulier sous l'effet de l'application de forces électrostatiques (*electrostatic droplet generation* [174]), sous l'effet mécanique d'un fil coupant le flux (*jet-cutter* [175]), sous l'effet de la vibration de la buse (*jet-excitation* [176]) ou sous l'effet des différences hydrodynamiques avec le milieu de solidification (*flow focusing* [177]). Ces méthodes fournissent des microsphères monodisperses. La méthode de *jet-excitation*, communément appelé prilling, a retenu notre attention. Elle a déjà démontré ses possibilités de production à l'échelle industrielle en conditions aseptiques [178,179] et permet l'obtention de microsphères de taille compatible avec l'adhésion des cellules souches en surface [176,180]. Enfin, le procédé de prilling permet de produire des microsphères et des microcapsules [179].

Au cours du procédé de prilling, la phase organique est composée d'une suspension de protéine dispersée dans la solution de polymère. Cette phase est extrudée à travers une buse au diamètre choisi. La buse est soumise à des vibrations dont la fréquence et l'amplitude sont adaptées. Le flux laminaire en chute libre est clivé à chaque vibration de la buse, fournissant des microgouttelettes. Celles-ci sont solidifiées avant d'être récupérées. Les microsphères sont ensuite lavées, filtrées puis lyophilisées.

La figure 5 présente les principes de fonctionnement des procédés s/o/w et prilling utilisés dans le laboratoire U1066 MINT.



Figure 5. Schémas des méthodes de formulation par (a) procédé d'émulsion *solid in oil in water* et (b) prilling.

#### **3 OBJECTIFS DU TRAVAIL DE THESE**

Ce travail de thèse vise à développer les microsphères composants de base des microcarriers pharmacologiquement actifs. Le but final était de proposer un système à libération prolongée, optimal pour une protéine modèle et apte au transfert.

Le système doit permettre une encapsulation, une conservation et une libération de la protéine sous forme active. La libération est souhaitée complète et continue sur un mois. Afin de produire un tel système deux stratégies d'amélioration sont mises en œuvre. La première stratégie consiste à apporter des additifs protégeant la protéine encapsulée pendant sa libération. Dans ce but, le premier chapitre sera consacré à l'étude bibliographique d'une nouvelle catégorie potentielle d'additifs. Trois additifs seront retenus : la protéine de choc thermique Hsp27, l'héparine et la fibroïne de soie. La deuxième stratégie consiste à modifier la composition du copolymère afin de moduler le profil de libération de la protéine encapsulée. Le deuxième chapitre sera consacré à l'application des trois additifs retenus et de la modulation de la composition du copolymère.

Le système doit être contrôlable et transférable à la production pharmaceutique en conditions aseptiques. Le troisième chapitre sera consacré au développement du procédé de prilling pour la formulation des microsphères, sans solvant toxique, adapté à la nanoprécipitation protéique.

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# **CHAPITRE I**

## Les additifs protéiques,

## outils thérapeutiques

## et agents de stabilisation

#### INTRODUCTION

Les microsphères sont des formes pharmaceutiques injectables pouvant être utilisées pour la délivrance de protéines thérapeutiques. Ces macromolécules étant fragiles, leur activité biologique doit être préservée lors de l'étape de formulation. De plus, les nombreux stress auxquels les protéines sont soumises lors du processus de libération pouvant entraîner une dénaturation [1,2].

De nombreuses solutions existent pour tenter de préserver l'activité biologique des protéines. Dans la majorité des cas des excipients (ou additifs) sont ajoutés à la formulation. Ces additifs peuvent être classés suivant deux principes généraux de protection. Dans le premier cas l'additif permet de diminuer le stress responsable de la déstabilisation. Nous retrouvons notamment les antioxydants, les sels tampons, les tensioactifs, les agents chélateurs [3–6]... Dans le second cas l'additif subit le stress à la place de la protéine thérapeutique. Des stabilisants tels des sucres, des sels, des polyols ou encore des protéines neutres apportent cette protection [7–10]. L'ensemble de ces additifs permet de conserver une partie de l'activité biologique de la protéine thérapeutique. Néanmoins leur impact reste limité et très dépendant de la forme pharmaceutique.

L'article de synthèse suivant traite de l'utilisation de protéines spécifiques pour une protection rapprochée et spécifique de la protéine thérapeutique. Plusieurs mécanismes sont proposés. Certains font déjà l'objet de recherches expérimentales en tant qu'additif [11] ou de système à libération prolongée [12,13]. D'autres mécanismes sont envisagés via l'utilisation de protéines reconnues pour leurs actions de protection dans divers environnements [14–16].

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#### **ARTICLE SOUMIS À BIOMACROMOLECULES**

# Prospective protein additives for the delivery of therapeutic proteins

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#### Abstract

Preservation of the activity of a therapeutic protein is a key factor in medicine. Proteins are one of the most fragile components administrable in the human body. They encounter various stresses and denaturing phenomena during their formulation, conservation and administration. The irreversible aggregation is most often the final step of their misfolding. According to the pharmaceutical form, different excipients are commonly added without allowing a full preservation or restoration of the protein activity. This review focused on specific proteins considered as new excipients. They would protect the therapeutic protein from the misfolding, or/and prevent the aggregation phenomenon. Different protective mechanisms are highlighted. The nature of the excipient determines the mechanism, the way and the possible contexts to be used. Promising strategies are demonstrated.

#### Key words

Aggregation, entrapment system, protein excipient, recognition system, therapeutic protein

#### **1** INTRODUCTION

Proteins were first described in 1838 by Mulder and Berzelius [1]. 60 years later, Pauling *et al.* [2] explained their structural analysis, and in 1949 Sanger *et al.* reported the first protein sequencing [3].

Proteins are biological polymers of aminoacids. They govern the overwhelming majority of the reactions in the cell. During the recent decades they have been commonly used as therapeutic agents against numerous diseases. Since the first injection of insulin in 1922 [4] to recombinant proteins [5], their pharmaceutical use is of continual increasing interest.

The protein activities depend on the easily fragile spatial configurations, which are easily fragile. In fact, to prevent the denaturation or restore the activity, the protein must be protected. Many strategies are used to preserve the activities during the formulation process. From their synthesis to the final administration form, excipients and chemical additives are employed. Unfortunately, these excipients do not lead to a total conservation of the therapeutic activity [6–8]. New protein excipients could bring more specific, diversified protections. In this review we will first introduce the folding and misfolding protein mechanisms and the necessity of developing a new type of protection, this review will then focus on potential protein additives and their mechanisms of action.

#### **2 PROTEIN FOLDING AND MISFOLDING**

#### 2.1 Globular protein folding

Protides are one of the major components in the living world with lipids, sugars and nucleic acids. They are composed by 20 different essential amino acids (called residues when polymerized) in the human body. Protides are called proteins with more than one hundred residues, called peptides on the contrary.

The specificities of a globular protein are especially linked to its tridimensional structure, resulting from the linear sequence. Thus the sequence of residues constitutes "the second half of the genetic code" [9], and provides at least one active spatial configuration [10]. The configuration can be predicted *in silico* using recurrent domains; however only experiments as NMR assess the real configuration [11].

The native state is the configuration of a protein with the lowest free-energy level [12]. It is the most thermodynamically stable configuration under physiological conditions. This state is the active state for the vast majority of the proteins. It is obtained by folding the protein with noncovalent intramolecular interactions (*e.g.* hydrogen bonds, electrostatic interactions, Van der Waals interactions). First these interactions fold localized domains of the protein into secondary structures (*e.g.*  $\alpha$ -helix,  $\beta$ -sheet...). Second these domains interact amongst themselves to produce a tertiary structure. An optional quaternary structure is constituted by the intermolecular association between protein chains [13].

The Levinthal's paradox represented the first hypothesis of the folding mechanism. The lowest free energy level would be reached by random structuration. However proteins took too long to fold compared to the biological process [14]. In reality  $\alpha$ -helixes and  $\beta$ -sheets need respectively 100ns and 1µs to be made [15]. Predetermined transition states were known to constitute the way to speed up towards the completed folding [16]. The preponderant one (called  $\alpha/\beta$  intermediate) is the first gathering of secondary structures. The folded structure is reached in 50µs on average. Proteins used as therapeutic agents are most often globular; they are composed of a hydrophobic core (a majority of  $\beta$ -sheets) and a hydrophilic shell (a majority of  $\alpha$ -helices).

Figure 1 ([12,17–22]) shows the energy landscape illustrating the free-energy levels of the folding states (unfolded protein,  $\alpha/\beta$  intermediate and folded protein) [12,23]. Multiple ways and intermediates could be represented [24]. Numerous reviews permit to explore theoretical and experimental approaches of the protein folding mechanisms [12,21,25,26].



Figure 1. Schematic free-energy surface of folding and misfolding steps of a globular protein. In blue for reaching a native configuration; in red the misfolding steps. Adapted from ref. [12,17–22].

#### 2.2 Globular protein misfolding

Many proteins constitute a therapeutic product commonly used in the pharmaceutical field. They are administrated in solubilized or lyophilized forms. In both cases phenomena can cause a loss of their biological activity during the formulation, the conservation and the administration. Many excipients are added for a general protection. However the best understanding of the misfolding causes and consequences is required in order to guarantee the best preservation of the biological activity of the therapeutic protein.

#### 2.2.1 Structuration of denatured state

Because of their fragility and their great importance, the misfolding mechanisms of the globular proteins are greatly studied. In 2013, 529 articles whose 108 reviews explored the causes and consequences of the "protein misfolding" (Pubmed data).

The failure of one of the steps described above or a stress on the folded protein leads to a misfolding, mainly causing an aggregation. Stress conditions supply energy to the protein and the configuration is destabilized. Depending on the stress nature and intensity, hydrophobic domains become abnormally exposed. The protein is misfolded in a prefibrillar state (figure 1). The

hydrophobic domains bind together or with other hydrophobic components in another prefibrillar protein. The chain reaction drives to a collapsed hydrophobic cluster [27]. The final amyloid fibril possesses a lower free-energy state than the native protein. This chain reaction is called aggregation [28–30]. The aggregation phenomenon constitutes one of the greatest challenge in the pharmaceutic field; it is largely explained in the literature [12,18,31–39].

Other stresses as a mutation in the residue sequence induce a direct misfolding. Some of them lead to more intermediate states. The lower the difference between free-energy levels, the easier the transition between states. Thus the native state is reached more quickly. However the denatured states also can appear [34,40].

According to the nature of the protein, the denaturant factor and the environment, different phenomena could lead to a protein denaturation. The misfolding possibilities are called "the denatured state ensemble". All mechanisms of the denaturation are not yet understood [31].

#### **2.2.2 Degradation factors**

*In vivo* well-known phenomena misfold a protein. Disrupted transcription, mutated sequence, or postsynthetic damages are one of the most important factors of denaturation [41].

*In vitro* pharmaceutical researches highlight notorious stress factors. Proteins are partially denatured by mechanical stresses (pumping, filtration, stirring, shaking), physical stresses (heating or freezing, UVs, X-rays) and chemical stresses (oxidation, high or low pH, detergents, hydrophobicity or hydrophilicity). Hawe *et al.* suggest a classification of these types of stresses [42].

#### 2.2.3 Misfolding consequences

In the human body the first misfolding steps are commonly reversible, or quickly degraded by the cellular machinery. However in some cases the misfolding leads to irreversible phenomena as the aggregation.

Sometimes the aggregation constitutes an expected mechanism for producing fibrillar proteins. These proteins are involved in the cell skeleton [39]. On the contrary diseases are induced by abnormal aggregation. Neurodegenerative diseases (Huntington's, Alzheimer's, Parkinson's or Creutzfeldt-Jakob's diseases), hypercholesterolemia, cystic fibrosis, phenylketonuria, stickle cell anemia or cataract are the subjects of numerous studies focusing on protein aggregation [37,43,44].

Additional phenomena appear in the biotechnology field. A recombinant protein can be synthetized in large quantity which leads to their intracellular aggregation, called inclusion bodies [45,46]. Chemical molecules, surfaces and leachables can induce the same issues [47].

Thus a therapeutic protein has to be protected from irreversible misfolding. The preservation of one of its reversible state with the active one is necessary to recover the activity.

#### **3 PROSPECTIVE PROTEIN ADDITIVES: THE NATURAL STRATEGIES FOR A PRESERVATION OF THE ACTIVE, THERAPEUTIC PROTEIN**

Nowadays numerous excipients are employed for improving the stability of the therapeutic protein during the manufacturing. Especially the thermodynamic transitions between states, the adsorption and the aggregation phenomena constitute a large area of research [47,48]. They reduce the denaturing hazard by stabilizing the protein environment or preventing the stresses. Jorgensen *et al.* [49] presented a classification of these excipients by molecular type and stabilizing effect. Sugars, polymers, polyols, amino acids, salts or surfactants are introduced as anti-adsorbent, anti-oxidant or pH stabilizer.

However the specific preservation of the active configurations, and the closest protection possible, are usually missing. For these purposes protein-based systems are emerging as a new class of excipients. They are commonly encountered in living cells for refolding or eliminating the misfolded proteins [50,51]. Because a therapeutic protein needs a specific environment to be misfolded (by modifying its configuration), another protein could act as a protector. The suggested proteins are biocompatible with no or small secondary effects.

The protein-based systems could be transferred into the formulations of therapeutic proteins as new excipients. Figure 2 illustrates mechanisms of preservation that could be encountered. They are classified in two main groups: the recognition systems and the entrapment systems. The following list illustrates these mechanisms.



Figure 2. Schematic view of the studied protective mechanisms of a target protein. The first principal mechanism recognizes (a) the misfolded target or (b) the active target. The second principal mechanism entraps the target (c) in a complex or (d) in a matrix. (e) The misfolding is recognized then entrapped.

#### **3.1 Recognition systems**

Some prospective proteins act by recognition of a specificity. The specificity can be held by the active state of a protein target, as a catalytic domain, or by the misfolded state of numerous proteins. Figure 3 summarizes the recognized state of a protein undergoing a misfolding, the linked potential excipients of the following list and their mechanisms of recognition.



Figure 3. Schematization of the appropriate use of recognition systems.

#### **3.1.1 Small heat shock proteins**

Most of the recognition systems possess a chaperoning activity. They could constitute in general one of the most promising excipients. They permit the closest protection possible of a large number of proteins. Moreover they can be specific or with a wide spectrum. They exhibit a large variety of mechanisms. Some of them recognize a misfolded state and bind to it, while others entrap a specific protein [52,53]. By definition, a chaperone is "a protein that binds to and stabilizes an otherwise unstable conformer of another protein [...]" [53]. By extension the chaperone-like activity (CLA) is the protection of the protein from instability, whatsoever the state of the protein. Chaperoning begins with the protection of another protein.

The chaperoning activity is essential for any living cell. In the bacterial world [54], the plant kingdom [55] or the animal kingdom, the chaperones represent a conserved class of proteins, demonstrating its prominent role [56–58].

In the human body, Heat shock proteins (Hsp) represent the main example of CLA proteins. Hsp constitute a common response to all stresses which could also be encountered during a formulation process. They are overexpressed when the cellular environment induces misfolded proteins [59]. Hsp include small Hsp (sHsp) or HspB family. sHsp are the first and preponderant response during stresses [38], notably linked to formulation processes. Their main benefits are the simplest (so easiest) preventive mechanism and the endogenous criteria of all human cell type.

Hsp27 is the preponderant example of sHsp. Without stress Hsp27 is found in every human cell as inactive 12 or 24-mer peptides. During a stress Hsp27 is reconfigured in active dimers that recognize and bind to misfolded proteins. A steric hindrance is created on the abnormal domain [52], which inhibits the aggregation as described above [60]. Hsp27 can not refold by itself the misfolded target. *In vivo* a refolding complex (as foldosome) collects the association Hsp27-misfolded target. It can also be taken in charge by a lysosome and degraded. So Hsp27 acts as shown on figure 2 (a).

Hsp27 is already introduced into drug delivery systems. It is co-encapsulated with a growth factor in a microsphere/hydrogel hybrid system for its anti-apoptotic activity [61,62]. With a close conservation and release Hsp27 can act as excipient for the other encapsulated protein.

Other sHsp were found out, possessing the same preventive mechanism with advantageous aspects.

Clusterin is one of them. It recognizes and holds abnormal hydrophobic domains [63]; the resulting association is taken in charge [64]. An advantage is the localization of it action. It was identified in all extracellular matrices in the mammalian species [65], in a variety of biological fluids [66], etc. [67,68]. Its preventive activity was already demonstrated *ex vivo* [38,65]. Moreover clusterin is a better efficient chaperone than Hsp27. It possesses a higher ratio of the chaperone / misfolded protein binding. This protection is effective for a large-scale protein type. So clusterin constitutes a remarkable candidate. It was not used in drug delivery systems yet; however it could be introduced in the same way as Hsp27.

 $\alpha$ -crystallin is another example. With the same protective mechanism, it is the most important protein in the mammalian lens. It prevents the aggregation of misfolded lens proteins, among others. These actions permit to conserve a cleaned and transparent lens [69–71]. It is naturally composed by the subunits A and B. The modification of the quantity of each subunit authorizes the modulation of its properties (e.g. half-life time [72–75]) and the specific action according to the tissue [76]. Such a possibility of modulation and environment could allow preventing the aggregation of therapeutic proteins in the *ex vivo* pharmaceutical formulations.

Many other sHsp have been discovered and could bring more special features. However their mechanisms are not well understood. PDC-109 illustrated that. It is a CLA protein for the

prevention of aggregation in the bovine seminal plasma and many other environments. It is not identified as Hsp yet, but possesses a Hsp homolog in the equine species. During a stress it acts as holdase for misfolded proteins in a seminal plasma [77–79] (without foldase activity). Its mechanism was not yet understood.

#### 3.1.2 Redoxin

Redoxin families are an example of proteins well-known for another predominant activity. They exhibit a main redox activity in an oxidative stress.

For instance, the peroxiredoxins family play an antioxidant role during an oxidative stress induced by reactive oxygen species [80,81]. Under stress they adopt a five-dimer configuration with chaperoning activity by hydrophobic bonds [82]. The protected protein is preserved from aggregation [83], but the mechanism is not yet understood. Other examples can be found, such as the protein disulphide isomerase exhibiting holdase and foldase activities for neosynthetized proteins [84], or in the plant world with the tobacco plastid thioredoxin [85–87]. The antioxidant/chaperone activities could improve a pharmaceutical formulation of specific therapeutic proteins that may be subjected to an oxidative stress.

#### 3.1.3 Nanobodies

Nanobodies constitute one of the novel promising approaches acting in a similar manner as the prevention mechanism of sHsp.

Nanobodies derive from Camelid antibodies. In this species antibodies do not possess light variable fragments [88]. The variability is brought by a specific heavy fragment called  $V_HH$  [89].  $V_HH$  can recognize its antigen, binds and creates a steric hindrance. It acts as a chaperone described in figure 2 (b).

 $V_HH$  can not prevent the aggregation of misfolded proteins [90], it acts beforehand by protecting from the misfolding. Moreover the complex  $V_HH$ /antigen is resistant against numerous chemical and physical stresses [91] and due to its low molecular weight,  $V_HH$  is a stable protein. Its main disadvantage is the specificity of each VHH towards only one protein, so it has to be produced for each target.

V<sub>H</sub>H is already incorporated into drug delivery systems being studied [92–94].

#### 3.1.4 Heparin

Heparin is the only non-protein described here. However it possesses a protective activity analogous to the nanobodies, with a specific interaction with certain growth factors presenting a heparin-binding site. Heparin directly binds and creates a steric hindrance preventing the misfolding of the growth factors. It is classified as shown on figure 2 (b). Some examples are given with the growth factor BMP-2 involved in osteogenesis [95,96].

So heparin could be an interesting excipient for the protection of formulated growth factors. Moreover its glycosaminoglycan nature makes it a cheap product compared to recombinant proteins. However its use must deal with its anticoagulant effect. It is already commonly introduced into pharmaceutical formulations for different improving activities [97–99].

#### 3.1.5 Prospective applications of mechanisms (a) and (b)

Additives classified as (a) mechanism recognize then protect a misfolded state of any globular protein. In this way, the best way to use them can be the co-dissolution of the therapeutic protein and the protein additive before the formulation. There is no association between an additive and an active protein; as soon as a misfolding appears the protein is recognized and protected by the additive. The aggregation chain reaction is prevented. The protein solution can be conserved as is or freeze-dried. However, the biological activity cannot be restored. The association therapeutic protein – protectors has to be taken in charge by the intracellular refolding pathway. The anabolism of the host cell can be harness. So this mechanism has to be used for a delivery of the therapeutic protein to the cells. In this way, some specific additives can bring advantages. For example clusterin and PDC-109 possess the holdase activity in extracellular medium [64,79].

With the type of mechanism (b) the active state of the therapeutic protein is recognized and protected. Compared to the mechanism (a) the main advantage is to conserve the biological activity. The therapeutic protein can be delivered in any media. So the additive has to be mixed with the therapeutic protein before any stress. Two major disadvantages could be found. First the additive protects only its specific target. Second the misfolding is not taken in charge; if proteins are engaged in fibrillary chain reactions, the aggregation cannot be avoided.

The types of mechanism (a) and (b) prevent the therapeutic protein to a greater destabilization of its ternary structure without entrapping it. So it is protected against physical stresses but remains sensitive to chemical stresses. The next mechanisms permit a global protection by entrapping the therapeutic protein.

#### **3.2 Entrapment systems**

The next entrapment systems reflect a manageable encapsulation of the therapeutic protein with a sustained protection. Preventing the first step of its misfolding permits to conserve all biological activities without dealing with deleterious phenomena. Entrapment systems could be used directly as the delivery system or constituting a sublayer of the organization close to the therapeutic protein.

The most widely entrapment systems encountered constitute the bionanoparticles. A bionanoparticle is a nanometric capsule produced by self-organized proteins. The capsule preserves a non-modified inner environment, when the outer environment is undergoing a stress. The best known bionanoparticles (ferritin and VLP) are described below.

