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Thérapie génique à l'aide de nanocapsules lipidiques PEGylées

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Thérapie génique à l'aide de nanocapsules lipidiques PEGylées

Thèse de doctorat
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Ecole doctorale : Biologie santé

Présentée et soutenue publiquement par

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INTRODUCTION GÉNÉRALE

1 - Introduction générale

1.1 - Le gène comme principe actif

Parallèlement aux thérapies conventionnelles, qui traitent les symptômes des maladies génétiques, le concept de thérapie génique déjà imaginé il y a une trentaine d'années, consiste à traiter la base de l'anomalie génétique en utilisant le gène comme agent thérapeutique.

Au cours des multiples divisions cellulaires, le matériel génétique est théoriquement recopié à l'identique. Chaque cellule reçoit ainsi normalement la même information codant pour une protéine fonctionnelle. Mais, des erreurs peuvent subvenir durant ces phénomènes et persister par la suite, malgré les mécanismes de contrôle cellulaire. Ces mutations sur la molécule d'ADN, qui peuvent aussi être induites par des agents extérieurs (UV, produits chimiques, radiations, ...) sont majoritairement muettes et n'ont aucune incidence sur l'intégrité des protéines produites. Cependant, il arrive que la mutation touche une partie codante du gène entraînant la perte d'activité ou l'absence d'une protéine. Les conséquences sont la plupart du temps graves car elles entraînent des pathologies d'origine génétique, héréditaires ou acquises. Par exemple, la mucoviscidose est la plus fréquente des maladies héréditaires mortelles des populations européennes, et touche en France 1/3000 naissances environ. La mutation du gène sur le chromosome 7, la plus fréquemment identifiée (75% des cas), correspond à la délétion sur la protéine CFTR (Cystis Fibrosis Conductance Transmembrane Regulator) d'un seul acide aminé, une phénylalanine [1].

Parmi les pathologies acquises, les cancers issus de la division anarchique des cellules tumorales, représentent les plus importantes causes de décès dans les pays

développés. Cette pathologie est le résultat de mutations et de phénomènes complexes qui va aboutir à la division incontrôlée des cellules, selon un schéma expliqué de manière succincte par la suite. Hors de toute pathologie, dans un processus de développement classique, il existe un ensemble de gènes nommés « proto-oncogènes » qui agissent essentiellement lors de la période embryonnaire et participent aux phénomènes de multiplication et de différenciation cellulaire, par la production de diverses protéines (facteurs de croissance, protéines activant le cycle cellulaire, ...). Après cette période de développement, leur rôle est quasiment nul et très régulé. Cependant, ils peuvent être activés, par exemple à la suite d'une mutation, et sont susceptibles d'entraîner une synthèse excessive de la protéine correspondante, ainsi qu'une multiplication anarchique des cellules. Ces gènes sont alors nommés « oncogènes » [2]. Parallèlement, il existe un système de contrôle, au travers de gènes « sentinelles » qui vont détecter les mutations et le comportement anormal de la cellule pour induire soit une réparation du matériel génétique, soit le déclenchement du processus de mort cellulaire ou apoptose. Ces gènes appelés « suppresseurs de tumeurs », peuvent entraîner une évolution tumorale lorsqu'ils sont mutés à leur tour, la cellule étant libre de tout contrôle. Simultanément à la création de la masse tumorale, les cellules tumorales vont induire l'expression de facteurs stimulant la création de vaisseaux sanguins pour lui apporter oxygène et nutriments ; ce phénomène est nommé néo-angiogénèse. Une meilleure compréhension des gènes impliqués dans le développement et la croissance du cancer a donc permis d'envisager le traitement de ce genre de pathologies complexes par la thérapie génique.

Ainsi, alors que le concept de thérapie génique est né sur l'idée de traiter des pathologies héréditaires, il s'est rapidement orienté vers le traitement de toutes les

affections, héréditaires ou non. Depuis le premier essai clinique, on observe qu'environ 10% des essais se sont focalisés sur des affections diverses comme les infections virales, 20% sur le traitement de maladies héréditaires classiques, et 70% sur le traitement du cancer [3] (<http://www.wiley.co.uk/genmed/clinical/>) (Figure 1). Contrairement aux pathologies génétiques comme la mucoviscidose, la thérapie génique anticancéreuse ne nécessite pas obligatoirement une correction à long-terme des cellules [4]. En effet, de récents essais ont démontré une efficacité anti-tumorale suite, par exemple, à l'apport d'un plasmide codant pour le gène suppresseur de tumeur p53 [5, 6], ou en inhibant certains oncogènes [7, 8], ou encore en prévenant l'expression de facteur stimulant l'angiogénèse [9].

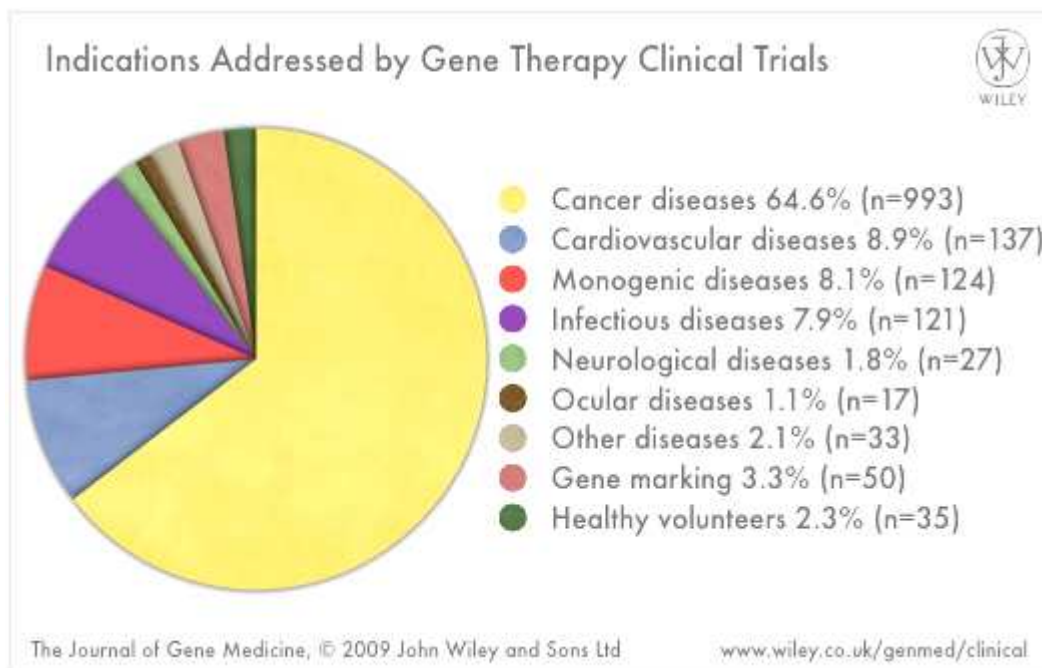


Figure 1. Répartition par type de pathologie des essais cliniques de thérapie génique en 2008.
Source : www.wiley.co.uk/genmed/clinical, 17/09/09.

1.2 - Les différentes stratégies de thérapie génique

Il existe quatre grands types de stratégies possibles en thérapie génique:

- **Apport d'une copie normale d'un gène muté**

Cette approche est la plus adaptée pour une mutation aboutissant à une perte de fonction et fut le centre d'intérêt de la majorité des essais cliniques menés à ce jour (www.willey.co.uk/genmed/clinical). C'est par exemple, le cas de certaines maladies monogéniques, telles que la mucoviscidose (mutation de la protéine CFTR), les myopathies (mutation de la distrophine pour la myopathie de Duchêne) ou les problèmes de déficience immunitaire (SCID Severe Combined Immuno Deficiency).

- **Modification de l'ARN messenger dans le but d'éviter les conséquences de la mutation**

Dans le cas de la thalassémie β , forme héréditaire de l'anémie, des mutations dans le second intron du gène de la β globuline vont créer un site d'épissage aberrant en 5' et activer un site cryptique d'épissage normalement inactif en amont. Ainsi, le pre-ARNm de la β -globuline thalassémique va être épissé presque exclusivement par ces sites d'épissages aberrants, aboutissant à une déficience en ARNm correct de la β -globuline, et donc en β -globuline elle-même. Des oligonucléotides ciblant les sites d'épissages aberrants générés par la mutation dans l'intron 2 du gène de la β -globuline ont été utilisés pour bloquer ces sites et restaurer un épissage correct en forçant la machinerie d'épissage à re-sélectionner les sites existants d'épissage [10]. Cette correction s'est accompagnée d'une traduction de l'ARNm et d'une β -globuline synthétisée sur toute sa longueur.

- Inhibition de l'expression d'un gène muté

Cette méthode est utile pour prévenir l'expression d'une protéine surexprimée. L'inhibition de l'expression d'un gène peut passer par différents types d'outils : les oligonucléotides antisens ADN (AS-ODN) ou ARN (ARN antisens), les ribozymes, l'interférence ARN (siRNA, miRNA, shRNA) [11]. L'utilisation de siRNA semble à ce jour être la plus utilisée pour éteindre un gène, ou plutôt son ARNm.

- Réparation d'un gène

Cette stratégie ultime et délicate a pour but de « reverser » une mutation. La technologie est basée sur l'utilisation d'une protéine chimérique composée d'un site de liaison spécifique à l'ADN et d'une endonucléase capable d'induire une coupure spécifique dans le double brin d'ADN. Simultanément, la séquence sauvage correspondant à la partie mutée de l'ADN est introduite dans la cellule et agit comme un substrat pour effectuer la réparation par recombinaison homologue [12].

1.3 - Le transfert de gène *in vivo*

Une fois le gène sélectionné pour son potentiel thérapeutique face à une pathologie, une étape cruciale de la thérapie génique est d'acheminer la nouvelle information génétique au plus près de son lieu d'action.

De nombreuses approches existent pour introduire un transgène chez un patient. Les stratégies les plus courantes sont les méthodes *ex vivo* et *in vivo*. La méthode *ex vivo* est basée sur la technique de transplantation cellulaire et celle-ci est applicable à tous types de tissus transplantables. Elle consiste à prélever des cellules cibles chez un patient, à les

cultiver de manière appropriée et à les traiter dans les conditions de culture cellulaire [13]. Les cellules ainsi transfectées sont réimplantées chez le patient. Cette méthode s'est révélée efficace pour transférer de nombreux types cellulaires (hépatocytes, kératinocytes, cellules endothéliales [14], fibroblastes). C'est la technique la plus couramment adoptée dans les essais cliniques. Elle a notamment été utilisée pour le traitement des déficits immunitaires sévères chez les "enfants bulles" [15]. Par ailleurs, elle permet de contourner les nombreux obstacles rencontrés lors du trafic extracellulaire du vecteur lorsqu'il est administré *in vivo*. Néanmoins, cette approche reste compliquée et coûteuse, de part les différentes technologies employées (chirurgie, culture cellulaire, ...).

L'approche *in vivo* consiste à administrer directement le gène médicament au patient. Diverses voies d'administration (systémique, locale) peuvent être envisagées. Cependant, l'efficacité de cette méthode est fortement compromise par les multiples barrières physiologiques que doit franchir le transgène pour atteindre sa cible. De plus, l'expression du gène est généralement diffuse (cas d'une injection par voie systémique) ou, au contraire, localisée au niveau du site d'injection. Par ailleurs, elle est souvent transitoire et nécessite des injections répétées.

1.4 - Les obstacles du transfert de gène *in vivo*

Le succès de la stratégie de thérapie génique repose en grande partie sur l'efficacité du transfert du gène médicament (transgène) vers son site d'action. L'idéal serait qu'il traverse efficacement et sans dégradation les nombreuses barrières biologiques jusqu'au noyau, afin de s'insérer en lieu et place du gène défaillant.

Cependant, quelque soit la voie d'administration (locale ou intraveineuse), le gène peut difficilement franchir seul les obstacles physiologiques rencontrés avant d'atteindre sa cible. En effet, les acides nucléiques sont des molécules polyanioniques hydrophiles de grande taille qui ne sont pas aptes à traverser les membranes plasmiques des cellules constituées d'une bicouche lipidique, hydrophobe et chargée négativement [16].

Ainsi, afin de parvenir à sa cible, le gène doit être associé à un système de vectorisation capable de le protéger des agressions du milieu biologique (en particulier des nucléases) et de le véhiculer au travers des différentes barrières physiologiques vers son site d'action. Enfin, le complexe vecteur/gène doit adhérer aux cellules, pénétrer dans celles-ci et délivrer le gène dans le noyau cellulaire. D'autre part, il doit préférentiellement cibler les tissus ou les cellules en dysfonctionnement et non les cellules saines.

Ce système vecteur est l'outil indispensable au succès d'une thérapie génique. Nous présenterons dans la partie suivante (1.5) les différents outils et systèmes vecteurs qui permettent de réaliser le transfert de gènes.

1.5 - Les outils du transfert de gènes

1.5.1 - Transfert d'ADN nu, les méthodes physiques

La première approche consiste en l'utilisation d'ADN nu, directement au niveau de la zone à traiter [17, 18]. Cependant, cette stratégie semble être limitée à une application aux organes accessibles par injection directe, tels que la peau et les muscles. Le transfert d'ADN nu peut toutefois être amélioré par différentes méthodes physiques tels que l'électroporation (perméabilisation de la membrane cellulaire par un courant électrique) [19], la micro-injection cellulaire ou tissulaire, ou encore le « gène gun » (bombardement d'ADN fixé sur des

particules d'or ou de tungstène vers la cellule)[18, 20]. L'injection de l'ADN *in vivo* sous la forme d'un grand volume d'ADN nu pour la transfection hépatique s'est montrée relativement efficace en utilisant une construction plasmidique comportant des éléments régulateurs spécifiques du foie [21]. Toutefois, cette méthode semble difficile à valider cliniquement compte-tenu du lourd volume à injecter (2ml en 5 à 7 secondes pour une souris de 20g). Excepté ce dernier exemple, l'utilisation d'ADN nu *in vivo* semble plutôt limité aux tissus accessibles, mais n'est pas adapté à la délivrance systémique [22] en raison de la présence des nucléases plasmatiques.

1.5.2 - Les vecteurs viraux

L'utilisation de virus modifiés pour transporter un gène thérapeutique repose sur le constat d'efficacité des virus pour transférer leur propre matériel génétique dans les cellules humaines. Pour produire des vecteurs viraux, on utilise des virus modifiés génétiquement, dits sécurisés. Le principe consiste à éliminer les séquences du virus qui codent des protéines, notamment celles associées à un éventuel comportement pathogène, et à ne conserver que celles qui sont utilisées pour construire la particule virale et assurer le cycle d'infection. Le génome du virus est reconstruit pour porter les séquences du gène thérapeutique. Les protéines virales qui potentiellement manqueraient à la formation des particules virales thérapeutiques sont fournies par des cellules dites productrices ou « d'encapsidation » lors de la phase de production des vecteurs. Différents types de vecteurs viraux sont utilisés.

Rétrovirus

Les rétrovirus possèdent un patrimoine génétique particulier sous forme de double brin d'ARN, ainsi qu'une enzyme spécifique, la transcriptase reverse (TR), qui permet le passage de l'ARN à l'ADN proviral [23]. Ces virus sont impliqués dans de graves pathologies humaines telles que les leucémies, ou le SIDA. C'est pourquoi les rétrovirus recombinants (généralement issus de virus murins) servant de vecteurs sont modifiés de manière à conserver leur capacité d'intégration du génome à la cellule hôte, tout en inhibant leur capacité de réplication. Le nouveau gène se transmet alors de cellules mères en cellules filles de manière égale, sans « dilution » de l'information génétique dans le temps. Cependant, de nombreux inconvénients sont liés à leur utilisation. Parmi eux, nous pouvons citer la faible capacité d'incorporation d'un exogène (8 kb) et le manque de spécificité cellulaire. En effet, les protéines de l'enveloppe sont capables de se lier à de nombreux récepteurs portés par différents types de cellules. De plus, l'intégration aléatoire de leur génome peut engendrer une mutagenèse conduisant à une anomalie du cycle cellulaire, et à l'activation d'oncogènes, comme dans le cas des "enfants bulles", ayant comme résultante le développement de leucémies [24]. Enfin, la plupart de ces virus n'infectent que des cellules en division, ce qui compromet fortement leur utilisation, étant donné que les cellules cibles de la thérapie génique (cellules souches sanguines, neurones, cellules musculaires, cellules du foie, etc.) sont le plus souvent des cellules qui ne se divisent pas ou très peu.

Cependant, des vecteurs dérivés du VIH totalement sécurisés, nommés lentivirus, permettent de pallier ce dernier inconvénient. En effet, ceux-ci sont capables de modifier génétiquement des cellules au repos, ouvrant ainsi des possibilités de manipuler toute une

gamme de populations cellulaires inaccessibles aux vecteurs rétroviraux dérivés de virus murins [25].

Adénovirus

L'adénovirus est un virus non enveloppé, de forme icosaédrique, possédant un double brin d'ADN. Il présente la caractéristique de faire pénétrer son matériel génétique dans la cellule cible sans attendre la mitose (division cellulaire) et sans insérer la nouvelle information génétique dans le génome de la cellule cible, permettant ainsi une transfection des cellules quiescentes, tout en évitant les processus de mutagenèse. L'inconvénient majeur lié à l'utilisation de ces vecteurs est la forte réaction immunogène de l'hôte, qui développe des anticorps anti-adénovirus empêchant ainsi une administration répétée de ces vecteurs [26].

Virus adénoassociés (AAV)

Les AAV possèdent un simple brin d'ADN et sont non pathogènes pour l'homme. Pour cette raison, leurs applications en thérapie génique n'ont cessé d'augmenter ces dernières années [26, 27]. Le principal avantage de ces virus est leur forte capacité à intégrer leur patrimoine dans la cellule hôte, permettant une expression prolongée du gène. Par ailleurs, ils sont capables d'infecter à la fois des cellules en division et des cellules quiescentes. Cependant, ces virus présentent des inconvénients majeurs, comme leur capacité de stockage d'un matériel génétique exogène limitée à 4,5 kb et une difficulté de production.

Autres virus

Au-delà des vecteurs précédemment décrits et fréquemment utilisés en clinique, de nombreuses tentatives d'utilisation de vecteurs à partir de virus sont décrits dans la littérature. Divers travaux concernant l'utilisation du virus Herpes Simplex (HSV) [28], des poxvirus (actuellement en développement clinique) [29], de virus animaux apparentés au VIH [30], ou du virus de la grippe, sont décrits dans la littérature.

Conclusion sur les vecteurs viraux

Les vecteurs viraux, du fait de leur profil naturel, sont très efficaces à la fois en terme de délivrance de gène et en terme d'expression. Pour cette raison, ils sont utilisés dans de nombreux essais cliniques (Figure 2). Cependant, ils souffrent de sévères inconvénients. En effet, l'intégration d'un exogène de grande taille est difficile. Certains engendrent des réactions immunitaires, d'autres peuvent redevenir pathogènes après mutagenèse. Enfin, leur production et leur manipulation sont compliquées et coûteuses. Tous ces facteurs ont encouragé les chercheurs à trouver une autre alternative, plus sûre.

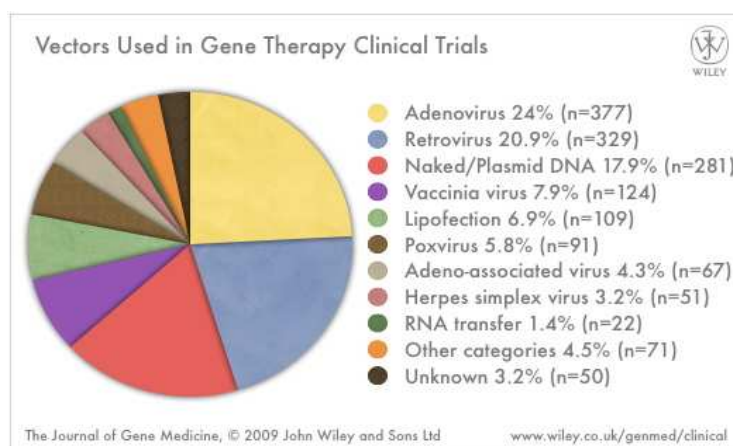


Figure 2 : Types de vecteurs utilisés lors des essais cliniques de thérapie génique en 2008.
Source : www.wiley.co.uk/genmed/clinical, 17/09/09.

1.5.3 - Les vecteurs synthétiques

Les vecteurs non viraux, également appelés vecteurs synthétiques, présentent plusieurs avantages dans le transfert de gènes comparativement aux vecteurs viraux. Ils sont peu toxiques, peu immunogènes, plus simples à élaborer et moins coûteux.

Vecteurs cationiques

Ces vecteurs synthétiques sont souvent basés sur le principe de complexation de l'ADN négatif par des molécules cationiques. Les vecteurs synthétiques peuvent être classés en deux grandes familles : les systèmes lipidiques et les systèmes polymériques, nommés respectivement lipoplexes et polyplexes. Les différents types de systèmes cationiques, les barrières rencontrées *in vivo* après une injection systémique, ainsi que les barrières intracellulaires seront plus largement développés dans la partie revue bibliographique [31]. Brièvement, l'ADN est compacté dans des objets globalement positifs qui vont présenter une bonne capacité de transfection *in vitro*, par le biais d'interactions électrostatiques. Cependant, cette charge positive peut représenter un inconvénient pour leur application *in vivo*. En effet, un objet chargé positivement est plus facilement reconnu par les cellules du système immunitaire car les protéines sériques vont venir s'adsorber à sa surface (Figure 3), aboutissant à la déstabilisation et/ou à l'élimination du vecteur. De ce fait, une dissimulation de la charge de surface des vecteurs est essentielle pour envisager une injection par voie systémique.

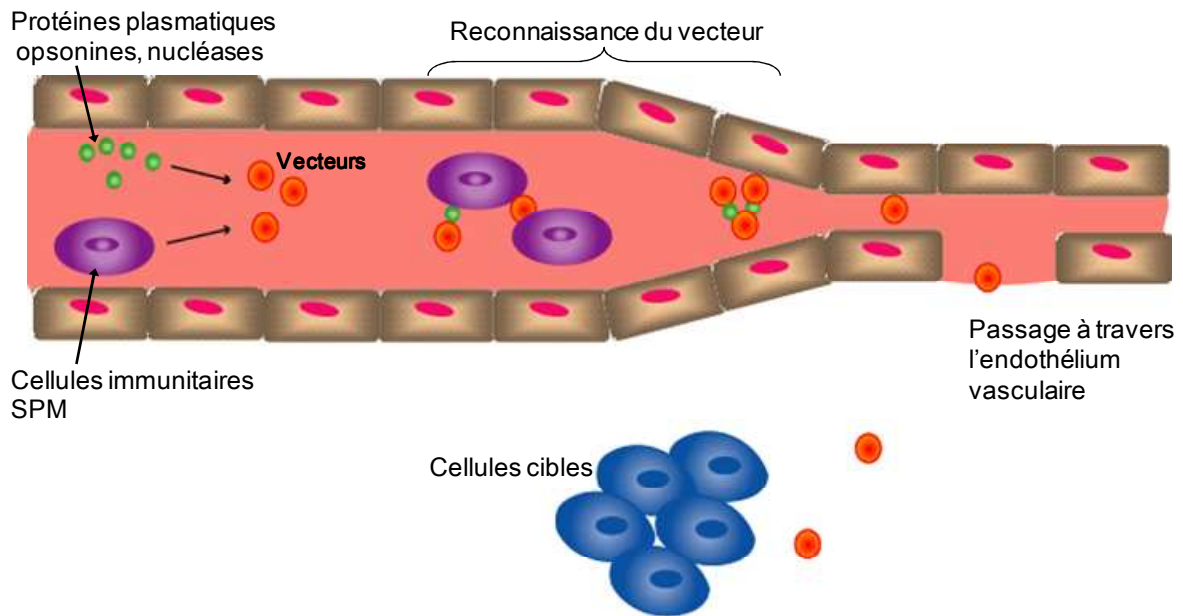


Figure 3. Représentation schématique des barrières rencontrées par un vecteur après son injection dans la voie sanguine.

Modification de surface, vers les vecteurs furtifs

En 1990, Klibanov *et al.* [32] démontrent pour la première fois que la présence de PEG (polyéthylène glycol) au sein de liposomes peut améliorer de manière significative leur temps de circulation. Suite à cette étude, de nombreux vecteurs furent recouverts de PEG, selon diverses méthodes, dans le but d'améliorer leur cinétique sanguine (Cf. Revue bibliographique, Table 2). En effet, ce recouvrement forme un réseau hydrophile à la surface des vecteurs, plus ou moins dense selon la concentration, et agit comme une barrière stérique vis-à-vis des protéines. En limitant les interactions hydrophobes ou électrostatiques avec le milieu extérieur et en augmentant ainsi la biodisponibilité des vecteurs, ce recouvrement les rend moins visibles aux yeux du système immunitaire, et en fait donc des vecteurs « furtifs » (exemple Figure 4).