#### 3.2.1 Ferritin

Ferritin, as ribosomes, redoxins or IDE, was first known for its iron storage activity[100]. Moreover it possesses the ability to self-assemble into bionanoparticles that could encapsulate many products. They are highly adjustable for creating different vectors [101–103].

According to the internal space it would be conceivable to encapsulate small therapeutic proteins [104]. Hybrid nanostructures of ferritin-polymer can give larger cages adapted for big proteins and protein complexes. Such a cage could participate in a pharmaceutical formulation [103,105].

#### 3.2.2 Virus-like particles

VLP (virus-like particles) bring many different bionanoparticles according to the nature of the virus. They are highly symmetric, the architecture being monodispersed and reproducible [106,107]. The shell of the capsule (called capsid) resists against many stresses, and the inner environment permits the choice of the inner medium for the preservation of any protein [108].

VLPs are already used as complete drug delivery systems, as for example the cowpea chlorotic mottle virus [109–111], and the P22 bacteriophage capsid permitting the encapsulation of only one protein [112] or complexes conserving their interactions [113,114]. So VLPs constitute a promising excipient for the preservation of a quaternary conformation of a protein complex. Finally, VLP permits a targeted vectorization [115,116].

With the type of mechanism (c) the therapeutic protein is entrapped in a well-structured complex bionanoparticles). Contrary to the precedent additive, a bionanoparticle is specifically designed for protecting, among other things, the activity of a therapeutic protein. Without holdase or refoldase activities, the active state is required; as (b) this system can not prevent an aggregation phenomenon from a misfolded state. A ferritin complex and VLP are examples of this system. A ferritin complex is a self-organized protein potentially obtained by recombination. The viral capsids are commonly obtained from viral infection, and the recombination was succeeded [117]. However

two disadvantages could be notified. First the structuration of the bionanoparticles was breakable under freeze-drying; only the soluble pharmaceutic form is conceivable. Second the release of the entrapped therapeutic protein has to be specified according to the therapeutic goal. The specific release could be an advantage if the delivery target is specific too; engineering studies permit to vectorize the bionanoparticles [118].

#### 3.2.3 Silk Fibroin

The example of the silk fibroin constitutes a final different approach of the bionanoparticles for an entrapment system. Here the protein is encapsulated not into a well structure but into a matrix.

The silk fibroin is a protein isolated from cocoons of the silkworm *Bombyx mori*. It is considered as a hydrophobic biopolymer with numerous  $\beta$ -sheets and few  $\alpha$ -helixes. It is modulated to produce a delivery system of proteins for cartilage- [119], muscle- [120] or bone-repair [121,122], mostly encapsulating growth factors [119,121,123–125] or antibodies [126] sustainably released. It acts as described in figure 2 (d).

The silk fibroin is negatively charged and highly hydrophobic. The most common therapeutic proteins are negatively charged (at a physiological pH) and hydrophilic on their surface (globular). The unsolicited interactions as hydrophobic bonds have to be considered as stresses. However studies demonstrated the preservation of the activity of the encapsulated proteins [127].

The silk fibroin constitutes an interesting approach for preserving a hydrophobic therapeutic protein, or non-sensitive to the hydrophobicity. It entraps the protein in a polymeric matrix. It is adapted for a sustained release.

#### **3.2.4** Prospective applications of mechanisms (c) and (d)

With the type of mechanism (d), the therapeutic protein is not protected by recognition or entrapment into a bionanoparticle. The fibroin could protect an entrapped protein by the creation of a non-deleterious, solid microenvironment. So the silk fibroin can be used only in a freeze-drying way. The formulation of silk fibroin encapsulating a protein was already studied and constitutes a research area as drug delivery system [128]. The encapsulation of a recombinant protein could even be realized from the silk cocoon [129]. Moreover SF is the only one not synthesizable additive. As the use of collagen and fibrin in the same way [130–133], their animal origins imply a specific purification. SF could be preferred for its great availability and easier extractability. However SF from *Bombyx mori* could possess a small quantity of immunogenic sericin after the purification. If

this problem persists, SF from other species (as spiders) could be an alternative because of its lack of sericin coating [134,135].

#### 3.3 Systems acting by recognition and entrapment

The next systems combine a protection effect by recognition, as mechanism (a), with an entrapment into a protective cavity as mechanism (c).

#### **3.3.1** Big heat shock proteins

The most known Hsp are big Hsp with the HspA family. Its first representative is Hsp70. Without stress Hsp70 is expressed ubiquitously for guiding neosynthetized proteins to fold into the native state. During a stress globular proteins exhibit the consensus sequence NRLLLTG (on hydrophobic domains). Hsp70 recognizes it and holds the protein. This holdase activity prevents the formation of prefibrillar states and consequently the aggregation. Hsp70 then forms the foldosome with co-chaperones. The foldosome entraps the misfolded protein into a cavity. It then refolds it via an ATP-dependent process [136,137]. The foldosome possesses a holdase activity one thousand times faster than Hsp70 alone [138]. So Hsp70 acts principally as shown on figure 2 (a) and its foldosome as figure 2 (e).

Other big Hsp families exist as Hsp90 or Hsp60. They work along similar lines to Hsp70 [139].

Big Hsp are already used in pharmaceutical formulation as vaccines either on a soluble form or nano-encapsulated [140]. They can stimulate the immunogenic response against the infection [141,142] and prevent heat-induced aggregation [143]. In this way, Hsp70 and other big Hsps could be introduced as excipients of a therapeutic protein. Its holdase activity could play a protective role against the aggregation during the formulation process and its foldase activity could be exhibited after *in vivo* administration and delivery. However, this activity is not found in all the tissues so the target site has to be considered.

#### 3.3.2 Caseins

With a similar activity to the Hsps without overexpression under stressful conditions, caseins represent a potential valued excipient. They prevent the aggregation of misfolded proteins and refold them with net-zero ATP consumption.

Caseins represent the major protein source in mammal milks [144]. They are intrinsically unstructured proteins without specific secondary and tertiary structures [29,145].

 $\alpha$ -casein and  $\beta$ -casein act as micelles from 150 to 300nm [146] exhibiting hydrophobic and hydrophilic domains. The hydrophobic domains bind to misfolded proteins during the aggregation process [147]. The misfolded protein is thus entrapped into the micelle and the aggregation and the denaturation processes are halted. This mechanism comprises the holdase activity [147–149]. A refolding activity of the  $\alpha$ -casein micelles would permit the misfolded protein to reach its active state. The current hypothesis of its mechanism is linked to the highly flexible structure of the micelles, thus favoring the lowest free-energy level [150]. The  $\beta$ -casein micelles possess more hydrophobic domains and its holdase activity seems to be more efficient [148,151,152]. However no refoldase activity is reported.

As an excipient, caseins may prevent the aggregation and refold the misfolded proteins. In a pharmaceutical formulation without ATP it would constitute the best candidate for conserving the total activity of the therapeutic protein. Interestingly caseins are already used as drug delivery systems [153]. They composed the matrix of hydrogels or nanoparticles for preserving the activity of proteins [154,155] and many other bioactive molecules [156–158].

So, big Hsp and caseins are considered as bionanoparticles with a CLA.

#### 3.3.3 Ribosomes

Ribosomes are well-known complexes of different ARNs and proteins involved in protein translation. In particular, ribosomes help the neosynthetized protein to reach its native state, during the translation [159]. This second activity prevents the misfolding then the aggregation.

Interestingly, ribosomes possess some properties of casein micelles: the ribosomal micelles exhibit a hydrophobic surface involved in the protective mechanism [160], but the misfolded protein refolding is not yet proved.

Ribosomes are commonly used as *in vitro* ribosome display technology for promoting, protecting and translating an mRNA to a protein [161,162]. Even though they have not yet been used in therapeutics for their micelle characteristics, they act as scaffolds for siRNA delivery systems [163,164]. The therapeutic protein can be synthetized *in vitro* by the ribosome display technology. Then the complex mRNA-ribosome can be targeted and the therapeutic protein directly synthetized in the desired place.

#### **3.3.4 Insulin-Degrading Enzyme**

Insulin-degrading enzyme (IDE) is an ubiquitous zinc metallopeptidase first identified as a degrading agent for peptides such as insulin [165,166] by initiating their catabolism [165,167,168]. Recently IDE showed an over-expression during stresses in a similar manner as Hsp [169]. Its

catalytic chamber can recognize peptides in a prefibrillar state, opens its door subdomain and entraps them before closing the door. In this way amyloidogenic peptides could be degraded [170,171]. The use of IDE as an excipient has to be considered when the pharmaceutical formulation permits such a system to act (as big Hsp). It would be interesting for therapeutic peptides.

#### **3.3.5** Prospective applications of mechanism (e)

With the type of mechanism (e) the misfolded therapeutic protein is recognized then entrapped. As (a), the misfolding can not lead to an aggregation. Some protectors (IDE, ribosomes) can not refold the therapeutic protein without an external help. Big Hsp can refold the target by supplying it with cofactors and ATP. In this case two strategies can be employed. First only big Hsp is added to the therapeutic protein and holds a misfolded protein; the refolding will be performed in the targeted cells by using the host metabolism. In this case any formulated form was possible. Second the formulation contains the required additional components and refolds directly the target. In this case only a soluble pharmaceutic form is possible. The solution has to allow the refoldase activity (pH, temperature, buffer...).

#### **4 CONCLUSION**

According to the pharmaceutical formulation expected, the desired objective and the nature of the therapeutic protein, different protein additives could be employed.

Into the general mechanisms by recognition or/and entrapment, specificities are encountered as the chaperone-like activity (CLA), the holdase or the refoldase activities. Figure 4 proposes a classification of the detailed mechanisms of protection revealed and their examples studied. Some interactions are revealed, as the implication of a refolding activity for a recognition system acting as a bionanoparticle, or an entrapment system possessing a CLA.



Figure 4. Classification of the prospective protein additives.

Moreover, some of them possess other activities than the protection one for an improvement of the formulation (*e.g.* Hsp, ribosomes, redoxins, IDE, ferritin and silk fibroin).

Finally, some innovative strategies combine two mechanisms of protection in one formulation. The resulting protection is enhanced. Such a bi-chaperone system of  $\alpha$ -crystallin and  $\beta$ -casein permits to prevent synergistically the aggregation of insulin [172].

The protein additives carry the closest possible protection of a protein and preserve them from an irreversible misfolding. The entrapment systems (bionanoparticles and silk fibroin) have already demonstrated their efficacy. The recognition systems (proteins with a CLA or specific to a protein type) are in early studies or hypothetic. The global protection by protein additives is a promising and emerging strategy.

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## **CHAPITRE II**

# Les microsphères, formes à libération prolongée de protéine : application,

### optimisation, structure

#### **1** APPLICATION A LA MICROENCAPSULATION DE **BDNF**

Les maladies neurodégénératives constituent aujourd'hui un enjeu de taille mondial. Elles affectent un nombre toujours croissant de la population des plus de 65 ans (source : Organisation Mondiale de la Santé, 2013). Aucune thérapie curative n'existe, seuls les symptômes tentent d'être atténués. Les thérapies alternatives comme la régénération tissulaire sont à l'étude pour apporter un traitement efficace. Les MPA (microcarriers pharmacologiquement actifs) sont développées dans ce but.

Les MPA fournissent un microenvironnement permettant la survie et la différenciation de cellules souches tout en encapsulant un facteur de croissance protéique. Dans le cadre du traitement de la maladie de Parkinson, les MSC sont de type MIAMI (Marrow-Isolated Adult Multilineage-Inducible) et le facteur de croissance est le NT-3. Ces MPA ont prouvé leur efficacité sur des modèles *in vitro* [1,2].

Dans le cadre du développement des MPA pour le traitement de la maladie de Huntington, le BDNF (Brain Derived Neurotrophic Factor) constituerait un choix plus approprié du facteur de croissance. Structurellement proche du NT-3, il induit la différenciation de cellules souches, comme les MSC, en cellules neuronales [3–5]. Son administration sous forme de solution permet de ralentir la progression de la lésion et d'induire une régénération neuronale dans un modèle de la maladie de Huntington [6–8].

Afin de formuler des microsphères de BDNF, la première étape consiste à nanoprécipiter le BDNF selon le procédé en vigueur au sein de l'unité U1066 [9]. Cette nanoprécipitation réversible permet de protéger la protéine des stress physicochimiques. Elle crée une barrière physique entre la protéine et le polymère composant les microsphères, ce dernier étant susceptible de dénaturer la protéine. Enfin, elle permet de moduler le profil de libération de la protéine afin d'atteindre plus facilement une libération continue et prolongée [10].

La première étape du procédé de nanoprécipitation consiste à dissoudre la protéine en solution saline, puis d'y ajouter une solution de poloxamère 188 (P188). Les actions combinées du NaCl et du P188 favoriseraient les interactions protéine-protéine et protéine-P188. Enfin l'ajout du glycofurol, non-solvant de la protéine, conduit à la formation de nanoprécipités protéine-P188 par désolvatation. Un tampon peut être ajouté avant l'ajout du poloxamère afin de stabiliser le pH proche du pHi de la protéine. Celle-ci se retrouve sous forme zwitterionique, présente moins de

répulsions intra- et intermoléculaires et est plus favorable à la nanoprécipitation. Le procédé est ensuite récupéré par centrifugation à 10000g, 4°C pendant 30 minutes.

Par ses structures primaire, secondaire et tertiaire, chaque protéine possède un comportement propre au cours de la nanoprécipitation. Ce processus nécessite une adaptation des conditions opératoires. La nature et/ou la concentration des composants sont modulées afin d'atteindre une nanoprécipitation maximale.

Des protéines physicochimiquement proches dont la nanoprécipitation a été optimisée peuvent servir de modèles à la nanoprécipitation de nouvelles protéines. Le BDNF est un dimère de 247 résidus d'acides aminés possédant une masse molaire de 27,8 kDa et un point isoélectrique prédit de 9,6 [11,12]. Le NT3 est une protéine qui a été nanoprécipitée dans un travail antérieur [2]. Elle se compose d'un dimère de 257 résidus d'acides aminés possédant une masse molaire de 29,4 kDa et un point isoélectrique prédit de 9,5 [11,13,14]. Les conditions de nanoprécipitation optimales du NT3 servent de base pour la mise au point de la noprécipitation du BDNF.

Le tableau suivant présente les rendements de nanoprécipitation du BDNF en fonction de la composition du mélange. Le volume final de la solution de BDNF était de 2 µL avant l'ajout du glycofurol. Le BDNF était dosé par la technique d'ELISA (Enzyme-Linked ImmunoSorbent Assay) en sandwich.

Conditions de nanoprécipitation							BDNF
DDUE	<b>D</b> 100	N. Cl	-		**	1 6 1	nanoprécipité
BDNF	P188	NaCl	Tampon		pН	glycofurol	
			nature	concentration			
μg/μL	μg	mol/L		g/L		g	%
5	0	0,3	abs.	abs.	ind.	0,2154	3,6
5	0	0,05	abs.	abs.	ind.	1,077	0,2
5	0	0,1	abs.	abs.	ind.	1,077	0,8
5	0	0,2	abs.	abs.	ind.	1,077	1,1
5	0	0,3	abs.	abs.	ind.	1,077	1,9
5	0	0,5	abs.	abs.	ind.	1,077	0,0
5	0	1	abs.	abs.	ind.	1,077	2,0
5	0	2	abs.	abs.	ind.	1,077	0,4
5	0	3	abs.	abs.	ind.	1,077	0,3
2,75	0	1	glycine	0,6	9,6	1,077	7,2
2,75	0	0,25	glycine	0,2	9,6	1,077	3,8
2,75	0	0,5	glycine	0,2	9,6	1,077	34,4
2,75	0	1	glycine	0,2	9,6	1,077	0,7
2,75	0	2	glycine	0,2	9,6	1,077	11,6
2,75	0	3	glycine	0,2	9,6	1,077	41,8
5	0	1,5	glycine	0,1	9,6	1,077	4,9
5	0	0,5	glycine	0,2	9,6	1,077	34,6
5	0	0,5	glycine	0,2	10	0,215	19,2
5	0	0,5	glycine	0,4	9,6	0,215	11,8
5	0	0,5	glycine	0,6	9,6	0,215	0,4
5	0	0,5	glycine	0,8	9,6	0,215	0,4
5	100	1,5	glycine	0,1	9,6	1,077	21,3
5	100	0,5	glycine	0,2	10	0,215	18,9
5	200	0,3	abs.	abs.	ind.	0,215	90,6

Tableau 1.Rendements de nanoprécipitation du BDNF en fonction de la composition du<br/>mélange. *abs* = absent ; *ind*. = indéterminé.

Pour évaluer le potentiel de nanoprécipitation du BDNF par ce procédé, une série d'expériences a été réalisée sans ajout de P188. En modifiant la concentration en NaCl sans utilisation de tampon, un maximum de 3,6% de BDNF nanoprécipité a été obtenu. Sans poloxamère et sans pH stabilisé au pHi, le BDNF ne présenterait pas suffisamment de liaisons intermoléculaires avant sa désolvatation par le glycofurol. Puis un sel de glycine a été ajouté afin de tamponner la solution au pH isoélectrique du BDNF. 41,8% de BDNF étaient au maximum nanoprécipités. Cependant la modification de la quantité de glycofurol et la concentration en tampon, la force ionique du tampon, les concentrations en sel et en BDNF ne permettaient pas d'excéder cette valeur. Seul un ajout de 200 µg de P188 (soit un ratio masse/masse BDNF/ P188 de 1/20) a permis d'atteindre 90,6% de BDNF nanoprécipité. Les conditions de cette nanoprécipitation correspondent

aux conditions optimales de nanoprécipitation du NT3. L'utilisation d'un ratio massique 1/20 de BDNF/P188 apparait comme nécessaire.

Notons que le test ELISA ne peut reconnaître que le BDNF exhibant les sites antigènes. Seul le dosage de la protéine totale permet de connaître la quantité de BDNF nanoprécipitée, quel que soit sa configuration. Cependant ces tests n'ont pas pu être interprétés à cause d'interférences entre le dosage et les composants du mélange (poloxamère, glycofurol).

Notons que le BDNF non mesuré peut également être interprété comme nanoprécipité sous une configuration irréversible non reconnue par les anticorps du test ELISA.

La suite des études a été réalisée par Saikrishna KANDALAM (doctorant au sein de l'unité U1066). La répétition des conditions optimales a permis d'obtenir 92,2  $\pm$  10,3% de rendement de nanoprécipitation. Le BDNF a été ensuite encapsulé dans des microsphères de PLGA-P188-PLGA avec une efficacité d'encapsulation de 76,9  $\pm$  0,4%. L'étude de sa libération *in vitro* est en cours.

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## **2 OPTIMISATION DE LA COMPOSITION DES MICROSPHERES**

#### **INTRODUCTION**

Les microsphères composant les microcarriers pharmacologiquement actifs assurent la libération prolongée de protéine thérapeutique. Ces microsphères sont composées de PLGA et de poloxamère. Le profil de libération de la protéine est souhaité continu sur 30 jours avec 100% de l'activité biologique conservée. Cependant, la protéine piégée dans une matrice de PLGA subit une dénaturation partielle au cours du processus de libération.

Des travaux précédents ont étudié les possibilités de protection de la protéine par des additifs couramment utilisés dans les microsphères de PLGA. L'inefficacité d'agents stabilisants (sucres et autres polyols, acides aminés, polymère) et l'efficacité d'additifs anti-adsorption (tensioactifs, protéines, autres polymères) ont été démontrées [1]. De plus, le remplacement du PLGA par un copolymère PLGA-PEG-PLGA puis PLGA-P188-PLGA, possédant des segments hydrophiles, prévient également l'adsorption de la protéine encapsulée [1–3]. Abordée au cours de la discussion de l'article précédent, la stratégie de protection globale et non spécifique de la protéine ne serait pas suffisante. Seules la diminution du stress hydrophobe et la protection spécifique contre ce stress préserveraient l'activité biologique de la protéine.

La modulation de l'hydrophobie du copolymère restent néanmoins à améliorer afin de recouvrer la totalité de l'activité biologique de la protéine lors du processus de libération. Dans ce chapitre deux stratégies ont été retenues.

La première consiste à modifier la composition du copolymère. Cette modification doit réduire le stress hydrophobe et augmenter la pénétrabilité de l'eau dans les microsphères par augmentation de l'hydrophilie du copolymère. La figure 1 présente les caractéristiques des copolymères étudiés. Trois paramètres seront modifiés : la masse molaire du PLGA, le ratio LA/GA et la nature du segment central (poloxamère ou poloxamine). Les formules topologiques des polymères soulignent les monomères hydrophiles (bleu) et hydrophobes (jaune).



Figure 1. Représentation schématique de la composition des copolymères étudiés.

La deuxième stratégie consiste à introduire trois nouveaux additifs abordés au cours du Chapitre I. Ces additifs permettraient une protection spécifique de la protéine encapsulée. Les trois additifs retenus sont la fibroïne de soie, la protéine de choc thermique Hsp27 et l'héparine ; ils sont représentés sur la figure 2.



Figure 2. Représentations des additifs étudiés. (a) Structure tertiaire de la fibroïne de soie [4] (b) Structures tertiaires (hexamérique et dimérique) de la protéine Hsp27 [5] (c) Motif de répétition de l'héparine représenté selon la projection de Haworth.

L'article suivant présente cette stratégie et les résultats obtenus.

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## **ARTICLE EN COURS D'ÉCRITURE**

# Microspheres for regeneration therapy – improvement of protein release profile regarding the polymer and additives

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## Abstract

Pharmacologically active microcarriers constitute a promising tool for the regeneration of injured tissues. A polymeric microsphere mimics a microenvironment favorable for the survival of adhered stem cells. A growth factor sustained release from microspheres makes the stem cells differentiating and proliferating. The use of a copolymer poly(D,L lactic-co-glycolic acid) (PLGA) – poloxamer 188 – PLGA improve the release profile of the lysozyme (modeling a growth factor) but the release was not complete at one month due to the inactivation of the protein. In this study the nature of the copolymer and the use of additives were investigated. Different characteristics of the copolymer were modified to provide a range of properties. The subtle modifications of the polymer characteristics led to high different behaviors of the encapsulated lysozyme. Heat shock protein 27 (Hsp27), heparin and silk fibroin were introduced in the microsphere to increase the protection of the lysozyme. Hsp27 and heparin enable a sustained release of 75% active lysozyme in 42 days. They highlighted the potential of improving the protein stability.