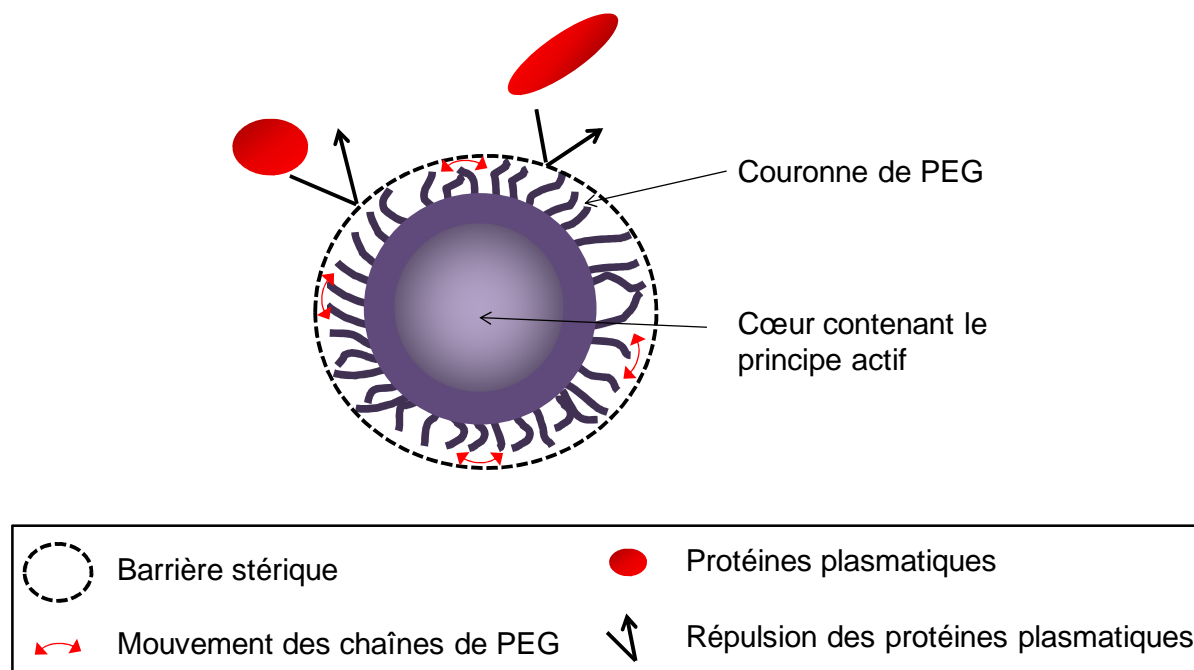


Figure 4 : Représentation schématique d'un vecteur furtif

Une dizaine d'année après la découverte de Klibanov, l'équipe de Campbell [33] découvre que des lipides cationiques stabilisés par des molécules de PEG sont capables de s'accumuler dans les tissus tumoraux à travers leur vascularisation lacunaire grâce à un phénomène de rétention propre aux tumeurs nommé effet EPR (Enhanced Permeability and Retention effect) (Figure 5) [34]. En effet, les tissus tumoraux sont caractérisés par plusieurs propriétés distinctes, telle qu'une hyper-vascularisation (permettant d'augmenter l'apport en oxygène et en nutriments nécessaires au métabolisme élevé des cellules cancéreuses), une architecture vasculaire déficiente, ainsi qu'un drainage lymphatique insuffisant. Ces caractéristiques font qu'un vecteur, s'il n'est pas immédiatement reconnu par le système immunitaire, aura plus de chance de s'accumuler et d'être retenu dans les tissus tumoraux que dans les tissus sains [34]. Cependant, il est également important de noter que le masquage des charges des polyplexes ou lipoplexes par les PEG se traduit aussi par une

réduction des interactions électrostatiques entre les complexes et les cellules, et diminue significativement les niveaux de transfection, aussi bien *in vitro* qu'*in vivo* [35, 36]. Ce point sera largement repris dans la partie discussion

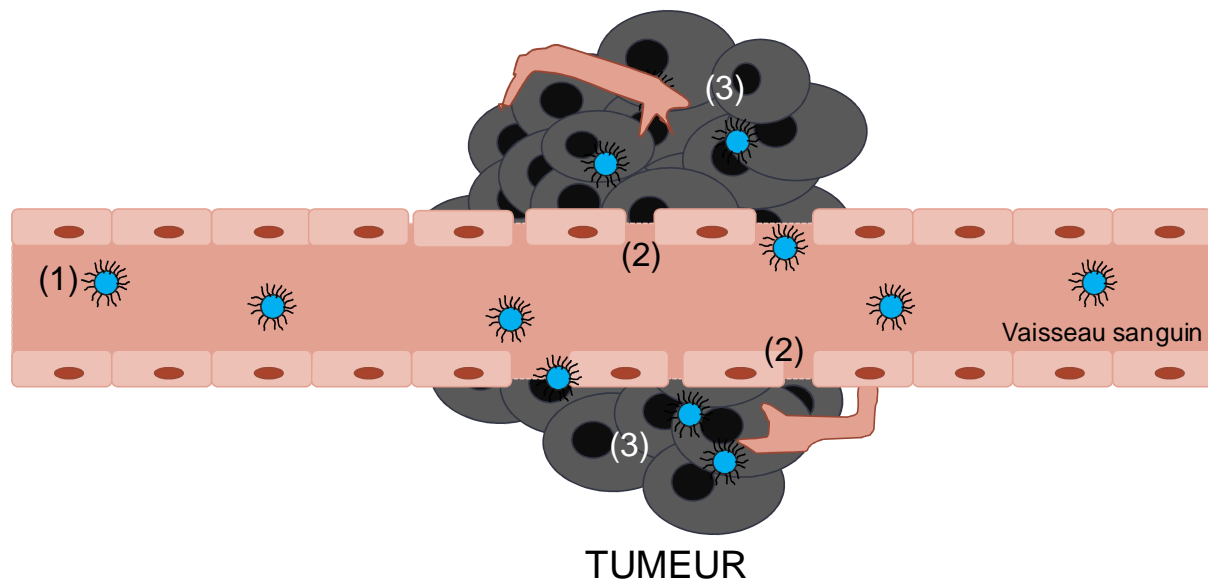


Figure 5. Représentation schématique de l'effet EPR. Les vecteurs à long temps de circulation (1) pénètrent à travers les jonctions endothéliales lacunaires au niveau de la tumeur (2) et y sont retenus du fait de la faible efficacité du drainage lymphatique, ce qui aboutit à une forte accumulation tumorale de ces vecteurs (3).

Conclusion sur les vecteurs synthétiques

La facilité de synthèse des vecteurs synthétiques fait que l'on peut créer des systèmes modulables, adaptables à chaque type de thérapie, de voie d'injection souhaitée, et de cible visée. Cependant, le transfert optimal du gène par ce type de vecteurs reste encore tributaire de nombreux obstacles, les vecteurs synthétiques ne représentant qu'un

faible pourcentage au sein des vecteurs utilisés en essais cliniques de thérapie génique (Figure 2).

Par ailleurs, lorsque les organes devant subir un traitement sont inaccessibles, la seule alternative est l'utilisation de la voie systémique. Or, l'injection par voie intraveineuse est un véritable challenge : la molécule injectée doit survivre dans la circulation, sans être dégradée ou capturée par les cellules du système immunitaire [37, 38] et atteindre son site d'action. Une fois arrivée sur le site, la molécule doit pénétrer la membrane cellulaire et passer les barrières intracellulaire (échappement endosomal, trafic cytoplasmique, entrée dans le noyau selon l'acide nucléique utilisé) (Cf. Revue bibliographique, Figure 4) [39, 40]. A ce jour, de nouvelles stratégies pour vectoriser les acides nucléiques sont donc attendues.

2 - Stratégies mises en œuvre dans le cadre de ce travail

2.1 - Objectifs

Ce travail de thèse a pour but de mettre au point des vecteurs d'ADN plasmidique efficaces pour le transfert de gène après injection par voie systémique, dans le but d'atteindre :

- **Les tumeurs**, par ciblage passif grâce à une accumulation par effet EPR dans les tissus tumoraux. Le gène porté au cœur de notre vecteur pourrait alors coder pour un gène suppresseur de tumeur dans le but d'obtenir un effet anti-cancéreux.
- **Le foie**, dans un essai d'application de ciblage actif, grâce à des vecteurs décorés de galactose, qui pourront cibler de manière spécifique les récepteurs à

l'asialoglycoprotéine (ASPGR) surexprimé sur la membrane cellulaire des hépatocytes [41]. Ces vecteurs pourraient alors s'accumuler au niveau du foie afin que la protéine traduite soit sécrétée par les hépatocytes.

2.2 - L'utilisation de nanocapsules lipidiques (LNC)

Dans ce but, nous avons travaillé à la synthèse et l'évaluation de deux types vecteurs (passif et actif), issus d'un système déjà connu et développé au laboratoire: les nanocapsules lipidiques [42].

Les nanocapsules lipidiques sont composées d'un cœur lipidique liquide de triglycérides entouré par une coque de tensioactifs amphiphiles et dispersées dans un milieu aqueux. Ces nanoparticules sont préparées suivant une méthode basée sur la variation de température autour de la zone d'inversion de phase d'une émulsion. Dans le but d'adapter ces vecteurs à la visée thérapeutique souhaitée, leur taille peut être contrôlée entre 20 et 100nm. Ces particules, préparées sans solvant organique, présentent une alternative intéressante aux systèmes de nanoparticules de polymère, émulsions et autres liposomes, notamment de part une très faible polydispersité et peuvent être utilisées par injection intraveineuse [43].

Au cours d'une thèse précédente, ces nanocapsules ont été légèrement modifiées afin de recevoir de l'ADN [44]. L'ajout de Plurol®, huile insaturée aux propriétés tensioactives, s'est révélé nécessaire à l'encapsulation d'ADN. Dans un premier temps, afin d'être encapsulé dans le cœur hydrophobe des LNC, l'ADN hydrophile doit être complexé à des liposomes cationiques de DOTAP/DOPE à un rapport de charge +/- de 5. Ces lipoplexes sont ensuite introduits dans la phase aqueuse en association avec les différents composés

de la formulation. Un remaniement se produit alors entre les lipides cationiques, l'ADN, et les lipides présents dans la formulation des LNC pour obtenir une encapsulation efficace de l'ADN dans le cœur des LNC, prouvée par électrophorèse et microscopie électronique (cryoTEM) [44]. Les objets obtenus, d'une taille de 100nm environ et d'une monodispersité suffisante ($PDI < 0.3$), sont nommés LNC ADN.

2.3 - Modification de surface des LNC

Afin de créer les différents types de vecteurs furtifs adaptés au ciblage passif et actif, la surface des LNC ADN a été modifiée par ajout de longues chaînes de PEG. L'objectif était alors de diminuer leur charge positive et d'augmenter leur temps de circulation sanguine, initialement trop insuffisant pour une application *in vivo* par voie systémique, quel que soit l'organe ciblé.

Au cours de cette étude, deux sortes de polymères ont été utilisées et comparées pour modifier la surface des LNC ADN :

- Le DSPE-mPEG₂₀₀₀ (Figure 7) :

Ce polymère est constitué d'une chaîne de PEG₂₀₀₀ (45 unités de PEG) liée d'un côté à une partie hydrophobe phospholipidique (distéaroylphosphatidyléthanolamine, DSPE) et portant à l'autre extrémité un groupement méthyle, ou une fonction réactive telle qu'une amine ou un hydroxyle permettant la fixation d'un ligand. L'insertion de DSPE-mPEG₂₀₀₀ dans les bicouches phospholipidiques procure aux liposomes de phosphatidylcholine un temps de demi-vie sanguine de plus de 20h après injection IV chez la souris. Chez l'homme,

l'aire sous la courbe (ASC) de liposomes pegylés chargés en doxorubicine est alors augmentée de 10 fois en comparaison à des liposomes classiques [45].

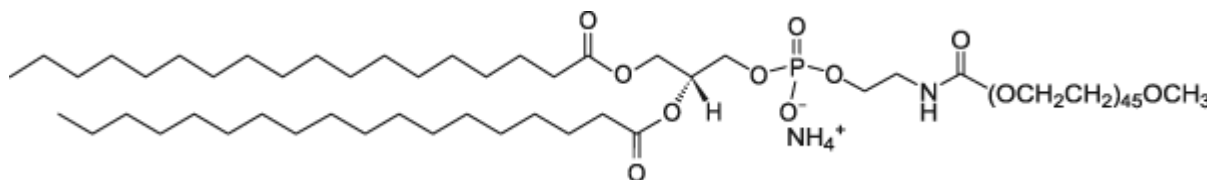


Figure 7. Structure du DSPE-mPEG₂₀₀₀

- Les copolymères à blocs F108 (Figure 8) :

Les copolymères amphiphiles de type poloxamère sont synthétisés par addition séquentielle de monomères d'oxyde de propylène (OP) et d'oxyde d'éthylène (OE) en présence d'un catalyseur alcalin [46]. Leur structure tri-sequencée est de type A-B-A : OE_x-OP_y-OE_x [32] avec des valeurs variables de x et y, respectivement de 132 et 50 unités dans le cas du F108.

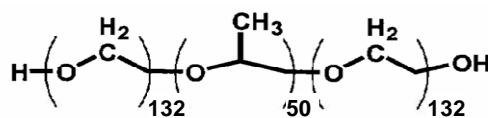


Figure 8. Structure du F108

Ce type de polymère, greffé à des polymères cationiques de type poly (éthylènimine) (PEI), permet de former des complexes stables donnant lieu à l'expression efficace d'un

transgène *in vivo* dans la rate, les poumons, le cœur et le foie après injection systémique [47, 48]. La stabilité de ces complexes est due à la formation d'une couronne d'OE à la surface des complexes qui va assurer leur stabilité dans les fluides biologiques. Par ailleurs, la partie hydrophobe OP est capable d'interagir avec les membranes lipidiques pour faciliter l'entrée des complexes dans les cellules [46]. En conséquence, l'utilisation de ce type de polymère à la surface des LNC ADN pourrait présenter de nombreux avantages en termes de stabilité et d'efficacité de transfection.

La première partie de ce manuscrit est consacrée à une **REVUE BIBLIOGRAPHIQUE** concernant la thérapie génique et les vecteurs de transfert de gènes, et plus particulièrement les vecteurs cationiques pour l'injection intraveineuse. Les divers obstacles rencontrés par un vecteur cationique dans la circulation, puis au niveau intracellulaire seront ici développés, ainsi que les solutions envisagées permettant de franchir ces barrières, et les adaptations possibles des vecteurs.

Les deux types de polymères choisis donneront lieu à deux types de vecteurs qui seront comparés tout au long de la **PARTIE EXPERIMENTALE**. Tout d'abord, les caractéristiques physico-chimiques des LNC ADN et la conformation des polymères à leur surface seront étudiées (**Publication n°1**). Puis, la capacité de ces vecteurs à s'accumuler de manière passive au sein de tissus tumoraux par effet EPR sera examinée dans la **Publication n°2**.

Dans un second temps, les deux types de polymères seront fonctionnalisés avec des motifs galactose permettant de cibler l'ASPGR. En effet, le greffage de galactose sur des systèmes multimodulaires a montré une spécificité de transfection au sein d'hépatocytes primaires de rat [49]. Cette spécificité sera évaluée sur nos vecteurs (**Publication n°3**).

Une **DISCUSSION GÉNÉRALE** sur l'ensemble de ces travaux permettra de faire un bilan de nos résultats, de présenter les résultats complémentaires non publiés et d'énoncer les perspectives de recherche à venir.

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REVUE BIBLIOGRAPHIQUE

Progress in developing cationic systems for non-viral vector systemic gene therapy against cancer

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SUMMARY

1 – Introduction

2 – Non viral vectors : current cationic systems

2.1 – Cationic polymers

2.1.1 – Poly (ethyleneimine) (PEI)

2.1.2 – Poly (L-Lysine) (PLL)

2.1.1 – Chitosan

2.1.1 – Dendrimers

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Abstract:

Initially, gene therapy was viewed as an approach for treating hereditary diseases, but its potential role in the treatment of acquired diseases such as cancer is now widely recognized. The understanding of the molecular mechanisms involved in cancer and the development of nucleic acid delivery systems are two concepts that have led to this development. Systemic gene delivery systems are needed for therapeutic application to cells inaccessible by percutaneous injection and for multi-located tumor sites, i.e. metastases. Non-viral vectors based on the use of cationic lipids or polymers appear to have promising potential, given the problems of safety encountered with viral vectors. Using these non-viral vectors, the current challenge is to obtain a similarly effective transfection to viral ones. Based on the advantages and disadvantages of existing vectors and on the hurdles encountered with these carriers, the aim of this review is to describe the "perfect vector" for systemic gene therapy against cancer.

1. Introduction

Cancer has become the first killer in developing countries and is on the verge of becoming the first cause of death in industrialized countries. Due to its invasive, aggressive growth profile as well as the complex mechanisms involved in cancer development and propagation, classical treatments such as surgery, chemotherapy and radiotherapy are still insufficiently effective in many cases, and are often up against resistant and infiltrating tumors. New anticancer strategies are thus urgently required.

A better understanding of the genes involved in the development and growth of cancer is leading to new approaches to treat this disease. Oncogenes and tumor suppressor genes, not working properly in most cancers, play a crucial role in the beginning and growth of the cancerous processes [1]. The approach of gene therapy provides a promising tool to eradicate this disease by treating it at its source. It can be particularly advantageous in that a relatively short expression of therapeutically active proteins may be sufficient to eradicate tumors, unlike genetic diseases such as cystic fibrosis in which there is a need for long-term expression [2]. Interestingly, between 1989 and 2004, cancer was the first candidate for gene therapy clinical trials (66% of all gene therapy trials) [3] (<http://www.wiley.co.uk/genmed/clinical/>). In the field of cancer gene therapy, four major targets have to be reached by vectors: turning off oncogene expression, enhancing tumor suppressor expression to induce apoptosis of cancer cells, inhibiting neoangiogenesis, and stimulating immune system against tumors cells.

Historically, there have been three different approaches applied to gene delivery. The first approach consists of the use of naked DNA. Direct injection of free DNA to the tumor site

has been shown to produce high levels of gene expression and the simplicity of this approach led to its use in a number of experimental protocols [4,5]. This strategy appears to be limited to tissues that are easily accessible by direct injection such as the skin and muscles [6] and is unsuitable for systemic delivery due to the presence of serum nuclease. The second approach involves using genetically altered viruses. Viral vectors are biological systems derived from naturally evolved viruses capable of transferring their genetic materials into the host cells. Many viruses including the retrovirus, adenovirus, herpes simplex virus (HSV) and adeno-associated virus (AAV) have been modified to eliminate their toxicity and maintain their high capacity for gene transfer [7] hence presenting various advantages [8–10]. Viral vectors are very effective in achieving high efficiency for both gene delivery and expression. However, the limitations associated with viral vectors, in terms of safety, immunogenicity, low transgene size and high cost, have encouraged researchers to focus on alternative systems. The third approach for delivery systems concerns non-viral vectors, which are mainly of a cationic nature: cationic polymers and cationic lipids. They interact with negatively charged DNA through electrostatic interactions leading to polyplexes and lipoplexes, respectively. The advantages associated with these kinds of vectors include their large scale manufacture, their low immunogenic response, the possibility of selected modifications and the capacity to carry large inserts (52 kb) (Table 1) [11,12]. While the transfection efficiency of nonviral vectors is still lower than that for their viral counterparts, a number of adjustments (e.g. ligand attachment) could improve this category of carriers which are, thus far, believed to be the most promising of gene delivery systems. Nonetheless, this class of vectors has to be modified to make systemic delivery possible. To date, systemic

administration has resulted in a toxic response (linked to their positive charge), incompatible with clinical applications.

Currently, the main objective in gene therapy via a systemic pathway now is the development of a stable and non-toxic gene vector that can encapsulate and deliver foreign genetic materials into specific cell types such as cancerous cells with the transfection efficiency of viral vectors.

In parallel to existing review in non-viral gene delivery against cancer ([13–16]), the aim of this work is to provide a nonexhaustive list of the cationic vectors currently developed for systemic delivery (for other administration pathways see reviews in Refs. [17–19]). The obstacles to their systemic injection and cell trafficking will be described. The possible strategies to overcome these problems will be argued thereafter.

2. Non-viral vectors: current cationic systems

2.1. Cationic polymers

2.1.1. Poly(ethyleneimine) (PEI)

PEI can be synthesized in different lengths, be branched or linear (Fig. 1), and undergo functionalized group substitution or addition. It is a versatile polymer which has a privileged place in the components of non-viral gene delivery, due to its superior transfection efficiency in a broad range of cell types compared to other systems described later. PEI polymers are able to successfully complex DNA molecules, leading to homogeneous spherical particles (Table 1) [20]. Studies showed that linear PEI with low molecular weight was the most efficient in transfection and the least cytotoxic [21]. Non-protonated amines

with different pKa values gave the PEI a buffering effect in a wide range of pH levels. This buffering property enabled the PEI to escape from the endosome due to the mechanism known as the 'proto-sponge' effect (enlightened later in this review in Section 3.2.2) [22]. However, the high amount of positive charges and their non-biodegradability resulted in fairly high toxicity of PEI polymers *in vivo* [23,24].

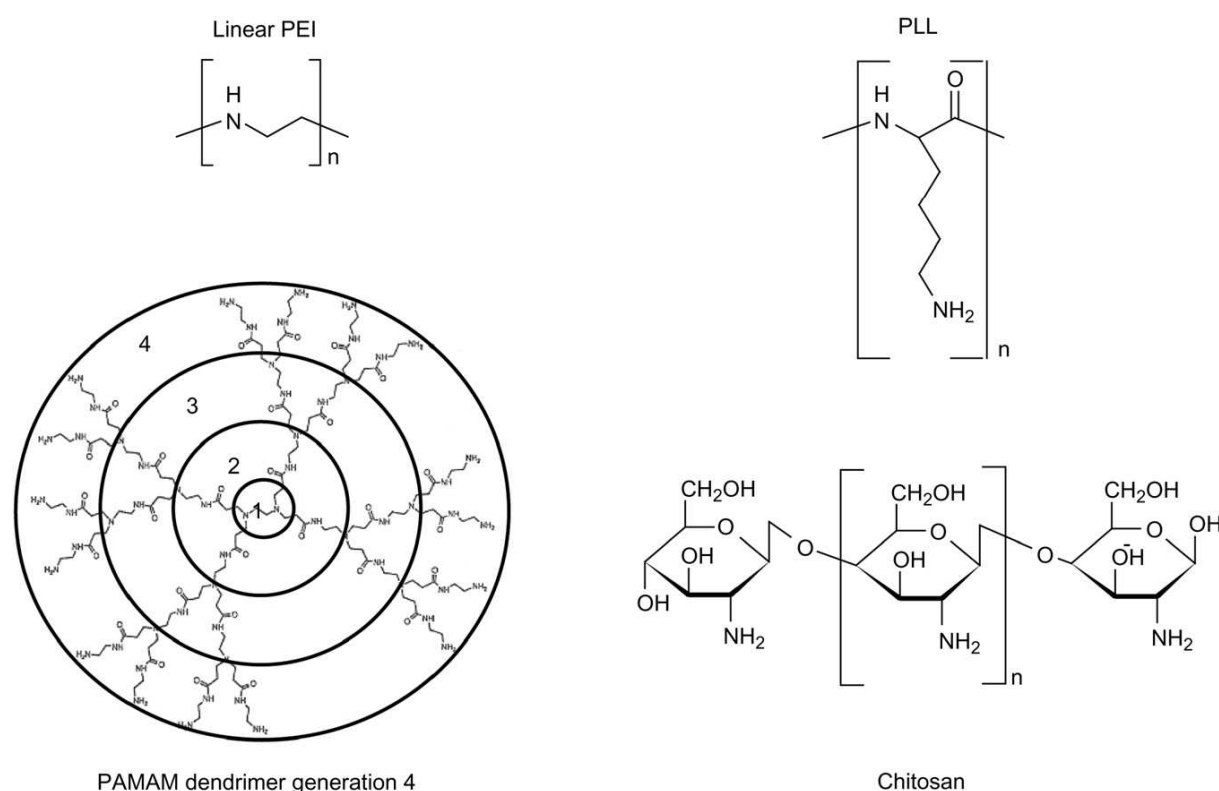


Fig. 1. Structures of current cationic polymers used in gene therapy. PEI $\frac{1}{4}$ poly(ethyleneimine), PLL $\frac{1}{4}$ poly(L-lysine), PAMAM $\frac{1}{4}$ poly(amidoamine).

2.1.2. Poly (L-lysine) (PLL)

Because of its peptide structure, PLL has a biodegradable nature, which is an advantage for *in vivo* use (Fig. 1). Until 2000, PLL was one of the most used cationic polymers for DNA delivery (Table 1) [25,26]. Having a low molecular weight (less than 3 kDa)

PLL cannot form stable complexes [27]. Furthermore, it appears that high molecular weight PLL is more suitable for gene delivery via systemic injection, with PLL 211 kDa/DNA complexes displaying levels in the blood up to 20-fold higher after 30 min compared to PLL 20 kDa/DNA complexes. Indeed, the complexes formed with low molecular weight PLL are fixed by the complement system *in vitro*, are less soluble *in vivo* (aggregation), and are thus rapidly removed by the Kupffer cells of the liver. Destabilization of these constructs in the blood is described as a possible mechanism for their removal from the blood circulation [28].

Cationic systems *	Particle Charge **	Particle size Range of diameter ***	Degradability	References
PEI - DNA	+30mV	20 to 130nm	No	[20-21]
PLL- DNA	+40mV	60 to 140nm	Yes	[25-28]
Chitosan - DNA	+25 to +37mV	20-500nm	Yes	[34, 37-42]
PAMAM dendrimer - DNA	+ 9 to +20mV	50–100 nm (generation 6-7)	Yes	[45-47]
DOPE/DOTAP	+ 30 to +50mV	60 – 120nm	Yes	[60]
DOPE/DC-Chol	+20 to +50mV	70 - 120nm	Yes	[60]
DOTAP/Chol	+50 to +60mV	100 - 126nm	Yes	[76]

Table 1: Physicochemical characteristics of current cationic systems

* PEI = poly(ethylenimine), PLL = poly(L-Lysine), PAMAM = poly(amidoamine), DOPE = 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine, DOTAP = 1,2-dioleoyl-3-trimethylammonium- propane, DC-Chol [N-(N',N' dimethylaminoethane)-carbamoyl] cholesterol, Chol = cholesterol.

** Depending on the +/- charge ratio

*** Depending on the method of preparation

The PLL/DNA polyplexes are internalized in a way comparable to PEI/DNA complexes, but their transfection efficiency is weak (due to a lack of amino groups allowing endosomolysis [15], cf Section 3.2.2).

A degradable PLL analogue, poly (α -[4-amino-butyl]-L-glycolic acid) (PAGA), showed significantly higher transfection efficiency than PLL, while no measurable cytotoxicity was detected [29]. For example, PAGA was used to deliver plasmid DNA (pDNA) encoding murine interleukin 10 (IL-10), via systemic injection in NOD (Non- Obese Diabetic) mice: the peak level of IL-10 expression was achieved at Day 5 after injection and gene expression lasted for more than 9 weeks [30]. The combined systemic administration of plasmid encoding IL-4 and IL-10 using PAGA to NOD mice also showed good expression levels [31].

2.1.3. Chitosan

Chitosan is a biodegradable and biocompatible linear aminopolysaccharide composed of 1-4 linked N-acetyl-D-glucosamine and D-glucosamine subunits (Fig. 1), obtained by deacetylation of chitin (a polysaccharide found in the exoskeleton of crustaceans and insects [32,33]). It can complex pDNA and is capable of forming stable, small (20–500 nm) particles depending on the molecular weight and the degree of deacetylation (Table 1) [34]. Its cationic polyelectrolyte nature provides strong electrostatic interaction with mucus, negatively charged mucosal surfaces and other macromolecules such as DNA [33,35]. This cationic polymer provides protection against DNase degradation that is comparable to PEI's one, and displays a significantly better biocompatibility [36]. Consequently, several groups have conducted studies using chitosan/ DNA nanoparticles, including use of galactosylated chitosan [37], galactosylated chitosan-graft-poly(vinylpyrrolidone) (PVP) [38], trimethylated

chitosan oligomers [39], N-dodecylated chitosan [40], deoxycholic acid modified chitosan [41] or ligand attached chitosans for targeting cell membrane receptors [42].

Cellular RNA interference machinery is now used in cancer gene therapy to turn off oncogene expression, for instance. This strategy is promising but there is a strong need to find an efficient vector enhancing the bioavailability of these small interfering RNA (siRNA) molecules. Chitosan nanoparticles have therefore been used to transport siRNA. Katas and Alpar [43] first studied the behaviour of interaction between siRNA and chitosans given that the structure and size of siRNA are quite different to that of pDNA. The ability of these nanoparticles to mediate gene silencing was assessed *in vitro* on CHO K1 and HEK 293 cells. Furthermore, an *in vitro* study revealed that the transfection efficiency of siRNA depended on its association with chitosan. Indeed, entrapping siRNA using ionic gelation showed a better biological effect than simple complexation or siRNA adsorption onto the chitosan nanoparticles. This might be attributed to strong interactions between the chitosan and siRNA (determined by gel retardation assay) and a better loading efficiency when using ionic gelation [43].

2.1.4. Dendrimers

Dendrimers are spherical, highly branched polymers. Dendrimers (from the greek dendron: 'tree' and meros: 'part') [44] are specific in that they have a hierarchical, three-dimensional structure. The heart of the molecule provides a central point from which monomers will ramify in a well-ordered and symmetrical manner. The tree-like construction is made by the repetition of the same sequence of reactions until the formation, at the end of every reaction cycle, of a new generation with an increased number of identical branches.

The most currently used dendrimers are polyamines, polyamides or polyesters, but the most commonly encountered is polyamidoamine (PAMAM) because of its high transfection efficiency (Fig. 1) (Table 1) [45–47]. Dendrimers bear primary amine groups on their surface and tertiary amine groups inside. The primary amine groups participate in DNA binding, compact it into nanoscale particles and promote its cellular uptake, while the buried tertiary amino groups act as a proto-sponge in endosomes and enhance the release of DNA into the cytoplasm. The size and diameter of dendrimers have an influence on their transfection efficiency. Thus, the transfection efficiency obtained with high generation dendrimers (10) is clearly superior to low generation dendrimers (5) [48]. Partially degraded PAMAM dendrimers are reported to have more flexible structures than intact dendrimers and therefore to interact more efficiently with DNA [49]. A fragmentation step consisting of hydrolytic cleavage of the amine bonds is needed to enhance the transfection efficiency [46,49–51].

2.2. Cationic lipids

Cationic lipids represent the second group of synthetic vectors commonly used in gene delivery. Since first being used for gene therapy in 1987 by Felgner *et al.* [52], numerous cationic lipids (also called cytofectins or lipofection reagents) have been synthesized and used for delivery in cell culture, animals, and patients enrolled in phases I and II of clinical trials (<http://www.wiley.co.uk/genmed/clinical/>).

Cationic lipids are technically simple and quick to formulate, readily available commercially, and may be tailored for specific applications. Cationic lipids are made up of a cationic head group attached by a linker to a lipid hydrophobic moiety. The positively charged

head group is necessary for the binding of nucleic acid phosphate groups. All cationic lipids are therefore positively charged amphiphile systems. They can be classified into various subgroups according to their basic structural characteristics (Fig. 2):

(1) monovalent aliphatic lipids characterized by a single amine function in their head group, e.g. N[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy-1-propanaminiumbromide) (DMRIE),

(2) multivalent aliphatic lipids whose polar head groups contain several amine functions such as the spermine group, e.g. dioctadecylamidoglycylspermine (DOGS),

(3) cationic cholesterol derivatives, e.g. 3β-[N-(N,N-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), bis-guanidium-tren-cholesterol (BGTC).

In general, reports indicate that the myristoyl (C14) chain is optimal for transfection (compared to C16 or C18 compounds), followed by the oleoyl chain (C18:1) [53,54]. Increasing the aliphatic chain length for amphiphatic compounds of this type is known to increase both the phase transition temperature and the bilayer stiffness of the resulting vesicles, and having a stiff bilayer is unsuitable for membrane fusion (which is an important step in DNA delivery mechanism) [53].

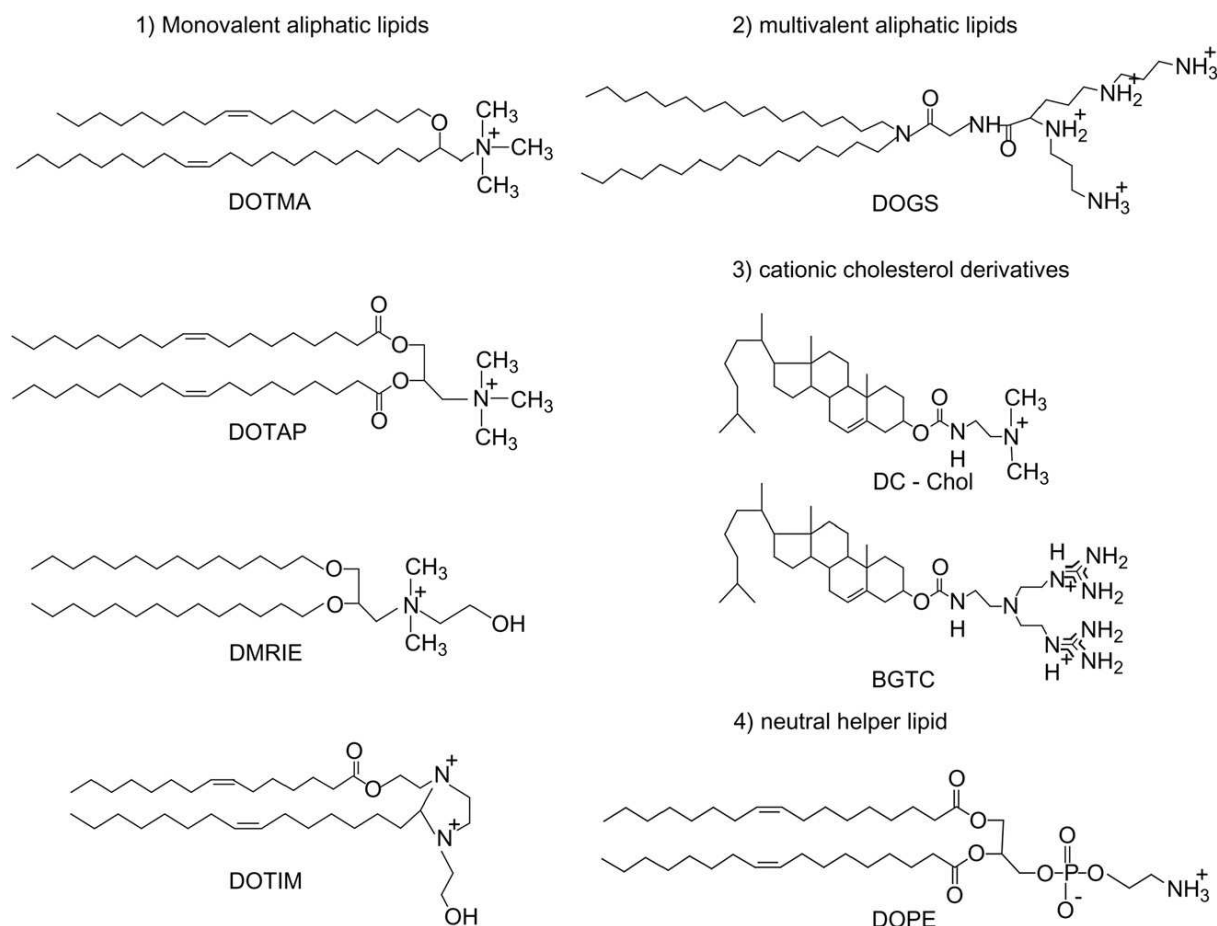


Fig. 2. Structure of current cationic lipids used in gene therapy and the helper lipid DOPE. DOTMA: N[1-(2,3 dioleyloxy) propyl]-N,N,N-trimethylammonium chloride, DOTAP: 1,2-dioleyl-3-trimethylammonium-propane, DMRIE: N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy-1-propananiam bromide), DOTIM: 1-[2-(oleoyloxy)ethyl]-2 oleyl-3-(2-hydroxyethyl)imidazolium chloride, DOGS: dioctadecylamidoglycylspermine, DC-Chol: [N-(N0,N0 dimethylaminoethane)-carbamoyl]cholesterol, BGTC: bis-guanidium-tren-cholesterol, DOPE: 1,2-dioleyl-sn-glycerol-3-phosphoethanolamine.

Adding DNA to cationic liposomes results in either lamellar or inverted hexagonal phase structure (Fig. 3). The lamellar form is a condensed and globular structure, consisting of DNA monolayers, characterized by uniform inter-helical spacing, sandwiched between cationic lipid bilayers [55], while the inverted hexagonal phase structure consists of DNA coated with cationic lipid monolayers arranged on a two-dimensional hexagonal lattice [56,57]. For transfection application, cationic lipids are often mixed with so-called helper

lipids, like DOPE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine) (Fig. 2) or cholesterol, both potentially promoting conversion of the lamellar lipoplex phase into a hexagonal structure, which is known to improve transfection efficiency [58,59]. It is worth noting that the ratio and combination of cationic/ helper lipids are important factors for transfection efficiency and toxicity [60,61]. In 1993, Zhu *et al.* [62] reported a relatively low *in vivo* gene expression by intravenous (i.v.) injection of pDNA complexed with DOTMA/DOPE liposomes. Additionally, DMRIE and DC-Chol were tested in clinical trials but the resulting therapeutic effects were disappointing and the formulation was hampered by toxicity [63]. After these first *in vivo* studies, several studies showed that various factors could enhance gene expression *in vivo*: a high cationic charge [64–67], cholesterol-containing liposomes [66,68–70], a high dose of pDNA [64,65,69,71–73], the absence of non-methylated CpG (dinucleotides composed of a cytosine followed by a guanine) otherwise recognized by the human immune system and that induces an inflammatory cytokine expression [74,75].

Systemic application of an improved extruded DOTAP/cholesterol cationic liposome formulation loaded with therapeutic tumor suppressor p53 pDNA resulted in successful treatment of primary and disseminated lung cancers in a mouse model [76]. More recently, a polycationic sphingolipid analogue was synthesized, containing a spermine head group. Interestingly, it was shown that the capacity of this compound to deliver antisense oligodeoxynucleotides (AS-ODNs) into cells, as reflected by an efficient anti-bcl-2 (apoptosis inhibitor) effect, was superior to that of vectors prepared from DOTAP or DC-Chol [77].

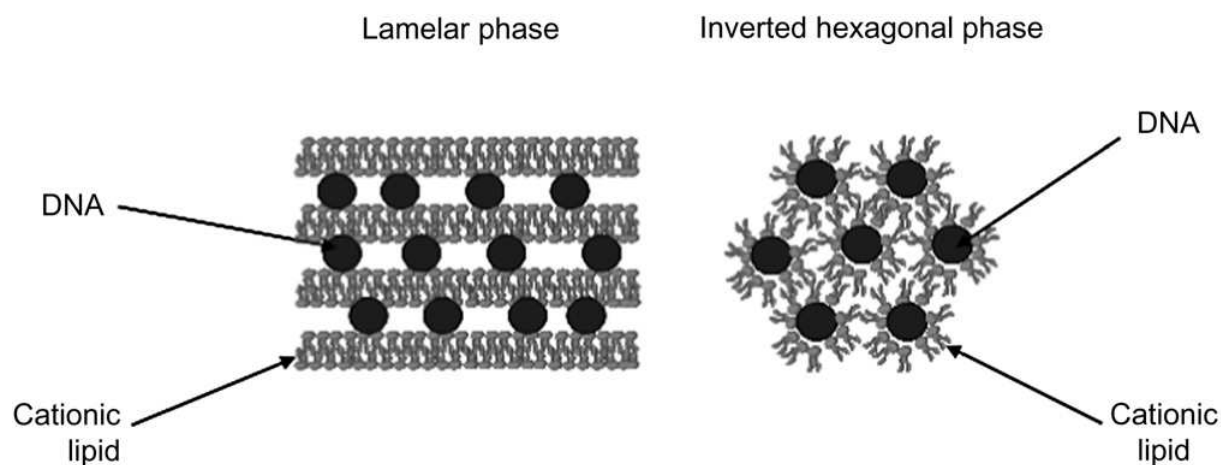


Fig. 3. Schematic representation of lamellar or inverted hexagonal phase structure in the formation of lipid/DNA complexes (lipoplexes).

Lipoplexes have recently been applied for siRNA delivery *in vivo* [78–80]. One example is the silencing of the hdm2 oncogene in p53 dependent human breast cancer [81]. Cationic lipoplex (prepared with cationic liposome containing 2-O-(2-diethylaminoethyl)-carbamoyl-1,3-O-dioleoylglycerol and egg phosphatidylcholine) formulations of siRNA targeting bcl-2 showed antitumor activity in two different tumor mouse models [82]. Peritumoral administration of these lipoplexes inhibited tumor cell growth of subcutaneously established prostate cancer, whereas an i.v. injection had strong antitumor activity in a mouse model of liver metastasis or lung carcinoma.

Landen *et al.* [83] encapsulated siRNA into neutral dioleoylphosphatidylcholine (DOPC) liposomes, which delivered siRNA efficiently not only into the tumor but also into other major organs, after i.v. infusion. In nude mice bearing intraperitoneal ovarian tumors, DOPC-encapsulated siRNA targeting the oncogene EphA2 was highly effective in reducing *in vivo* EphA2 expression 2 days after a single injection. Four weeks of treatment with EphA2 siRNA liposomes reduced tumor growth by about 50%. These results were reduced again by 15% when administered in association with the anticancer drug paclitaxel.

Another new approach used in clinical trials for cancer gene therapy is to employ immunological strategies, using the transfer of gene for cytokines. This concept was further developed toward the systemic application of lipoplexes. Dow *et al.* [84] evaluated repeated i.v. delivery of IL-2 pDNA lipoplexes [cholesterol/DOTIM (1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolium chloride)] (Fig. 2) in a pre-clinical phase I study in 20 dogs with chemotherapy-resistant osteosarcoma metastases. These injections were well tolerated, resulting in detectable IL-2 transgene expression in lung tissues and significantly increased overall survival times in treated dogs compared with historical controls at the same stage of disease. Three of the 20 dogs experienced partial or complete regression of lung metastases.

3. Principal hurdles for cationic systems

For disseminated cancer diseases, such as many cancer forms, treatment needs to be administered systemically and therefore must be targeted to the cancer cells. Systemic targeting of tumors via the bloodstream is a real challenge: the cationic systems have to survive in the bloodstream without being degraded or captured by cellular defense mechanisms [85–90]. Once at the tumor site, they have to extravasate into the tissue and bind specifically to the target cells. After their cellular internalization, intracellular barriers (endosomal escape, cytoplasm trafficking, nucleus entry) are additional hurdles, in which each of the listed steps can be a major bottleneck for the efficiency of such a gene delivery system (Fig. 5) [91,92].

3.1. Barriers to systemic gene delivery for cationic systems

The main obstacles in the use of polyplexes or lipoplexes via systemic delivery are their aggregation, instability, toxicity and their propensity to be captured by the mononuclear phagocyte system (MPS).

Numerous biodistribution studies show an accumulation of cationic systems in the lungs, liver and spleen. This accumulation can be explained by the fact that these non-viral gene delivery systems at therapeutic doses require high concentrations of lipoplexes or polyplexes and these positively charged particles readily aggregate as their concentration increases. These aggregates which are generally ineffective gene delivery agents can be toxic due to embolization of the particulates in the lung. Classical studies of colloid behaviour assume that particles of similar charge, possessing an electrostatic repulsion that is greater than the Van derWaals forces, are stable against aggregation. But in the case of DNA/cationic (lipid or polymer) complexes, this classical electrostatic stabilization model seems to be inadequate. A more complex model such as that of bridging gap between particles by extent polymerloops or by collision between electrostatic surfaces of opposite charges on the particles may be required [50]. The surface charge of the complex depends on the nature of the condensing agent and also on the ratio of the condensing agent to DNA. Numerous studies expose that the stability of DNA complexes under physiological buffer conditions is an important consideration in the design of particles, with particles presenting no aggregate showing the best transfection efficiency [50].

Furthermore, an accumulation of gene delivery vectors in the lungs could be explained by the electrostatic interaction of cationic systems with negatively charged

erythrocyte membranes [93]. In the same way, positively charged particles can be opsonized with plasma proteins such as immunoglobulin M, complement C3 and proteins of the coagulation cascade [20] leading to their rapid clearance by phagocytic cells of MPS in the liver, spleen, lungs, and bone marrow [94]. This opsonization can also activate the complement system, one of the innate immune mechanisms against 'foreign' particles within the bloodstream [95], which in turn activates the phagocytosis and initiates an inflammatory response against positively charged particles [96]. The clearance rate of these vectors from the circulatory system in fact depends on their physicochemical surface characteristics. To be 'stealthy' (undetectable by macrophages) [97], vectors have to be as small and neutral as possible [98].

At the same time, other serum proteins, in particular albumin, lipoproteins (HDL and LDL) and macroglobulin, interact with cationic lipid/nucleic acid complexes, and can alter the complex diameter and zeta potential (from positive to negative values) [99]. These interactions can provide destabilization of the system and nucleic acids' release which could be recognized by Toll-like receptor 3 (TLR3) (which recognizes double stranded RNA [100]), TLR7 and TLR8 (single stranded RNA [101]) or TLR9 (bacterial DNA [102]) expressed in B cells and plasmacytoid dendritic cells, inducing cytokine production at the origin of their toxicity [103]. A dose-dependent toxicity of different kinds of lipoplexes (GL-67 (N4-spermine cholesterylcarbamate) and GL-62 (N1-spermine cholesterylcarbamate), DMRIE (1,2-dimyristoyloxypropyl-3-dimethyl- hydroxyethylammonium bromide), DOTMA/DOPE) was observed, for instance, after injection in a mouse presenting hair erection and lethargy. Clinical studies have shown dose-dependent haematological and serological changes

typified by profound leukopenia, thrombocytopenia, and elevated levels of serum transaminases indicative of hepatocellular necrosis [103].

However, this strong immune response could be used to develop plasmid DNA vaccines for cancer [104]. As an example, systemic administration of lipoplexes [DOTIM (1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolium chloride) and cholesterol associated to DNA] can trigger strong activation of the innate immune response and release of high concentrations of T helper type-1 cytokines like IL-12 and interferon (IFN- γ) providing an enhancement of the immune reaction against tumor cells [104]. Additionally, systemic administration of cationic lipid/DNA complexes can display potent antitumor activity depending on both NK cells and IFN- γ . [105,106]. Moreover, lipoplex vaccination can elicit large numbers of functionally active and tumor-specific infiltrating CD8+T cells. Such an advance could be considered for patients whose tumors express known tumor antigens including melanoma, renal cell carcinoma, prostate cancer and colon cancer. This approach needs to be applied clinically and an optimization of the balance between induction of T cell-mediated antitumor immunity and induction of toxicity by over-stimulation of innate immune responses needs to be achieved [105,106].

3.2. Barriers to intracellular trafficking in gene delivery

Once the vector is at the tumor site, the tumor matrix, consisting of collagen and other proteins, is a further barrier to gene delivery, as the target cannot easily be reached because of limited diffusion of the vector within the tissue [107]. Depending on the type of tumor, the influence of the extracellular matrix composition and structure on macromolecule mobility can

vary significantly. For example, a 5 to 10-fold faster diffusion of large molecules (such as non-specific IgM, FITC/dextran 2,000,000MW or liposomes) was evidenced in cranial window tumor models compared to dorsal chamber tumor models [107]. Moreover, following this extracellular barrier, there are several intracellular hurdles (Fig. 4) as described below.

3.2.1. Internalization

The pathway followed by the cationic carriers, from the exterior of the cell to the nucleus, is not yet fully understood, but the fusion of vesicles with the plasma membrane is perceived as a better route, since it avoids the endolysosomal compartment (with its acidic environment resulting in DNA/RNA degradation). However, studies of electron and fluorescence microscopy have shown that lipoplexes and polyplexes can be detected in intracellular vesicles beneath the cell membrane, suggesting that they enter cells by endocytosis and will thus be directed toward the endolysosomal compartment [108–110].

There are a multitude of endocytic pathways that can be processed by the carrier systems: clathrin-mediated endocytosis via coated pits (adsorptive or receptor mediated), lipid-raft mediated endocytosis (caveolae-mediated or not), phagocytosis, macropinocytosis (Fig. 4) [111,112].

The predominant way of entry of cationic gene delivery systems seems to be by non-specific adsorptive endocytosis followed by the clathrin-coated pit mechanism, because negatively charged glycoproteins, proteoglycans and glycerophosphates, present on the cell membrane, are able to interact with the positively charged systems [113,114]. Using specific inhibitors of different endocytosis pathways, Rejman et al. [115] conclude that lipoplex (DOTAP/DNA) uptake can be proceeded only by clathrin-mediated endocytosis, while

polyplexes (PEI/DNA) can be taken up by two mechanisms, one involving caveolae and the other clathrin-coated pits. However, the internalization pathway seems to be dependent on the system used and the cells to transfect [116,117]. Carrier systems containing a specific targeting moiety, which are specifically recognized by a cell surface receptor, could enter cells via both adsorptive endocytosis and receptor-mediated endocytosis [118].