## Key words

Microsphere, drug delivery system, protein, PLGA, Hsp27, heparin

### **1** INTRODUCTION

Nowadays numerous degenerative diseases remain untreated due to a lack of knowledge of the causes of the diseases and the difficulty to find appropriated therapeutic tools [1,2]. This observation was met more and more because of its links with the population ageing (World Health Organization, 2013). In particular the neurodegeneration constitutes one of the most studied medical issues of recent decades, as Alzheimer's, Parkinson's and Huntington's diseases [3,4]. These diseases force the clinical research to find new therapeutic ways. The gene therapy constitutes one potential way for a regeneration from the injured tissue by a local genome manipulation [5–7]. The regenerative medicines are other promising approaches [8–10].

Pharmacologically Active Microcarriers (PAMs), a tool for regenerative therapy, are developing [11–17]. They permit the survival, the differentiation and the proliferation of stem cells through an adhesion on a platform of drug delivery system. This platform is composed by a microsphere PLGA-derived polymer encapsulating a growth factor and being covered with a biomimetic surface. The growth factor and the stem cell type depend on the type of tissue to regenerate. The factor has to be sustainably released over one month to improve the survival and the differentiation of the stem cells. The differentiated cells will colonize and regenerate the damaged tissue.

PLGA (poly(lactic-co-glycolic) acids) is a biocompatible and biodegradable polymer highly studied as a scaffolding material [18–21]. However a PLGA microsphere gives a low and incomplete release of the encapsulated therapeutic protein. Many interactions appear between the protein and its microenvironment and lead to an irreversible destabilization of its spatial conformation and to an irreversible entrapment into the microspheres. The protein is no longer available in the active state [22,23].

Some strategies were previously developed to improve the complete sustained release. The introduction of poloxamer 188 (P188) was one of them. Poloxamer is a linear biocompatible polymer of poly ethylene oxide (PEO) and poly propylene oxide (PPO) residues approved for the injectable route [24]. The protein was mixed with P188 and then nanoprecipitated before its encapsulation. The protein was in a reversible configuration of the active state and protected by P188 from the denaturation [25,26]. Moreover PLGA was covalently linked to P188. The copolymer PLGA-P188-PLGA (ABA) possessed different physicochemical properties; the resulting matrix improved the preservation of the activity and the kinetic of release of the protein [17,25,27–29]. However these modifications did not lead to a complete and sustained release of the protein.

In this study two strategies of enhancement were performed in parallel. The first one focused on the impact of new copolymers, the second one on the input of additives.

The previous copolymer was composed of a linear links between two extreme PLGA segments and a central poloxamer 188 segment. Three of its characteristics were modulated. The ratio lactic acid:glycolic acid [30–33] and MW of PLGA segments [17,34,35] were controlled according to what have been already reported in the literature. Then, Poloxamer 188 was replaced by tetronic 1107 to modify the structure and the hydrophobic property of the resulting copolymer. Tetronic have been selected due to its ability to improve drug delivery [36–39].

The input of additives would increase the protection of the encapsulated protein, during the storage of the microspheres and the sustained release. Silk fibroin (SF) is a fibrous protein acting as a highly modulable polymer. It was recently developed as drug delivery system in microspherical form for encapsulated proteins [40–42], with surviving adhered stem cells [43–46]. Its biocompatible and biodegradable properties were already proved [47–50]. For a closer protection of the encapsulated protein, heparin and heat shock protein 27 (Hsp27) were used. The low molecular weight heparin was known as Lovenox® for its anticoagulant effect; more it preserves the activity of encapsulated growth factors in polymeric microspheres [51–55]. Hsp27 is a protein implicated into the protection of proteins against any stress without energy consumption or cofactors [56,57]. It was preferred to other Hsp families as Hsp70, 90 or 100 which need a complexation with cofactors and a ATP hydrolysis activity [58–60].

## **2** MATERIALS AND METHODS

## 2.1 Materials

Lysozyme (chicken egg white) and its substrate *Micrococcus lysodeikticus*, sodium chloride (NaCl), glycofurol (tetraglycol or a-[(tetrahydro-2-furanyl)methyl]-x-hydroxy-poly(oxy-1,2ethanediyl)), dimethylsulfoxide (DMSO), methylene chloride (DCM), acetone, trizmabase (Tris), bovine serum albumin (BSA), poloxamer 188 (Pluronic<sup>®</sup> F-68, 8.400 g/mol), tin(II) 2ethylhexanoate (Sn(Oct)<sub>2</sub>, 95%), diethyl ether and tetrahydrofuran (THF) were obtained from Sigma-Aldrich (Saint Quentin-Fallavier, France). Poloxamine (Tetronic<sup>®</sup> 1107; 15,000 g/mol) was purchased from BASF (Levallois Perret, France). Polyvinyl alcohol (Mowiol® 4-88) was obtained from Kuraray Specialities Europe (Frankfurt, Germany). D,L-lactide (DL-LA) and glycolide (GA) were purchased (Purac, Lyon, France).

## 2.2 Methods

#### **2.2.1** Copolymer synthesis

PLGA-based copolymers were synthetized from the combination of PLGA segments at the extremities and poloxamer 188 (P188) or tetronic 1107 (T1107) as central segment. The PLGA segments were modulated according to the constituting ratio lactic acid:glycolic acid (LA/GA) and the segment  $M_W$  (10, 20 or 40 kDa theoretical).

P188 was a linear polymer of 150 poly(ethylene oxide) residues (PEO) and 30 poly(propylene oxide) (PPO) residues. Its use led to synthetize ABA copolymers. T1107 was a four-branched polymer of 244 PEO residues and 76 PPO residues. Its use led to synthetize AACAA copolymers.

Copolymers were prepared by ring-opening polymerisation of DL-lactide and glycolide using poloxamer 188 or tetronic 1107 as an initiator, and stannous octoate [Sn(Oct)2] as a catalyst[61]. Briefly, precise amounts of various poloxamer 188 or tetronic 1107, DL-lactide and glycolide were mixed and introduced into 100mL round-bottom flasks with the catalyst. The mixtures were heated to 140°C and degassed by 15 vacuum-nitrogen purge cycles in order to remove the moisture and the oxygen, inhibitors of this polymerisation. Flasks were then freeze at 0°C and sealed under dynamic vacuum at 10<sup>-3</sup>mbar. Polymerisation was allowed to proceed at 140°C under constant agitation. After 5 days, the products were recovered by dissolution in methylene chloride and then precipitated by adding the same volume of ethanol. Finally, the polymer was filtered, washed with cold ethanol and dried overnight at 45°C under reduced pressure, up to constant weight.

Two groups of polymers were constituted for studying the ratio LA/GA, the PLGA  $M_W$  and the nature of the central segment. In one group the ratio LA/GA was modified for ABA copolymers containing PLGA segments at 20 kDa. In the other group the nature of the central bloc and the size of the PLGA segments were modified; the ratio LA/GA was set at 25/50.

#### 2.2.2 Copolymer characterization

Copolymers were characterised by <sup>1</sup>H NMR spectra and Size Exclusion Chromatography (SEC). The molecular  $M_W$  of the PLGA block was determined by using the integration ratio of resonance of PEG units at 3.6ppm and PLGA blocks at 4.76ppm in the <sup>1</sup>H NMR spectra. The molecular  $M_W$  of the copolymers were determined by SEC using Waters Inc. equipment fitted with a Plgel 5µm mixed-C (60cm) column as the stationary phase and a Waters 410 refractometric detector, eluted with DMF at 1mL.min<sup>-1</sup>. Typically, samples were dissolved in DMF at 10mg/mL

and filtered on PTFE filter Millex<sup>®</sup>-FH (pore size  $0.45\mu$ m) from Millipore Corporation, prior to  $20\mu$ L of the solution of polymer being injected. The Mw was expressed according to calibration against poly(styrene) standards.

Differential scanning calorimetry (DSC) measurements were carried out under nitrogen on a Perkin Elmer Instrument DSC 6000 thermal analyser. Samples were subjected to the first heating scan from -50°C to 200°C (10°C.min<sup>-1</sup>) followed by cooling (10°C.min<sup>-1</sup>) and the second heating scan from -50°C to 200°C (10°C.min<sup>-1</sup>). The glass transition temperature (Tg) was measured on the second heating ramp.

#### 2.2.3 Protein nanoprecipitation

#### 2.2.3.1 Lysozyme nanoprecipitation

Nanoprecipitated lysozyme was prepared as described in Giteau et al. [25] and patented [26]. Briefly, 45  $\mu$ L of 0.3 M NaCl solution containing 900  $\mu$ g of lysozyme and 18 mg of poloxamer 188 (1/20 mol/mol protein/poloxamer respected) was added to 1.04 g of glycofurol to form a 1 mL suspension. After 30 minutes at 4°C the protein nanoparticles were recovered by centrifugation (10,000 g, 4°C, 30 minutes).

The amount of lysozyme can be changed to conserve 0.6 % w/w protein/polymer. The volume of glycofurol can also be adjusted.

#### 2.2.3.2 Lysozyme-heparin nanoprecipitation

 $22.5 \ \mu$ L of 0.3 M NaCl solution containing 900  $\mu$ g of lysozyme and 900  $\mu$ g of heparin were mixed to 22.5  $\mu$ L at 0.3 M NaCl solution containing 18 mg of poloxamer 188. Then 1.04 g of glycofurol was added to form a 1 mL suspension. After 30 minutes at 4°C the protein nanoparticles were recovered by centrifugation (10,000 g, 4°C, 30 minutes).

#### 2.2.3.3 Lysozyme-Hsp27 nanoprecipitation

Before the nanoprecipitation step, Hsp27 was added to the solution of lysozyme in a molar ratio 1/2 lysozyme/Hsp27 [62]. The solution was incubated at 40°C during one hour. Then it was cooled at 4°C before being nanoprecipitated.

 $22.5 \ \mu$ L of 0.3 M NaCl solution containing 900  $\mu$ g of lysozyme and 3.40 mg of Hsp27 (molar ratio lysozyme/Hsp27 ½ [62]). The solution was incubated at 40°C during 1 h, then cooled at 4°C. It was mixed to 22.5  $\mu$ L at 0.3 M NaCl solution containing 18 mg of poloxamer 188. Then 1.04 g of glycofurol was added to form a 1 mL suspension. After 30 minutes at 4°C the protein nanoparticles were recovered by centrifugation (10,000 g, 4°C, 30 minutes).

#### 2.2.4 Microsphere formulation

#### 2.2.4.1 PLGA-based formulations

Copolymer microspheres (diam.60  $\mu$ m) were prepared using a s/o/w emulsion solvent extraction-evaporation process described in [25], with some modifications. The nanoprecipitated proteins were dispersed in the organic phase (2000  $\mu$ L of 150 mg copolymer dissolved in a 3:1 methylene chloride:acetone solution), which was emulsified in a poly(vinylalcohol) (Mowiol<sup>®</sup> 4-88, Kuraray Specialities Europe, Frankfurt, Germany) aqueous solution (90 mL, 4 % w/v at 1°C) and mechanically stirred at 550 rpm for 1 min (Heidolph, RZR 2041, Merck Eurolab, Paris, France). After addition of 100 mL of deionized water and stirring for 10 min, the emulsion was added to 500 mL deionized water and stirred for 20 min to extract the organic solvent. Finally, the microspheres were filtered on a 0.45  $\mu$ m filter (HVLP type, Millipore SA, Guyancourt, France), washed and freeze-dried.

#### 2.2.4.2 PLGA-based mixed SF formulations

30 mg of SF powder was mixed with 120 mg of 25P40 dissolved in 2 mL of methylene chloride:acetone. Then this mix was used as the organic phase during the s/o/w emulsion solvent extraction-evaporation process.

## 2.3 Microsphere characterization

The average particle size and size distribution were determined using a Coulter® Multisizer (Coultronics, Margency, France). The microparticles were suspended in isotonic saline solution and sonicated for a few minutes prior to analysis. The mean particle sizes were expressed as volume distributions.

The surface particle morphology was investigated by two scanning electron microscopies (SEM and cryoSEM). SEM (JSM 6310F, JEOL, Paris, France) was used at room temperature. Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon using MED 020 (Bal-Tec, Balzers, Lichtenstein). CryoSEM (EVO LS10, Carl Zeiss, Oberkochen, Germany) was used with the observation chamber at -70°C and the acceleration voltage at 7 kV.

## 2.4 Protein analysis

The active protein rate was determined using a *Micrococcus lysodeikticus* test. The particles were dissolved in DMSO (1 mL) in PTFE tubes at room temperature under agitation during 1 h. Then 3 mL of 0.01 M HCL solution were added. The solution was left to stand for 1 h at room

temperature under agitation. The resulting solution was incubated with *Micrococcus lysodeikticus* for lysozyme activity determination.

The total protein rate was determined using a Molecular Probes' Nanoorange® Protein Quantitation Kit. The Nanoorange® reagent as fluorescent probe was mixed to the protein sample, heated at 95°C and then cooled at room temperature before being excitated at 485 nm and emitting at 590 nm.

## 2.5 In vitro release

The *in vitro* release of lysozyme was determined by adding 250µl of 0.05 M TRIS-HCl buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl to 5 mg of microspheres in capped tubes. At determined time intervals, the tubes were centrifuged for 5 min at 2,800g. 200 µL of the supernatant were collected for analysis and replaced by fresh buffer (250µl). The percentage of biologically active released lysozyme was measured by enzymatic assay previously described.

## **3 RESULTS**

## **3.1** Modulation of the copolymer

#### 3.1.1 Copolymer characterization

The NMR and SEC experiments determined the  $M_W$  of the segments constituting the copolymers. The results were shown in table 1. The differences between theoretical and experimental  $M_W$  of the PLGA segments were considered as non-significant.

Code name	Theoretical MW				LA/GA ratio	Experimental MW		Ір	Tg 1		Tg 2	
	PLGA	P188	T1107	Total		PLG A	Total		Tg	ΔCp	Tg	ΔCp
	kDa	kDa	kDa	kDa		kDa	kDa		°C	J/g	°C	J/g
		8.4							-27.9	0.70	53.9	161.52
			15						-30.0	0.48	50.1	131.48
25P10	10	8.4		28.4	25/50	9.8	27.9	1.37	-24.1	0.11	-2.8	0.21
25T10	10		15	55	25/50	6.7	41.9	1.20	-21.4	0.42	/	/
25P20	20	8.4		48.4	25/50	21.3	51.0	2.03	-24.5	0.15	9.6	0.28
30P20	20	8.4		48.4	30/40	24.2	56.8	2.21	-27.4	0.14	9.1	0.19
37.5P20	20	8.4		48.4	37.5/25	18.7	45.7	1.49	-22.7	0.12	13.1	0.25
25T20	20		15	95	25/50	17.3	84.0	1.35	-14.7	1.10	/	/
25P40	40	8.4		88.4	25/50	41.9	92.2	1.54	-26.1	0.29	21.1	0.33
25T40	40		15	175	25/50	29.8	134.0	1.44	14.5	0.196	/	/

 Table 1. Physicochemical characterization of the studied copolymers.

The Tg (glass transition temperature) represented a preponderant property resulting from the nature of the copolymers. Two Tg were observed for ABA copolymers. The low Tg (Tg 1) corresponded to the transition from the solid state to the glass state of the central segment, poloxamer or poloxamine. The high Tg (Tg 2) was linked to PLGA segments. It was modulated according to its  $M_W$  and ratio LA/GA. Smaller the  $M_W$  of the PLGA segment lower the Tg; the lengths decreased, the chains were less entangled. Increasing the ratio increased the Tg. PGA polymers possessed lower Tg than PLA polymers. So increasing the percentage of LA units made the Tg tend towards a higher value. The ratio LA/GA presented a smaller effect than the  $M_W$  on the Tg.

No second Tg was measured for AACAA copolymers. The single Tg was linked to the entire copolymer. The Tg increased with the  $M_W$  of PLGA segments. The effect of the  $M_W$  of PLGA segments was considerable regarding ABA copolymers. Each AACAA copolymer possessed four segments of PLGA, only two for ABA.

#### **3.1.2** Microsphere characterization

The ABA and AACAA copolymers were used to produce microspheres. The microspheres were formulated by emulsion / solvent extraction process as described previously. They were

characterized regarding to the manufacturing yield, the diameter and the encapsulation efficiency. Each copolymer was used to formulate 3 batches of microspheres. All batches were stored at -80°C.

The manufacturing yield corresponds to the percentage of the microspheres recovered after freeze-drying. All manufacturing yields were on a range of 60 - 85% with no significant difference between the copolymers.

In figure 1 were shown the diameters of the microspheres according to the type of copolymer. Modifying only the ratio LA/GA did not induce significant diameter modification (Figure 1 (a)). In figure 1 (b) all diameters were significantly different except between 25P10 and 25T20, 25P20 and 25P40, 25P20 and 25T40, 25P40 and 25T40.



Figure 1. Mean diameters (± SD) of the microspheres according to (a) the ratio LA/GA for ABA copolymers (b) the PLGA MW for ABA and AACAA copolymers.

The  $M_W$  of the PLGA segment and the type of the central bloc acted significantly (Figure 2 (b)). AACAA microspheres were bigger than ABA microspheres. An AACAA branched molecule needed more space for its four PLGA segments. Moreover a high  $M_W$  of PLGA segment led to more hydrophobic intermolecular interactions, so closer chains, so smaller microsphere diameters. Increasing the  $M_W$  of PLGA led to a less heterogeneous copolymer with a bigger homogeneous sequence. So for the same  $M_W$ , high PLGA segments occupied less space than small PLGA segments.

Copolymer name	PLGA MW	Nature central segment	LA/GA ratio	Encapsulation efficiency	
	kDa				
25P10	10	P188	25/50	83.69 ± 11.7	
25T10	10	T1107	25/50	$88.77 \pm 7.7$	
25P20	20	P188	25/50	$75.67\pm3.1$	
30P20	20	P188	30/40	$80.70\pm2.1$	
37.5P20	20	P188	37.5/25	$77.21 \pm 16.6$	
25T20	20	T1107	25/50	$83.51 \pm 9.1$	
25P40	40	P188	25/50	$72.9\pm7.4$	
25T40	40	T1107	25/50	$74.43 \pm 9.0$	

Table 2. Mean encapsulation efficiencies (± SD) of lysozyme into microspheres according to (a) the ratio LA/GA for ABA copolymers (b) the PLGA MW for ABA and AACAA copolymers.

All the encapsulation efficiencies were comprised between 72.9 and 88.8%. There was no significant difference. The same tendencies as diameters could be observed. Better encapsulations were observed for AACAA copolymers than ABA copolymers; better encapsulations were observed for small segments of PGLA than high M<sub>W</sub> PLGA segments. Increasing the diameters would permit better encapsulation efficiency.

The microspheres were observed by SEM (room temperature) and cryoSEM at -70°C (Figures 2 and 3).For ABA microspheres no significant difference was observed between different ratios of LA/GA. The size of PLGA segments showed a dominant effect. Big PLGA segments implicated a smooth surface; small PLGA segments implicated a rough surface. This effect was more pronounced for AACAA microspheres. 25P10 microspheres possessed a smooth surface because its global Tg was much lower than the temperature of observation. The observation of the internal structures highlighted microsphere lattice. The smooth standard 25P40 microspheres possessed a uniform internal structure; the 25P20 microspheres irregular on surface possessed specific structures like holes of networks inside.

CHAPITRE II



Figure 2. Comparative observation (magnification x800) according to (a) the ratio LA/GA for ABA microspheres by cryoSEM (b) the ratio LA/GA for ABA microspheres by SEM (c) the PLGA MW for ABA microspheres by SEM (d) the PLGA MW for AACAA microspheres by SEM.



Figure 3. Observation by cryoSEM of the internal structure of 25P20 and 25P40 microspheres. Magnificence x800.

## 3.1.3 In vitro release

Figure 4 compared the in vitro release of the lysozyme when encapsulated into different microspheres. The control 25P40 microspheres presented a sustained release associated to a low total amount of active lysozyme released. Regarding to the ratio LA/GA for ABA microspheres, 30P20 microspheres reached a similar total amount without a sustained release. 37.5P20 microspheres released more than 50% of the encapsulated lysozyme after 40 days without a sustained release. 25P20 appeared to be the best candidate with the same total amount released more continuously.



Figure 4. In vitro cumulative release profile of lysozyme according to (a) the ratio LA/GA in ABA microspheres (b) the PLGA MW in ABA microspheres (c) the PLGA MW in AACAA microspheres. n = 3.

Regarding the size of the PLGA segments and the nature of the central segment, only 25P10 microspheres gave a total protein release of 80% more or less continuously. However as shown on figure 4 its low Tg provided molten microspheres at room temperature. They were not sufficiently easy to handle.

## 3.2 Studies about additional products

#### **3.2.1** Microsphere formulation

The additives were added during the formulation of 25P40 microspheres in order to be compared to the precedent study. Each additive was used for formulating 3 batches of microspheres. All batches were stored at -80°C.

#### 3.2.2 Microsphere characterization

The manufacturing yield was between 60 and 78.5% without significant difference between all formulations.

In figure 5 were shown the diameters and the encapsulation efficiencies per additive. There is no significant difference for the diameters. Encapsulation efficiencies showed different results according to the additives. The use of SF maximized the encapsulation. The dissolved SF into the organic phase permitted to capture more nanoprecipitates than the copolymer alone. The uses of HP and Hsp decreased strongly the encapsulation.



Figure 5. (a) Mean diameters and (b) encapsulation efficiencies of the microspheres per type of additives.