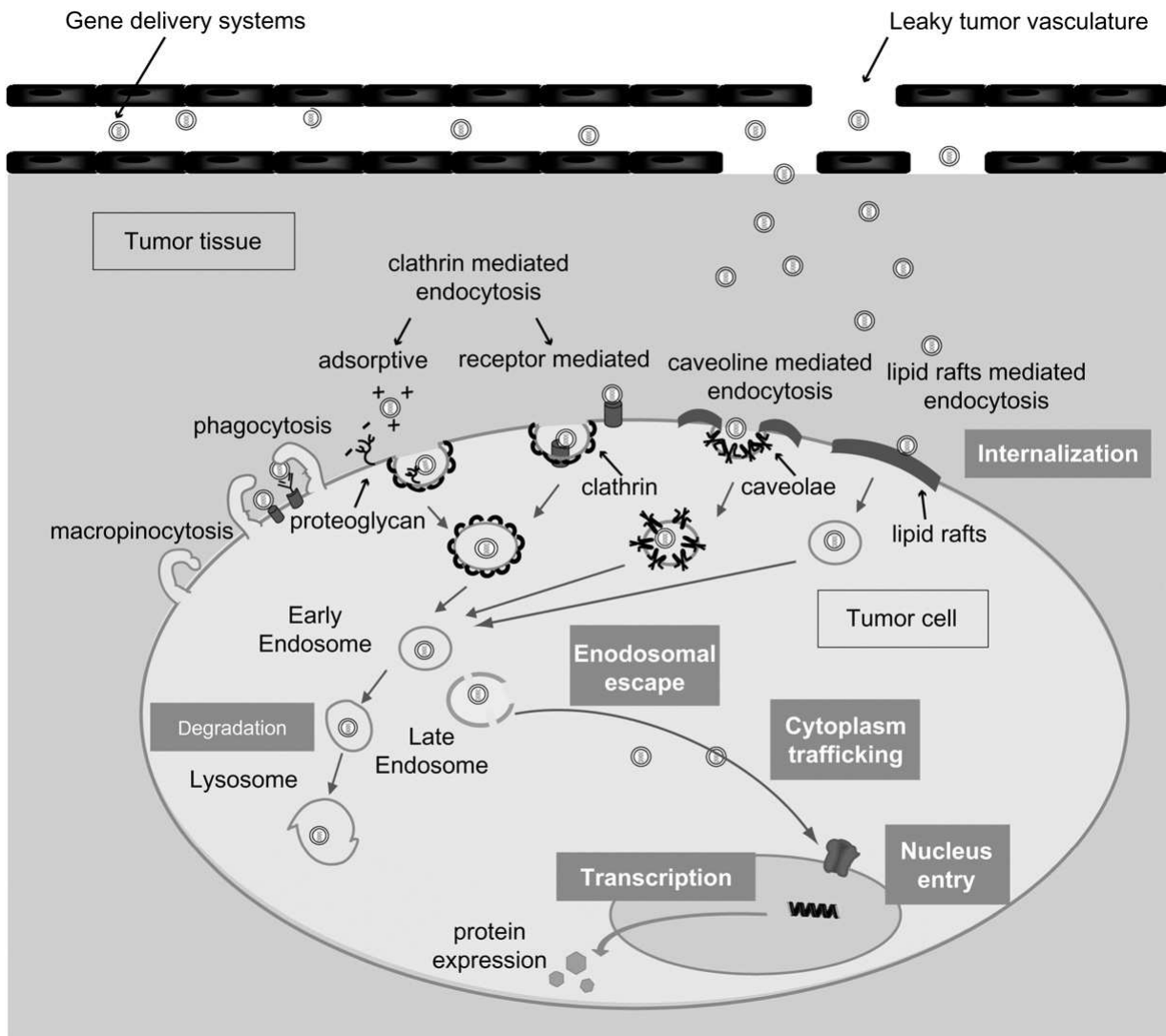


Fig. 4. Schematic representation of the different hurdles encountered by a gene delivery system to enter and traffic into a tumor cell.

Macropinocytosis can also mediate the uptake of cationic carriers because of its ability to internalize large structures such as bacteria [119]. Phagocytosis of lipoplexes and polyplexes, even in cell lines that are not professional phagocytes, has also been evidenced [120,121].

The relative contribution of each pathway in the internalization of synthetic vectors is poorly defined, given the large variety of carriers [122]. Therefore, factors such as cell membrane composition or surface charge and the size [123] of complexes may influence the balance in favour of either one or the other pathway.

3.2.2. Endosomal escape

If the gene delivery system enters the cells via an endocytosis pathway, endosome capture will represent a major barrier to efficient transfection. In order to be effective, the vector, or at least its content, has to be released from this compartment, preferably at an early stage.

The mechanisms involved in endosomal release of DNA by cationic polymer-based vectors are unsure. Two hypotheses have been suggested to explain this escape. The first one is based on the idea that a physical disruption of the negatively charged endosomal membrane occurs on direct interaction with the cationic polymer. Such a mechanism has been suggested for both PAMAM dendrimers and PLL [124]. Interestingly, this mechanism seems to depend on the target membrane composition (cell type). The second hypothesis used to explain endosomal disruption by cationic polymers with ionizable amine groups has been termed the “proton- sponge” hypothesis (Fig. 5) [22]. Endosomal membranes possess an ATPase enzyme that actively transports protons from the cytosol into the vesicle resulting

in acidification of the compartment [125]. The proton-sponge hypothesis assumes that polymers such as PEI and PAMAM, containing a large number of secondary and tertiary amines, can buffer the pH, causing the ATPase to transport more protons to reach the desired pH. The accumulation of protons in the vesicle results in an influx of counter ions which causes osmotic swelling and rupture of the endosomal membrane, in turn releasing the polyplexes into the cytoplasm [22,126,127].

In the case of cationic lipid-based vectors, another model has been proposed for local endosomal membrane destabilization, in which electrostatic interactions between the cationic lipids and the endosomal membrane induce the displacement of anionic lipids from the cytoplasm-facing monolayer of the endosomal membrane, by way of the so-called flip-flop mechanism (Fig. 5). The formation of a neutral ion pair between anionic lipids present in the endosomal membrane and the cationic lipids of the vector will then cause subsequent decomplexation of the DNA and finally its release into the cytoplasm [128,129]. Additionally, non-cationic helper lipids such as neutral DOPE facilitate membrane fusion and help destabilize the endosomal membrane [60,130,131]. It is indeed known that DOPE has a tendency to form an inverted hexagonal phase, often observed when membranes are fused.

Interestingly, it has been suggested that transfection with the multivalent lipopolyamine DOGS [22,132] or with BGTC containing a tertiary amine with a low pKa [133] involves escape from the endosome to a similar process to that proposed earlier for cationic polymers, PEI: the “proton-sponge” mechanism.

Despite these escape hypotheses, the majority of gene delivery systems seems to be stopped at this stage.

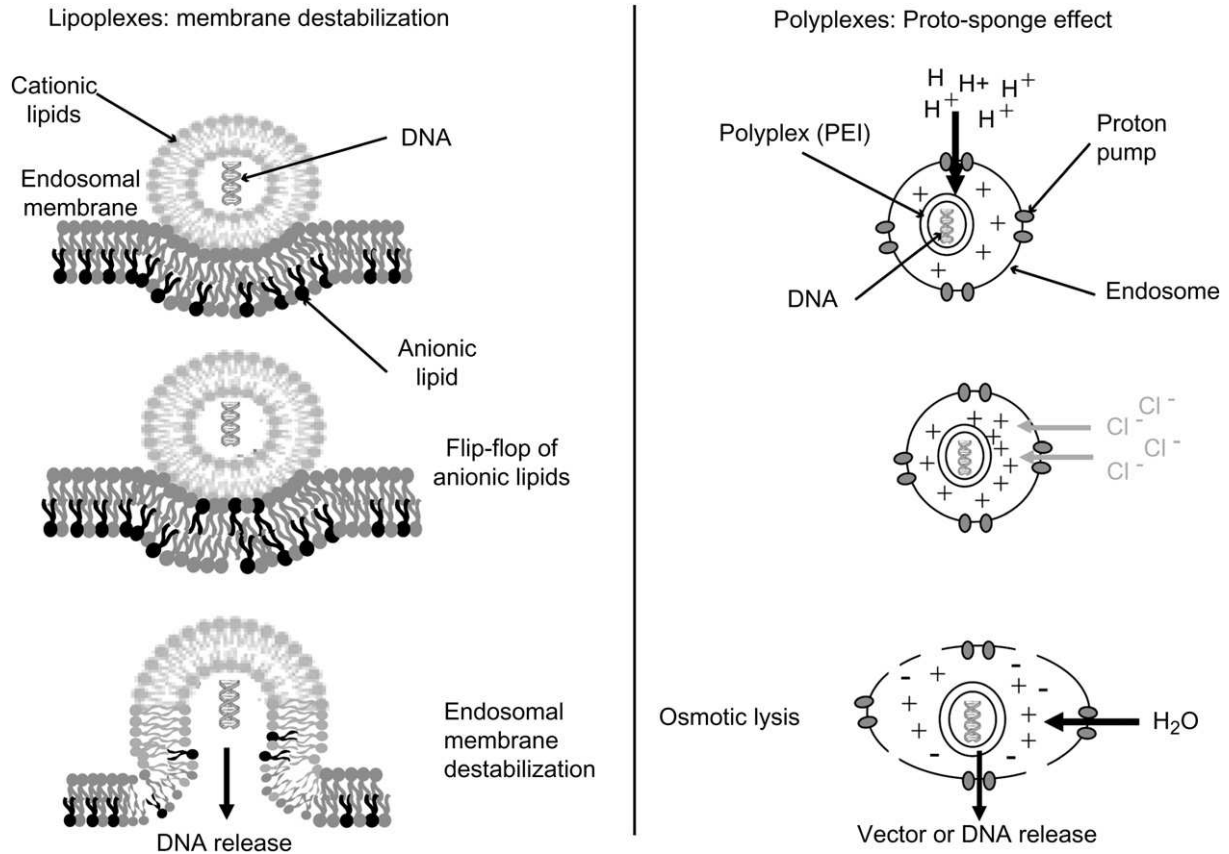


Fig. 5. Hypothesis of endosomal escape of lipoplexes' and polyplexes' gene delivery systems.

3.2.3. Nucleus entry

After endosomal escape, the nucleic acid must traffic through the cytoplasm and enter the nucleus. We could hypothesize that in the case of use of siRNA, this hurdle can be overcome since the interference mechanism arises in the cytoplasm. The mobility of large molecules, such as pDNA, is extremely low in the cytoplasm, making them an easy target for cytoplasmic nucleases [134]. Lechardeur *et al.* [135] reported that the half-life of a naked pDNA in the cytoplasm of Cos-7 and HeLa cells is 50–90 min. Thus pDNA has to be both protected and also available to enter the nucleus.

An important factor in nucleic acid transport through the cytoplasm is the rate of mobility which depends on the size and spherical structure of the molecule (circular plasmid DNA > linear DNA) [28]. But in the case of DNA complexed with gene delivery systems, the state of DNA when present in the cytoplasm is poorly documented and almost unknown. Indeed, the DNA molecules could be free or still associated to their carrier, and in a compacted state. This compaction could lead to increased cytosolic mobility or could offer increased stability against cytoplasmic nucleases. In the case of polyplexes, the microinjection of PEI/pDNA complexes resulted in a 10-fold higher levels of transgene expression compared to naked DNA, and showed that the enhanced expression may be the consequence of increased cytoplasmic mobility, due to the smaller size of the compacted DNA [136].

It is known that plasmids that are microinjected in the cytoplasm of cultured cells are poorly expressed whereas those that are microinjected directly in the nucleus are highly expressed [137]. These results indicate that nucleus entry is another important barrier (the final one) for efficient transfection. Indeed, DNA needs to access transcription machinery which is present inside the nucleus.

The nuclear envelope, a double membrane, is interrupted by large protein structures called nuclear pore complexes (NPCs). These proteins allow the passage of molecules up to 9 nm in size (40–60 kDa), but in the case of larger macromolecules, the transfer needs shuttle molecules and is energy-dependent [138]. The NPC is able to mediate the transport of ions, small molecules, proteins, RNA, and ribonucleoproteins in and out of the nucleus. Specific sequences on proteins expected to enter the nucleus, named Nuclear Localization Sequence (NLS), allow intracellular protein trafficking toward the nuclear pores [139]. The

first NLS described was the derived sequence of the simian cancer virus large T antigen [140]. These NLSs are recognized in the cytoplasm by a soluble protein, importin-a [139]. The complex of NLS/importin-a connects to another protein, importin b, and this trimeric complex then docks at the NPC and can enter the nucleus (Fig. 6) [141].

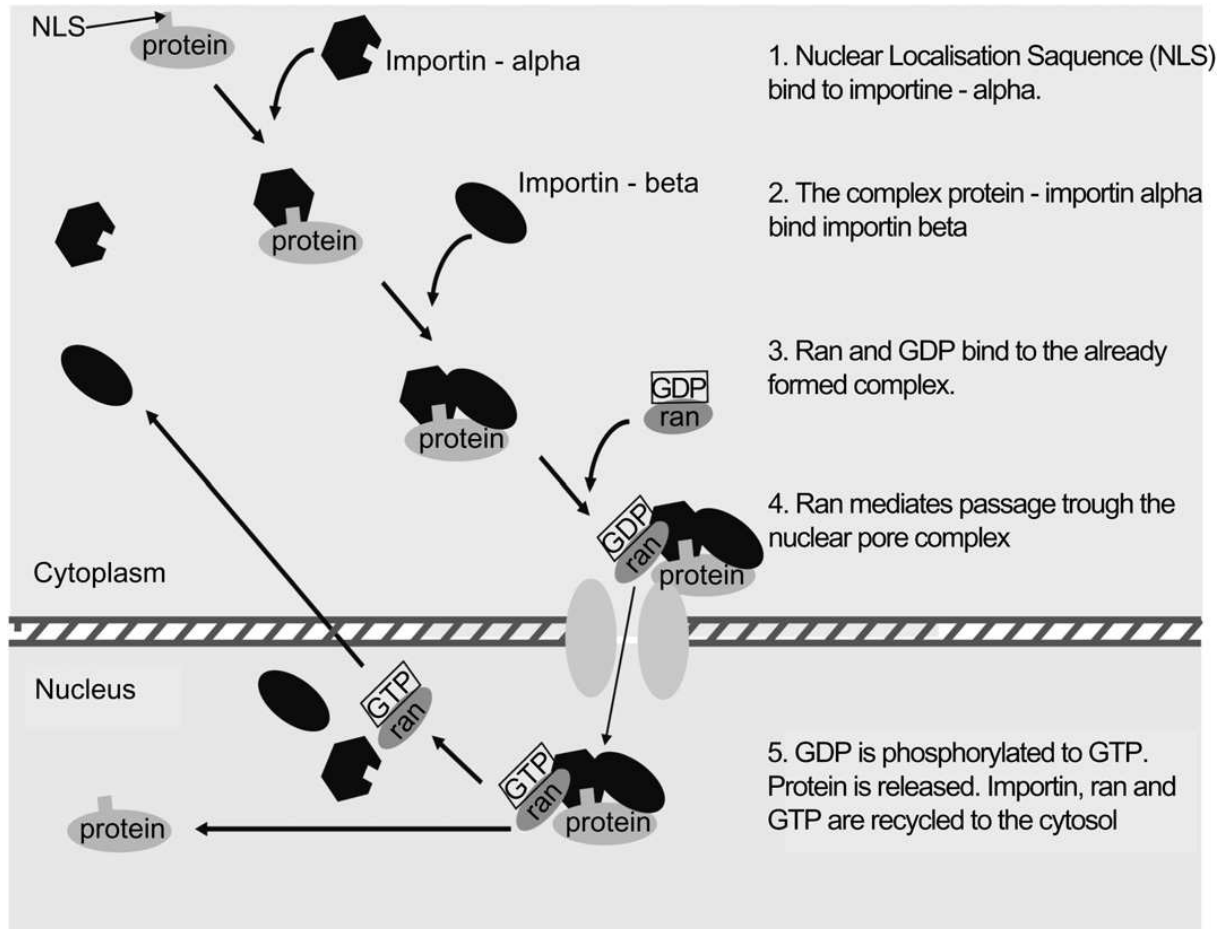


Fig. 6. Schematic representation of nuclear entry mechanism through nuclear pore complexes (NPCs).

Cytoplasm mixing and the loss of nuclear membrane during mitosis could be a way to overcome this problem. Consistent with this hypothesis, gene transfer in cultured cells has been shown to be greatly enhanced by mitotic activity for both lipoplexes [142,143] and polyplexes [143]. This would mean that non-dividing cells are rarely transfected, and this

could be a positive point for targeting tumoral cells, especially in the brain where healthy cells have no or low dividing activity.

4. Strategies to improve systemic delivery and intracellular trafficking of cationic systems

As seen previously (Section 3.1), systemic delivery represents a daunting hurdle in gene therapy and nowadays there is no general approach to systemic gene delivery. However, numerous efforts have been made to obtain effective and stable gene delivery systems and interesting and promising solutions have been found, as described below (Table 2).

4.1. Systemic delivery

4.1.1. DNA condensation in ternary system

These systems consisting of hybrid constructs with different polymers or between polymers and lipids have been synthesized by the precondensation of nucleic acids with a number of different possible polycationic agents. They have been developed to allow a smaller size and a better stability of the colloids during their stay in the blood.

An approach to ameliorate the complexation of nucleic acids developed by Hwang *et al.* [144] consists in associating cationic polymers including cyclodextrin rings within them. The cationic nature enables DNA complexation whereas cyclodextrin rings allow PEG binding.

In the same way, the interaction of pDNA with protamine sulfate, followed by the addition of DOTAP cationic liposomes, allows the creation of LPD (liposome/protamine/DNA)

to take place. Protamine is a naturally occurring polycation which condenses DNA in the head of spermatozoa. The nuclear localizing property of protamine makes it particularly attractive for transfection applications. Also, protamine sulfate is a small defined peptide system (4–4.25 kDa), that possesses a high affinity for DNA structure [145] and contains a short runs of basic amino acids known to act as NLS [89]. This small polycationic agent would be expected to show low probability of immunogenic responses in the target tissue due to the absence of aromatic amino acids and the lack of a rigid structure [146]. Compared to classic DOTAP/DNA lipoplexes, LPD offers better nuclease protection and gives consistently higher gene expression in mice via tail vein injection [147]. The E1A protein has been demonstrated to elicit antitumor effects through various mechanisms, including the induction of apoptosis and also sensitization to chemotherapeutic agents and radiation. The systemic delivery of LPD lipopolyplexes, encoding the E1A protein, to human xenograft tumor models for breast, head and neck cancer resulted in reduced tumor growth in both models [148]. The combination of systemic E1A pDNA therapy and paclitaxel chemotherapy strongly enhanced therapeutic effects and dramatically repressed tumor growth, in the case of a breast cancer model [149].

Several ternary systems, using other cationic condensing molecules, have been described in the past few years including systems based around mu peptide [150,151], PEI [152,153], PLL [154,155], spermidine [156], lipopolylysine [110], histone proteins [157,158], chromatin proteins [159], human histone derived peptides [90], oligo-L-lysine [160,161], L-lysine containing synthetic peptides [162] and a histidine/lysine (H-K) copolymer [163]. For example, a significant decrease in p53 protein biosynthesis in HepG2 and hepatoma 2.2.15 cells was only seen with PEI/DOTAP/Chol formulation but not with cationic liposomes or PEI

alone [153]. However, these systems are, like lipoplexes, positively charged and as seen previously (Section 3.1), when injected systemically, they risk to be sensitive to plasmatic proteins that lead to their destabilization and consequently the confrontation of nucleic acids to nucleases and/or their clearance by the MPS. The first *in vivo* results concerning LPD confirmed this hypothesis [64,164].

4.1.2. DNA encapsulation

4.1.2.1. *Polymer systems.* In parallel to the studies based on polyplexes, nanoparticle-based systems were developed. Nanoparticles are colloidal drug carriers ranging from 10 to 1000 nm in which a biologically active substance is entrapped, encapsulated or adsorbed onto the surface.

Nanoparticles formulated from biodegradable polymers such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acids) (PLGA) were extensively investigated as non-viral gene delivery systems due to their biocompatibility [165,166]. PVA (poly (vinyl) alcohol)/chitosan blends can be used to stabilize the PLGA nanospheres and form a homogeneous cationic population that can bind DNA on its surface by electrostatic interactions [165]. They can be used in association with PEI to decrease particle size [167,168]. Nevertheless, results of *in vitro* transfection and cell viability on HEK 293 cells [167] and in the human bronchial cell line Calu-3 [168] exhibited weak transfection as compared to PEI alone [167].

In 1991, Bertling *et al.* [169] first introduced cationic polyalkylcyanoacrylate (PACA) nanoparticles as drug delivery systems for pDNA delivery, where nucleic acids were also adsorbed on the surface. Later, a large variety of cyanoacrylate polymers were associated with nucleic acids: polybutylcyanoacrylate (PBCA), polyisobutylcyanoacrylate (PIBCA) [170–

172], polyisohexylcyanoacrylate (PIHCA) [170,171,173,174], and polyhexylcyanoacrylate (PHCA) [175].

Systems	Particles Size ^a	Particles Charge ^b	Coating PEG	Targeting Ligands	Applications ^{***}	Réf
Condensing systems						
Lipoplexes-protamine- DNA (LPD)	98-144 nm	+ 25 mV	×	×	E1A protein (<i>in vitro</i>)	[147-149]
Lipoplexes-mu-DNA (LMD)	90-150 nm	Not informed	×	×	Reporter gene (<i>in vivo</i> : local lung application)	[150,151]
Encapsulating systems						
PIBCA nanoparticles	250-450 nm	-40 mv	×	×	AsODN against EWS Fl-1 (<i>in vivo</i> : intratumorale injection)	[176,177]
Calcium phosphate nanoparticles	23-34 nm	+16 mV	×	×	Chimeric suicide gene yCDglyTK (<i>in vitro</i>)	[178]
Sponglike alginate nanoparticles	320nm	-34 mV	×	×	Radiolabeled ODN (<i>in vivo</i> : systemic injection)	[179]
Solid lipid nanoparticles (SLN)	300-800 nm	+40 mV	×	×	Reporter gene (<i>In vitro</i>)	[181-182]
Cationic copolymer (P123-g-PEI)-DNA complexes	110nm	Close to zero	PEO moiety of P123 blocks copolymer	×	Reporter gene (<i>In vivo</i> : systemic injection)	[204]
Stabilized plasmid lipid particles (SPLP)	90-110 nm	Not informed	PEG-Cer or PEG-DAG	×	Reporter gene (<i>In vivo</i> : systemic injection)	[208, 221-224,228]
Stable nucleic acids lipid particles (SNALP)	129-153nm	Not informed	PEG-C-DMA	×	siRNA against ApoB mRNA (<i>in vivo</i> : systemic injection)	[210]
Stabilized antisense-lipid particles (SALP)	80-140 nm	-1,8 to - 0,8 mV	PEG -Cer	×	AsODN against ICAM-1, c-myc and c-myb (<i>in vivo</i> : systemic injection)	[213]
Lipoplexes (AtuFECT01 + DPhyPE)	117 nm	+46 mV	DSPE-PEG	×	siRNA against PTEN siRNA against CD31 (<i>in vivo</i> : systemic injection)	[211-212]
PEGylated gelatin nanoparticles	290-320 nm	-7 mV	PEG-succinimidyl glutarate	×	pDNA VEGF-R1 (<i>in vivo</i> : systemic delivery)	[201]
Targeted systems						
Coated cationic liposomes (CCL)	70-100 nm	+7 to +10 mV	DSPE-PEG	mAb against-GD2 (Neuroectodermal tumor)	AsODN against c-myb (<i>in vivo</i> : systemic injection)	[214-215]
Sterically stabilized immunolipoplexes	89 ± 6.6 nm	+9,7 to 24,1mV	MAL-PEG-NHS	scFv against -Transferrin receptor	P53 gene (<i>in vivo</i> : systemic injection)	[238-240]
Pegylated PEI	90 ± 10 nm	+5 to +7 mV	PEG	RGD peptides (targeting tumor vasculature)	siRNA (VEGF R2) (<i>in vivo</i> : systemic injection)	[241]
Gal-C4-Cholesterol lipoplexes	141-235 nm	Not informed	×	Galactose (targeting hepatocytes)	pCMVluc (<i>in vivo</i> : intraportal injection)	[242, 243]
Lipoplexes-protamine- DNA (LPD)	Not informed	Not informed	DSPE-PEG	Anisamide (targeting sigma receptor over-expressed in human lung tumor cells)	AsODN and siRNA against survivin (<i>in vitro</i>)	[250]
PEG/PEI/DNA complexes	620-640 nm	+0,3 to 4,3 mV	PEG	Tumor-specific CNGRC peptide	pCMVp53 including SV40 DNTS and NLS binding site (<i>in vivo</i> : systemic injection)	[248,294]

Table 2. Example of promising systems for systemic gene delivery

mAb: monoclonal antibody, scFv: single-chain antibody fragment, pDNA: plasmid DNA, as-ODNs: antisense oligodeoxynucleotides, siRNA: small interfering RNA, DSPE-PEG : 1,2-distearoylglycero-3-phosphatidylethanolamine-N-polyethylene glycol, PEG-Cer: PEG-ceramide, PEG-DAG: PEG-diacylglycerol, PEG-C-DMA: 3-N-[(methoxypoly(ethyleneglycol)2000)carbamoil]-1,2-dimyristyloxy-propylamine, MAL-PEG-NHS: Maleimide-PEG-hydroxysuccinimide, AtuFECT01: cationic lipid, DPhyPE: 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine, DTNS: DNA nuclear targeting signal, NLS: nuclear localization signal peptide, SV40 : nuclear localization signal peptide.

^a Absence.

^b Depending on the method of preparation.

^c Depending on the +/- charge ratio.

^d The application to cancer therapy is indicated when it exists; otherwise, reporter genes are used (e.g. plasmid encoding luciferase, beta galactosidase, green fluorescent protein).

These cationic nanoparticles allow the neutralization of the negative charge of nucleic acids, allowing an efficient interaction with cellular membranes. As an example, Chavany *et al.* [173] carried cell uptake studies of ODN adsorbed onto PIHCA nanospheres. They showed that the uptake of the ODNs was dramatically increased when associated with nanospheres. After 24 h incubation, uptake of oligonucleotide was 8 times higher when adsorbed to nanospheres than when incubated as an ODN free. Regrettably, up to now, very little is known about *in vivo* applications of these drug carriers.

These kinds of adsorbing systems lead to a better cellular internalization compared to DNA alone, nevertheless the fact of exposing nucleic acid adsorbed on the nanoparticle surface prevents from the use of a systemic injection since the DNA will not be protected from the hostile DNase environment, and opsonins. Consequently, for systemic gene delivery, the idea of encapsulating nucleic acids inside polymer-based systems was rapidly developed to provide an efficient protection of nucleic acids and a long-term release in the required environment. Therefore, the development of nanocapsules containing nucleic acids in their aqueous core was considered. Nanocapsules were prepared by interfacial polymerization of isobutylcyanoacrylate (IBCA) in a W/O emulsion. The nanocapsules displayed a size of 350 ± 100 nm, a zeta potential of +40 mV and were able to encapsulate efficiently high amounts of phosphorothioate oligonucleotides (ODNs) directed against EWS Fli-1 chimeric RNA (a translocation found in 90% of both Ewing's sarcoma and primitive neuroectodermal tumor). Moreover, it permitted to obtain inhibition of Ewing sarcoma-related tumor in mice after intratumoral injection of a cumulative dose as low as 14.4 nmol [176,177].

Liu *et al.* [178] chose to formulate ultra low size calcium phosphate nanoparticles entrapping DNA molecules. The average diameter of the particles was approximately $23.5 \pm$

34.5 nm and they displayed a zeta potential of +16.8 mV. *In vitro* studies showed that these nanoparticles efficiently encapsulated the DNA, protected it from DNase degradation. Assays on suicide gene therapy (the delivery of a gene able to transform a pro-drug into an active drug, injected second time) displayed good results. However, these nanoparticles were not tested via systemic injection.

Aynie *et al.* [179] developed a process which led to the preparation of sponge-like alginate and polylysine nanocapsules. The resulting so-called nanosponges displayed a size of 320 nm and a zeta potential of -34 mV. Alginate nanosponges lead to an important protection of AS-ODN: 80% remained undegraded after 1 h incubation in fetal calf serum.

4.1.2.2. Lipid-based systems. A novel pDNA/nanoparticle delivery system (synthesized from emulsion) was developed by entrapping hydrophobized pDNA inside nanoparticles engineered from microemulsion precursors. Plasmid DNA was hydrophobized by complexing with cationic surfactants DOTAP and DDAB. Warm microemulsions were prepared at 50–55°C with emulsifying wax, Brij® 78, Tween® 20, and Tween® 80. Nanoparticles were engineered by simply cooling the microemulsions containing the hydrophobized pDNA in the oil phase to room temperature while stirring. Biodistribution studies have showed a long circulation time and, 30 min after tail vein injection to mice, only 16% of the ‘naked’ pDNA remained in the circulating blood compared to over 40% of the entrapped pDNA [180]. However, no transfection studies have been carried out *in vivo*.

For a decade, trials have been undertaken to utilize solid lipid nanoparticles (SLNs) as alternative drug delivery systems [181]. The suitability of cationically modified SLN as a novel transfection agent was investigated. Only one SLN batch composed of 4%

Compritol®ATO 888 (as matrix lipid), 4% Tween®80/Span® 85 (as surfactant) and 1% EQ1 (N,N-di-(b-steaorylethyl)-N,N-dimethylammonium chloride) (as charge carrier) was able to form stable complexes with DNA. Typical complexes were 300–800 nm in size, with a surface charge around +40 mV. Cytotoxicity and transfection studies exposed a good tolerance of the complexes, and an efficient transfection [181]. However, this transfection remained very low in comparison to conventional agents [182].

Taking into account the large size and the high charge of these encapsulating systems, their *in vivo* behaviour is uncertain. In fact, as seen previously (Section 3.1), size and charge are important factors influencing the activation of the immune system. Thus, for the majority of the cited encapsulating systems, biodistribution studies displayed a localization in the MPS organs and a subsequent distribution in the other organs [171,172,179]. These systems are therefore weakly efficient for the systemic delivery of nucleic acids; nevertheless it may be a useful method for local administration of a therapeutic gene, such as the pulmonary route or directly into a solid tumor.

4.1.3. Positive charge dissimulation

For increasing the efficiency of gene therapy via systemic delivery, it is necessary to mask the net positive charge of these carriers to enable the circulation of complexed DNA in the bloodstream. To avoid non-specific interaction with blood components, modification can be accomplished by “shielding” the vector surface with a hydrophilic and flexible polymer such as PEG. In 1990, Klivanov *et al.* [183] exposed for the first time that PEG use could enhance the circulation time of liposomes. After this, Campbell *et al.* [184] found that cationic lipids stabilized with the addition of PEG could accumulate in a tumor through its leaky

vasculature according to the “enhanced permeability and retention (EPR) effect”. Indeed, tumor tissues display several distinct characters such as hypervascularization, defective vascular architecture and a deficient lymphatic drainage system, which lead carriers to accumulate preferentially and to be more retained in tumor tissues than in normal tissues [185,186]. Today, numbers of the gene delivery systems (including lipoplexes, as well as polyplexes or nanoparticles) aimed at systemic injection are PEGylated to provide enhanced circulation time in the bloodstream [187]. This effect is the result of a high hydrophilic profile, combined with brush type polymer crowding and flexibility [98], which prevents from opsonisation and capture by macrophages. PEG capacity for repelling proteins and not interacting with macrophage plasma membrane is largely dependent on different parameters such as the molecular weight (MW), the density, the conformation and the flexibility of chain (for review see Ref. [98]). Most of the authors advocate an efficient MW in the range of 1500–3500 Da for decreasing protein adsorption *in vitro*, but concerning the macrophage uptake, chains have to be very long to be efficient (20,000 Da)[188]. To resume, the MW and the density are important criteria that can compensate each other and are related to each other in order to create a sufficient thickness limiting interaction with proteins and/or macrophages [189]. Moreover, the PEG molecules can be modified to improve the target specificity of the PEGylated particles [190].

4.1.3.1. PEGylated polymer-based systems. Numerous sort of modified PEIs have been synthesized in order to increase the polyplex circulation time within the bloodstream, and to decrease its toxicity [191–193]. Results showed that the degree of PEGylation and the molecular weight of PEG strongly influenced the properties of the resulting PEG/PEI

conjugates [194]. While most of the groups grafted the PEG onto PEI first and formed the DNA complexes in a subsequent step, Wagner *et al.* first condensed DNA with PEI 800 kDa and subsequently grafted on the hydrophilic polymer to provide a better condensation of the DNA with the polycations [20,93].

The covalent coupling of PEG can be carried out via the primary amino groups of the PEI molecules by reaction with the succinimidyl derivative of PEG. Recently, the PEGylation of dendritic PLL caused great changes in enhancing blood residence and reducing hepatic accumulation [195,196]. Another promising coated type of gene delivery system is a polyelectrolyte complex (PEC) micellebased vector. PEC micelles are the result of interactions between PEG conjugated oligonucleic acids (PEGylated ODNs) and a polycations (e.g. PEI). The negatively charged antisense c-Raf ODN interacts with polycations to form a neutral charged hydrophobic inner core of PEC micelles, while PEG segments are localized outside the core to form a hydrophilic shell. Thus, compared to ODN alone, the micelles showed a superior antiproliferative activity against ovarian cancer cells *in vitro* and *in vivo* when injected intratumorally [197].

Gelatin, a compound currently employed in pharmaceuticals and cosmetics, as well as food products, was also used to form 200 nm nanoparticles containing DNA. The amine residues of basic amino acids can be easily modified with PEG derivatives to confer long-circulating properties to the nanoparticles [198,199]. *In vitro* studies showed that these nanoparticles were internalized by nonspecific endocytosis pathway and able to deliver their content in the peri-nuclear region within 12 h. The transfection efficiency of these PEGylated gelatin nanoparticles was significantly higher than that for classic gelatin nanoparticles [200]. Very recently, Kommareddy and Amiji [201] showed an efficient *in vivo* expression of a pDNA

encoding for the soluble form of the extracellular domain of vascular endothelial growth factor receptor-1 (VEGF-R1 or sFlt-1) after intravenous administration in female nude mice bearing orthotopic MDA-MB-435 breast adenocarcinoma xenografts. These PEG-modified gelatin-based nanovectors are therefore promising systems for an effective systemically administered gene delivery vehicle in solid tumor.

Another important and promising example of polymer therapeutics concerns polymer micelles formed by amphiphilic block copolymers. As an example, Pluronic® block copolymers consist of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure: EO_x-PO_y-EO_x. These amphiphilic copolymers self-assemble into micelles in aqueous solution. Interestingly, *in vitro*, the transfection efficiency of poly (N-ethyl-4-vinylpyridinium bromide)/pDNA complexes and asialo-oroso-mucoid-poly (L-lysine)/ pDNA complexes was significantly increased in the presence of free Pluronic® F85 and Pluronic®F127, respectively [202,203]. In another study, a block graft copolymer synthesized by covalent conjugation of Pluronic® P123 and branched polyethyleneimine [P123-g-PEI (2K)] formed a stable complex with pDNA (110 nm), and exposed higher or similar gene expression than well-known lipid transfection reagents (Lipofectin®, Lipofectamine™, CeLLFECTIN®and DMRI-C) in the spleen, the lung, the heart and the liver 24 h after i.v. injection and produced relatively low toxicity [204]. Similarly, a system of P85-g-PEI (2K) was used for preparing ASODN complexes. This construct also accumulated almost exclusively in the liver, predominantly in hepatocytes, whereas only a small amount was found in the lymphocytes/monocytes' population [205]. The high stability is due to the formation of micellelike structures: electrostatic binding of the nucleic acids and PEI chains of the P123-g-PEI (2K) or P85-g-PEI (2K) results in the neutralization of positive charges of the cationic

polymer PEI and as a result, the formed particles bear a surface charge close to zero. Therefore, it is likely that these systems were stabilized in dispersion by the EO corona in a manner similar to classic Pluronic® micelles. Moreover, the PO chains in Pluronic® molecules are known to interact with lipidic membranes and induce their structural rearrangement. Contrary to EO chains, the presence of PO chains allows the carriers to translocate the carriers. Pluronic® based polyplexes, such as P123-g-PEI (2K) graft block polymer complexes, are promising vectors for gene delivery which have many advantages compared to 'simple' polyplexes (such as high stability in dispersion and high transfection activity).

PEG is currently the most used polymer in coating methods, but other hydrophilic polymers can be employed. Bharali *et al.* [206] synthesized poly (vinylpyrrolidone) (PVP) nanoparticles and demonstrated that these particles on i.v. administration could evade the MPS and remain in circulation for a considerable period of time. This hydrophilic polymer is known to be biocompatible, non-antigenic and is therefore safe for animal experiments [206]. DNA could be successfully encapsulated in PVP nanoparticles and could be protected from nucleases. The reporter gene pSVb-gal was encapsulated and the *in vitro* transfection efficiency of this system was found to be nearly 80% compared to the highly successful *in vitro* transfection reagent Polyfect® (derived from dendrimers). Further *in vivo* biodistribution studies have indicated that this system could be used safely for effective gene delivery [207].

4.1.3.2. PEGylated lipid-based systems. Stabilized plasmid lipid particles (SPLPs) are systems associating small size (100 ± 10 nm) and encapsulation. They are synthesized by a detergent dialysis procedure and consist of a unilamellar lipid bilayer containing (usually) the cationic lipid DODAC (N,N-dioleoyl-N,N-dimethylammonium chloride), the neutral helper lipid

DOPE, and PEG conjugated ceramides (PEG-Cer), surrounding a single copy of plasmid DNA [208]. Nevertheless, low transfection activity was observed.

Moreover, although SPLP showed potential for systemic gene transfer, the detergent dialysis method of preparation suffered from a number of limitations making any clinical application impossible [209]. A fully scalable and extrusion-free method (by spontaneous vesicle formation) was developed to rapidly prepare reproducible stabilized plasmid lipid particles. Additionally, this method accelerated SPLP formulation, enabling the rapid development and evaluation of novel carrier systems, also called as stabilized nucleic acid lipid particles (SNALP) when encapsulating siRNA. Using these systems, Zimmermann *et al.* [210] reported for the first time an siRNA-mediated silencing of the apolipoprotein B (ApoB) in response to systemic delivery in non-rodent species. In this study, SNALP were composed of lipids 3-N-[(methoxypoly(ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio. SNALP containing ApoB-specific siRNAs were administered by intravenous injection to cynomolgus monkeys at doses of 1 or 2.5 mg kg⁻¹. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. This study exposed for the first time a clinically relevant systemic RNAi-mediated gene silencing in non-human primates [210].

A novel cationic lipid AtuFECT01 (β -L-arginyl-2,3-L-diaminopropionic acid-N-palmityl-N-oleyl-amide trihydrochloride, Atugen AG), the neutral phospholipid 1,2-diphytanoyl-sn-

glycero-3-phosphoethanolamine (DPhyPE) (as fusogenic lipid) and the PEGylated lipidN-(carbonyl methoxy polyethylene glycol - 2000) - 1,2 - distearoyl - sn - glycerol - 3 - phospho ethanolamine sodium salt (DSPE-PEG) were mixed in a molar ratio of 50/49/1 to form a complex with siRNA named AtuPLEX (with a size of 117.8 nm and a surface charge of 46.4 mV). By using siRNA molecules for targeting endothelial specific expressed genes, such as CD31 and Tie2, Santel *et al.* [211] demonstrated downregulation of the corresponding mRNA and protein *in vivo* after repeated systemic i.v. administration (1/day for 4 days) in mice [211,212].

Other particle models were synthesized, such as the stabilized antisense-lipid particles (SALP), that have characteristics close to SPLP ones, but are constituted of multilamellar vesicles resulting in an ionizable aminolipid (1,2-dioleoyl-3-dimethylammonium propane, DODAP) and an ethanol-containing buffer system for encapsulating large quantities (0.15–0.25 g ODN/g lipid) of polyanionic ODN [213]. In the same way, Pagnan *et al.* [214] chose to formulate coated cationic liposomes (CCL) composed of (hydrogenated soy phosphatidylcholine (HSPC)), cholesterol, DSPE-PEG 2000, and DOTAP. These systems resulted from the shielding of cationic lipid/DNA complexes by a neutral liposomal membrane where PEG residues are inserted. This CCL associated with monoclonal antibodies targeting disialoganglioside GD2, extensively expressed in neuroectodermal tumors, can encapsulate AS-ODN against c-myc proto-oncogene and displays interesting antitumor effects *in vivo* [215].

4.1.4. Solutions to the limitations of PEG coating

On one hand, the PEG coating increases blood circulation time, which consequently leaves time for the objects to reach tumors thanks to the EPR effect [20,27,216,217]. But on the other hand, the PEG shield represents a major barrier for internalization and endosomal escape [218–220].

4.1.4.1. Use of removable PEG. One solution is to use a dynamic environment-responsive, movable PEG as exploited by one of the most promising vector, the SPLP. It has been established that the *in vitro* transfection potency of SPLP is dependent on the hydrophobic Cer group anchoring the PEG polymer to the SPLP, where Cer groups, containing shorter acyl groups, exhibited improved transfection properties [208]. This is attributed to the ability of PEG-Cer molecules with shorter acyl groups to dissociate from the SPLP, thereby destabilizing the particle and improving association with and uptake into target cells [221]. The transfection levels achieved for SPLP containing PEG-CerC8 were substantially higher than those for SPLP containing PEG-CerC14 or PEG-CerC20, this being consistent with the necessity for the PEG-Cer to dissociate itself from the SPLP surface for maximum transfection efficiency [222]. It should be noted that the SPLPs were recently prepared using a series of PEGdiacylglycerol lipids (PEG-S-DAG). These constructs are more easily synthesized and exhibited extended circulation lifetimes and tumor- selective gene expression compared to SPLP containing the PEG-Cer [223]. This kind of SPLP (PEG-S-DAG or PEG-Cer) has been shown to bypass the so-called 'first pass' organs (including the lung, liver and spleen) and elicit levels of gene expression in distal tumor tissues 100 to 1000-fold greater than that observed in normal tissues. Furthermore, some improvements in

transfection were noted when short PEG polymers (PEG 750) were incorporated into the PEG-Cer rather than PEG 2000 or PEG 5000 polymers [222]. SPLP systems are an example of the ambiguous role of PEG coating. A compromise has to be found between a sufficient circulation time in the bloodstream and good transfection efficiency [224].

Additional to the internalization problems engendered, PEG coating could also impede the endosomal escape of the gene delivery system. To resolve this problem, the neutralizing PEG shield could be attached to the polyplex core via an acid labile linkage that, confronted with the acidic environment of the endosome compartment, will be hydrolyzed, providing the shielding destabilization. Chemical linkages that may display pH-dependent hydrolytic degradation once internalized into endosomal and lysosomal compartments include acetals [225,226], vinyl ether [227], ortho esters [228] and hydrazones [229].

To improve internalization and endosomal escape, Walker *et al.* [230] chose to link PEG to PLL and PEI via a pH-sensitive hydrazone bond. The targeting ligand transferrin was also included to the polyplexes. Their *in vitro* and *in vivo* studies showed that receptortargeted polyplexes generated with this kind of PEG led to dramatically higher transfection efficiency in targeted cells compared to those generated with stable shielded control polyplexes. In the same way, Choi *et al.* [228] chose to use an acid labile PEG-diorthoester- distearoylglycerol lipid (POD) mixed with a cationic lipid (DOTAP) and phosphatidylethanolamine (PE) to prepare SPLP (POD-SPLP) that could mediate *in vitro* gene transfer by a pH-triggered escape from the endosome. This POD-SPLP exposed up to 3 times greater gene transfer activity *in vitro* than pH-insensitive nanoparticle. Moreover, both the pH-sensitive and the pH-insensitive nanoparticles were internalized to a qualitatively similar extent, underlining that increased gene transfer of the POD-SPLP was due to a faster

escape from the endosome rather than to greater cell association of these nanoparticles [228].

4.1.4.2. Cell-specific targeting, ligand attachment. To avoid the problem of non-specific interaction and to overcome the difficulties encountered with PEG coating, a target-specific ligand can be added to the gene delivery system (Table 2) resulting in active targeting and receptor-mediated endocytosis [218,231,232]. Gene delivery vectors should allow specific cell types to be targeted by utilizing the interactions between cell surface receptors and ligands present on their surface. These ligands can be small molecules (e.g. folate, galactose, low-density lipoprotein (LDL), fibroblast growth factor (FGF) receptor binding peptides, etc.) [196,233–235] or peptides and proteins (e.g. transferrin or antibodies). Numerous studies have been carried out. For example, transferrin is a common ligand used to target tumor cells [236,237]. Repeated systemic application of surface shielded transferrin-polyethyleneimine (Tf- PEI)/DNA delivery systems promoting the expression of the tumor necrosis factor (TNF)- α (carrying immunostimulatory and cytotoxic properties) into tumor-bearing mice induced tumor necrosis and inhibition of tumor growth in four murine tumor models [238]. Because the expression of TNF- α was largely localized within the tumor, no significant systemic TNF-related toxicities were observed. In the same way, Xu *et al.* [239] chose to incorporate an anti-human transferrin receptor single-chain antibody Fv fragment (TfRscFv) into the lipoplexes (DOPTAP/DOPE/DOPC) as an alternative targeting ligand. More recently, they developed an improved formulation with a sequential conjugation of lipoplexes with PEG and TfRscFv, and demonstrated enhanced expression *in vivo* of genes encoding p53 and green fluorescent protein (GFP) in prostate tumors [240].

With the goal of targeting tumor vasculature, RGD peptides (specifically targeting the up-regulated integrin receptors in certain tumors) were attached to a PEGylated PEI system containing siRNA against vascular endothelial growth factor (VEGF) receptor-2 [241]. Intravenous administration into tumor-bearing mice resulted in selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor and the inhibition of both tumor angiogenesis and growth rate.

Lipoplexes grafted with galactose (Gal-lipoplexes) were used to target hepatocytes through specific recognition by the asialoglycoprotein receptor and to reduce non-specific uptake by Kupffer cells [242,243]. In the same way, Reddy *et al.* [244] and Hofland *et al.* [245] chosen to use folate to target folate receptor known to be over-expressed in a large variety of human tumors and basically expressed in normal tissues. They succeeded in developing folateconjugated lipoplexes that exposed *in vivo* transgene expression in mice tumors.

Epidermal growth factor (EGF)-receptor expression is often increased in breast or prostate cancers, making it a good candidate for targeting gene-transfer complexes. Blessing *et al.* [246] used an EGF-targeted PEI-based system and obtained highly increased transgene expression *in vitro*. ErbB2, a tumor marker that is highly up-regulated in many human breast and prostate cancers, was targeted with a delivery system (protamine and cationic lipids) containing a single-chain antibody [247]. It should be noted that when using antibody, the lack of the Fc-fragment of the antibody can avoid recognition by macrophages and thus clearance from the bloodstream. In order to target the tumor-specific marker PSMA (prostate-specific membrane antigen), an anti-PSMA monoclonal antibody, J591, was generated and showed cellular internalization. Therefore, Moffatt *et al.* [248] envisaged that a

J591/PEI/DNA β -gal vector could be harnessed in order to target PSMA in the prostate tumor. The *in vivo* organ distribution profile revealed gene expression predominantly in the tumor. Whatever the choice of ligand, it seems important to place it several nanometers away from the surface of the particle to provide an effective binding to cell surface receptors. Several investigators have already applied this concept, and projected the targeting ligand away from polyplexes through PEG linkers, where the ligand is attached at the distal end of the PEG [236,246,249].

As seen before (Section 4.1.1), 'naked' LPD nanoparticles were developed for pDNA delivery, but these carriers tend to aggregate in the presence of serum protein due to their high surface charge [147]. Recently, Li and Huang [250] formulated AS-ODN and siRNA in LPD, and further introduced PEGylated lipid by the post-insertion method to induce serum stabilization. Anisamide, a compound that specifically binds to sigma receptor (over-expressed in human lung cancer cells) was added to the distal end of PEG for tumor targeting. *In vitro* studies exposed that this ligand increased the delivery efficiency by 4 to 7-fold for sigma receptor overexpressed cells. Therefore, the PEGylated, anisamide-targeted LPD showed a strong potential to deliver systemically oligonucleotides for cancer therapy.

Recently, Green *et al.* [251] linked their ligand by electrostatic interactions with cationic polymeric gene delivery nanoparticles. This kind of RGD coated nanoparticle enabled effective ligandspecific gene delivery to human primary endothelial cells in serumcontaining media, suggesting promising results for an *in vivo* application.

To further increase specificity and safety of gene therapy, the expression of therapeutic genes can be tightly controlled within the targeted tissue. The presence of tissue or environment-specific promoter can allow the activation of gene expression in diseased

states, or in unfavourable tumor-associated microenvironment, for example, hypoxia (for review see Ref. [252]).

4.2. Intracellular trafficking

4.2.1. Internalization

With the goal of improving transfection efficiency, the use of protein transduction domain (PTD) has been tested to mediate an endocytosis-independent cellular uptake of proteins as well as large molecules [253]. Several sequences exist that have been identified as possessing transducing efficiency, among them is the TAT protein derived from the HIV-1 virus [254]. As proof of the concept, the SLN transfection efficiency was significantly improved *in vitro* by pre-complexing DNA with a dimer of the HIV-1 TAT peptide, which contains a cell-penetrating domain for the improvement of cellular uptake and a nuclear localization sequence for the translocation of DNA into the nucleus [255]. It was demonstrated that liposomes (200 nm) injected at the tumor site can be delivered directly into cytoplasm without causing major damage of the vesicles by attaching TAT peptides to their surface via a PEG spacer in order to decrease steric interaction [256]. This study showed that the attachment of TAT peptides led to significantly higher transducing efficiency. It can be noted that the TAT peptide, because of its viral origin, still has the risk of being recognized by the immune system in the case of a systemic injection.