## 3.2.3 In vitro release

Figure 6 compared the *in vitro* release of the encapsulated lysozyme according to the type of additive. The release from 25P40 microspheres was considered as control. 25P40-SF microspheres did not present any improvement. 25P40-HP and 25P40-Hsp microspheres presented a near to first-order release until 42 days with a total amount of 75 to 80% lysozyme released. Specifically a burst effect of 12% at 24 hours, a sustained release during 27 days then a plateau phase until 42 days was observed for 25P40-HP microspheres. No burst and a near-to-complete sustained release at 42 days

were observed for 25P40-Hsp microspheres. These two last formulations provided a great improvement.



Figure 6. In vitro cumulative release profile of lysozyme per type of the additives.

### **4 DISCUSSION**

In this work, Pharmacologyically Active Microcarriers (PAM) loaded with a model growth factor (lysozyme) were designed and developed as an innovative tool to regenerate an injured tissue. To obtain a good regeneration [63], it is important to obtain a complete and sustained release of the grow factor over one month. The aim of this work is improve the lysozyme release from PAM controlling the composition of the copolymer that constitutes the microspheres.

The modulation of the nature of the copolymer led to synthetize height different copolymers. The first group of copolymers differentiated three values of the ratio LA/GA in the PLGA segment. The MW of the PLGA segments and the nature of the central bloc were respectively set at 20kDa and P188. The second group differentiated three values of the MW of the PLGA segments and two natures of the central bloc. The ratio LA/GA was set at 25/50.

Characteristics of the copolymer and the resulting microspheres followed expected phenomena. The Tg evolved according to the modifications of the copolymer: it decreased with the decrease of the MW of PLGA segments and with the increase of the ratio LA/GA [64,65]. The use of T1107 could be considered as a doubling of the MW of the PLGA segments compared to the use of P188. Although the Tg could vary and be under 0°C, the uses of T1107, high MW or a low

LA/GA ratio permitted the formulation of microspheres at 4°C. The resulting copolymers possessed a global Tg compatible with the particle solidification during the solvent extraction steps. The microspheres were washed, sieved and lyophilized without aggregation.

The modulation of the copolymers provides different microspheres too. Increasing the MW of PLGA segments led to decrease the particle diameters. The intermolecular interactions of the chains in organic solution were more numerous with closer chains. However the comparison between ABA and AACAA microspheres brought the most significant differences. The branched structure of AACAA copolymers compensated the effect of twice as PLGA segments as ABA copolymers. T1107 was heavier than P188, leading to reduce the possibility of intermolecular interactions, so to a slacker matrix. Moreover the less hydrophobic properties of copolymers containing small PLGA segments could catch and keep water during the formulation. Then this water was extracted by freeze-drying. So the resulting microspheres possessed a looser network [66].

The SEM images permitted to investigate this phenomenon. The structure of the surface of the microspheres could be explained by the hydrophilicity/hydrophobicity balance of the copolymers. With more hydrophobic copolymers the surfaces were smooth without many visible structures. More hydrophilic copolymers possessed a rough surface. So the surface was shaped by the behavior of the copolymer during the formulation. A more hydrophobic copolymer interacts to a smaller degree with the aqueous phase, being largest buried into the organic phase during its extraction. A less hydrophobic copolymer let their hydrophilic segments (central blocs) interact with the aqueous phase during the formulation and create after extraction this specific structure.

The cross-section of the 25P40 and 25P20 microspheres confirmed this observation. The "holes" or "network" observed into the 25P20 microspheres could be explained by the water retention during the formulation, due to the less hydrophobic property of the copolymer. In the 25P40 microspheres this structure was not observed. The structure of the interne microsphere could be confirmed.

The hydrophilicity/hydrophobicity balance showed its limit. During the SEM observation the 25P10 microspheres were aggregated with a smooth surface. Only a cryoSEM observation permitted to observe isolated microspheres with a high rough surface. So these non processable microspheres had to be excluded of this study. A same effect could be observed for 25P20, 30P20 and 37.5P20 microspheres; however these microspheres conserved their particularities under the experimental conditions, they could be further explored.

The *in vitro* studies explored the release of the encapsulated lysozyme during 42 days. Here were the expected conditions: a small "burst", a near to zero order of the release and a complete release at 30 days. In these conditions 25P20 microspheres is the best candidate. Increasing the rate of lactic acids led to stop the release after approximately 10 days with the same (37.5P20) or smaller (30P20) total amount. The use of T1107 led to stop the release after 8 days, with different total amounts which depend on the MW of PLGA segments. Finally the use of 25P40 microspheres presented a wanted profile but only 33% of active lysozyme were released after 42 days.

According to the conditions and the previous results some interesting profiles could be identified. Low MW of PLGA segments gave non processable microspheres (25P10) or a maximum active lysozyme released of 31% at 8 days (25T10). A high MW of PLGA segments gave a lower total protein release. The high hydrophobicity of these chains did not allow the water to penetrate and the lysozyme to diffuse sufficiently through the matrix. The combined effect of branched chains of AACAA copolymers blocked the lysozyme release after some days, while the linear ABA copolymers lead to a sustained release. An inactivation of the lysozyme by hydrophobic interactions with the PLGA segments could also explain this phenomenon, considering the twice quantity of PLGA segments for a same MW. So a slacker microsphere did not lead necessary to a higher and quicker release, it depended on the properties of the copolymer. So the use of P188 as central bloc had to be conserved and the T1107 excluded; the use of 20kDa of PLGA segments decreased the total hydrophobicity while creating an organized structure improving the release profile. Moreover a pronounced porosity, driven by a low hydrophobicity, would produce a better scaffold for the survival of adhered stem cells [67].

The release profiles possessed one similarity: the maximal amount of active lysozyme released could not exceed 50% of the active lysozyme encapsulated. So half of lysozyme was still encapsulated or released in an inactive form. The next three additives were tested notably for improving this part. The lysozyme should be protected to permit all encapsulated amount to be released in its active form, until 42 days.

Silk fibroin, heparin and Heat shock protein 27 were successfully introduced in the microspheres during the formulation of 25P40 microspheres. SF constituted a part of the matrix. HP and Hsp27 were nanoprecipitated with lysozyme.

The use of SF decreased a slightly the microsphere diameter. An observation under optical microscope showed inclusion bodies in the microspheres. Their size (5 to  $10\mu$ m) corresponded to the SF particles before formulation. So the formulation step did not mix SF and 25P40 chains but solidified the 25P40 copolymer around the SF particles during the organic phase extraction. The encapsulation efficiency presented an improvement. The nanoprecipitates could be adsorbed on the inclusions. However its release profile was deteriorated. These results could be explained by hydrophobic bonds between the lysozyme during its release and the SF. In this state SF chains were mostly folded by beta-sheets; they exhibit a high hydrophobicity [68–70]. So before any release the nanoprecipitation protected the lysozyme; during the release SF denatured the lysozyme. SF should not be used in this state but has to be undergo a physical modification in order to become more hydrophilic [71].

The microspheres containing HP or Hsp27 presented low encapsulation efficiencies and diameters. The nanoprecipitation parameters should not be adapted to the complexes lysozyme-additive. Indeed each protein possesses specificities and need an optimization of its nanoprecipitation. The complex HP – protein or Hsp27 – protein could also hide the lysozyme activity. This phenomenon led to denatured, nanoprecipitated lysozyme or/and to partially nanoprecipitated lysozyme. However 35 to 42% of active lysozyme were encapsulated. The release profiles presented a great improvement. With a near to zero order profile, the total amount released was 75 to 78% of active encapsulated lysozyme after 42 days. 25P40-HP microspheres still possessed a small burst and reached a plateau at 35 days. 25P40-Hsp27 microspheres did not possessed a burst anymore and no plateau was reached at 42 days. So the two strategies of close protection of the lysozyme are successful. Hsp27 and heparin would bind the lysozyme, Hsp27 by recognition of abnormal hydrophobic areas, heparin by electrostatic interactions [72]. They would limit the irreversible denaturation of the lysozyme during its release without entrapping it.

Hsp27 possesses interesting activities: it prevents the protein aggregates in some neurodegenerative diseases [73] and reduces the cell death due to polyarginine aggregates [74]. Moreover Hsp27 was endogenous of the cellular model used in the PAMs. It was suggested that secreted Hsp27 helps to repair an injured tissue with a neuroprotection activity and a protection from senescence [75–79]. Thus Hsp27 could improve the global properties of PAM. However Hsp27 introduced was a recombinant human protein. So its use led to produce expensive microspheres.

Heparin was known to preserve the biological activity of encapsulated growth factors [51– 55] in polymeric microspheres. It decreased the burst and the hydrolysis of PLGA-based microspheres by opposing its high negative charge against the lactic and glycolic acids generated by the polymer degradation [80]. A burst initiated the differentiation of the adhered stem cells; their proliferation will be improved. So heparin could be preferred for two reasons

Thus silk fibroin was excluded because of its negative action on the release of the lysozyme. Heparin and Heat shock protein 27 were protective additives for the encapsulated lysozyme; their use has to be optimized in order to limit the loss of lysozyme during the formulation.

## **5** CONCLUSION

Pharmacologically Active Microcarriers were designed for proposing an adapted regenerative therapy for degenerative diseases, especially neurodegenerative ones. Using a microspherical drug delivery system with a PLGA-based copolymer and an encapsulating lysozyme as protein model, this study brought new strategies for improving the PAM. The exploration of the two strategies permitted to highlight their potentialities.

The exploration of the nature of the copolymer exposed the crucial roles of its hydrophobic properties, glass transition temperature and spatial configuration on the inner and outer organization of the microspheres. This led to modulate the release of the lysozyme. Thus the linear intermediate 25P20 copolymer appeared to represent the best candidate.

In parallel, two additives acted as protectors on the lysozyme during its encapsulation and/or release. They drastically improved the release profile permitting obtaining a sustained release over 42 days of more than 75% of the active lysozyme.

These two strategies will be further studied for producing the best microsphere candidate for the PAM project.

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## **3** STRUCTURES INTERNE ET EXTERNE DES MICROSPHERES

## 3.1 Introduction

L'article précédent présente des microsphères fournissant un outil adéquat à la libération prolongée d'une protéine (lysozyme) encapsulée. Afin d'atteindre une libération prolongée et complète de la protéine sous forme active, les microsphères sont formulées à partir de différents copolymères. La modulation de la composition du copolymère entraîne la modification recherchée du profil de libération du lysozyme, mais également la modification des structures interne et externe des microsphères.

Parmi les trois paramètres de la composition du copolymère abordés précédemment, la nature du segment central (P188 ou T1107) et la masse molaire des segments PLGA présentent les effets les plus importants. Leurs modulations impliquent une modification de leur balance hydrophile/hydrophobe et de leur configuration spatiale. Ces nouvelles propriétés induisent des modifications de la structure et du comportement des microsphères, modifiant les mécanismes de libération de la protéine encapsulée.

Ces mécanismes sont complexes et ne sont pas totalement connus [1]. Il serait donc intéressant de pouvoir relier directement la structure des microsphères et la libération protéique à travers la modulation de la composition du copolymère. Ainsi nous pourrions prévoir la structure des microsphères adéquate en fonction du profil de libération souhaité. Afin d'approfondir notre compréhension de la structure des microsphères en fonction de cette composition, des études morphologiques complémentaires ont été réalisées.

En parallèle, une observation de la distribution de la protéine au sein des microsphères de PLGA-P188-PLGA a été réalisée afin de compléter une étude précédente démontrant l'amélioration de cette distribution grâce à la nanoprécipitation de la protéine dans des microsphères de PLGA [2]. Une distribution homogène est souhaitable pour un profil de libération optimisé et reproductible [3,4].

## **3.2 Matériels et Méthodes**

#### 3.2.1 Matériels

Le lysozyme (originaire de blanc d'œuf de poule) et son substrat *Micrococcus lysodeikticus*, fluorescéine isothiocyanate FITC (F7250,  $\geq$  90%), chlorure de sodium (NaCl), glycofurol (tétraglycol ou  $\alpha$ -[(tetrahydro-2-furanyl)methyl]-hydroxy-poly(oxy-1,2-ethanediyl)), diméthylsulfoxide (DMSO), dichlorométhane (DCM), acétone, trizmabase (Tris), BSA, poloxamère 188 (Pluronic® F-68 ; 8,400 g/mol), tin(II) 2-éthylhexanoate (Sn(Oct)<sub>2</sub>, 95%), éther diéthylique et tétrahydrofurane (THF) sont fournis par Sigma-Aldrich (Saint Quentin Fallavier, France). La poloxamine 1107 (Tetronic® 1107 ; 15,000 g/mol) est fournie par BASF (Levallois Perret, France). L'alcool polyvinylique (Mowiol® 4-88) est fourni par Kuraray Specialities Europe (Francfort, Germany). Le D,L-lactide (LA) et le glycolide (GA) sont fournis par Purac (Lyon, France).

## 3.2.2 Méthodes

#### 3.2.2.1 Synthèse des copolymères

Les copolymères résultent d'une combinaison de segments de PLGA aux extrémités et d'un segment central de P188 ou de T1107. Les segments de PLGA sont modulés en fonction du ratio LA/GA et de la masse molaire désirés.

Le P188 est un copolymère linéaire composé de 75 résidus d'oxyde de polyéthylène (PEO) à chaque extrémité et de 30 résidus d'oxyde de polypropylène (PPO) au centre. Les copolymères correspondants sont notés ABA. Le T1107 est un polymère à quatre branches composé de 61 résidus de PEO et de 19 résidus de PPO à chaque extrémité. Les copolymères correspondants sont notés AACAA.

Les copolymères sont préparés par polymérisation par ouverture de cycle, avec les DLlactide et glycolide utilisant le P188 ou le T1107 comme initiateurs, et l'octanoate stanneux [Sn(Oct)2] comme catalyseur. Brièvement, des quantités précises de P188 ou de T1107, de DLlactide et de glycolide sont mélangées et introduites dans un ballon à fond rond de 100 mL avec le catalyseur. Les mélanges sont chauffés à 140°C et dégazés par 15 cycles de purge sous vide dans le but d'éliminer l'humidité et l'oxygène, inhibant la polymérisation. Les ballons sont ensuite refroidis à 0°C et scellés sous un vide dynamique de 10<sup>-3</sup> mbar. La polymérisation se poursuit à 140°C sous agitation constante. Après 5 jours, les produits sont récupérés par dissolution dans du DCM puis précipités par ajout d'un volume identique d'éthanol. Enfin, le copolymère est filtré, lavé avec de l'éthanol froid et séché à 45°C sous pression réduite.

#### **3.2.2.2** Production de lysozyme-FITC

Le marquage fluorescent du lysozyme par le FITC est réalisé par Bezemer *et al.* [5]. Brièvement, 33 mg de FITC dissous dans 1,65 mL de DMSO sont lentement mélangés à une solution de lysozyme (250 mg) dans du Na<sub>2</sub>Co<sub>3</sub> (25 mL, 0,1 M, pH 8). Après 2 heures d'incubation dans le noir à température ambiante, la solution est dialysée afin de ne récupérer que le lysozyme marqué par le FITC. Le lysozyme marqué est ensuite nanoprécipité dans les conditions identiques au lysozyme non marqué. La formulation et la conservation des microsphères s'effectuent à l'abri de la lumière.

#### 3.2.2.3 Nanoprécipitation de la protéine

La nanoprécipitation du lysozyme (ou du lysozyme-FITC) est réalisée comme décrit par Giteau *et al.* [6]. Brièvement, 1,04 g de glycofurol sont ajoutés à 45 µL d'une solution de 0,3 M NaCl contenant 900 µg de lysozyme et 18 mg de P188, pour former une solution de 1 mL. Après 30 minutes à 4°C les nanoparticules protéiques sont récupérées par centrifugation (10000 g, 4°C, 30 minutes).

#### **3.2.2.4** Formulation des microsphères

Les microsphères de copolymères (diamètre recherché 60 µm) sont préparées par le procédé d'émulsion s/o/w décrit dans la littérature [19, 21]. Le nanoprécipité protéique est dispersé dans la phase organique (2000 µL d'une solution dichlorométhane :acétone 3:1 contenant 150 mg de copolymère dissous). Cette solution est émulsionnée dans une phase aqueuse (90 mL, 4% w/v PVA, 1°C) sous agitation mécanique à 550 rpm. Après l'addition de 100 mL d'eau désionisée en maintenant l'agitation pendant 10 min, l'émulsion est versée dans 500 mL d'eau désionisée et agitée pendant 20 min pour extraire les solvants organiques. Enfin, les microsphères sont filtrées sur 0,45 µm –type HVLP, Millipore SA, Guyancourt, France), lavées, lyophilisées et conservées à -20°C.

#### 3.2.2.5 Caractérisation morphologique des microsphères

La morphologie de la surface des microsphères est examinée par microscopie électronique à balayage (SEM) et microscopie à force atomique (AFM). La SEM (JSM 6310F, JEOL, Paris, France) est réalisée à température ambiante. Les microsphères lyophilisées sont fixées sur un plot métallique à l'aide d'un scotch double-face puis métallisées sous vide via un MED 020 (Bal-Tec, Balzers, Lichtenstein). Elles sont observées sous une accélération électronique de 11 kV. L'AFM (Nanowizard II, JPK Instruments, Berlin, Allemagne) est également réalisée à température ambiante. Les microsphères lyophilisées sont également fixées sur un plot métallique à l'aide d'un scotch double-face. À l'air libre, une pointe pyramidale Bruker (r = 7 mm) est montée sur un levier (cantilever) en nitrure de silicium. La pointe balaie la surface des microsphères en mode contact et contact intermittent (*tapping*). La surface est examinée par des carrés de 5  $\mu$ m de côté. La rugosité de surface est calculée à partir des images à l'aide du logiciel WSxM [7].

La distribution des nanoprécipités protéiques est examinée par microscopie confocale. Un microscope Olympus FV300 Laser Scanning Confocal Imaging System (Olympus, Japon) équipé d'un laser (Melles Griot, Voisins Le Bretonneaux, France) et un microscope Olympus BX50

(Olympus, Japon) sont utilisés. Toutes les images de fluorescences confocales sont prises avec un objectif x60, à immersion dans l'huile, ouverture numérique 1,40 (zoom numérique de 2). Le programme utilisé est Fluoview® version 3.3 (Olympus, Japon). Le laser est ajusté en deux modes. Le mode fluorescence verte est obtenu avec un laser ion Argon, avec une longueur d'onde d'excitation de 488 nm. Le mode fluorescence rouge est obtenu avec un laser Hélium-Néon, avec une longueur d'onde d'excitation de 543 nm.

## 3.3 Résultats

#### 3.3.1 Distribution de la protéine au sein des microsphères

Deux lots de microsphères sont formulés par le procédé d'émulsion s/o/w avec un taux théorique de 0,6% de lysozyme-FITC. Deux types de polymères sont utilisés : PLGA et PLGA-P188-PLGA. Les segments de PLGA sont de 40kDa, ratio LA/GA 25/50 (25P40). Les diamètres moyens sont respectivement de 64 et 75  $\mu$ m et les efficacités d'encapsulation de 81 et 85%. Les deux formulations sont jugées acceptables pour la suite de l'étude.

Les microsphères sont observées en microscopie confocale. Les images par microscopie confocale sont prises par tranche de 1 µm d'épaisseur puis compilées. Les figures 1 et 2 présentent les images issues des observations des deux lots de microsphères.



Figure 1. Images en microscopie confocale des microsphères de PLGA. (a) Observation à 543 nm (b) Observation à 488 nm (c) Images a et b compilées.



Figure 2. Images en microscopie confocale des microsphères de PLGA-P188-PLGA, ratio LA/GA 25/50, M<sub>W</sub> PLGA 40 kDa (25P40). (a) Observation à 543 nm (b) Observation à 488 nm (c) Images a et b compilées.

Les figures 1 (a) et figure 2 (a) présentent une émission fluorescente non recherchée des composants de la microsphère. Les figures 1 (b) et figure 2 (b) présentent l'émission fluorescente recherchée du FITC. Les spots fluorescents observés correspondent aux nanoprécipités protéiques, ceux-ci sont distribués de manière homogène dans les deux lots.

Une étude quantitative de l'intensité totale de fluorescence par microsphère est réalisé par ImageJ [8]. La distribution des nanoprécipités semble plus homogène dans les microsphères de 25P40 ; cependant le manque d'observations ne permet pas de connaître la significativité.

## 3.3.2 Morphologie globale des microsphères

Des images par SEM complémentaires à celles présentées au cours de l'article précédent sont réalisées à température ambiante. La nature du segment central et la masse molaire des segments PLGA varient ; le ratio LA/GA est fixé à 25/50. Différents grossissements sont présentés.



Figure 3. Observations en microscopie électronique à balayage des microsphères (grossissement x30). La nature du copolymère varie de gauche à droite en fonction de la

masse molaire du segment PLGA (40, 20 et 10 kDa), de haut en bas en fonction de la nature du polymère central (P188 ou T1107).

La vue d'ensemble des lots de microsphères (figure 3) permet d'appréhender leur agrégation. Les copolymères 25P40, 25P20, 25T40 et 25T20 fournissent des microparticules principalement sphériques, polydisperses et non agrégées. Les copolymères 25P10 et 25T10, associés à une masse molaire de PLGA de 10 kDa, fournissent des microparticules sphériques, polydisperses et peu agrégées (25T10) ou très agrégées (25P10). Ce résultat est corrélé avec la température de transition vitreuse (Tg) des copolymères abordées dans l'article précédent. La Tg de ces deux derniers copolymères est suffisamment basse pour que les chaînes polymériques dans les microsphères résultantes soient mobiles à température ambiante, ce qui conduit à l'agrégation des microsphères.

#### 3.3.3 Morphologie détaillée des microsphères

Les figures suivantes présentes des vues à plus fort grossissement des surfaces des microsphères par SEM (figures 4 et 5) et des reconstitutions en trois dimensions de la surface par AFM (figure 6). La figure 7 est une représentation graphique des rugosités calculées à partir de 12 microsphères, 3 lots différents, pour chaque type de copolymère.