The knowledge of how PTDs mediate cell entry is currently discussed. Until recently, it was widely assumed that the internalization of cationic PTDs was an energy and receptor-independent process based on direct transport through the lipid bilayer [257–259]. On the

other hand, there have been indications that the uptake of full-length TAT protein occurs via endocytosis and depends on cell surface heparan sulfate receptors [260]. Recently, numerous possible internalization routes for TAT have been proposed, such as lipid-raft mediated macropinocytosis [261], caveolae-mediated endocytosis [262,263] and clathrin-dependent endocytosis [264]. However, the uptake characteristics of the TAT peptide alone and of TAT-conjugated cargoes have been demonstrated to differ significantly [265–267]. Furthermore, the TAT-mediated internalization process has been proposed as being dependent on the properties of the cargo molecule, TAT concentration and the cell line [265,268]. To conclude, the mechanism of PTD-mediated delivery remains elusive, and may depend on the vector associated with PTDs.

A new concept for the development of vectors is to overcome the limitations of individual vectors by combining them. In this way, virosomes were synthesized, resulting in a mix between viral glycoprotein and anionic liposomes, in which DNA is encapsulated under a protein complex form to allow better intracellular trafficking and nuclear entry. One example concerns the HVJ (haemagglutinating virus of Japan) liposome (or fusogenic liposome) which is composed of HJV glycoproteins. Indeed, some proteins of this virus possess fusogenic properties which facilitate cellular uptake by interacting with sialic residues of the cellular membrane. Such systems had efficient transfection activity *in vitro* and *in vivo* following intratissular injection [269,270]. Recently, to facilitate the usefulness of the HVJ-liposome for hepatic gene therapy, Kaneda's group evaluated the efficacy of the total vascular exclusion (TVE) technique during the portal vein injection (PVI) of the gene transfer vector regarding its transfection efficiency in rat liver [271]. This consists of a surgical procedure in which the liver is isolated from the surrounded tissues; HVJ-liposome solution

was then infused selectively into the liver through an inserted catheter. The results indicated that PVI + TVE might facilitate the liver-specific gene delivery using the HVJ-liposome method, avoiding the extra-hepatic transgene expression. Nevertheless, this technique requires a surgical handling as opposed to a 'simple' systemic injection, and this seems to be a limitation for a clinical use.

In the sameway, combinatorial nanotechnology using fusogenic liposomes (having a Sendai virus envelope, glycoprotein on the surface) encapsulating poly(vinyl amine) nanoparticles seems to be a valuable system for regulating the intracellular pharmacokinetics of gene-based drugs [272]. This class of construct is able to efficiently deliver the encapsulated contents to the cytoplasm through its direct fusion with the cytoplasm membrane.

This kind of combining systems could be the beginning of the promising "virus like vector" that will be able to mimic the efficient transfection of viruses. But these systems have to be improved for *in vivo* application, particularly as far as immune recognition is concerned.

4.2.2. Endosomal escape

With the objective of enhancing endosomal escape, pH-sensitive endosomolytic peptide can be attached to polyplexes or lipoplexes. Viruses have developed clever mechanisms to overcome the endosomal barrier: viral proteins often contain membrane active domains mediating the delivery of the viral genome to the cytoplasm after their activation in the endosome (for review see Ref. [273]). Among these virus peptides, we can find peptides derived from the N-terminal domain of *Haemophilus influenzae* haemagglutinin-2 peptide [274], or synthetic peptides such as GALA [275], or KALA [276]. As a proof of the

concept, fusogenic peptides were incorporated in chitosan/plasmid complex which significantly increased transfection efficiency [277]. Also, other naturally occurring peptides and small proteins, such as those present in the venom of vertebrates and invertebrates, can be highly membrane-destabilizing (for review see Ref. [278]). Both types of compounds were already used to enhance the intracellular delivery of transferred DNA, such as influenza-derived peptides [279] or melittin (derived from bee venom) [280] which were applied *in vivo* to facilitate the delivery of double stranded RNA to glioblastoma [281]. However, melittin also showed pronounced lytic activity at neutral pH, which is undesirable and responsible for toxic side effects. To overcome this limitation, Rozema *et al.* [282] chose to modify the lysines of melittin with a dimethylmaleic anhydride derivative, which can mask the lytic activity at neutral pH. Concerning endosomal acidification, the masking groups are removed and the lytic activity of melittin is restored. Other approaches to pH-dependent lytic activity include acidic melittin analogs [283] or the incorporation of melittin into bioreducible copolymers [284].

An original concept was proposed to disrupt endosomal and lysosomal membranes in a desired location (e.g. tumor), called photochemical internalization (PCI). Indeed, the cytoplasmic delivery of macromolecular compounds can be enhanced by the photochemical disruption of the endosomal membrane using light and a hydrophilic photosensitizer. This smart concept is, in principle, applicable to *in vivo* gene delivery in a light-sensitive manner [285]. However, the cytotoxicity is due to photochemical reactions in the cell, and this might need to be reduced before considering further applications of this technology. If not, damage to organelles other than the endosomal membranes, for example plasma or mitochondria membranes, could occur. Nishiyama *et al.* [286] chose to develop a light-responsive carrier

based on a ternary complex of pDNA, cationic peptides, and anionic dendrimer-based photosensitizer (dendrimer phthalocyanine). This sophisticated system seems to reduce the toxicity and to enhance the efficiency of transfection, leading to a 100-fold improvement in the level of transgene expression using light irradiation. Polyplexes consisting of pDNA and glycosylated PEI were used for *in vivo* p53 gene transfer in mice bearing head and neck squamous cell carcinoma xenografts. The treatment was combined with PCI technology. PCI led to a 20-fold increase of sustained transgene expression. Weekly treatment repeated for 7 weeks resulted in tumor growth inhibition in all animals and cured 83% of the mouse population [287].

4.2.3. Nucleus entry

Interestingly, viral particles can travel through the cytoplasm by interaction with the micro-tubular network and have various mechanisms for traversing the nuclear membrane. The observation that linear PEI seemed to assist these transport processes, whereas branched PEI did not, was of interest [143,288].

The NLS concept has been used to improve the uptake of plasmid DNA into the nucleus. Several groups demonstrated that the covalent attachment of an SV40 nuclear localization sequence (SV40NLS) either directly to DNA or to polymers that form complexes with DNA led to an increase of nuclear import resulting in enhanced protein expression [289–291]. Branden *et al.* [292] demonstrated that a PNA (peptide nucleic acid) molecule linked to an SV40 NLS peptide can work as a nuclear targeting signal when hybridized to a fluorescence-labeled oligonucleotide or to a plasmid. Similar results were obtained using DOTAP or 25 kDa PEI as transfection reagents in HeLa, NIH-3T3, or Cos-7 cells [292].

Recently, a bifunctional targeting vector was made, consisting of a recombinant reovirus type 3 σ 1 attachment protein modified with an SV40-derived NLS covalently attached to the PEI to create an efficient DNA-delivery vehicle. This allowed these carriers to specifically bind to plasma membrane cell surface receptors when located outside the cell and engage the nuclear import machinery for enhanced nuclear translocation after uptake into cells. Currently, pDNA complexed with the PEI- σ 1-NLS delivery vehicle resulted in substantially greater levels of *in vitro* gene expression [293].

Moffatt *et al.* [294] newly synthesized a multi-functional PEIbased polyplex for systemic p53-mediated gene therapy. This PEGylated vector attached with a CNGRC peptide for CD13 targeting in tumors also carries two systems targeting the nucleus: a Simian Virus (SV) 40 peptide (nuclear localization signal) and an oligonucleotide based nuclear signal (DNA nuclear targeting signal). This promising vector exposed a significant tumor regression and 95% animal survival after 60 days.

5. Conclusions

Incontestably, recombinant viral vectors are still the most efficient systems for gene transfer in comparison to lipoplexes or polyplexes [295,296]. Although considerable improvement has been made over the last few years, the standard requirements for clinical use have not been reached in terms of efficiency and specificity, in particular via systemic injections. Gene delivery is a multi-step process that needs a multi-functional carrier to go through each step. For this reason, research should focus on the synthesis of a vector, attached with different ligands to mimic a virus: this system could be called the “artificial virus” and each component would be able to help the vector overcome the various barriers it

could meet. All these improvements should decrease the toxicity of the initial cationic complexes, but can, however, modify the interactions with biological systems. It is thus necessary to carry on meticulous biocompatibility studies for each new system in each application.

Finally, it seems clear that the expression of a single transgene is unlikely to be sufficient to eradicate a tumor, in particular when it is diagnosed late in disease progression. Hence, multimodality therapy, including conventional therapy (surgery, chemotherapy and radiotherapy) with one or more transgenes will have to be considered to provide a “chance” of success.

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TRAVAIL EXPERIMENTAL

Publication n°1

Conception et caractérisation d'un vecteur furtif d'ADN

L'utilisation de polymères hydrophiles comme le poly (éthylène glycol) (PEG) à la surface de systèmes colloïdaux constitue une des approches les plus efficaces pour augmenter le temps de circulation des vecteurs dans le sang. Le PEG génère une barrière stérique autour de la particule qui empêche l'adsorption d'opsonines impliquées dans les mécanismes de phagocytose. Cet effet est notamment influencé par la masse moléculaire du polymère hydrophile, ainsi que par la densité de chaîne présente à la surface des particules. Dans ce contexte, deux types de polymères, le DSPE-PEG₂₀₀₀ et le copolymère à bloc F108, ont été post-insérés aux LNC ADN. Les modifications de surface engendrées par cette association ont été mises en évidence grâce à une étude de mobilité électrophorétique, permettant de caractériser la conformation des chaînes nouvellement ajoutées à la surface des LNC ADN. L'influence de cette conformation a été corrélée avec la capacité du vecteur à échapper d'une part à la capture macrophagique et, d'autre part, à celle des cellules du système des phagocytes mononucléés *in vivo* après une injection intraveineuse.

“Preparation and characterisation of coated DNA lipid nanocapsules : the influence of amphiphilic PEG conformation on macrophages uptake and biodistribution”

Publication soumise à Journal of Controlled Release

Preparation and characterisation of coated DNA lipid nanocapsules: the influence of amphiphilic PEG conformation on macrophage uptake and biodistribution

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Abstract

With the goal of creating an efficient vector for systemic gene delivery, a new kind of nanocarrier consisting of lipid nanocapsules encapsulating DOTAP/DOPE lipoplexes (DNA LNCs), was used. It is now well established that PEG addition modifies surface characteristics. Indeed, important factors such as its conformation, electrostatic features, and hydrophobicity, can result in an improvement in the pharmacokinetic behaviour of the vector. The aim of this study was to modify the coating of DNA LNCs in order to enhance their blood-circulation time. DNA LNCs were therefore pegylated by the post-insertion of two kinds of amphiphilic and flexible polymers: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀) and F108 poloxamer (poly(ethyleneoxide)₁₃₂-poly(propyleneoxide)₅₀-poly(ethyleneoxide)₁₃₂). The surface structure characteristics of the newly pegylated DNA LNCs were studied by measuring electrophoretic mobility as a function of ionic strength. This allowed us to establish a correlation between surface properties and the *in vivo* performance of the vectors, and provided evidence of a brush conformation of DSPE-mPEG₂₀₀₀ coating, leading to its efficient removal by the liver.

1 - Introduction

Gene therapy via a systemic pathway still requires an efficient vector suitable to carry the therapeutic genes safely and efficiently to the target tissue. Indeed, viral vectors are still the vectors of choice for efficient gene expression, but they suffer from important disadvantages such as risks of mutagenesis, immunogenicity, and high production costs. These problems have forced researchers to focus on alternative pathways, such as synthetic vectors. These vectors based on the use of electrostatic interactions between cationic lipids or polymers and anionic DNA molecules are efficient *in vitro* due to their global, positive charge, but are not suited to *in vivo* transfection [1, 2]. Their positive charge provides adsorption by seric proteins and elimination from the blood circulation. Indeed, when injected intravenously, a nanocarrier has to be small (50-200nm) and neutral or weakly charged if it is to escape recognition by cells of the mononuclear phagocyte system (MPS).

Lipid nanocapsules (LNCs) covered with poly (ethylene glycol)₆₆₀ hydroxystearate (HS-PEG₆₆₀) have been developed according to a solvent-free process based on emulsion phase-inversion [3]. These standard LNCs have been modified to allow the encapsulation of positively-charged DOTAP/DOPE-DNA lipoplexes, providing nanocarriers named DNA LNCs which efficiently protect DNA in their lipid cores [4]. Nevertheless, the encapsulation of these complexes still results in systems carrying a positive surface charge, and this is incompatible with an intravenous *in vivo* injection.

In order to extend the disappearance half-life time, by dissimulating the surface charge and avoiding opsonisation, the surface of DNA LNCs was modified by the post-insertion of longer PEG chains on their surface [5]. Two kinds of polymers were chosen: amphiphilic PEG derivative 1,2-distearoyl-*sn*glycero-3-phosphoethanolamine-N-

[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀) and poloxamer F108 (poly(ethyleneoxide)₁₃₂-poly(propyleneoxide)₅₀-poly(ethyleneoxide)₁₃₂). PEG density, thickness and length, are important parameters to consider to avoid opsonisation [6]. Furthermore, it has been reported that the dominant factor to control the interactions with the biological cell surface is not only linked to the surface charge carried by the particle but also to the softness of the polymer surface [7].

Thus, the surface of DNA LNCs, covered or not with DSPE-mPEG₂₀₀₀ and F108 block copolymers, was analysed using Ohshima's electrokinetic theory for "soft" or "hairy" particles [8]. This theory applies to a spherical, hard, colloidal particle coated with a layer of polyelectrolytes, and is based on the ion permeability of the polymer layer present in the outer part of the particle shell. It has already been applied to LNCs without DNA [9, 10]. The spatial charge density (ZM) and softness ($1/\lambda$) of the surface layer of our different particles were determined. The influence of the coating was then tested on *in vitro* macrophage uptake and a biodistribution study by *in vivo* fluorescence imaging.

2- Material and methods

2.1 - Preparation and characterisation of the nano-colloids

2.1.1 - Liposomes

DOTAP (1,2-DiOleoyl-3-TrimethylAmmonium-Propane) and DOPE (1,2-DiOleoyl-sn-glycero-3-PhosphoEthanolamine) (Avanti Polar Lipids, Inc, Alabaster, USA) were first dissolved in chloroform (Sigma, Saint-Quentin Fallavier, France) and then dried by an

evaporation process under vacuum. The lipid film that was formed was hydrated with deionised water. The liposomes were then sonicated for 20 minutes. Lipoplexes were prepared by mixing DOTAP/DOPE (1/1, M/M) liposomes with 660µg of luciferase-encoding plasmid [11] (pgWIZ-luciferase amplified and research grade purified by GENEART, Regensburg, Germany) at a charge ratio of 5 (+/-) in 150mM NaCl.

2.1.2 - DNA-loaded lipid nanocapsules (DNA LNCs)

The formulation of LNCs was based on a phase-inversion process described by Heurtault *et al.*[12]. LNCs were composed of lipophilic Labrafac® WL 1349 (caprylic-capric acid triglycerides, European Pharmacopeia, IVth, 2002) and oleic Plurol® (Polyglyceryl-6 dioleate) which were kindly provided by Gatefossé S.A. (Saint-Priest, France) and Solutol® HS-15 (30% of free polyethylene glycol 660 and 70% of polyethylene glycol 660 hydroxystearate (HS-PEG) European Pharmacopeia, IVth, 2002) which was a gift from BASF (Ludwigshafen, Germany). Briefly, 3.9 % of oleic Plurol® (w/w), 5.9 % of Solutol® (w/w), 9.9 % of Labrafac® (w/w), 78.9 % of water (w/w) and 1.4 % of NaCl, were mixed together under magnetic stirring. DNA LNCs were synthesised as described previously [4]. Fluorescent lipid nanocapsules were obtained by a previously-described method [13]. Briefly, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, excitation wavelength (exc.) = 549nm; emission wavelength (em.) = 565nm) or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD, exc. = 644nm; em.= 665nm) (Invitrogen, Cergy Pontoise, France) was dissolved in acetone at 0.6% (w/w) and the resulting DiI or DiD stock solution was incorporated in Labrafac® (1:10 (w/w)). Finally, acetone was evaporated before use.

2.1.3 - Preparation of pegylated nanocapsules by post-insertion

Two kinds of polymers were used for post-insertion: 1,2-DiStearoyl-*sn*-glycero-3-PhosphoEthanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀) (Mean Molecular Weight (MMW) = 2,805g/mol) (Avanti Polar Lipids, Inc, Alabaster, USA) or Pluronic® F108 (poly(ethyleneoxide)₁₃₂-poly(propyleneoxide)₅₀-poly(ethyleneoxide)₁₃₂) (MMW = 14,600g/mol) kindly provided by BASF. These polymers were added to LNCs in order to obtain a final concentration of 2, 5 and 10mM for DSPE-mPEG₂₀₀₀ and 1, 2, 3mM for F108. Prior to the post-insertion, the LNCs were purified thanks to the use of PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France) and then concentrated by ultrafiltration with Millipore Amicon® Ultra-15 centrifugal filter devices (Millipore, St Quentin-Yvelines, France). Since this purification step results in a desalting effect, the salt concentration of the suspension was therefore adapted to obtain a physiologic concentration of NaCl (150mM). Preformed LNCs and DSPE-mPEG₂₀₀₀ or F108 polymers were co-incubated for 4h at 30°C. The mixture was vortexed every 15 minutes and then quenched in an ice bath for 1 minute. To provide controls, the same thermal treatments were applied to LNC suspensions without polymers.

2.1.4 - Size measurement and stability study

The average hydrodynamic diameter of the LNCs was determined by dynamic light scattering (DLS) using a Malvern Zetasizer® (Nano Series DTS 1060, Malvern Instruments S.A., Worcestershire, UK). A 1:100 dilution of the nanoparticles in deionised water was

processed and size measurements were performed at 25°C (in triplicate). A stability study was performed by monitoring mean diameter evolution for 12h at 37°C of non-coated DNA LNCs and coated DNA LNCs at the maximum concentration of post-inserted polymers (10mM DSPE-mPEG₂₀₀₀ and 3mM F108).

2.2 - Electrophoretic mobility measurements

DNA LNCs and coated DNA LNCs were diluted to obtain different ionic strengths (0.5, 1, 1.5, 2, 2.5, 5, 10, 25 and 50mM NaCl) at pH 7.4 and an electrophoretic mobility measurement was performed using a Malvern Zetasizer® (Nano Series DTS 1060, Malvern Instruments S.A., Worcestershire, UK). Each measurement was repeated at least 3 times.

The surface properties of the nanoparticles were studied by soft-particle analysis using the Ohshima theory [8, 14]. In this model, it is supposed that the particle having an ionised group of valency Z on the surface and that is uniformly distributed at a number density of N (m⁻³) moves in a liquid containing a symmetrical valency ν in the applied electric field. The electrophoretic mobility μ is then expressed by Eq. (1):

$$\mu = \frac{\varepsilon_r \varepsilon_0}{\eta} \frac{\psi_o / \kappa_m + \psi_{DON} / \lambda}{1 / \kappa_m + 1 / \lambda} + \left(\frac{zeN}{\eta \lambda^2} \right) \quad (1)$$

$$\kappa_m = \kappa [1 + A^2]^{1/4} \text{ with } A = \frac{ZN}{2zn} \quad (2)$$

$$\kappa = \left(\frac{2Z^2 e^2 n}{\varepsilon_0 \varepsilon_r kT} \right)^{1/2} \quad (3)$$

$$\psi_{DON} = \frac{kT}{ze} \ln [A + (A^2 + 1)^{1/2}] \quad (4)$$

$$\psi_0 = \frac{kT}{ze} \left[\ln \{A + (A^2 + 1)^{1/2}\} + \frac{1}{A} \times \{1 - (A^2 + 1)^{1/2}\} \right] \quad (5)$$

In Eq. (1), η is the viscosity of the medium, λ characterises the degree of friction exerted on the liquid flow in the surface layer, ϵ_r the relative permittivity of the solution, ϵ_0 the permittivity of a vacuum. κ_m (Eq. (2)) can be interpreted as the Debye-Hückel parameter of the shell, where κ is the Debye-Hückel parameter (the reciprocal of the Debye length). T is the thermodynamic absolute temperature, k (Eq. (3)) is the Boltzmann constant and n is the bulk concentration of the electrolyte solution. Ψ_{DON} (Eq. (4)) is the Donnan potential of the surface layer, Ψ_0 (Eq. (5)) the potential at the boundary between the surface layer and the surrounding solution.

2.3 - Macrophage uptake evaluation and the biodistribution study

Cell culture

THP-1 cells (from a human monocyte/macrophage cell line obtained by ATCC, Manassas, VA, USA) were grown in suspension in a humidifier-incubator (5% CO₂) at 37°C in RPMI 1,640 supplemented with 10% foetal bovine serum (FBS), 10mM HEPES, 1mM sodium pyruvate, 1.5g/l bicarbonate (Lonza, Verviers, Belgium), 0.05mM 2-mercaptoethanol, and 100U/mL penicillin G and 100µg/mL streptomycin (Sigma, Saint-Quentin Fallavier, France). Cells were cultured in the same medium with 200mM Phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 24h to allow adherence and differentiation [15]. The medium was then aspirated (to eliminate non-adhered cells) and the

cells were subsequently incubated in a new medium for an additional 24h prior to uptake studies. The cells were harvested and counted using a Trypan blue exclusion assay with a haemocytometer. Cells ($0.8 \times 10^6/\text{ml}$) were plated on sterile, 24-well cell culture clusters, and then allowed to grow for 24h at 37°C.

Internalisation study by flow cytometry

Dil-labelled LNCs (coated or not) were incubated with adherent cells in serum-containing media. After 2h of incubation, the cells were harvested and centrifuged. They were then resuspended in a 0.4% (w/v) trypan blue solution in Hanks Balanced Saline Solution (HBSS, Lonza, Verviers, Belgium) to quench the extra cellular fluorescence[16], thus enabling the determination of the fraction that was actually internalised. Experiments were carried out in parallel at 4°C to inhibit phagocytosis and in order to evaluate nanocapsule adsorption on cell membranes (data not shown). After this, cells were washed three times in HBSS and were finally fixed in 1% formaldehyde/azide/PBS. Analysis of the internalised nanoparticles was performed with a BD FACSCalibur fluorescent-activated flow cytometer and BD CellQuest software (BD Biosciences). Cell profiles were constructed according to the parameters of granularity (side scatter) and size (forward scatter). This region was gated, thereby isolating cellular fluorescence from that of unphagocytosed LNCs and dead cells. A total of 10,000 events were analysed for each sample and experiments were performed in triplicate. The statistical significance in comparing the different uptakes was determined by Dunnett's Test.

In vivo fluorescence imaging

Female NMRI *Nude* mice (6-8 weeks old, JANVIER) were injected intravenously in the tail vein with 200 μ L of LNC suspension. They were then illuminated with 660nm light-emitting diodes equipped with interference filters. Fluorescence images as well as black and white pictures were acquired by a back-thinned CCD camera at -80°C (ORCAII-BT-512G, Hamamatsu) fitted with an RG 9 high-pass filter (Schott) [17, 18]. All the animal experiments were performed in agreement with the EU guidelines and the “Principles of Laboratory Animal Care” (NIH publication no. 86 -23, revised 1985). Image display and analysis were performed using Wasabi software (Hamamatsu).

3 – Results and discussion**3.1 - Polymer post-insertion and nanoparticle characterisation**

PEG derivatives were associated to LNCs by post-insertion on pre-formed LNCs, a method usually used to synthesise stealth liposomes [19] and recently applied to classic LNCs [20]. This method consists of a co-incubation step of pre-formed DNA LNCs with different concentrations of PEG derivatives, followed by a cooling step which stabilises the system [20].

The co-incubation step has to be done at a temperature slightly higher than the gel/liquid transition of the polymer, but also at a lower temperature than the phase inversion temperature (PIT) of LNC formulation to avoid any disorganisation due to the phase inversion process [20]. In the case of DNA LNCs, the co-incubation step was performed at 30°C since the PIT of the nanoparticles is weak (around 35°C)[4].

The evolution of the hydrodynamic diameter after post-insertion showed a DSPE-mPEG₂₀₀₀ and F108 bond with the LNC shell at each concentration (Figure 1). Depending on the polymer structure (Figure 2A) and based on previous studies [19-22], two different associations can be hypothesised: an anchorage of the lipid part of DSPE-mPEG₂₀₀₀ (DSPE) into the core of the LNC or a physical adsorption of PPO parts of the F108 block copolymer by hydrophobic interaction (Figure 2B). Compared to lipoplex-loaded LNCs (DNA LNCs) (112.8 ± 5.8), the DSPE-PEG coating provided a significant size increase (124 ± 5 nm, 135 ± 2 nm and 136 ± 9 nm for 2, 5 and 10mM respectively). The mean size obtained after the post-insertion of the F108 block copolymers was also enhanced, between 127 and 130nm, whatever the concentration. This size increase was stable for 12h at 37°C, without swelling, micelle apparition, or aggregation (data not shown).