Figure 4. Observations en microscopie électronique à balayage des microsphères (grossissement x800). La nature du copolymère varie de gauche à droite en fonction de la masse molaire du segment PLGA (40, 20 et 10 kDa), de haut en bas en fonction de la nature du polymère central (P188 ou T1107).


Figure 5. Observations en microscopie électronique à balayage des microsphères (grossissement x2500). La nature du copolymère varie de gauche à droite en fonction de la masse molaire du segment PLGA (40, 20 et 10 kDa), de haut en bas en fonction de la nature du polymère central (P188 ou T1107).



Figure 6. Observation AFM des microsphères. La nature du copolymère varie de gauche à droite en fonction de la masse molaire du segment PLGA (40, 20 et 10 kDa), de haut en bas en fonction de la nature du polymère central (P188 ou T1107).



Figure 7. Rugosités moyennes (nm) de la surface des microsphères en fonction de la nature du copolymère. Les moyennes de rugosité sont significativement identiques (p < 0,05, test Mann-Whitney) entre les microsphères 25P40 et 25T40, et 25P20, 25T20 et 25T10.

En fonction de la composition du copolymère, différentes rugosités et porosités sont obtenues.

Le paramètre conduisant aux différences principales est la masse molaire des segments PLGA. Les copolymères à PLGA de 40 kDa fournissent des surfaces plutôt lisses. Leur rugosité moyenne est de 52 nm (25T40) et 64 nm (25P40). Les pores, autre structure observable, sont peu nombreux et ne dépassent pas une amplitude de 82,2 nm (25T40) et 196,3 nm (25P40) pour un diamètre inférieur à 1  $\mu$ m. La diminution de la masse molaire du PGLA entraîne une amplification des structures. Les copolymères à PLGA de 20 kDa fournissent des surfaces présentant de plus grandes protubérances. La rugosité moyenne est de 132 nm (25T20) et 181 nm (25P20). Les pores sont également plus nombreux, majoritairement plus larges (entre 1 et 4  $\mu$ m) et plus profonds. Leur amplitude maximale est de 694,2 nm (25T20) et 885,0 nm (25P20). Les microsphères de 25P10 sont lisses sans pore apparent. La grande mobilité de leurs chaînes polymériques à température ambiante est mise en cause. Elle entraînerait un lissage de la surface. Le copolymère 25T10 fournit des microsphères avec une rugosité moyenne de 149 nm et des pores possédant une amplitude de 727,5 nm.

La nature du segment central conduit à des différences structurelles moins marquées. L'utilisation du T1107 entraîne une légère diminution de la porosité et de la rugosité.

## 3.4 Discussion

Les observations par microscopies confocale, à balayage électronique et à force atomique sont réalisées afin de compléter les études présentées au cours de l'article précédent. L'objectif principal de cette étude est donc de comprendre comment une microsphère se structure et comment la modifier afin d'influencer ses mécanismes de dégradation et, par voie de conséquence, la libération de la protéine encapsulée.

Les observations par microscopie confocale ne montrent pas de différence significative de la distribution du nanoprécipité protéique dans des microsphères de PLGA et de PLGA-P188-PLGA (de type 25P40). Associées aux résultats d'Alexandra PAILLARD-GITEAU [2] ces observations prouvent que l'homogénéité de la distribution de la protéine est principalement due à l'état nanoprécipité de la protéine. Une homogénéisation plus conséquente pourrait être favorisée grâce à l'hydrophilie du polymère. Un échantillon plus large permettrait de connaître la significativité de ce résultat.

Les observations aux microscopes électronique à balayage et à force atomique fournissent une caractérisation de la surface des microsphères en fonction de la composition des copolymères. Mis à part le cas des microsphères 25P10, les interprétations convergent. Plus la masse molaire de PLGA est faible, plus la surface est rugueuse et possède des pores. Le remplacement du poloxamère par de la poloxamine entraîne une légère diminution de la rugosité et de la porosité en surface.

Ces phénomènes pourraient être expliqués par deux propriétés physicochimiques des chaînes de copolymères ; la balance hydrophile / hydrophobe et la configuration spatiale. Ces propriétés modifieraient le comportement des chaînes lors de la formulation des microsphères.

Pour un segment central déterminé, un copolymère est globalement plus hydrophobe pour une masse molaire des segments PLGA augmentée. Lors de la formation de l'émulsion, le polymère a plus d'affinité avec le solvant organique. Lorsque le solvant est extrait les chaînes de copolymère se figent en exhibant les interactions avec les phases aqueuse et organique. Sans interaction avec la phase aqueuse, la microsphère est lisse. Lorsque la masse molaire des segments PLGA diminue, le copolymère est moins hydrophobe. Dans ce cas les segments hydrophiles du copolymère (unités PEO du P188 et du T1107) ne seraient plus suffisamment enfouis par les segments PLGA mais pourraient interagir avec la phase aqueuse. Les chaînes sont davantage « attirées » vers la phase aqueuse et pourraient former des micro- ou nanostructures à l'interface. La solidification des microsphères conduirait à une rugosité et une porosité exacerbées. La rugosité augmenterait donc avec la diminution de la masse molaire des segments PLGA. Dans la littérature, différents degrés de rugosité des microsphères ont été obtenues de manière similaire. La création d'interactions à l'interface des phases aqueuse et organique conduit à l'augmentation de la rugosité [9,10].

Le remplacement du poloxamère par de la poloxamine est plus complexe car entraîne trois modifications physicochimiques. Les deux premières modifications portent sur la balance hydrophobie / hydrophilie : le T1107 possède un ratio PEO/PPO plus faible que le P188 donc globalement plus hydrophobe; le T1107 implique une structure à quatre branches donc quatre segments de PLGA alors que le P188 est linéaire (donc deux segments de PLGA par chaîne). Cela augmente l'hydrophobie globale de l'utilisation du T1107. Troisième modification, la structure à quatre branches impliquerait une configuration spatiale et des interactions intermoléculaires différentes. En ne prenant en compte que les modifications de la balance hydrophobie / hydrophilie, et en se basant sur les différences de rugosité entre les microsphères 25P40 – 25P20, ou 25T40 – 25T20, le remplacement du P188 par du T1107 impliquerait une division par 2 à 3 de la rugosité moyenne. Cependant la diminution de la rugosité est moins conséquente (exemple : entre les microsphères 25P40 et 25T20). Il est possible que les interactions intermoléculaires soient moins nombreuses avec des chaînes branchées qu'avec des chaînes linéaires. Les segments hydrophiles seraient moins enfouis dans la phase aqueuse. Ainsi, l'utilisation de copolymères contenant du T1107 fournirait des microsphères globalement plus hydrophobes tout en présentant une surface rugueuse et poreuse. La figure 8 suivante schématise les différentes hypothèses.



Figure 8. Schématisation des hypothèses de l'arrangement des chaînes de copolymères à la surface des microparticules.

Ces résultats peuvent être mis en relation avec les observations par cryoSEM. En profondeur, les microsphères 25P20 sont plus poreuses que les microsphères 25P40. En extrapolant 106

les hypothèses précédentes, nous pourrions imaginer que les segments hydrophiles des copolymères peu hydrophobes créent des micro- ou nano-environnements internes emprisonnant la phase aqueuse, telles des micelles.

Ces hypothèses peuvent également être reliées avec le profil de libération du lysozyme encapsulé. Les meilleurs profils de libération (souhaitée continue et complète) sont obtenus avec les microsphères 25P20 et 25P10. Elles sont constituées des copolymères les moins hydrophobes. Cependant les microsphères 25P10 ne sont pas processables à cause de leur Tg basse. La meilleure solution est donc d'utiliser le copolymère le moins hydrophobe possible et possédant une Tg permettant la processabilité des microsphères.

Cette hydrophobie modérée conduirait à deux phénomènes majeurs recherchés. Le premier phénomène serait de créer un microenvironnement interne plus propice à la conservation de l'activité biologique de la protéine. En effet une hydrophobie exacerbée du polymère entraîne une dénaturation irréversible de la protéine encapsulée [11–14]. Le deuxième phénomène serait la création d'un réseau de pores internes améliorant les mécanismes de libération de la protéine, notamment sa diffusion à travers ce réseau [15].

Des études complémentaires permettraient de lever les hypothèses de l'organisation des chaînes de copolymères. Citons notamment la méthode de spectroscopie infrarouge. La caractérisation des liaisons ester (entre résidus du PLGA) et des liaisons éthers (entre résidus du P188 et du T1107) nous fournirait des informations de localisation des différents segments composant le copolymère.

### 3.5 Conclusion

Les microsphères biocompatibles et biodégradables peuvent fournir un système adéquat pour la libération prolongée de protéines encapsulées. La modulation de la nature du copolymère composant les microsphères induit des morphologies externes et internes différentes. Ces morphologies différentes induisent à leur tour des profils de libération de la protéine différents.

Afin d'appréhender les différentes relations existant entre la nature du copolymère et le profil de libération, des observations sont réalisées en complément des études présentées au cours de l'article précédent. Ces observations mettent en évidence le rôle prépondérant des propriétés physicochimiques des copolymères utilisés. Ces propriétés influenceraient de manière prévisible les structures des microsphères résultantes.

Des études complémentaires sont nécessaires afin de confirmer les résultats. Elles permettraient de prévoir la composition du copolymère à utiliser pour un profil de libération de la protéine souhaité.

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# **CHAPITRE III**

# Production de microsphères par le

# procédé de prilling sans solvant

halogéné

## **PROCEDE DE PRILLING ET GLYCOFUROL : FAISABILITE DE PRODUCTION DE MICROSPHERES DE PLGA**

Les microsphères composant les microcarriers pharmacologiquement actifs sont classiquement formulées par la technique d'émulsion / extraction de solvant. Cette technique conduit notamment à produire des microsphères polydisperses en utilisant un solvant halogéné [1]. Le taux résiduel de ce type de solvant dans une forme pharmaceutique injectable est réglementé en fonction de la dose et de la fréquence d'administration.

La formulation par le procédé du prilling fournit une alternative intéressante. Il permet de formuler des particules de 60 µm [2] sous forme de microsphères ou de microcapsules [3]. Il peut être appliqué pour la production de lots cliniques [3,4]. La figure 1 représente le Spherisator® S, pilote commercialisé par la société Brace® GmbH. Ce pilote est utilisé au sein du laboratoire pour la formulation par prilling.



Figure 1. Photographie de l'unité de production Spherisator® S (Brace® GmbH, Alzenau, Allemagne).

Les recherches précédentes portées par Van-Thanh TRAN ont permis de formuler des microsphères à partir de PLGA dissous dans de l'acétone. Ces microsphères contiennent du lysozyme. Celui-ci est préalablement nanoprécipité dans du glycofurol, centrifugé, puis re-dispersé

dans la phase organique acétone/PLGA. L'abandon de l'étape de centrifugation fournirait un procédé entièrement réalisable en conditions aseptiques.

Le glycofurol fait notamment l'objet d'études en tant que solvant du PLGA pour différentes techniques de formulation [5–7]. L'étude suivante porte donc sur le développement du procédé de prilling, pour la production de microsphères de PLGA en utilisant une phase organique composée de PLGA dissous et de la protéine dispersée directement dans cette phase organique en utilisant le glycofurol comme agent de précipitation.

Dans ces conditions, de nombreuses contraintes techniques ont nécessité des études de faisabilité avant de permettre la production de microsphères. Les premières études se sont intéressées à la mise en place de solutions simples. L'acétone a été étudiée comme agent nanoprécipitant du lysozyme en remplacement du glycofurol ; l'activité biologique de cette protéine n'était pas préservée. Ensuite l'utilisation du glycofurol comme solvant unique du PLGA ne permettait pas une extrusion à travers la buse pour une concentration supérieure à 5% (m/v). Cette concentration est trop faible pour permettre la solidification des microgouttelettes formées. Le mélange acétone/glycofurol a été finalement retenu.

Les paramètres de production fournissant des microsphères acceptables ont été établis. La plupart des combinaisons testées fournissaient des microparticules déformées et agrégées. Des plans d'expériences ont donc été mis en place afin d'identifier l'ensemble des possibilités de formulation.

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# Development of prilling process for biodegradable microspheres through experimental designs

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## Abstract

The prilling process proposes a microparticle formulation easily transferable to the pharmaceutical production, leading to monodispersed and highly controllable microspheres. PLGA microspheres were used for carrying an encapsulated protein and adhered stem cells on its surface, proposing a tool for regeneration therapy against injured tissue. This work focused on the development of the production of PLGA microspheres by the prilling process without toxic solvent. The required production quality needed a complete optimization of the process. Seventeen parameters were studied through experimental designs and led to an acceptable production. The key parameters and mechanisms of formation were highlighted.

## Key words

Prilling, PLGA, microsphere, experimental design, glycofurol.

### **1** INTRODUCTION

The prilling or "laminar jet break-up technology" is a process used for the production of uniform particles from 10 to 10,000 micrometers. Liquid droplets are produced by extrusion of a flux through a vibrating nozzle; then the droplets are solidified by different ways. The prilling presents many advantages comparing to other formulation techniques, as an one-step process, a highly control of the narrow-size distribution of the prills [1,2], the possibility to create core-shell particles [3], and the easy transfer of the manufacture of particles in sterile chamber [4] with the required Good Manufacturing Practices.

The prilling technique was developed from the first theoretical approach by Joseph Plateau and Lord Rayleigh at the end of the 19<sup>th</sup> century [5]. It is essentially used in the agronomic and ceramic industries. Fertilizers are produced with ammonium nitrate, calcium nitrate, urea [6].... These droplets are dried during the fall of over 50 meters in environmental controlled towers, for the production of 2 mm prills.

One of the most other used materials is sodium alginate for the easily production and controllability of biocompatible prills for a drug delivery system. In this case alginate is solidified after a few centimeters fall into ethanol or aqueous calcium chloride solution. These prills are used and developed in phytosanitary [7–10] and pharmaceutical [11–15] fields. For example, Del Gaudio *et al.* [16] presents alginate core-shell particles (produced from a double-nozzle) encapsulating piroxicam (PRX). The *in vitro* studies show a complete release of PRX before the 7<sup>th</sup> day.

Other materials are used to develop a drug-loaded system for a sustained release over one month. Poly(lactic-co-glycolic) acids or PLGA is a FDA-approved polymer commonly used as biocompatible and biodegradable microspheres. Microparticles are mainly produced by emulsion – solvent extraction [17–19]. In few cases PLGA microparticles are prepared by break-up and extrusion processes [20]. All of these techniques need a previous PLGA organic solution introduced by different ways into a non-solvent of the PLGA for solidification. Different extrusion techniques lead to objects with diameters in the order of magnitude of 1 mm [21–23]. The only one jet break-up technique used in this case is the flow focusing, giving microsphere diameters about 2-3  $\mu$ m or more with increasing the size distribution [24,25].

Moreover, PLGA was commonly used as drug delivery system. PLGA microspheres allow many advantages as scaffolds for regeneration therapy [26] supporting adhered stem cells [28] and for the encapsulation, the conservation and the sustained release of protein [27,28]. Proteins are considered as highly breakable products.

In order to create spherical scaffolds for the encapsulation of therapeutic proteins and for the survival of adhered stem cells [28–30], the prilling would be an alternative to produce narrow-size microspheres with an intermediate diameter of 50-100  $\mu$ m. According to a potential manufacturing scale-up, some parameters have to be highlighted. Halogenated solvents appropriated to PLGA, are toxics. Glycofurol, acetone and ethyl acetate are acceptable solvents as class 3 according to the European Pharmacopoeia. For the protein microencapsulation, a nanoprecipitation step is performed to improve the protein stability [31]. The protein is nanoprecipitated in glycofurol, pelleted by centrifugation then resuspended in the PLGA solution. Nevertheless, the centrifugation step should be avoided for the pharmaceutical manufacturing in sterile chambers. So the development of a centrifugation-free process makes easier the scale-up. This is possible if the protein is directly nanoprecipitated in the PLGA solution, given that glycofurol is also a PLGA solvent [32,33]. All these requirements need a radical process adjustment.

The aim of this study is to develop protein loaded PLGA microspheres by the prilling process. Experimental designs were chosen to consider all the process parameters simultaneously.

## **2** MATERIALS AND METHODS

## 2.1 Materials

Lysozyme (chicken egg white) and its substrate *Micrococcus lysodeikticus*, glycofurol (tetraglycol or  $\alpha$ - [(tetrahydro-2-furanyl) methyl]- $\omega$ -hydroxy-poly (oxy-1, 2-ethanediyl)), hydrochloric acid (HCl), acetone, ethanol, dimethyl sulfoxide (DMSO), Pluronic® F68 (Poloxamer P188), were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Uncapped PLGA 37.5/25 (M<sub>w</sub> 24kDa, Ip 1.8) was obtained from Evonik Corporation (Birmingham, UK).

## 2.2 Methods

#### 2.2.1 Characterization of the extraction medium

The surface tension was measured as described in Hirsjärvi *et al.*[34] using a drop tensiometer device (Tracker Teclis, Longessaigne, France). The surface tension was evaluated for the interface of a rising air bubble (5  $\mu$ L, controlled all along the experiment), in 3 mL of the studied solvent combinations, using the Laplace equation.

The dynamic viscosity was measured as described by Moysan *et al.* [35] using a Kinexus® rheometer (Malvern Instruments, S.A., United Kingdom), with a cone plate geometry (diameter 40 mm, angle  $2^{\circ}$ ) at room temperature.

The density was directly calculated with the measure of the weight of 1.000 mL of the mixture of components using an analytical balance, sensitivity 0.01 mg.

#### 2.2.2 Formulation of the microparticles

#### 2.2.2.1 Protein nanoprecipitation

Nanoprecipitated lysozyme was prepared as patented and described by Giteau *et al.* [31]. Briefly, 45  $\mu$ L of 0.3 M NaCl solution containing 900  $\mu$ g of lysozyme and 1.8 mg of poloxamer 188 were added to 1.04 g of glycofurol to form a 1 mL suspension. After 30 minutes at 4°C the protein nanoparticles were recovered.

The amounts of lysozyme, poloxamer 188 and glycofurol were modulated for the necessity of the studies. The quantity of lysozyme was modulated, for a constant lysozyme/PLGA ratio of 0.6% w/w. The quantity of poloxamer 188 was modulated to retain a constant ratio lysozyme/poloxamer 1/10 mol/mol). The quantity of glycofurol was modulated independently.

#### 2.2.2.2 Preparation of the organic phase

190 mg of PLGA were dissolved into 1 mL of acetone or ethyl acetate. The nanoparticles were gently suspended into the PLGA solution. The organic suspension was filtered through a 0.2  $\mu$ m Minisart Sartorius RC25 sieve (Aubagne, France). The amount of acetone or ethyl acetate was modulated.

#### 2.2.2.3 Jet break-up / solvent extraction

The microspheres were formulated using a Spherisator® S apparatus (Brace GmbH, Alzenau, Germany). Briefly, the organic suspension was poured into a sealed tank. A constant level of air pressure pushed the mix through a 100  $\mu$ m nozzle subjected to a vibration to break the resulting jet into a chain of homogeneous falling microdroplets. The practical efficacy of the vibration frequency and amplitude were determined with the help of a stroboscopic lamp. The microdroplets were recovered and solidified by solvent extraction into an extraction medium under stirring. The resulting microparticles were sieved at 150  $\mu$ m and filtered with a Durapore® Membrane Filter 0.45  $\mu$ m (Merck Millipore, Billerica, MA, USA). The filtrated fraction was called "Isolated microparticles", the residual fraction on the sieve the "Aggregated microparticles".

The prilling process was described in three steps (Figure 1). Step 1 related to the production of microdroplets from the extrusion of the organic phase. Step 2 concerned the recovering and the hardening of the microdroplets into an extraction medium. Step 3 concerned a second time of hardening into a second aqueous phase.



Figure 1. Schema of the prilling process and input variables.

### 2.2.3 Characterization of the microspheres

The particle circularity was determined by image analysis with the Zeiss Axioskop II optical microscope (Carl Zeiss, Oberkochen, Germany). The images were treated with ImageJ [36]. The circularity was analyzed for a minimum of 100 microparticles. A microparticle was considered as spherical for a circularity equal or above 0.8. Results showed the amount of spherical microparticles (called microspheres) expressed in comparison to the total amount of microparticles recovered.

The average particle size and size distribution were determined using a Coulter® Multisizer (Coultronics, Margency, France). The microparticles were suspended in isotonic saline solution and sonicated for a few minutes prior to analysis. The results were expressed in diameter means ( $\mu$ m) and standard deviation or SD ( $\mu$ m).

The surface particle morphology was investigated by scanning electron microscopy (SEM, JSM 6310F, JEOL, Paris, France). Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon using MED 020 (Bal-Tec, Balzers, Lichtenstein).

#### **2.2.4 Determination of the activity of the lysozyme**

The encapsulation active protein yield was determined using a *Micrococcus lysodeikticus* test. The particles were dissolved in DMSO (1 mL) in PTFE tubes at room temperature under agitation during 1 h. Then 3 mL of 0.01 M HCL solution were added. The solution was left to stand for 1 h at room temperature under agitation. The resulting solution was incubated with *Micrococcus lysodeikticus*. A standard curve was obtained by incubation of a concentration range of lysozyme from 0 to 100 ng/mL with dissolved blank microspheres. The determination of the quantity of active lysozyme was obtained by comparison with the standard curve.

#### **3 RESULTS**

The aim of the study was to adapt the prilling process to the production of spherical and isolated PLGA microparticles. Feasibility studies permitted to list 17 factors considered as potentially influent on the production quality (as shown on figure 1). Seven of them are implicated in the composition and the extrusion of the organic phase (step 1), six in the recovering and the extraction step into the extraction medium (step 2) and four in the optional solidification into the second aqueous phase (step 3).

Designs of experiments were performed to study the different factors and for that, different values (levels) were respectively given to each factor. Three factors needed preliminary studies to define their limit values. Two of them were associated to the organic phase, the third one to the extraction medium.