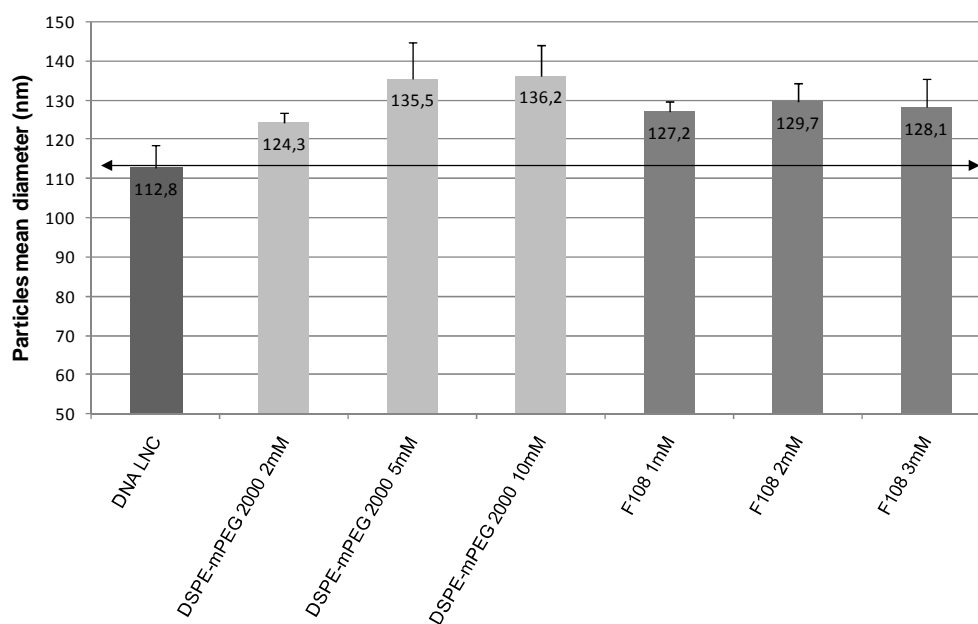


Figure 1. Size evolution after the post-insertion of DSPE-mPEG₂₀₀₀ and F108 block copolymer at different concentrations.

Previous studies have shown that the overall lengths of a coiled and an extended PEG₂₀₀₀ chain is about 5 and 10-15nm, respectively [23]. The size variation when adding DSPE-mPEG₂₀₀₀ to the DNA LNC surface, whatever the concentration, was from +11nm (2mM DSPE-mPEG₂₀₀₀) to +22nm (10mM DSPE-mPEG₂₀₀₀), indicating a probable extended chain conformation (Figure 2B). By contrast, the size increase provided by F108 post-insertion was smaller (around 15nm), whereas F108 PEG chains are 3 times longer than DSPE-mPEG₂₀₀₀ ones (132 vs. 45 EG units) (Figure 2). These results suggest a probable coiled configuration of the F108 chains, as schematically represented in Figure 2B.

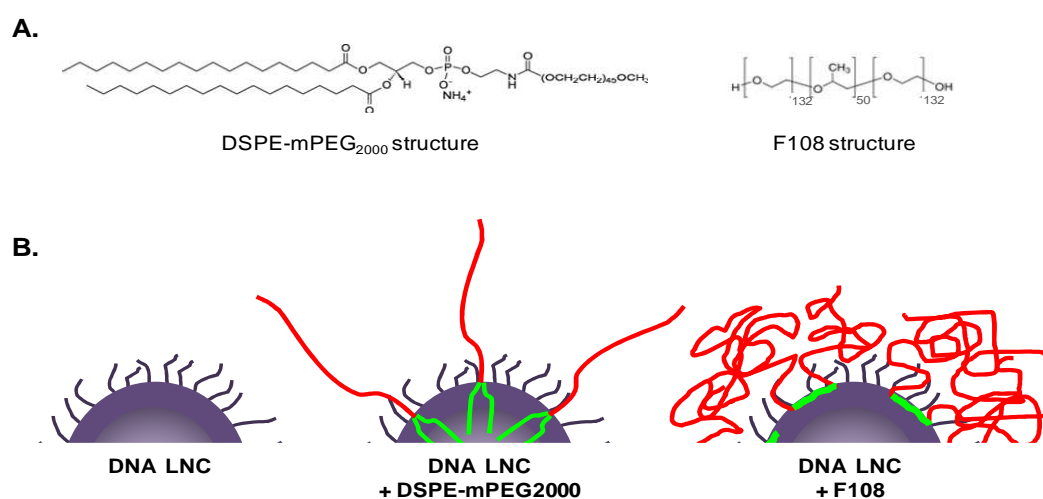


Figure 2. Chemical structure of the different post-inserted polymers (A) and schematic representation of the polymer association to DNA LNCs (B).

3.2 - Surface properties of DNA LNCs and coated DNA LNCs

Information about the surface properties of DNA LNCs, covered or not with DSPE-mPEG₂₀₀₀ (Figure 3A) or F108 (Figure 3B), were obtained from the electrophoretic mobility measurements. Nanoparticles were dispersed in electrolyte solutions with various ionic

strengths. With increasing ionic strength, the absolute mobility values of all the nanoparticles decreased because the shielding effect of electrolyte ions in the medium increased.

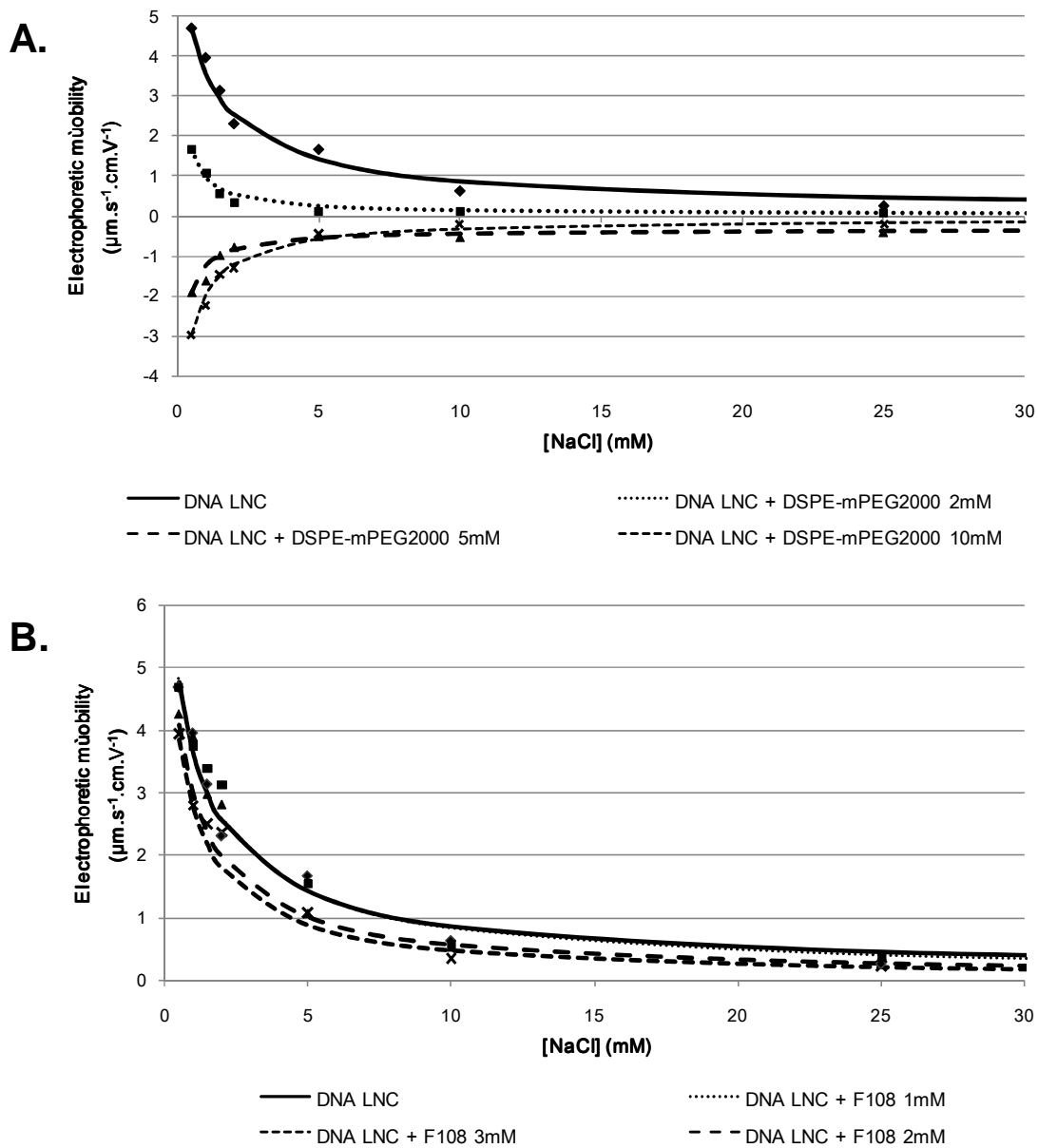


Figure 3A. (A) Electrophoretic mobility of DNA LNCs and DSPE-mPEG₂₀₀₀-coated DNA LNCs as a function of NaCl concentration. Symbols represent the experimental data with: \blacklozenge for DNA LNCs, \blacksquare for DNA LNCs + DSPEmPEG₂₀₀₀ 2mM, \blacktriangle for DNA LNCs + DSPEmPEG₂₀₀₀ 5mM, \blacktimes for DNA LNCs + DSPEmPEG₂₀₀₀ 10mM. Solid lines represent the theoretical data calculated according to zN and $1/\lambda$ presented in Table 1. **(B.) Electrophoretic mobility of DNA LNCs and F108-coated DNA LNCs as a function of NaCl concentration.** Symbols represent the experimental data with: \blacklozenge for DNA LNCs, \blacksquare for DNA LNCs + F108 1mM, \blacktriangle for DNA LNCs + F108 2mM, \blacktimes for DNA LNCs + F108 3mM. Solid lines represent the theoretical data calculated according to zN and $1/\lambda$ presented in Table 1.

The values obtained with DNA LNCs remained positive at all ionic strengths implying that their surfaces have net positive charges, as already described [24]. The presence of DSPE-mPEG₂₀₀₀ induced a decrease in electrophoretic mobility down to negative values for concentrations of DSPE-mPEG₂₀₀₀ of 5 or 10mM. Concerning F108-coated DNA LNCs, the electrophoretic mobility was close to that obtained with non-coated DNA LNCs when adding 1mM of poloxamer. A more pronounced decrease is observed when DNA LNCs are coated with 2 and 3mM, but the values remain positive. In all cases, the electrophoretic mobility is modified by the coating and tends to non-zero values, even when the ionic strength is 50mM NaCl (data not shown). This means that the surfaces of DNA LNCs and coated DNA LNCs are soft and that their properties can be analysed by the electrokinetic mobility theory for soft surfaces [8].

Equation (1) (described in section 2.4) involves two unknown parameters, ZN and $1/\lambda$, which represent the fixed charge density in the polymer layer and its softness, respectively. Values of ZN and $1/\lambda$ were determined by a curve-fitting procedure already described [7]. The theoretical values of Eq. (1) (solid lines in Figure 3) were plotted versus the ionic strength in comparison with the experimental data (symbols) (Figure 3). The best fit values of the charge density ZN and the softness parameter $1/\lambda$ are shown in Table 1.

The spatial charge density (ZN) present in the layer of DNA LNCs was higher than that of the 100nm LNC (+1.44 compared to $-0.64 \times 10^6 \text{C.m}^{-3}$) (Table 1). This can be linked to two simultaneous effects: the first could be due to the absence of lecithin in the formulation (compared to classic LNCs). Indeed, phospholipids of lecithin can be considered as electric dipoles in water due to the low level of electronegative behaviour of

the phosphoric groups [9]. The second, and probably the more influential effect, can be the presence of highly, positively-charged lipoplexes in the core of DNA LNCs.[25]

	$ZN (10^6 \text{ C.m}^{-3})$	$1/\lambda \text{ (nm)}$
100nm LNCs [8]	-0.64	0.1
DNA LNCs	1,44	1.5
DNA LNCs + DSPE-mPEG2000 2mM	0,16	1
DNA LNCs + DSPE-mPEG2000 5mM	-0,12	1.5
DNA LNCs + DSPE-mPEG2000 10mM	-0,48	1
DNA LNCs + F108 1mM	0.88	0.7
DNA LNCs + F108 2mM	0.72	0.7
DNA LNCs + F108 3mM	0,48	0.5

Table 1. ZN and $1/\lambda$ parameters for classic LNCs of 100nm, DNA LNCs and coated DNA LNCs

As observed by several authors [26-29], it was found that PEGylation decreased the surface charge density of the outer layer. This effect was observed for F108 in proportion to its concentration (0.88, 0.72, 0.48 $\times 10^6 \text{ C.m}^{-3}$). Moreover, the increase of DSPE-mPEG₂₀₀₀ concentration at the surface of DNA LNCs induced a decrease of the charge from positive to negative values. Vonarbourg *et al.* [9] demonstrated that PEG chains carry negative dipolar charges and that the higher the charge density, the more ordered the chain configuration. Furthermore, the similarity of $1/\lambda$ values between DSPE-mPEG₂₀₀₀ and DNA LNCs implies that the DSPE-mPEG₂₀₀₀ chains do not disturb counter-ion penetration, Na⁺ and Cl⁻ respectively (comparison between Figures 4A and 4B). So, ZN and $1/\lambda$ values

indicate a DSPE-mPEG₂₀₀₀ coating organised in a brush conformation, as represented in Figure 2B and 4B.

$1/\lambda$ values obtained with F108 are inferior to DNA LNC and DSPE-mPEG₂₀₀₀-coated DNA LNC ones (0.5 to 0.7nm compared to 1 to 1.5nm, Table1). Therefore, the accessible layer to ions is thinner for F108-coated DNA LNCs. This tends to confirm that there is a super-coiled conformation of the long F108 chains at the surface of LNCs, preventing the deep penetration of counter-ions into the layer (Figure 4C). This result correlates to the previous result linked to size measurement.

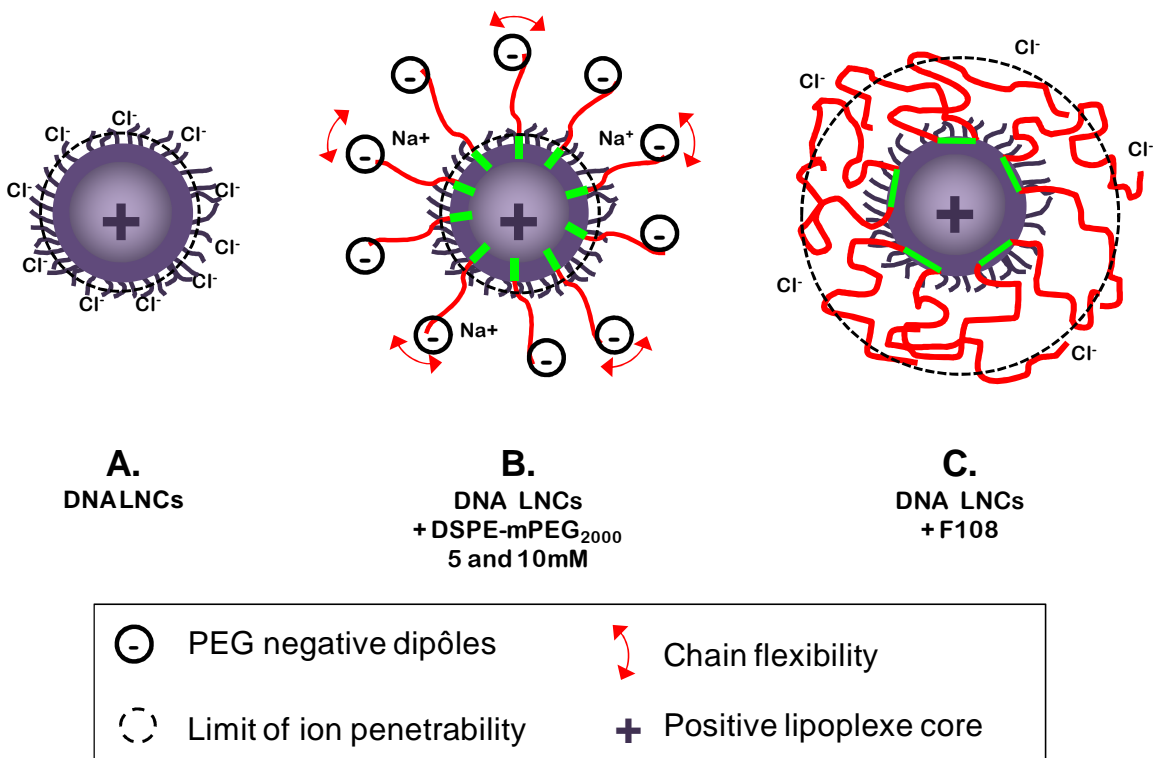


Figure 4. Schematic representation of the PEG chain conformation at the DNA LNC surface in the case of DNA LNCs (A.), DNA LNCs + DSPE-mPEG₂₀₀₀ at 5 or 10mM (B.), and DNA LNCs + F108 at 2mM (C.).

3.3 - *in vitro* macrophage uptake and *in vivo* distribution study

The mean fluorescence intensity of LNCs inside THP-1 cells was normalised to allow for comparisons: DNA LNC uptake was taken as the 100% uptake reference. As seen in Figure 5, the DSPE-mPEG₂₀₀₀ coating on DNA LNCs significantly decreased internalisation with the increase of DSPE-mPEG₂₀₀₀ concentration, from 16, 34, and 33% for 2, 5 and 10mM respectively (Figure 5). Particles coated with F108 were less phagocytosed by THP-1 cells only at the 3mM concentration (24%). As expected, the addition of long chains of PEG at a sufficient concentration improved the macrophage escape of DNA LNCs, especially in the case of extended DSPE-mPEG₂₀₀₀ chains (Figure 5). The decrease in charge density present at the DNA LNC surface, thanks to the use of PEG polymers, most probably allows a decrease in the pegylated nanoparticle removal *via* non-specific interactions with macrophages [30, 31].

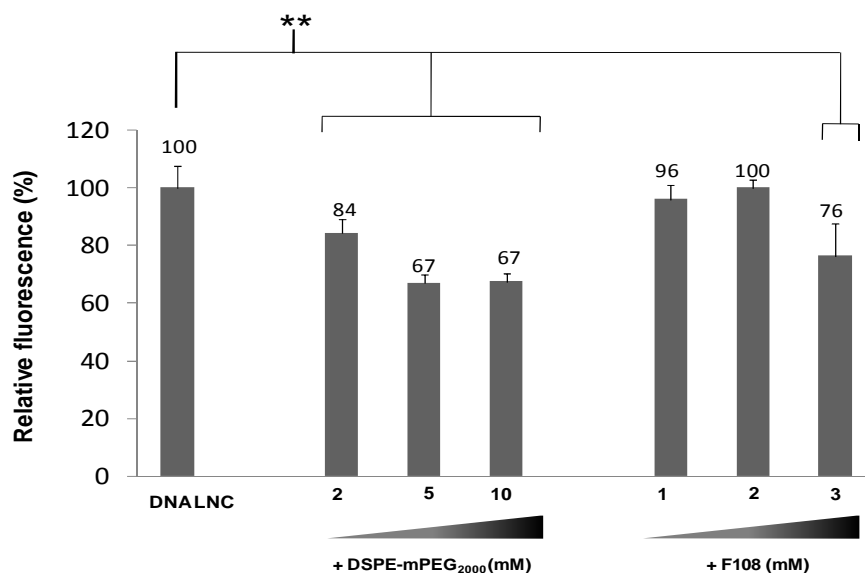


Figure 5. Influence of the different coatings of DNA LNCs on phagocytosis by THP-1 macrophages. Flow cytometry analysis of Dil-labelled LNC phagocytosis in THP-1 cells in serum containing medium after 2h of incubation. For quantification represented on the histogram, the mean cell fluorescence intensity in THP-1 cells was normalised: the DNA LNC uptake was taken as the 100% uptake reference.

To estimate *in vivo* tissue distribution, the more promising nanoparticles (i.e. 10mM DSPEmPEG₂₀₀₀-coated DNA LNCs and 3mM F108-coated DNA LNCs compared to DNA LNCs) were stained with a near infra-red fluorochrome, DiD (Ex=644nm; Em=665nm), to allow fluorescence imaging in living mice after intravenous injection. As early as 1h30 after injection, they displayed different fluorescence intensities and localisations (Figure 6). Indeed, for DNA LNCs an intense liver relocation of the fluorescent signal was observed. This was also detected with F108-coated DNA LNCs but at 3h after injection, and in a less intense way. By contrast, liver localisation was never observed for DSPEmPEG₂₀₀₀-coated DNA LNCs throughout the study (48h).

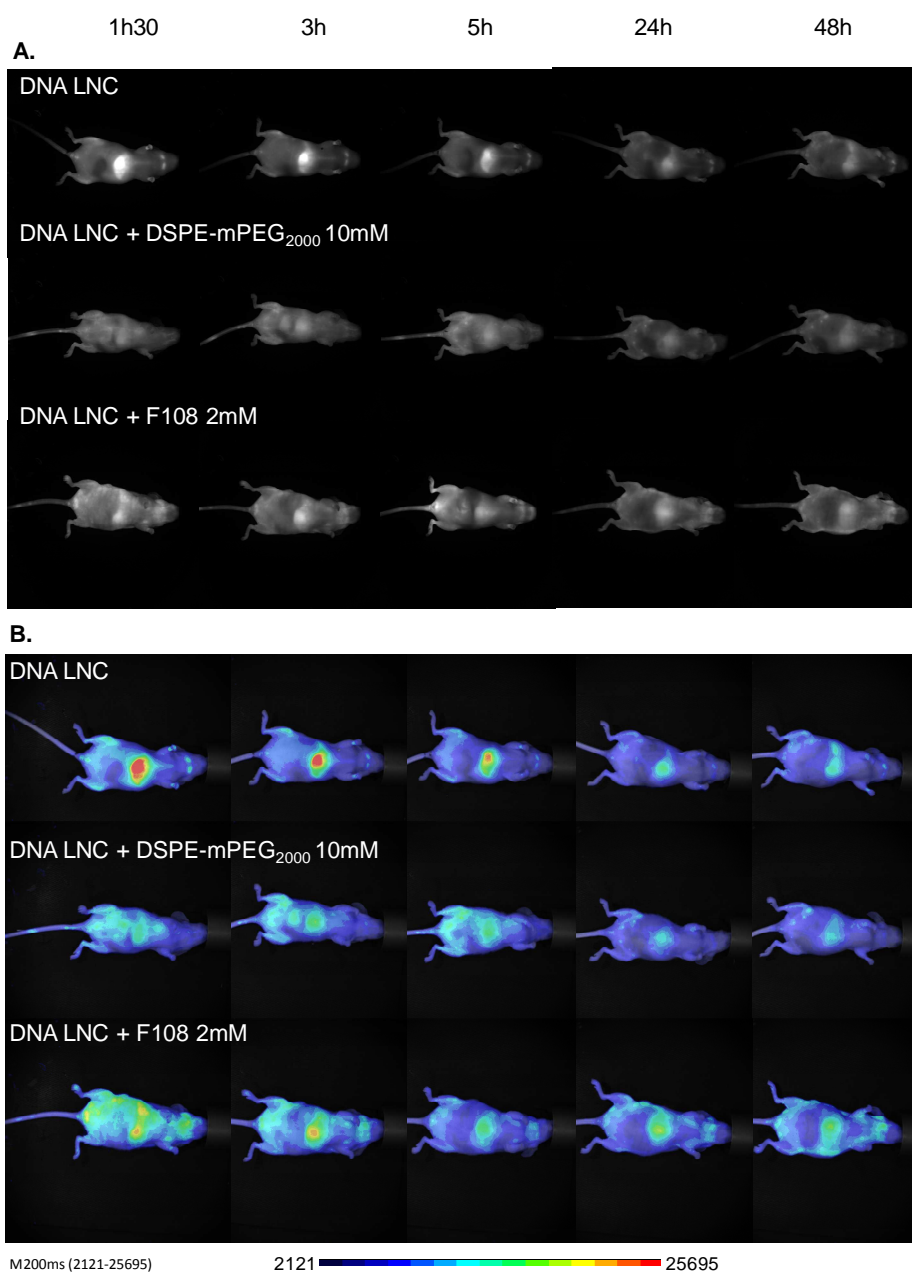


Figure 6. *in vivo* fluorescence imaging (A, black and right; B, colored) of athymic nude mice after intravenous injection of DNA LNCs, DSPE-mPEG₂₀₀₀-coated DNA LNCs or F108-coated DNA LNCs. The colour bar on the lower part of the pictures indicates the signal strength of the fluorescence emission coming from the animal.