Then the prilling process was studied through two experimental designs. All the experimental designs were analyzed using NemrodW® Software [37].

## 3.1 Preliminary studies

#### **3.1.1** Focus on the organic phase

The factors PLGA concentration and ratio organic solvent/glycofurol, involved in the prilling process, result respectively from the concentration of lysozyme and the volume of glycofurol, involved in the protein nanoprecipitation. The goal was to study the nanoprecipitation parameters in order to know the acceptable values of the prilling factors. In this part, factors are thus the concentration of protein and the volume of glycofurol and their domain of interest is shown on table 1.

Table 1.Experimental	Experimental domain for the nanoprecipitation experimental design				
Factors	Undimensional variables	Domain of interest			
U <sub>1</sub> : Concentration of lysozyme (mg/m	L) $X_1$	20 mg/mL to 30 mg/mL			
U <sub>2</sub> : Volume of glycofurol ( $\mu$ L)	$X_2$	250 μL to 1000 μL			

The experimental response is the nanoprecipitation efficiency (Y) measured by a *Microccocus lysodeikticus* test [38] and expressed in comparison to the lysozyme activity before nanoprecipitation. A response surface methodology was chosen to establish a relation between the variation of the response and the variation of the factors [39]. A second order polynomial model was postulated and a full factorial design  $3^{1}4^{1}$  was performed to calculate the coefficients of the model and to predict the response all over the experimental domain with a good quality.

From the results of the twelve experiments, with two in triplicate (Appendix 1), the coefficients were estimated by multilinear regression and the model for nanoprecipitation efficiency is:

 $Y = 96.5 + 20.1X_1 + 14.9X_2 - 8.2X_1^2 - 19.2X_2^2 - 7.2X_1X_2$ 

The variance analysis permitted to validate the model: the lack of fit and regression tests are satisfactory.

The analysis of this response (Figure 2) showed that the nanoprecipitation efficiency varied from 27.2 to 109.6%. The experimental errors allowed the 109.6% response. More the concentration of lysozyme increased, more the precipitation was efficient; the same relation was observed for the volume of glycofurol. The optimal nanoprecipitation efficiency (more than 90%) was obtained for 25 to 30 mg/mL of lysozyme and 500 to 100  $\mu$ L of glycofurol.

These limit values gave by extrapolation the lower and upper limits of the factors PLGA concentration and ratio organic solvent/glycofurol for the next step. The levels of PLGA concentration are 9.5 and 11%. The levels of ratio organic solvent / glycofurol are 25/75 and 75/25. For the latter, the feasibility studies demonstrated a potential major influence on the production quality. The ratio 25/75 would tend towards aggregation of spherical microparticles after few minutes. The ratio 75/25 rather would lead to shapeless microparticles after few seconds. The extraction of glycofurol would seem to be the key factor of this phenomenon. Thus the intermediate level 50/50 was added to the studies.



Figure 2. Contour plot of the variation of the nanoprecipitation efficiency (Y, %) as a function of the concentration of lysozyme (mg/mL) and the volume of glycofurol ( $\mu$ L).

### **3.1.2** Focus on the extraction medium

The aim was to define the physicochemical properties of the extraction medium. A wide range of its characteristics was expected. According to the library data, surface tension, viscosity and density are potentially the most influent physicochemical properties of the extraction medium during break-up processes [2,40–44]. Water, ethanol, polyethylene glycol 400 (PEG400) and propylene glycol (PG) were selected as potential components of the extraction medium for their following characteristics:

- they are miscible with polymer solvents (acetone, ethyl acetate, glycofurol);
- they possess complementary physicochemical properties (described in table 2);
- they are approved for parenteral administration.

Solvent	Surface tension (mN/m at 20°C)	Dynamic viscosity (mPa.s at 20°C)	Density
Water	72.8	0.89-1	1.000 (20°C)
Ethanol	22.8	1.22	0.789 (20°C)
Polyethylene glycol 400	44.0	105-130	1.128 (25°C)
Propylene glycol	40.1	58.1	1.036 (25°C)

Table 2. Physicochemical properties of the PLGA non-solvents [45].

To study these 4 components and the possible mix of them, a specific experimental design was performed because the measured responses, surface tension (Y<sub>1</sub>, mN/m), viscosity (Y2, mPa.s) and density (Y<sub>3</sub>), are supposed to be related to the proportions of the components, Water (x<sub>1</sub>), Ethanol (x<sub>2</sub>), PEG400 (x<sub>3</sub>) and PG (x<sub>4</sub>). To know the responses of interest for all the possible mixtures, an empirical mathematical model was postulated and more precisely, a reduced third order polynomial model. To estimate the coefficients a Scheffé simplex centroid design with some check points was performed [46], as shown on appendix 2. Some formulas were replicated to evaluate the experimental error. The analysis of the results shows a large ratio *maxY/minY* for the surface tension and the viscosity. In this case, a suitable transformation is required and for these two responses, a Box-Cox transformation, 1/Y, was done.

The considered responses were described by the following models:

 $10^{3}/Y_{1} = 14.46X_{1} + 43.44X_{2} + 23.19X_{3} + 33.56X_{4} + 35.27(X_{1}X_{2}) + 6.13(X_{1}X_{3}) + 9.94(X_{2}X_{3}) - 4.28(X_{1}X_{4}) - 0.83(X_{2}X_{4}) - 11.96(X_{3}X_{4}) + 28.04(X_{1}X_{2}X_{3}) + 4.15(X_{1}X_{2}X_{4}) + 8.73(X_{1}X_{3}X_{4}) + 64.72(X_{2}X_{3}X_{4})$ 

 $10^{3}/Y_{2} = 574.83X_{1} + 573.72X_{2} + 13.83X_{3} + 24.16X_{4} - 974.51(X_{1}X_{2}) - 817.72(X_{1}X_{3}) - 423.50(X_{2}X_{3}) - 527.44(X_{1}X_{4}) - 387.10(X_{2}X_{4}) + 16.47(X_{3}X_{4}) + 46.41(X_{1}X_{2}X_{3}) + 519.85(X_{1}X_{2}X_{4}) - 127.57(X_{1}X_{3}X_{4}) - 278.31(X_{2}X_{3}X_{4})$ 

$$\begin{split} Y_3 &= 0.995X_1 + 0.783X_2 + 1.115X_3 + 1.028X_4 + 0.050(X_1X_2) + 0.080(X_1X_3) - 0.089(X_2X_3) \\ &+ 0.078(X_1X_4) - 0.047(X_2X_4) - 0.011(X_3X_4) + 0.070(X_1X_2X_3) + 0.159(X_1X_2X_4) + 0.042(X_1X_3X_4) - 0.010(X_2X_3X_4) \end{split}$$

The analyses of variance show that these models are significant and the lack of fit tests are acceptable.

From these equations, the three physicochemical properties can be calculated for any extraction medium: Figure 3 shows the isoresponse curves of the responses in function of the

composition of the mixture. The analysis of different contour plots shows that PEG400 and PG present similar effects and values on the three physicochemical characteristics of the mixture.



Considering its extraction function, PG solidified faster the PLGA, so it was selected and PEG400 was set at 0.

Figure 3. Ternary diagrams of the models of the responses as function of the composition of the extraction medium with PEG400 = 0 (a) variation of the  $10^3$ /surface tension ( $10^3$ /Y<sub>1</sub>, m/N) (b) variation of the  $10^3$ /viscosity ( $10^3$ /Y<sub>2</sub>, Pa<sup>-1</sup>.s<sup>-1</sup>) (c) variation of the density (Y<sub>3</sub>). The physicochemical properties incompatible with the formation of microparticles in the prilling process were marked in grey. The circles pointed the selected mixtures.

Some impossible combinations of properties were revealed through the three ternary diagrams. For example a mixture could not possess a low surface tension with a high viscosity or a high density. A mixture could not possess a high viscosity with a low density.

The feasibility studies established some restrictions about the extraction medium: no microparticle was recovered when the viscosity is higher than 7 mPa.s  $(10^3/Y_2 = 142.9 \text{ Pa}^{-1}.\text{s}^{-1})$  and the microdroplets could not penetrate the reception medium for a surface tension higher than 33.33 mN/m  $(10^3/Y_1 = 30.00 \text{ m/N})$ . The areas of no feasibility were marked in grey on figure 3.

To avoid these phenomena the presence of ethanol was required with a minimum of 45%. The goal was to establish some mixtures with the widest possible diversity of physicochemical properties. One or two properties would want to be identical between two mixtures in order to compare them.

A minimum of 45% of ethanol followed closely the isocurve of density at 0.91. Using this limit, a smaller ternary diagram was drawn delimited by pure ethanol in one corner, a mixture of ethanol and water on the second corner and a mixture of ethanol and propylene glycol on the third

corner. The three corners possessed the most different possible properties. They constituted the selected mixtures. They were produced and measured, their experimental properties fitted with the predicted values. They were described in table 3 and represented as circles on figure 3.

Name	Solvents (proportions in the mixture)		Predicted responses			
	Water	Ethanol	Propylene Glycol	Surface tension	Viscosity	Density
				mN/m	mPa.s	
Eth	0	1	0	23.00	1.736	0.7830
Eth/W	0.516	0.484	0	27.30	3.165	0.9057
Eth/PG	0	0.454	0.546	26.50	5.625	0.9056

 Table 3. Composition and physicochemical properties of selected mixtures for the extraction medium.

## 3.2 Process study

The two preliminary studies permitted to precise the tested levels for the factors considered as potentially influent. Table 4 describes the seventeen factors and associated levels. The next studies focused on the development of the prilling process itself. The first step consisted in obtaining an overview of the influences of the factors on the production quality to rank their importance. The second step consists in focusing on the influential factors, taking into account the interaction effects.

Facto	pr	Unity	Step	Level 1	Level 2	Level 3	Additional description
U1	PLGA concentration	%	1	9.5	11		Cf. §3.1.1
U2	Organic solvent		1	acetone	ethyl acetate		
U3	Temperature 1	°C	1	4	37		
U4	Time 1	S	1	10	30		
U5	Surfactant	%	2	0	1		Nature: Span® 80
U6	Volume 2	mL	2	100	400		
U7	Temperature 2	°C	2	4	37		
<b>U8</b>	Time 2	min	2	1	20		
U9	Volume 3	mL	3	0	500		
U10	Temperature 3	°C	3	4	37		
U11	Time 3	min	3	10	20		
U12	Agitation 3		3	without vortex (WO)	with vortex (W)		
U13	Organic solvent / glycofurol	v/v	1	25/75	50/50	75/25	Cf. §3.1.1
U14	Extraction medium		2	Eth	Eth/W	Eth/PG	Cf. §3.1.2
U15	Agitation 2		2	no agitation (NO)	without vortex (WO)	with vortex (W)	
U16	Drop height	cm	1	5	30		
U17	Additional water	v/v	1	100/0	85/15		

Table 4. Experimental domain of factors potentially influent on the prilling process.

#### **3.2.1** Screening step

The first study was a screening design to identify the influential factors on the quality of the recovered microparticles.

The factors drop height  $(U_{16})$  and additional water  $(U_{17})$  were excluded from this study. The drop height could not be studied associated to the volume 2  $(U_6)$  because of a steric hindrance of the material. The factor additional water was conceived to allow a faster solidification of the PLGA during the recovering of the microdroplets. This phenomenon was considered as hugely influent.

The first fifteen factors were studied (Table 4) and an asymmetric screening design  $2^{12}3^3//24$  was performed as shown on appendix 3 [47]. The weight yield of the "Isolated microparticles" and the circularity of both fractions were considered as the experimental responses. The circularity response showed the expected sphericity. The weight response showed the quantity of microparticles recovered. The goal was to maximize both responses. Great sphericities display an expected solidification of the microdroplets, even if the microparticles were aggregated or not.

From the experimental results, the coefficients of the screening model were estimated for each response and figure 4 represents the behavior of the different tested levels for each factor. Statistical tests allowed to conclude about the significant effects and therefore to identify the influential factors. The analysis of the results shows a large ratio maxY/minY for the weight response. In this case, a suitable transformation is required and for these two responses, a log transformation was done.



Figure 4. Graphical representation of the effects of the studied factors (a) On the circularity of isolated microparticles  $(Y_1)$  (b) On the circularity of aggregated microparticles  $(Y_2)$  (c) On the weight yield of the isolated microparticles  $(\log(Y_3))$ . For each factor, the last level is normalized (last bar per factor) and a star means a significant variation of the response between the levels of the factor on the considered response (p<0.05).

The compilation of these effects leads to conclude about the importance of each factor.

Five factors were non-influent on any response; six other factors were influent on one or two responses and their effects considered as non-ambiguous. These 11 factors were set in order to increase the stability of the encapsulated protein (e.g. temperatures at 4°C) or to facilitate the process. Some of them showed significant influences on the production: temperature 2, time 2, agitation 2 and volume 3 for the circularity of the aggregated microparticles; surfactant and time 1 for the weight yield of the isolated microparticles. However they did not show a great influence on the other responses. They were set according to their influences to improve the responses.

The other four factors were considered as influent on a minimum of one response and their effects considered as ambiguous: PLGA concentration  $(X_1)$ , organic solvent  $(X_2)$ , organic solvent / glycofurol  $(X_{13})$  and extraction medium  $(X_{14})$ . The improvement of the responses showed ambiguities about the recommended levels. The circularity of the isolated microparticles was higher with 11% of PLGA in a ratio 75/25 of acetone/glycofurol, but 9.5% in a ratio 50/50 of ethyl acetate/glycofurol were recommended for reducing the aggregated microparticles.

The last four factors were carried out for the next study. The domain of variation of factors PLGA concentration and solvent were not modified. The factor organic solvent / glycofurol was adjusted. The level 25/75 did not lead to any isolated microparticles so it was removed. The extraction medium was adjusted too. The extraction medium Eth/W did not bring any advantage; Eth and Eth/PG were conserved because they improved the circularity of the aggregated microparticles. A new level Eth/W/PG (ratio w/w/w 75.5/12.25/12.25) was chosen for the next study with the following physicochemical properties: a surface tension at 23.92 mN/m, a viscosity at 2.664 mPa.s and a density at 0.8437. These values gave properties of the new mixtures of the three solvents nearer to the ethanol.

#### 3.2.2 Study of factor effects and interactions

This part was designed for introducing the factors "drop height" ( $X_{16}$ ) and "additional water" ( $X_{17}$ ) in a quantitative study of the four factors revealed influential in the screening step, considering the potential interactions with the additional water. The six factors, PLGA concentration ( $X_1$ ), organic solvent ( $X_2$ ), organic solvent / glycofurol ( $X_{13}$ ), extraction medium ( $X_{14}$ ), drop height ( $X_{16}$ ) and additional water ( $X_{17}$ ) with their respective domain of interest are summarized on table 5.

Phase	Factors	Undimensional variable	Unit	Experimental domain			
				Level 1	Level 2	Level 3	
1	U1: PLGA concentration	$X_1$	%	9.5	11		
1	U2: organic solvent	$X_2$		acetone	ethyl acetate		
1	U13: organic solvent 1 / glycofurol	X <sub>13</sub>	v/v	50/50	75/25		
2	U14: extraction medium	$X_{14}$		Eth	Eth/PG	Eth/W/PG	
1	U16: drop height	X <sub>16</sub>	cm	5	30		
1	U17: additional water	X <sub>17</sub>	v/v	100/0	85/15		

Table 5. Experimental domain for the quantitative study

To quantify the influence of the six factors and the four interaction effects  $X_1X_{17}$ ,  $X_2X_{17}$ ,  $X_{13}X_{17}$  and  $X_{14}X_{17}$  (interaction with the additional water) a D-optimal design with 16 experiments (Appendix 4) was performed [48]. Some experiments were replicated and the studied responses are "Isolated microparticles" weight (Y<sub>1</sub>) and its circularity (Y<sub>2</sub>). The goal is to maximize the responses.

From the experimental results, the estimation of the coefficients (main effects and interaction effects) was calculated by multilinear regression and the values are reported on table 6, with the significance, calculated on the basis of replicates.

Coefficient	<b>y</b> 1	p-value	<b>y</b> <sub>2</sub>	p-value	
	(mg)		(%)		
<b>b</b> <sub>0</sub>	21.55		38,17		
$b_1$	-6.62	**	-2.95	3.08 *	
$b_2$	-8.67	***	11.50	***	
b <sub>13</sub>	-9.98	***	5.57	**	
b <sub>14a</sub>	0.20	91.3	-1.97	24.9	
b <sub>14b</sub>	5.33	*	-4.50	*	
b <sub>16</sub>	-0.14	91.1	3.23	*	
b <sub>17</sub>	-3.15	*	-12.91	***	
b <sub>1-17</sub>	-2.81	5.6	-3.95	**	
b <sub>2-17</sub>	6.49	**	-13.03	***	
b <sub>13-17</sub>	3.36	*	-4.00	**	
b <sub>14a-17</sub>	-0.72	69.3	1.44	38.5	
b <sub>14b-17</sub>	-2.28	22.5	3.78	4.35 *	

Table 6. Estimations of the model coefficients and significance test.

Because of the interaction effects, the factors cannot be interpreted independently, but with interaction graphs to visualize the influence of the factors depending on the level of the others. Figures 5 and 6 present these interaction diagrams. They were used for setting the qualitative variables and for studying the tendencies of the others.

The factors were classified in two groups. The first group gathered the factors improving both responses in the same way: PLGA concentration, drop height and additional water. PLGA concentration and additional water had to be set together at the high levels. These levels led PLGA to be the nearest to its saturation. A short drop height permitted to increase the circularity. The ratio organic solvent / glycofurol dominated for the weight response. As expected the presence of water in the organic phase improved the circularity. It was included into all interactions, so its actions had to be analyzed.

The presence of additional water improved the weight response with limitations according to the levels of the other factors. Only the association with use of ethyl acetate did not permit an improvement by the additional water (Figure 6); the association of both factors was excluded.



Figure 5. Interaction diagrams for response Y<sub>1</sub>: weight of the isolated microparticles (a) Additional water - PLGA concentration (b) Additional water - organic solvent (c) Additional water - organic solvent1/glycofurol (d) Additional water - extraction medium.



Figure 6. Interaction diagrams for response Y<sub>2</sub>: circularity of the isolated microparticles
 (a) Additional water - PLGA concentration (b) Additional water - organic solvent (c)
 Additional water - organic solvent1/glycofurol (d) Additional water - extraction
 medium.

The second group gathered the factors proposing opposing conclusions. Acetone was significantly recommended as solvent for increasing the circularity; ethyl acetate was significantly recommended for the weight. A ratio 50/50 for the factor organic solvent / glycofurol was non-significant for the circularity; a ratio 75/25 was significantly recommended for the weight. The level Eth/W/PG was non-significantly recommended for the circularity; Eth/PG was recommended with the organic solvent for the weight yield.

## **3.3 Optimized production: choice and characterization**

A production was performed with the selected levels of the studied factors. The levels were described on figure 7 (a). The circularity of the isolated microparticles was 78.6% with a mean diameter of 29.7  $\mu$ m. The encapsulation efficiency of the active lysozyme was 13.9%. SEM observations were performed and shown on figure 7 (b) and (c). The observations confirmed the circularity value (Figure 7b). Few microspheres presented a varying ellipsoid shape (Figure 7 (c)).



Figure 7. Production of microspheres by the prilling process (a) Selected levels of the studied factors (b) and (c) SEM observation of the resulting microspheres.

#### **4 DISCUSSION**

The goal of this work was to obtain spherical microparticles of PLGA easy to handle by the prilling process.

In this way some feasibility studies identified numerous parameters potentially involved, seventeen parameters were selected to be studied.

Three factors were defined by the preliminary studies. The first study explored the protein nanoprecipitation step by Giteau *et al* [31]. It revealed the possibility to modulate two parameters involved in the nanoprecipitation without altering the precipitation efficiency.

The second one consisted on using the response surface methodology to establish a mathematical relation between the physicochemical properties of a mixture (surface tension, the viscosity and the density) and its composition (water, ethanol, PEG 400 and propylene glycol). Three mixtures were selected to represent three levels (three physical properties) strongly different for the extraction medium.

The recovery of microdroplets in the extraction medium requires a surface tension under 33.33 mN/m and a viscosity under 7 mPa.s. Setting the amount of ethanol at 45% minimum permitted to avoid the surface tension and viscosity incompatibilities. The 45% minimum of ethanol limit followed a maximum density at 0.95. It should be noted that the surface tension, viscosity and density of the extraction medium values were close to the values of pure ethanol.

The surface tension incompatibility was explained by the Marangoni effect [49]. At the lowest surface tension (pure ethanol) no disturbance appeared at the interface organic phase – aqueous phase. When the surface tension of the extraction medium exceed 33.33 mN/m, unexpected interfacial turbulences appeared [50]. The microdroplet was strongly destabilized, broken in nanodroplets and quickly solidified in the extraction medium. The viscosity and density incompatibilities would be explained by the Rayleigh-Taylor instability [51] acting during the impact of the organic microdroplets on the reception medium. When the microdroplets are sufficiently light, they were destabilized then disrupted. The consequence was identical than under the Marangoni effect.

The two next experimental designs studied the process by the determination of the effects of the studied factors and the interactions between some of them.

These steps highlighted two possible mechanisms for recovering the microparticles. Some combinations of factors resulted in a slower particle solidification. When the extraction of the organic solvent was more difficult (high viscosities, small extraction volume, low temperatures...) the time required to obtain solid microparticles increases [52,53]. Theses experimental conditions matched with 9.5% PLGA in ethyl acetate/glycofurol and 100 mL of Eth/PG without agitation. Interestingly these conditions gave generally more isolated microparticles according to the screening design, contrary to the expectation. So increasing the solvent extraction speed would protect the microdroplets from the aggregation. Actually droplets were firstly aggregated in the extraction medium then disrupted before their solidification in the second aqueous phase. Such phenomena were similar for a formulation of PLA spheres obtained by direct dialysis [54]. So PLGA microparticles could be aggregated during their solidification then could be broken. The break leads to shapeless small microparticles: high weight values and low circularity values. This mechanism had to be prevented.

Opposite combinations of factors accelerated the solidification of the microdroplets: low viscosities, high extraction volume, high PLGA concentration, high temperatures, high agitation.... These modifications are supported in the literature [53,55,56]. These levels permitted to obtain circular but mostly aggregated microparticles. Especially with the use of pure ethanol for the extraction medium at 37°C during 1 min and 500 mL of water for the second aqueous phase. These results could be explained by a quick but not fast enough solidification of the microdroplets. During their hardening the PLGA chains of different soft microparticles can be entangled, leading to an aggregation. To accelerate the solidification, water was added to the organic phase at 15% (v/v). According to the quantitative study (D-optimal design), this additional water was necessary for the improvement of the circularity and the low aggregation of particles.

Other phenomena were highlighted. A short drop height was recommended for the circularity. A long drop could lead to higher destabilization of the microdroplets during the drop. The expected evaporation of acetone during the drop did not offset the turbulences. So no evaporation effect was observed. A longer drop than 30 cm could permit to evaporate acetone allowing a better stabilization by solidification.

With specific levels (PLGA 11%, ratio ethyl acetate / glycofurol 75/25, Eth/PG or Eth/W/PG) the organic and extraction media possessed high viscosities and densities. These characteristics allowed a stability of the microdroplets as explained before. However the extraction of the organic solvents need more time and their sphericity was not remained. Even if the use of acetone (instead of ethyl acetate) led to a higher extraction speed, decreasing the proportion of glycofurol to 25% did not speed up sufficiently this extraction. The majority of microparticles were aggregated. But aggregated or not they possessed a high circularity.

A production of microparticles was performed for testing the relevant levels of the studied factors. A part of the resulted microparticles possessed the expected characteristics: circular and isolated. Others were smaller and polydispersed; they underwent the Marangoni effect or the Rayleigh-Taylor instability.

Some microparticles were considered as spherical (circularity > 0.8) but showed a particular radius of curvature (Figure 7 (c)). During the penetration into the extraction medium, the droplet undergoes a strain jump. The energy of the collision was notably expressed as an oscillation of the interface organic phase – aqueous phase. This oscillation loosened in few seconds and the droplet configures as a sphere, according to the Young-Laplace equation [57]. This phenomenon could explain the specific radius of curvature when its solidification is faster than the required time required of a total relaxation.

The encapsulation efficiency was lower than expected. The studied factors allowed the highest nanoprecipitation yield. So the lysozyme was lost during the process. Further studies will be necessary to understand and improve it.

So the four experimental designs permitted first to complete the knowledge of the factors potentially involved in the prilling process. Second they gave much information for the improvement of the production regarding to its quality (circularities) and quantity (weights). Further studies will try to harmonize circularity and weight yield. With the comprehension of the phenomena, improvements can be found as the near-to-saturation of the organic phase or the adjustment of the organic phase and the reception medium viscosities.

## **5** CONCLUSION

The prilling process carries many promises for the production of one step and monodisperse microspheres in the pharmaceutical field. This study was based on the development of PLGA microspheres encapsulating a model protein without toxic product and centrifugation step. Experimental designs were performed to obtain an expensive overview of seventeen production parameters potentially involved.

The designs highlighted 4 to 6 influent factors with different possible ways of solidification of the microparticles. The fastest solidification was a priority. Acceptable microspheres were produced with the compliance of all constraints.

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## Appendix

Run	Experimen	tal design	Nanoprecipitation				
n	U <sub>1</sub>	$U_2$	— yield				
	mg/mL	μL	%				
1	20	250	27.2				
2	25	250	65.6				
3	30	250	69.2				
4	30	250	85.6				
5	30	250	84.1				
6	20	500	56.8				
7	25	500	93.6				
8	30	500	106.8				
9	20	750	67.5				
10	25	750	99.0				
11	30	750	109.6				
12	20	1000	76.9				
13	20	1000	66.6				
14	20	1000	77.4				
15	25	1000	85.0				
16	30	1000	98.3				

Appendix 1. Full factorial experimental design, experimental conditions and corresponding response (nanoprecipitation yield, %).

Run n°	Experime	ental design		Responses				
	$X_1$	$X_2$	X <sub>2</sub> X <sub>3</sub>		Y <sub>1</sub>	Y <sub>2</sub>	Y <sub>3</sub>	
	Water	EtOH	PEG400	PG	Surface tension	Viscosity	Density	
					mN/m	mPa.s		
1	1.000	0.000	0.000	0.000	71.4	1.60	0.9920	
2	1.000	0.000	0.000	0.000	70.4	1.78	1.0009	
3	0.000	1.000	0.000	0.000	21.7	1.74	0.7808	
4	0.000	0.000	1.000	0.000	44.4	95.20	1.1142	
5	0.000	0.000	0.000	1.000	25.2	44.12	1.0231	
6	0.000	0.000	0.000	1.000	35.9	46.18	1.0317	
7	0.500	0.500	0.000	0.000	26.8	2.98	0.9004	
8	0.500	0.000	0.500	0.000	50.4	11.01	1.0771	
9	0.500	0.000	0.000	0.500	43.7	5.87	1.0319	
10	0.000	0.500	0.500	0.000	25.4	5.34	0.9244	
11	0.000	0.500	0.000	0.500	25.9	5.02	0.8929	
12	0.000	0.000	0.500	0.000	39.1	57.28	1.0674	
13	0.333	0.333	0.333	0.000	28.9	7.21	0.9673	
14	0.333	0.333	0.333	0.000	32.7	6.57	0.9691	
15	0.333	0.333	0.333	0.000	28.8	6.84	0.9689	
16	0.333	0.333	0.000	0.333	29.2	4.86	0.9513	
17	0.333	0.333	0.000	0.333	29.3	4.90	0.9487	
18	0.333	0.000	0.333	0.333	44.3	17.10	1.0628	
19	0.000	0.333	0.333	0.333	28.1	9.59	0.9553	
20	0.625	0.125	0.125	0.125	38.5	4.24	1.0006	
21	0.625	0.125	0.125	0.125	38.6	4.72	0.9999	
22	0.125	0.625	0.125	0.125	24.9	3.75	0.8889	
23	0.125	0.625	0.125	0.125	24.7	3.54	0.8728	
24	0.125	0.125	0.625	0.125	35.5	27.15	1.0530	
25	0.125	0.125	0.125	0.625	33.1	14.69	1.0151	
26	0.000	0.800	0.200	0.000	27.9	2.54	0.8345	
27	0.200	0.600	0.200	0.000	25.0	3.87	0.9040	
28	0.600	0.200	0.200	0.000	34.1	4.18	0.9915	

Appendix 3. Asymmetric screening design  $2^{12}3^3//24$ , experimental conditions and responses: Y<sub>1</sub>:circularity of the isolated microparticles (%), Y<sub>2</sub>: circularity of the aggregated microparticles (%), Y<sub>3</sub>: weight yield of the isolated microparticles (mg).

Ru	Expe	rimental	design													Respo	onses	
n	U1	U <sub>2</sub>	U <sub>3</sub>	$U_4$	U <sub>5</sub>	U <sub>6</sub>	U <sub>7</sub>	U <sub>8</sub>	U <sub>9</sub>	U <sub>10</sub>	U <sub>11</sub>	U <sub>12</sub>	U <sub>13</sub>	U <sub>14</sub>	U <sub>15</sub>	Y <sub>1</sub>	$Y_2$	Y <sub>3</sub>
n°	%	2	°C	s	%	mL	°Ċ	min	mL	°C	min	12	D		10	%	%	mg
1	9.5	Ethylacetate	37	10	0	100	4	1	0	4	10	W	50/50	Eth/	NO	12.9	18.2	5.18
														PG				
2	9.5	Acetone	37	30	0.01	100	37	1	0	4	10	WO	75/25	Eth/ W	W	9.0	2.4	0.74
3	9.5	Ethylacetate	4	30	0	400	37	1	0	4	20	WO	25/75	Eth/ PG	WO	4.7	0.0	2.40
4	11	Ethylacetate	4	10	0.01	100	37	1	0	4	20	W	50/50	Eth	W	6.9	6.6	3.98
5	11	Ethylacetate	37	30	0.01	400	4	1	0	4	10	WO	25/75	Eth	NO	12.5	15.3	0.10
6	9.5	Acetone	37	30	0.01	100	37	20	0	37	20	WO	25/75	Eth	NO	6.7	6.3	0.36
7	9.5	Acetone	4	10	0.01	400	4	20	0	37	10	W	75/25	Eth	WO	36.8	0.0	0.26
8	11	Acetone	4	30	0	100	4	20	0	37	10	WO	25/75	Eth/ PG	W	4.9	2.9	0.84
9	11	Acetone	4	30	0	100	4	20	0	37	10	WO	25/75	Eth/ PG	W	7.3	1.3	1.36
10	11	Acetone	4	30	0	100	4	20	0	37	10	WO	25/75	Eth/ PG	W	5.3	7.3	5.57
11	11	Ethylacetate	4	10	0.01	100	37	20	0	37	10	W	25/75	Eth/ W	NO	0.0	0.0	0.19
12	11	Ethylacetate	4	10	0.01	100	37	20	0	37	10	W	25/75	Eth/ W	NO	0.0	0.0	0.27
13	11	Acetone	37	10	0	400	37	20	0	37	20	W	75/25	Eth/ PG	NO	36.1	9.7	0.68
14	11	Acetone	37	10	0	400	37	20	0	37	20	W	75/25	Eth/ PG	NO	39.3	0.0	0.42
15	11	Acetone	37	10	0	400	37	20	0	37	20	W	75/25	Eth/ PG	NO	23.9	7.1	0.40
16	11	Ethylacetate	37	30	0.01	400	4	20	0	37	20	WO	50/50	Eth/ W	WO	24.6	3.2	1.10
17	9.5	Ethylacetate	37	10	0	100	4	1	500	37	10	WO	25/75	Eth	WO	13.0	14.3	1.08
18	9.5	Ethylacetate	4	30	0	400	37	1	500	37	20	W	75/25	Eth	NO	37.2	31.3	15.84
19	9.5	Acetone	4	10	0.01	400	4	1	500	37	20	WO	50/50	Eth	W	8.8	8.8	0.18
20	11	Acetone	4	30	0	100	4	1	500	37	20	W	25/75	Eth/ W	WO	24.5	11.1	0.37
21	11	Acetone	37	10	0	400	37	1	500	37	10	WO	50/50	Eth/ W	NO	35.3	22.0	2.56
22	11	Ethylacetate	37	30	0.01	400	4	1	500	37	10	W	75/25	Eth/ PG	W	14.2	3.1	0.30
23	9.5	Ethylacetate	37	10	0	100	4	20	500	4	20	WO	75/25	Eth/ W	NO	6.7	3.9	4.04
24	9.5	Acetone	37	30	0.01	100	37	20	500	4	20	W	50/50	Eth/ PG	WO	16.3	6.6	8.80
25	9.5	Ethyl acetate	4	30	0	400	37	20	500	4	10	W	25/75	Eth/ W	W	14.4	9.5	25.93
26	9.5	Acetone	4	10	0.01	400	4	20	500	4	10	WO	25/75	Eth/ PG	NO	24.8	11.8	0.82
27	11	Acetone	4	30	0	100	4	20	500	4	10	W	50/50	Eth	NO	16.5	0.0	74.92
28	11	Ethylacetate	4	10	0.01	100	37	20	500	4	10	WO	75/25	Eth	WO	52.7	14.3	1.24
29	11	Ethylacetate	4	10	0.01	100	37	20	500	4	10	WO	75/25	Eth	WO	33.7	14.3	1.68
30	11	Acetone	37	10	0	400	37	20	500	4	20	WO	25/75	Eth	W	0.0	7.1	0.38

Run n°	Expe	erimental des		Responses				
	$U_1$	$U_2$	U <sub>13</sub>	U <sub>14</sub>	U <sub>16</sub>	U <sub>17</sub>	<b>Y</b> <sub>1</sub>	Y <sub>2</sub>
	%				cm	%	mg	%
1	11	Ethyl acetate	50/50	Eth	5	100/0	17.82	41.18
2	9.5	Ethyl acetate	75/25	Eth	5	100/0	17.11	19.79
3	9.5	Ethyl acetate	75/25	Eth	5	100/0	19.58	22.78
4	11	Acetone	75/25	Eth	5	85/15	28.68	62.99
5	11	Acetone	50/50	Eth	30	100/0	23.57	25.23
6	9.5	Acetone	75/25	Eth	30	100/0	10.50	10.96
7	9.5	Ethyl acetate	50/50	Eth	30	85/15	22.57	32.35
8	9.5	Acetone	50/50	Eth/PG	5	100/0	1.87	22.77
9	9.5	Acetone	50/50	Eth/PG	5	85/15	3.57	82.01
10	9.5	Ethyl acetate	75/25	Eth/PG	5	85/15	55.56	8.33
11	11	Ethyl acetate	75/25	Eth/PG	30	100/0	43.90	23.83
12	11	Ethyl acetate	75/25	Eth/PG	30	100/0	36.94	30.63
13	11	Acetone	50/50	Eth/PG	30	85/15	4.04	71.77
14	11	Ethyl acetate	75/25	Eth/PG	30	85/15	66.06	9.09
15	11	Acetone	75/25	Eth/W/PG	5	100/0	25.43	34.78
16	11	Acetone	75/25	Eth/W/PG	5	100/0	32.56	34.15
17	11	Ethyl acetate	50/50	Eth/W/PG	5	85/15	22.88	48.15
18	9.5	Ethyl acetate	50/50	Eth/W/PG	30	100/0	3.35	17.61
19	9.5	Acetone	75/25	Eth/W/PG	30	85/15	9.46	77.36

Appendix 4. D-optimal design, experimental conditions and responses: Y<sub>1</sub> weight yield of the isolated microparticles (mg), Y<sub>2</sub> circularity of the isolated microparticles.

## **DISCUSSION GENERALE**

Les maladies dégénératives en général et neurodégénératives en particulier constituent aujourd'hui un défi pour la recherche médicale. Les thérapies curatives classiques sont en échec. Des voies alternatives de traitement sont actuellement en cours d'études. L'une d'elles consiste en l'implantation de cellules souches au sein du tissu endommagé. L'objectif est de régénérer le tissu et permettre la prolifération et la différenciation des cellules greffées, par exemple en neurones dans le cadre d'une neurodégénérescence. De nouveaux outils font l'objet de recherche pour optimiser la survie, la différenciation et la prolifération des cellules greffées.

Au sein de l'unité U1066, les Microcarriers Pharmacologiquement Actifs (MPA) sont développés dans le but de proposer un outil qui potentialiserait la réparation tissulaire. Les cellules souches sont ainsi adhérées à la surface des microsphères d'un diamètre moyen de 60 µm. La surface des microsphères est recouverte de molécules de la matrice pour lui conférer des propriétés biomimétiques. De plus, les microsphères doivent libérer un facteur de croissance en continu, sur une période d'un mois, pour potentialiser la survie et la différentiation cellulaire.

Les microsphères doivent donc présenter une encapsulation maximale et une libération prolongée du facteur de croissance sur une période de 30 jours. Le système microsphère constitue un système très étudié pour assurer la libération prolongée de protéines thérapeutiques et ainsi réduire leur fréquence d'administration [1–4]. De plus, la nanoprécipitation de la protéine avant son encapsulation (mise au point au sein de l'unité) améliore sa stabilité au cours du processus [5].

Les microsphères doivent être biocompatibles et biodégradables pour permettre leur administration par voie parentérale. Pour cela, leur matrice est composée de copolymères de PLGA et de poloxamère 188 (P188). Ces copolymères permettent d'assurer une libération prolongée de la protéine encapsulée.

Grâce à l'ensemble des études précédemment réalisées, les MPA fournissent aujourd'hui une encapsulation de la protéine et une survie cellulaires acceptables. Cependant, deux aspects majeurs restent à améliorer. D'une part le profil de libération de la protéine est souhaité continu sur 30 jours, mais seuls 50% sont libérés sous forme active après 20jours ; s'en suit un arrêt de la libération [6]. D'autre part la formulation des microsphères est classiquement réalisée par émulsion – extraction de solvant nécessitant l'emploi de solvants halogénés toxiques et fournissant des diamètres polydisperses. L'utilisation d'une méthode alternative produisant des microsphères monodisperses et sans solvant halogéné serait un atout pour faciliter le transfert vers la clinique [7].

La première partie des études portent donc sur le profil de libération de la protéine encapsulée. Celui-ci est insuffisant principalement à cause de la dénaturation de la protéine au cours

de sa libération, donc la perte de son activité biologique. Cela se produit notamment par des phénomènes dénaturants induits par le microenvironnement (PLGA) [8–11]. Deux stratégies sont mises en place afin de pallier ces phénomènes. La première stratégie consiste en la recherche et l'utilisation de nouveaux additifs pour la formulation. La seconde stratégie s'intéresse à la modulation de la matrice des microsphères en s'inscrivant dans la continuité des travaux précédents.

## **1** STRATEGIES D'AMELIORATION DU PROFIL DE LIBERATION DE LA PROTEINE

### 1.1 Recherche de nouveaux additifs

Ainsi, de nouveaux additifs sont recherchés afin d'améliorer la préservation de l'activité biologique de la protéine encapsulée au cours de sa libération. Au sein des microsphères à base de PLGA, de nombreux additifs ont déjà été testés. Ils peuvent être classés selon deux approches principales : les additifs pour une protection globale contre tout type de phénomène dénaturant (ou stress), et les additifs pour une protection ciblée contre un stress particulier. La protection globale consiste en l'ajout de composés dits stabilisants, tels des acides aminés, des sucres ou des polyols. Testée au sein des MPA, cette approche ne préserve pas l'activité biologique de la protéine [12]. La protection ciblée consiste en la diminution des stress encourus ou la diminution de leur impact sur la protéine. Considérant l'hydrophobie du PLGA comme un stress [8–11], l'utilisation de copolymère PLGA-P188-PLGA et la nanoprécipitation de la protéine avec du P188 sont deux solutions préservant l'activité de la protéine [5,13]. Cependant la libération de la protéine engendre toujours une perte importante de son activité biologique. Il est donc nécessaire de considérer une nouvelle approche.

C'est pourquoi une étude bibliographique est réalisée afin d'identifier de nouvelles voies. Cette troisième approche est souhaitée spécifique, non pas au stress encouru, mais à la préservation de l'activité biologique de la protéine. Tout en étant ciblée vers la protéine, cette préservation serait donc globale contre tout stress. Les recherches se basent sur les phénomènes connus de protection des protéines *in vivo*. Progressivement, deux mécanismes principaux de stabilisation apparaissent de manière récurrente, basés sur les interactions et assemblages protéine-protéine. L'un des mécanismes révélés est la capture de protéines thérapeutiques à l'intérieur de complexes protéiques. Ce mécanisme est déjà à l'étude en tant que vecteurs de protéines thérapeutiques, illustré par les bionanoparticules et la fibroïne de soie [14,15]. L'autre mécanisme révélé est la reconnaissance spécifique d'une protéine en fonction de sa nature ou d'un état dénaturé. Ce mécanisme constitue une voie de recherche encore inexplorée pour une application pharmaceutique. Cette étude propose donc l'utilisation d'additifs protéiques pour préserver l'activité biologique du facteur de croissance au cours de sa libération. La difficulté principale réside en l'utilisation de ces additifs lors de la formulation des MPA, possédant chacun leurs avantages et inconvénients.

Certains additifs sont jugés incompatibles avec la formulation. Par exemple, les bionanoparticules possèdent des tailles moyennes de quelques dizaines à quelques centaines de nanomètres [16], comparables aux nanoprécipités protéiques, mais ne se dissolvent pas en milieux aqueux. La protéine thérapeutique « capturée » dans la cavité des bionanoparticules ne serait donc pas libérée par l'absorption d'eau dans les microsphères, mais uniquement lors de la dégradation des microsphères. La libération serait retardée et le facteur de croissance protégé de l'environnement interne des microsphères, ce qui constituerait un avantage. Cependant, leur structure est sensible à certaines étapes de formulation tels la centrifugation et la lyophilisation, incompatible avec la forme sèche. L'encapsulation des bionanoparticules dans les microsphères de PLGA risquent donc de déstabiliser les interactions protéine-protéine formant le la cavité. Ainsi, les bionanoparticules ne sont pas envisageables en conservant les caractéristiques classiques des microsphères actuelles.

D'autres additifs sont jugés compatibles mais peu adaptable. Par exemple, les systèmes associant la reconnaissance et la capture tels les foldosomes, les ribosomes et l'insulin-degradingenzyme. Dans le cadre des foldosomes, la Hsp70 reconnait la dénaturation d'une protéine, recrute des co-facteurs et forme le foldosome. Celui-ci capture la protéine dénaturée. La lyse d'une molécule d'ATP fournit l'énergie nécessaire au repliement correct de la protéine dénaturée. Ce mécanisme est peu envisageable au cœur des microsphères. Il faudrait encapsuler chacun des co-facteurs séparément et permettre les multiples interactions amenant à la formation des foldosomes.

Enfin, certains additifs assurent une préservation de la protéine encapsulée par les mécanismes plus simples et sont jugées compatibles et applicables en l'état. La Hsp27, l'héparine et la fibroïne de soie ont été sélectionnées pour leurs différents mécanismes d'action. La Hsp27 reconnait et protège le premier stade réversible de la dénaturation d'une protéine [17]. L'héparine reconnait et protège les facteurs de croissance actifs [18]. La fibroïne de soie fournit un microenvironnement sans stress majeur pour la protéine [19].

Des microsphères de PLGA-P188-PLGA ont été formulées pour l'encapsulation du lysozyme, protéine modèle des facteurs de croissance. Les trois additifs sélectionnés ont été

introduits dans les microsphères. La Hsp27 et l'héparine ont chacune été co-dissoutes avec le lysozyme avant l'étape de nanoprécipitation ; ainsi les additifs sont au plus près du lysozyme lors de la dissolution des nanoprécipités au cours de la libération. La fibroïne de soie a été dispersée dans la solution de copolymère PLGA-P188-PLGA ; ainsi l'environnement interne de la microsphère a été modifié.

Les ajouts de l'héparine et de la Hsp27 ont conduit à l'augmentation de la quantité totale de lysozyme actif libéré (de 50 à 75%). De plus les profils indiquent une libération continue.

L'héparine constitue une matrice de microsphères connue comme protecteur de protéines encapsulées. De par sa nature polymérique hydrophile et sa capacité à protéger les facteurs de croissance, plusieurs laboratoires s'y intéressent en tant que système à libération prolongée [20-23]. L'héparine coprécipitée pourrait, en plus de la protection de l'activité biologique du lysozyme, jouer un rôle matriciel et moduler également par cette voie la cinétique de libération. L'association héparine-lysozyme dans des microsphères est utilisable comme modèle de la relation héparine facteur de croissance [24]. Appliquée aux microsphères de PLGA-P188-PLGA, elle permet d'atteindre 75% de lysozyme actif libérés de manière continue. Cependant le rendement d'encapsulation de lysozyme actif a été évalué expérimentalement à 30%. La première hypothèse expliquant ce rendement serait une faible nanoprécipitation du complexe héparine-lysozyme. De plus il a été prouvé que plus de 95% du lysozyme est lié à l'héparine [24] via des sites de liaison hétérogènes [25]. Le complexe héparine-lysozyme nanoprécipité puis dissolu pourrait masquer le site actif du lysozyme pour son substrat Micrococcus lysodeikticus. Dans ce cas, le rendement d'encapsulation réel serait supérieur et la quantité relative libérée diminuée. D'autres études révèlent que l'association avec l'héparine ne masque pas l'activité biologique de la protéine associée [26]. Dans ce cas la nanoprécipitation ou l'encapsulation de l'association héparine lysozyme serait l'étape du procédé à optimiser. Avant d'envisager une utilisation courante de l'héparine, des études de dosage de protéines totales encapsulées ont été menées. Des biais expérimentaux, notamment des interactions entre l'héparine et les réactifs du test, empêchent pour le moment de conclure.

Des résultats similaires sont obtenus avec l'association Hsp27 - lysozyme. En plus de son activité de protection de l'activité biologique du facteur de croissance, l'ajout de l'Hsp27 dans des MPA pour le traitement d'une neurodégénération est envisageable en tant que protéine thérapeutique. En effet, l'Hsp27 permet de ralentir la formation d'agrégats protéiques induisant certaines maladies neurodégénératives, comme la maladie de Huntington [27–30]. L'encapsulation et la libération prolongée de l'Hsp27 a été démontrée avec la conservation de son activité protectrice [31,32]. L'amélioration du profil de libération du lysozyme permet de conclure que

l'association Hsp27 – lysozyme est effective au sein des microsphères. Cependant, comme dans le cas de l'utilisation de l'héparine, les rendements d'encapsulation du lysozyme actif sont faibles. D'autres phénomènes peuvent expliquer ce résultat. La prise en charge *in vivo* du complexe Hsp27 – protéine dénaturée est réalisé via deux mécanismes possibles : le repliement de la protéine via des foldosomes (*Micrococcus lysodeikticus* possède les gènes nécessaires à la formation des foldosomes [33–35]), ou la dégradation du complexe par des protéases [36,37]. Il est donc possible que le lysozyme inactif donc associé à l'Hsp27 ne récupère pas son activité. Une nanoprécipitation inadaptée expliquerait également le faible rendement d'encapsulation. Une étude est en cours afin d'apporter des compléments d'information.

La fibroïne de soie est obtenue par purification à partir des cocons de soie. Récupérée sous forme nanoparticulaire, elle présente une structure majoritairement fibrillaire en feuillets béta, exhibant une hydrophobie [38]. Les études utilisant la fibroïne procèdent à la modification de cette structure afin de diminuer l'hydrophobie donc fournir un environnement adéquat au maintien de l'activité biologique des protéines encapsulées [39–43]. Différents solvants organiques ont été testés afin de co-dissoudre la fibroïne et le copolymère. Cette co-dissolution n'ayant pas été obtenue, la fibroïne a été dispersée dans le dichlorométhane. Par microscopie optique, les nanoparticules de fibroïne s'observent à l'intérieur des microsphères de PLGA-P188-PLGA. Le profil de libération du lysozyme à partir de ces microsphères est ralenti. Il est supposé que les nanoparticules de fibroïne utilisées dans notre étude exhibent une configuration en feuillets béta hydrophobes. Le lysozyme dissous dans la microsphère, en cours de libération, interagirait par interactions hydrophobes dénaturantes avec la fibroïne. Le lysozyme inactif serait ensuite libéré ou adsorbé à la surface des nanoparticules [44]. Le profil de libération montrerait donc une saturation après 10 jours des sites d'adsorption avec une reprise de la libération du lysozyme actif.

### **1.2 Etudes de la nature du copolymère**

Ainsi, la libération de la protéine encapsulée active est améliorée également grâce à la mise en place d'une approche alternative. La seconde stratégie étudiée au cours de cette thèse s'intéresse à la modification de la composition du copolymère. Celui-ci est une chaine linéaire de deux segments de PLGA encadrant un segment de P188. Les modifications ont porté sur trois paramètres : le remplacement du P188 par la poloxamine 1107 (T1107, à quatre branches), les modifications du ratio acide lactique / acide glycolique (LA/GA) et de la masse molaire des segments PLGA. Ces modifications ont engendré différentes structures internes et externes des microsphères ainsi que différents profils de libération du lysozyme actif. Les différences de structures engendrent elles-mêmes des modifications du profil de libération [45]. Les meilleurs profils de libération permettent d'identifier les caractéristiques recherchées du copolymère et des microsphères. Moins le copolymère est hydrophobe, plus le profil de libération est amélioré et les microsphères possèdent des structures de surface importantes. Les microsphères doivent donc être préférentiellement rugueuses et poreuses. Cependant une diminution de la masse molaire du copolymère (engendrant une diminution de son hydrophobie) conduit également à la baisse de la température de transition vitreuse (Tg). Dans le cas du polymère 25P10, la Tg est trop basse (valeur ?) pour permettre une processabilité des microsphères.

L'hydrophobie du polymère est souvent mise en avant pour expliquer les pertes d'activités des protéines encapsulées. C'est pourquoi de nombreux travaux tentent de diminuer cette hydrophobie, afin de proposer au sein des microsphères des interactions hydrophiles entre la protéine encapsulée et le polymère [46–49]. Une hydrophobie exacerbée de la matrice (par les nanoparticules de fibroïne de soie) a précédemment démontré son effet néfaste sur la protéine encapsulée. Les interactions hydrophobes polymère – protéine seraient donc à l'origine de la perte de l'activité biologique d'une partie du lysozyme [50].

La diminution de l'hydrophobie du copolymère conduit à l'augmentation de la porosité et de la rugosité des microsphères, ainsi qu' à des profils de libération prolongée et des quantités totales récupérées augmentées [46,49,51,52].

La relation entre porosité exacerbée et libération améliorée peut être expliquée par les relations entre les mécanismes de libération, la nature du copolymère et le milieu de libération. Quatre mécanismes de libération principaux sont connus : la diffusion à travers les pores, la diffusion à travers le polymère, le gradient osmotique et l'érosion [45]. Au cours de la libération les microsphères sont en suspension dans un milieu aqueux. Par diffusion à travers les pores et à travers le copolymère, l'eau s'infiltre plus rapidement dans des microsphères poreuses. L'eau est davantage et profondément absorbée à l'intérieur des microsphères grâce à la diminution de leur hydrophobie. Ainsi, le milieu interne est plus favorable à la préservation de l'activité biologique de la protéine encapsulée ; la quantité totale de lysozyme actif libéré est plus conséquente [53,54]. Par les mêmes mécanismes de diffusions ajoutés au gradient osmotique, la libération du lysozyme est accélérée [45].

Dans ces travaux et dans la littérature, il n'existe pas de relation connue entre rugosité et libération. Une rugosité exacerbée est néanmoins souhaitable afin de faciliter l'adhésion des cellules en surface, donc une amélioration de l'utilisation des MPA [55–57].

#### DEVELOPPEMENT DU PROCEDE DE PRILLING

L'optimisation de la libération de la protéine encapsulée a constitué le premier volet des études menées dans ce travail. L'ensemble de ces études a été réalisé sur des microsphères formulées par la technique d'émulsion – extraction de solvant, technique couramment utilisée fournissant des diamètres de microsphères polydisperses et nécessitant l'emploi de solvant halogéné toxique tel le dichlorométhane (DCM). Le second volet s'intéresse au développement d'une technique de formulation alternative. Le but est de produire, par une technique facilement transposable vers la clinique et sans solvant halogéné, des microsphères aux caractéristiques contrôlables. Le procédé de prilling et les microsphères résultantes constitueraient une réponse à ce cahier des charges.

Dans le domaine pharmaceutique, le procédé de prilling est avant tout utilisé dans le cadre de la production de particules lipidiques [58–60]. Certains polymères d'origine animale ou végétale (alginate, dextran, chitosan) constituent également la matrice finale de la microsphère [61–65]. Ces produits sont le plus souvent utilisés car ils permettent une extrusion et une solidification facilitées. Cependant le PLGA, en tant que polymère synthétique biocompatible et biodégradable, fournit le support à la stratégie des MPA sous forme de microsphères injectables. La production de microsphères de PLGA par le procédé de prilling est breveté [66,67] et publié [68] en utilisant le DCM. L'emploi de solvant non toxique (de classe III selon la Pharmacopée Européenne) pour la production de microsphères de PLGA par le procédé du prilling constitue un travail original. Les travaux de thèse de Van-Thanh TRAN ont conduit à la production de microsphères à partir d'une phase organique PLGA/acétone.

De plus, l'encapsulation de protéines sous forme nanoprécipitée serait un avantage supplémentaire à la formulation de systèmes à libération prolongée. Afin d'anticiper la production en conditions aseptiques, l'étape de centrifugation des nanoprécipités doit être évitée. Le glycofurol, solvant non toxique du PLGA et utilisé comme agent de la nanoprécipitation protéique, est ainsi retrouvé dans la phase organique avant l'extrusion. L'ensemble des travaux réalisés ont porté sur le développement de la production de microsphères par le procédé de prilling, à partir d'une phase organique contenant le PLGA et le glycofurol.

Des études de faisabilités ont permis d'identifier 17 paramètres de production (facteurs) potentiellement influents. Deux plans d'expériences préliminaires ont été nécessaires à la détermination de l'ensemble des valeurs prises par les facteurs (niveaux). Deux plans d'expériences supplémentaires ont permis d'explorer les effets des facteurs et certains effets d'interaction. Des conditions opératoires ont pu ainsi être définies pour conduire à des particules acceptables.

Les difficultés rencontrées sont majoritairement liées à la stabilisation des microgouttelettes produites par extrusion de la phase organique, et à l'extraction des solvants organiques. Ces deux phénomènes découlent de la présence du glycofurol dans la phase organique. Le glycofurol est un solvant du PLGA étudié pour sa non toxicité et son injectabilité [69–71]. C'est également un solvant visqueux (13 mPa.s à 20°C) donc peu extrudable, en plus d'être un mauvais solvant du PLGA. Il n'est donc pas possible de réaliser une concentration élevée du PLGA dans la phase organique. Bien qu'il soit utilisé comme solvant extractible du PLGA [69,70,72], les propriétés physicochimiques du glycofurol en font davantage un agent de solubilisation de molécules hydrophobes en vue d'une administration par voie parentérale [73–78].

L'instabilité des microgouttelettes et la lente extraction des solvants organiques a conduit à l'agrégation des microparticules en cours de solidification et à leur rupture en microsphères plus petites. La stratégie utilisée a permis de résoudre en partie ces difficultés liés à l'introduction du glycofurol dans la phase organique. L'une des solutions révélées consiste à accélérer la solidification des microparticules en amenant le polymère dissous dans la phase organique à une concentration la plus proche de sa solubilité maximale.

Ainsi, la formulation de microsphères de PLGA par le procédé de prilling est réalisable dans le cadre d'une production en conditions aseptiques. Néanmoins, cette formulation nécessite une optimisation, la majorité des microsphères obtenues étant agrégées et le rendement d'encapsulation insuffisant. L'étape d'optimisation complétée, des ajustements des paramètres de production seront menés en fonction du diamètre des microsphères souhaité, de l'utilisation des additifs héparine et Hsp27, et de la nature des copolymères. Ce dernier point aura comme base de travail le copolymère 25P20 (copolymère de PLGA-P188-PLGA contenant des segments PLGA de 20 kDa et un ratio LA/GA de 25/50). La maitrise des paramètres de la production et de la stabilisation de la protéine devra être associée à un procédé simple et transposable.

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# **CONCLUSION & PERSPECTIVES**

Dans le cadre de la thérapie cellulaire, les microsphères à base de PLGA fournissent un outil pour la survie et la différenciation de cellules souches adhérées en surface ainsi que pour la libération prolongée d'un facteur de croissance. D'une part, le profil de libération de la protéine est souhaité complet et continu sur 30 jours afin de stimuler et entretenir correctement la différenciation cellulaire. Ces microsphères sont en phase d'optimisation. En effet, la conservation de l'activité biologique de la protéine au cours du processus de libération reste un défi majeur. D'autre part, la formulation classique par émulsion – extraction de solvant nécessite l'utilisation de solvant halogéné (DCM) et fournit des microsphères polydisperses. La technique du prilling contournerait ces problèmes tout en facilitant la transposition en conditions aseptiques vers la clinique. Les objectifs de ces travaux de thèse furent : l'amélioration du profil de libération d'une protéine modèle encapsulée (lysozyme) et le développement du procédé de prilling associé au glycofurol, solvant non toxique.

La libération du lysozyme est souhaitée continue pour aboutir à une libération complète sous forme active pendant un mois. L'activité biologique est conservée au cours de la formulation et de la conservation des microsphères, grâce notamment à la nanoprécipitation du lysozyme. Les stress physicochimiques amenant à la perte d'activité interviennent au cours du processus de libération.

Le profil de libération du lysozyme est amélioré par deux stratégies menées parallèlement. La première consiste à rechercher et appliquer de nouveaux additifs permettant la préservation de l'activité biologique de la protéine. L'étude bibliographique met l'accent sur des additifs essentiellement protéiques. Parmi les additifs adaptables à la formulation, trois d'entre eux sont séparément co-encapsulés avec le lysozyme. La fibroïne, introduite pour créer un environnement hydrophile adéquat à la préservation de la protéine, se révèle hydrophobe et néfaste. Des méthodes existent afin de modifier sa structuration pour la rendre hydrophile. Une modification de la structure de la fibroïne améliorerait ses propriétés et conduirait à la formulation de microsphères moins dénaturantes pour le lysozyme au cours de sa libération, tout en améliorant ses propriétés mécaniques [1]. Les ajouts de la protéine de choc thermique Hsp27 et de l'héparine conduisent à des rendements de nanoprécipitation du lysozyme de 30 à 35% ; cette étape reste à optimiser. Cependant la quantité libérée de lysozyme actif passe de 50 à 75% grâce à ces additifs. D'autres additifs révélés au cours de l'étude bibliographique sont envisageables. Par exemple, la caséine possède un fort potentiel grâce à ses propriétés d'assemblage spontané, de chaperonnage et de restauration spontanée de l'activité [2–4]. Son utilisation nécessitera des études approfondies.

La seconde stratégie est basée sur la modulation de la nature du copolymère composant la matrice de la microsphère. Suite aux travaux des thèses d'Alexandra PAILLARD-GITEAU [5] et Van-Thanh TRAN [6,7], le PLGA a été remplacé par un copolymère PLGA-PEG-PLGA puis PLGA-P188-PLGA. La modulation des caractéristiques de ce dernier met en évidence la prépondérance de deux de ses propriétés, la balance hydrophile/lipophile du copolymère et sa structure moléculaire. Cette modification permet d'obtenir une libération du lysozyme actif au-delà de 20 jours, impossible auparavant. La compréhension des structures interne et externe des microsphères met en évidence leur lien direct avec la nature du copolymère et par voie de conséquence le profil de libération du lysozyme ; des études complémentaires doivent être réalisées afin de relier précisément ces deux paramètres. Le meilleur profil de libération est obtenu avec le copolymère processable (à la température de transition vitreuse suffisamment élevée) possédant la plus faible hydrophobie. De nouvelles stratégies sont envisagées en mélangeant des copolymères de nature différente, afin d'obtenir les avantages sans les inconvénients.

Enfin, l'apport d'additifs et la modulation de la nature du copolymère pourront se faire simultanément. Des ajustements seront probablement nécessaires, les additifs et le copolymère agissant l'un sur l'autre. Au final, l'objectif sera de déterminer le compromis idéal entre libération prolongée, activité du lysozyme préservé, et structure de la microsphère.

La technique du prilling, introduite par Van-Thanh TRAN au cours de sa thèse, est établie dans le cas de la production de microsphères à partir d'une suspension de nanoprécipités de lysozyme dans la solution organique de PLGA. La suppression de l'étape de centrifugation pour récupérer les nanoprécipités autoriserait une production réalisable en conditions aseptiques. De plus, écarter l'utilisation de solvant halogéné entraîne la présence d'un volume de glycofurol dans la phase organique. L'association prilling – glycofurol conduisait à un verrou technologique. Ce verrou a été levé grâce à l'identification et l'analyse de 17 paramètres de formulation potentiellement influents. Deux plans d'expériences préliminaires ont établi les possibilités de formulation. Deux plans d'expériences supplémentaires ont désignés les paramètres influents et les mécanismes d'obtention des microsphères à préférer. Notamment, la diminution de la solvabilité du PLGA dans la phase organique avant extrusion a constitué une innovation-clé.

La meilleure production obtenue est constituée de microsphères isolées en petit nombre ; la majorité des microsphères récupérées restent agrégées. L'adéquation entre extraction du solvant organique et non-agrégation des microgouttelettes en cours de solidification n'est pas optimale. Des

études complémentaires seront nécessaires. Notamment, l'ajout d'un tensioactif ou tout autre élément est envisagé afin de stabiliser les microgouttelettes au cours de la solidification [8,9].

Le glycofurol est un composé amphiphile, mauvais solvant du PLGA. Il peut s'avérer être un meilleur solvant pour des copolymères amphiphiles de type PLGA-P188-PLGA. Dans ce cas la concentration du copolymère dans la phase organique pourrait être augmentée, ce qui contribuerait à une solidification plus rapide.

Ainsi, les trois éléments étudiés conduisant à la microsphère (additif à la protéine encapsulée, copolymère, technique de formulation) sont liés. Lorsque le(s) additif(s), la ou les nature(s) du copolymère et les paramètres du procédé de prilling seront établis, des microsphères optimales sur les plans de la libération de la protéine active et de la production adéquate à la clinique pourront être étudiées *in vivo* comme outil à la régénération tissulaire.

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# Thèse de Doctorat

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Développement d'une protéine à libération prolongée, mise au point du procédé d'encapsulation sans solvant halogéné et optimisation du profil de libération.

Development of microencapsulation process without toxic solvent, application to sustained protein release.

### **Résumé**

La régénération tissulaire est une voie prometteuse de thérapie dans le cadre des maladies dégénératives. Dans ce but sont conçus les microcarriers pharmacologiquement actifs (PAM). Ce sont des microsphères fournissant un environnement adéquat à la survie et la différenciation de cellules souches par la libération d'un facteur de croissance protéique encapsulé.

Pour potentialiser l'intérêt des PAM, les microsphères doivent (1) permettre la libération complète et prolongée de la protéine (2) être formulées sans solvant halogéné par un procédé transposable à l'échelle pilote.

Deux stratégies sont menées afin d'améliorer la stabilité et la libération de la protéine. La première consiste à utiliser de nouveaux additifs. Une étude bibliographique révèle le potentiel d'additifs protéiques ; leur application a permis d'augmenter significativement l'activité biologique de la protéine libérée. La seconde stratégie consiste à moduler la matrice de copolymère PLGA-P188-PLGA. La modification de ses propriétés physicochimiques (M<sub>w</sub>, hydrophobie...) a permis d'accéder à la compréhension de la structure des microsphères et d'obtenir une libération continue.

Le développement du procédé de fabrication des microsphères sans solvant toxique associe la technique du prilling avec le glycofurol comme solvant. Cette combinaison se heurte à de nombreux verrous technologiques. La mise au point du procédé a été réalisée à l'aide de plans d'expériences. Ils ont conduit à la production de particules grâce à la modélisation des propriétés physicochimiques du milieu de réception et à la prise en compte des différents paramètres du procédé.

#### Mots clés

Microsphère, Protéine, Libération prolongée, Prilling, PLGA, Poloxamère, Hsp27, Héparine.

### Abstract

Pharmacologically active microcarriers (PAM) have been developed as innovative tools for tissue regeneration. This microspherical platform provided an environment for the survival and the differentiation of stem cells through the release of encapsulated protein growth factor.

To improve the therapeutic efficacy of the PAM, the microspheres have to (1) provide the full and sustained release of the protein (2) be formulated without halogenated solvent by a process with an easy scale-up.

The protein release has been studied through two strategies. The first one was to look for a preservation of the biological activity of the protein during the release. A literature review highlighted protein additives. Some of them were incorporated into the microspheres and increased significantly the protein release. The second one was the modulation of the matrix copolymer PLGA-P188-PLGA. The modification of its properties (M<sub>W</sub>, hydrophobicity) permitted to reach a continuous release and to understand the structure of the microspheres.

The prilling technique and the use of glycofurol provide an easy transferable process without toxic solvent. Experimental designs were performed to overcome the technological barriers. Through the modeling the physicochemical properties of the reception medium and the study of the process parameters, the formulation has been improved to produce acceptable particles.

#### Key Words

Microsphere, Protein, Drug Delivery, Prilling, PLGA, Poloxamer, Hsp27, Heparin.