The difference of association of DSPE-mPEG₂₀₀₀ (anchorage) and F108 (adsorption) to DNA LNCs can be the cause of this difference of behaviour. Indeed, adsorption is a weak interaction which is known to be unable to resist a long time to in *in vivo* conditions [32].

However, the longer blood-circulation time and macrophage escape obtained with DSPE-mPEG₂₀₀₀ coating could also be due to a chain conformation that is better adapted to repulse protein opsonisation, hence providing higher flexibility thanks to extended chains of PEG compared to the super-coiled F108 ones. Indeed, even if the DSPE-mPEG₂₀₀₀ chain conformation allows the penetration of small ions (Figure 4), proteins are not able to access the LNC surface because of the undulating PEG chains. By contrast, the surface of DNA LNCs coated with F108 is even more inaccessible, but the entanglements of PEG chains probably do not provide enough flexibility, a feature that is crucial to allow efficient opsonin repulsion.

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Publication n°2

Les LNC PEGylées comme vecteurs d'ADN pour un ciblage passif des tumeurs

Seule l'utilisation de la voie systémique permet de traiter des organes ou tumeurs inaccessibles. Mais à ce jour, il n'existe pas de vecteur efficace en thérapie génique par injection intraveineuse. En effet, les vecteurs classiques de type polyplexes ou lipoplexes possèdent une forte charge positive induisant une toxicité et une élimination rapide de la circulation sanguine. L'encapsulation de lipoplexes de DOTAP/DOPE dans les LNC ADN a permis d'obtenir un vecteur moins toxique, mais avec un temps de circulation insuffisant pour une utilisation systémique efficace. Le recouvrement des LNC ADN par de longues chaînes de PEG a donc été réalisé (publication n°1). Cette seconde partie consiste en l'évaluation des propriétés furtives de nos nouveaux vecteurs, en terme d'activation par le système du complément, de capture macrophagique par un nouveau test, et de pharmacocinétique sanguine après injection intraveineuse. De plus, le potentiel d'accumulation tumorale de ces vecteurs par effet EPR a été évalué par un système d'imagerie non-invasif permettant, grâce à l'encapsulation d'un fluorochrome émettant dans le proche infra-rouge, un suivi de fluorescence chez l'animal vivant.

“Long-circulating DNA lipid nanocapsules as a new vector for passive tumor targeting”

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LONG-CIRCULATING DNA LIPID NANOCAPSULES AS NEW VECTOR FOR PASSIVE TUMOR TARGETING

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Abstract

Systemic gene delivery systems are needed for therapeutic application to organs that are inaccessible by percutaneous injection. Currently, the main objective is the development of a stable and non-toxic vector that can encapsulate and deliver foreign genetic material to target cells. To this end, DNA, complexed with cationic lipids i.e DOTAP/DOPE, was encapsulated into lipid nanocapsules (LNCs) leading to the formation of stable nanocarriers (DNA LNCs) with a size inferior to 130nm. Amphiphilic and flexible poly (ethylene glycol) (PEG) polymer coatings [PEG lipid derivative (DSPE-mPEG₂₀₀₀) or F108 poloxamer] at different concentrations were selected to make DNA LNCs stealthy. Some of these coated lipid nanocapsules were able to inhibit complement activation and were not phagocytised *in vitro* by macrophagic THP-1 cells whereas uncoated DNA LNCs accumulated in the vacuolar compartment of THP-1 cells. These results correlated with a significant increase of *in vivo* circulation time in mice especially for DSPE-mPEG₂₀₀₀ 10mM and an early half-life time ($t_{1/2}$ of distribution) 5-fold greater than for non-coated DNA LNCs (7.1h vs 1.4h). Finally, a tumor accumulation assessed by *in vivo* fluorescence imaging system was evidenced for these coated LNCs as a passive targeting without causing any hepatic damage.

1 - Introduction

For the treatment of unreachable organs and disseminated or metastatic cancer, it is now essential to develop intravenous forms of gene therapy. However, systemic targeting remains a real challenge. Synthetic vectors based on the use of cationic lipids or polymers associated to DNA appear to have promising potential, given the safety problems encountered with viral vectors. Nevertheless, the systemic injection of these synthetic carriers usually results in a toxic response linked to their strong positive charge, incompatible with clinical applications [1].

Furthermore, when injected intravenously, colloidal carriers are rapidly cleared by the mononuclear phagocyte system (MPS) mainly represented by Kupffer cells in the liver and spleen macrophages. The recognition of the carriers by macrophages usually occurs through specific recognition by cellular receptors specific for plasma proteins that have been adsorbed at the vector surface. Among them, the C3 protein of the complement system plays a major role in the immune system's recognition of foreign particles [2]. The concept of modifying the surface of vectors has therefore been applied in order to decrease the opsonisation process and the specific or non-specific recognition by MPS and blood components [3].

Heurtault *et al.*[4] developed lipid nanocapsules synthesised by a solvent-free method and covered by PEG₆₆₀ at high density, leading to really weak complement activation and low macrophage uptake [3, 5]. In a previous work, the formulation of these nanocapsules was adapted to obtain DNA nanocapsules (DNA LNCs) [6]. Thanks to the use of oleic Plurol® instead of Lipoid® in their formulation, the lipid core allowed the entrapment of plasmid DNA molecules via the formation of lipoplexes (cationic liposomes of DOTAP:DOPE complexed

with plasmid DNA). DNA LNCs were small ($117 \pm 10\text{nm}$), suitable for an intravenous injection, but *in vivo* stability and blood half-life remained low and were ill-adapted to efficient *in vivo* transfection [6].

To allow an extended circulation time, and consequently a higher tumor selectivity by passive accumulation through the EPR (enhanced permeability and retention) effect [7], we chose to modify the surface of our gene delivery systems, by inserting longer PEG chains at the surface of DNA LNCs between the already-existing, dense PEG₆₆₀ chains. This was carried out through the use of two kinds of amphiphilic and flexible polymers. The first one was F108 block copolymer, consisting of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure (EO₁₃₂-PO₅₀-EO₁₃₂). This kind of amphiphilic polymer recently demonstrated great promise for the delivery of pDNA, thanks to its proven *in vivo* transfection efficiency [8-11]. The second one was a lipid PEG derivative, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀). The ability of the different particles to escape complement activation and uptake by THP-1 macrophages was investigated. Then the long-circulating properties of these particles *in vivo* after intravenous injection in mice and their tumor accumulation ability by NIR fluorescence imaging system were evaluated. In parallel, blood samples were harvested to measure the hepatotoxic impact of the different formulations before and after injection.

2 - Materials and methods

2.1 - Preparation of the nano-colloids

2.1.1 - Liposomes

DOTAP (1,2-DiOleoyl-3-TrimethylAmmonium-Propane) and DOPE (1,2-DiOleoyl-sn-glycero-3-PhosphoEthanolamine) (Avanti Polar Lipids, Inc, Alabaster, USA) were first dissolved in chloroform (Sigma, Saint-Quentin Fallavier, France) and then dried by an evaporation process under vacuum. The formed lipid film was hydrated with deionized water. Then liposomes were sonicated for 20 minutes. Lipoplexes were prepared by mixing DOTAP/DOPE (1/1, M/M) liposomes with 660µg of luciferase-encoding plasmid [10] (pgWIZ-luciferase amplified and research grade purified by GENEART, Regensburg, Germany) at a charge ratio of 5 (+/-) in 150mM NaCl.

2.1.2 - DNA-loaded lipid nanocapsules (DNA LNCs)

The formulation of LNCs was based on a phase-inversion process described by Heurtault *et al.*[12]. LNCs were composed of lipophilic Labrafac® WL 1349 (caprylic-capric acid triglycerides, European Pharmacopeia, IVth, 2002) and oleic Plurol® (Polyglyceryl-6 dioleate) which were kindly provided by Gatefossé S.A. (Saint-Priest, France) and Solutol® HS-15 (30% of free polyethylene glycol 660 and 70% of polyethylene glycol 660 hydroxystearate (HS-PEG) European Pharmacopeia, IVth, 2002) which was a gift from BASF (Ludwigshafen, Germany). Briefly, 3.9 % of oleic Plurol® (w/w), 5.9 % of Solutol® (w/w), 9.9 % of Labrafac® (w/w), 78.9 % of water (w/w) and 1.4 % of NaCl, were mixed together under magnetic stirring. DNA LNCs were synthesized as already described[6]. Fluorescent lipid

nanocapsules (Dil or DiD empty LNCs and Dil or DiD DNA LNCs) were obtained by a previously-described method [13]. Briefly, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, emission wavelength (em.) = 549nm; excitation wavelength (exc.) = 565nm) or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiD, em.= 644nm; exc.= 665nm) (Invitrogen, Cergy Pontoise, France) was dissolved in acetone at 6 ‰ (w/w) and the resulting Dil or DiD stock solution was incorporated in Labrafac® (1:10 (w/w)). Finally, acetone was evaporated before use.

2.1.3 - Preparation of coated nanocapsules by post-insertion

Two kinds of polymers were used for post-insertion: 1,2-DiStearoyl-*sn*-glycero-3-PhosphoEthanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀) (Mean Molecular Weight (MMW) = 2,805g/mol) (Avanti Polar Lipids, Inc, Alabaster, USA) or Pluronic® F108 (Poly(ethyleneoxide)₁₃₂-poly(propyleneoxide)₅₀-poly(ethyleneoxide)₁₃₂) (MMW = 14,600g/mol) kindly provided by BASF. These polymers were added to LNCs in order to obtain a final concentration of 2, 5 and 10mM for DSPE-mPEG₂₀₀₀ and 1, 2, 3 mM respectively for F108. Prior to the post-insertion, the LNCs were purified thanks to the use of PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France) and then concentrated by ultrafiltration with Millipore Amicon® Ultra-15 centrifugal filter devices (Millipore, St Quentin-Yvelines, France). This purification step providing a desalting effect, the salt concentration of the suspension was therefore adapted to obtain a physiologic concentration of NaCl (150mM). Pre-formed LNCs and DSPE-mPEG₂₀₀₀ or F108 micelles were co-incubated for 4h at 30°C. The mixture was vortexed every 15 minutes and then

quenched in an ice bath for 1 minute. To provide controls, the same thermal treatments were applied to LNC suspensions without polymers.

2.1.4 - Polymethyl methacrylate nanoparticles (PMMA NP)

Polymethyl methacrylate (PMMA) nanoparticles were synthesized by the polymerisation of methyl methacrylate (MMA, Merck, Hohenbrunn, Germany) as described previously[14]. To obtain fluorescent PMMA NPs, the pre-formed PMMA particles were allowed to swell in methanol during an incubation period of 2h at room temperature with Dil dissolved in acetone. The water-insoluble Dil diffused into the PMMA nanoparticles and was entrapped when the solvent was removed through the evaporation process, for 30min. at 70°C.

2.2 - Characterisation of the nanoparticles

2.2.1 - Physico-chemical characteristics of coated DNA LNCs

The average hydrodynamic diameter and the polydispersity index (PI) of the LNCs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer® (Nano Series DTS 1060, Malvern Instruments S.A., Worcestershire, UK). A 1:100 dilution of the nanoparticle in deionized water was processed and size measurement was performed at 25°C (in triplicate). The measure of zeta potential was achieved on nanoparticle suspensions at 150mM NaCl diluted in deionized water at 1:100, providing a final salt concentration of 1,5mM.

2.2.2 - DNA stability study

The stability of nanocapsule suspensions during storage at 4°C was assessed by measuring the size distribution. The stability was also tested after centrifugation at 15,000g at room temperature for 20min in order to visualize any demixing among the components. The stability of encapsulation and the integrity of DNA molecules after the process of nanocapsule formulation, and post-insertion were evaluated by agarose gel electrophoresis. A volume of LNCs or lipoplexe suspension equivalent to 0.2µg of DNA before and after treatment with Triton® X100 (Sigma, Saint-Quentin Fallavier, France) was mixed with gel-loading solution (Sigma, Saint-Quentin Fallavier, France) and deposited in each well of 1% agarose gel containing ethidium bromide (Sigma, Saint-Quentin Fallavier, France). Controls were constituted by using 0.2µg of free DNA in solution or associated to cationic lipids. Samples were migrated 20min at 100V in a Tris- EDTA buffer.

2.3 - Macrophage uptake evaluation

2.3.1 - Cell culture

THP-1 cells (human monocyte/macrophage cell line obtained by ATCC, Manassas, VA, USA) were grown in suspension in a humidifier-incubator (5% CO₂) at 37°C in ATCC suggested medium. Cells were cultured in the same medium with 200mM Phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 24h to allow adherence and differentiation [15]. The medium was then aspirated (to eliminate non-adhered cells) and the cells were subsequently incubated in a new medium for an additional 24h prior to uptake studies. Cells were harvested and counted using Trypan blue exclusion assay with

a haemocytometer. Cells ($0.6 \times 10^6/\text{ml}$) were plated on sterile, 24-well cell culture clusters, and then allowed to grow for 24h at 37°C.

2.3.2 - Cytotoxicity assay

The 24-well plates were exposed to different suspensions (free DNA, free lipoplexes, empty LNCs, and DNA LNCs, at a DNA concentration equivalent to the DNA LNCs one, excepted for empty LNCs). Nanoparticles were prepared at a DNA concentration of 446µg/ml and 1:10 cascade dilutions were performed in culture (44.6, 4.46 and 0.446µg/ml). After 48h of exposure, cell viability was determined by the MTT test performed in triplicate according to the procedure described by Mosmann [16]. Briefly, 40µl of MTT solution at 5mg/ml in phosphate-buffered saline (PBS) 1X was added to each well, and then the plates were incubated at 37°C for 4h. The medium was removed and 200µl of 0.06N acid-isopropanol was added to each well and mixed thoroughly to completely dissolve the dark blue crystals. The optical density was measured at 580nm using a Microplate reader Multiskan Ascent (Thermo Fisher Scientific, Cergy-Pontoise, France).

2.3.3 - Internalization study by cellular imaging

Cells were incubated with a series of 2-fold dilutions of Dil-labelled LNC suspensions starting from 1/100. After 20min or 24h, macrophages were stained with Syto60 (Invitrogen, Cergy Pontoise, France). Confocal images were recorded on an automated fluorescent confocal microscope Opera™ (PerkinElmer) using a 20X-water objective (NA 0.70). Dil-labelled LNCs were detected using a 532nm laser coupled with a 565/50nm detection filter (green channel) and cells labelled with Syto60 were identified with a 635nm laser coupled

with a 690/40nm detection filter (red channel). Four independent pictures were taken for each plate well and each image was then processed in order to quantify the number of green LNCs and cells.

2.4 - Complement activation study

Complement consumption was assessed in normal human serum (NHS) (provided by the Etablissement Francais du Sang, CHU, Angers, France) by measuring the residual haemolytic capacity of the complement system after contact with the different particles[2]. The technique consisted in determining the amount of serum able to lyse 50% of a fixed number of sensitized sheep erythrocytes with rabbit anti-sheep erythrocyte antibodies (CH50), according to the procedure described elsewhere [5]. Complement activation was expressed as a function of the surface area in order to compare particles with different mean diameters. Nanoparticle surface areas were calculated as described elsewhere[14], using the equation: $S = \sqrt[3]{4Vr^2}$ and $V = n(4/3)(\pi r^3)$ leading to $S = 3m/\rho$ where S is the surface area (cm²) and V the volume (cm³) of n spherical beads of average radius r (cm), m the weight (µg) and ρ the volumetric mass (µg/cm³). All experiments were performed in triplicate and a t-test of non-matched samples was used to test for the statistical significance of the results.

2.5 - *In vivo* hepato-toxicity study after IV injection

Blood samples (~200 µL) were collected from the saphenous vein of the mice on a heparin tube before and 24 h after administration. Tubes were then centrifuged at 10000×g for 2 min at +4 °C and plasma were harvested to measure the activity of the ALAT (alanine

aminotransferase) and ASAT (aspartate aminotransferase) enzymes. For ALAT measurements, two solutions were needed (S1 = Tris buffer at pH 7.5: 125mmol/L ; L-aspartate: 680 mmol/L ; LDH>2000 U/L and S2 = α -cetoglutarate 97 mmol/L; NADH 1.1mmol/L). First, 200 μ L of S1 were mixed with 50 μ L of S2 during 20 sec. Then, 25 μ L of plasma were added to the mixture. After 50 sec of incubation at room temperature, the absorbance at 340 nm was measured immediately and 60 sec later. The ALAT activity was expressed in UI/L and resulted of the product between the variation of the absorbance ($\Delta A/\text{min}$) and a coefficient σ [$\sigma = (\text{final volume in mL} * 1000) / (\text{serum volume in mL} * \text{length of the optical distance} * 6.3)$ (6.3 corresponding to the absorption of NADH at 340nm)]. Here, the σ coefficient was equal to 1746. For ASAT evaluation, the protocol was the strictly same, except that solutions S1 and S2 have been adapted (S1 = Tris buffer at pH 7.8: 100 mmol/L ; L-aspartate: 330 mmol/L ; LDH> 2000 U/L ; MDH>1000 U/L and S2 = α -cetoglutarate 78 mmol/L ; NADH 1.1mmol/L). The measurements of the ALAT and ASAT transaminases released into the serum reflected the toxicity, notably the hepatological impact, of the various formulations. Our method adapted for small blood volume allowed performing a kinetic of the transaminase activity on each animal, limiting the device induced by inter-individual variations that resulted from the techniques that required sacrifice of the animals to get enough volume.

2.6 - Blood kinetic study

Animal care was administered in strict accordance to French Ministry of Agriculture regulations. One hundred and fifty microlitres of fluorescent LNCs were injected in the tail vein of six-week old female Swiss mice (20-22g) (Ets Janvier, Le Genest-St-ile, France). The

fluorescence was measured at Time 1, 5, 15, 30, 60, 120, 240, 300min. and 24h. At each time, blood sampling was performed by cardiac puncture on 3 mice and each sample was centrifuged for 10min at 2,000g in a venous blood collection tube (Vacutainer, SST II Advance, 5 ml, Becton Dickinson France SAS, Le Pont-De-Claix, France). One hundred and fifty microlitres of the supernatant were deposited in a black, 96-well plate (Greiner Bio-one, Frickenhausen, Germany). Empty samples were constituted by the supernatant of centrifuged blood taken from 3 mice injected with an isotonic solution (150mM NaCl), representing the residual fluorescence of the plasma. Dil fluorescence (ex: 544 nm, em: 590 nm) was counted by a Fluoroscan (Ascent FL, Thermo Fisher Scientific, Cergy-Pontoise, France) and the results were analyzed with the Ascent software for Fluoroscan (Thermo Fisher Scientific, Cergy-Pontoise, France). The blood concentration of the different particles at the various times was calculated on the assumption that blood represents 7.5% of mouse body weight[17]. Fluorescence was expressed in fluorescence units (FU) and was calculated as: $FU_{sample} - FU_{empty}$. 100% of fluorescence was considered as the value at $t=1$ min.

Pharmacokinetic data were treated by non-compartmental analysis of the percentage of the injected dose versus time profiles with Kinetica 4.1.1 software (Thermo Fisher Scientific, Villebon sur Yvette, France). The half-lives were calculated as following: $t_{1/2} = \text{Log}(2)/Lz$. The Lz was determined from linear regression using defined intervals (5h and 24h for $t_{1/2}$ distribution [0-5h] and $t_{1/2}$ elimination [0-24h] respectively). The trapezoidal rule was used to calculate the area under the curve (AUC) during the whole experimental period (AUC [0-24h]) without extrapolation, as well as the area under the first moment curve (AUMC). The mean residence time was calculated from 0 to 5h, from the following equation: $MRT [0-5h] = AUMC [0-5h] / AUC [0-5h]$.

2.7 - *In vivo* fluorescence imaging

Tumor bearing mice were prepared by injecting subcutaneously a suspension of 1×10^6 U87MG glioma cell line (ATCC, Manassas, VA) in 150 μ l of Hanks Balanced Saline Solution (HBSS) into the right flank of athymic nude mice (6 weeks old females, 20-24g, purchased from Charles Rivers, Wilmington, Ma). In order to evaluate the biodistribution of coated DNA LNCs and uncoated DNA LNCs in tumor bearing mice, LNCs were labeled with DiD, a near-infrared (NIR) fluorophore. After 21 days, 150 μ l of nanoparticles were injected via the tail vein of the mice presenting tumors on their right flank. Non-invasive fluorescent imaging was then performed 3h, 5h, 24h and 48h post-injection using the biofluorescence imaging (BFI) system of the LB 983 NightOWL II (Berthold France – Thoiry - France) equipped with cooled slow scan CCD camera and driven with the WINLIGHT software. (Berthold France, Thoiry, France). As DiD fluorescent tag was used to localize the nanoparticles, the 590nm excitation filter and the 655nm emission filter were selected. In parallel, the light beam was kept constant for each fluorescent measurement, which was ideal with the ringlight epi illumination. If the ringlight was always set at the same height, the excitation energy on the sample would always be the same.

Each mouse was anesthetized with a 4% air-isofluran blend. Once laid in the acquisition chamber, the anesthesia of the mice was maintained with a 2% air-isofluran mixture all along the experiment. With the BFI system, the fluorescent acquisition time was 2 sec and the fluorescent signal was then overlaid on a picture of the mice.

CCD camera collected light coming out from the skin of the animal without any *a priori* information regarding the deepness of the sources. However, excitation and emission

photons employed in our experiments have a mean path before absorption of 1-2 cm, and this property depends on the optical characteristic of tissues themselves. Thus, since the photons can pass up to 2 cm through the animal body, sources located up to 2 cm below the skin can be visualized. However, in order to unambiguously localize the fluorescent dye accumulated in specific anatomical areas, a much more detailed study should be performed.

3 - Results

3.1 - Preparation of stealth DNA LNCs and physicochemical characterization of the different coatings

The physico-chemical properties (Table 1) and the DNA encapsulation ability (Figure 1) of DNA LNCs were examined before and after the post-insertion of DSPE-mPEG₂₀₀₀ or F108. We used DSPE-mPEG₂₀₀₀ concentrations from 2 to 10mM, and F108 concentrations from 1 to 3mM. The high molecular weight of F108 (14,600Da) did not allow us to associate more than 3mM of F108 at the LNC surface. Above this concentration demixing was observed, indicating an excess of F108. A higher density of DSPE-mPEG₂₀₀₀ chains was therefore possible to obtain at the LNC surface.

Compared to empty LNCs ($48 \pm 4\text{nm}$), the lipoplexe-loaded LNCs (DNA LNCs) demonstrated a significant increase in size ($117 \pm 10\text{nm}$) and zeta potential measured in a final concentration of 1,5 mM NaCl ($+30 \pm 2\text{mV}$ versus $-14 \pm 1\text{mV}$ for empty LNCs) (Table 1).

LNC formulation	Mean size (nm)	Pdl	Zeta potential (mV)
Empty LNC	48 ± 4	0.020	-14 ± 1
DNA LNC	117 ± 10	0.255	+30 ± 2
DNA LNC + DSPE-PEG 2000 2mM	131 ± 10	0.230	+23 ± 8
DNA LNC + DSPE-PEG 2000 5mM	139 ± 19	0.374	-12 ± 3
DNA LNC + DSPE-PEG 2000 10mM	142 ± 20	0.250	-41 ± 11
DNA LNC + F108 1mM	129 ± 2	0.209	+14 ± 2
DNA LNC + F108 2mM	129 ± 4	0.256	+17 ± 3
DNA LNC + F108 3mM	132 ± 3	0.248	+22 ± 1

Table 1. Influence of the incorporation of DSPE-mPEG₂₀₀₀ and F108 at the surface of DNA LNCs on size, polydispersity and zeta potential. Results show the mean ± SD of at least 4 independent formulation measurements and 3 measurements per sample.

The mean size obtained after the post-insertion of DSPE-mPEG₂₀₀₀ was 131 ± 10, 139 ± 19 and 142 ± 20nm for 2, 5, 10mM respectively. When adding F108 block copolymers, the sizes were weakly increased with 129 ± 2, 129 ± 4, and 132 ± 3 for 1, 2, 3mM respectively. In all cases, size increase was between 130 and 142nm, whatever the concentration, without significant differences. The zeta potential values decreased progressively from +30mV for DNA LNCs to -41mV with increasing concentrations of DSPE-mPEG₂₀₀₀. DNA LNC zeta potentials decreased more weakly in the case of F108 incorporation, i.e. +14, +17 and +22mV for 1, 2 and 3mM, respectively.

Agarose gel electrophoresis experiments showed that DNA molecules did not migrate after the nanocapsule formulation process. By contrast, incubation of nanocapsules with Triton® led to the release of DNA molecules that migrated into the gel (Figure 1). These results clearly indicate that the addition of polymers at the surface of DNA LNCs does not disturb encapsulation (lanes 4 to 15) and that DNA molecules remain well encapsulated inside nanoparticles.

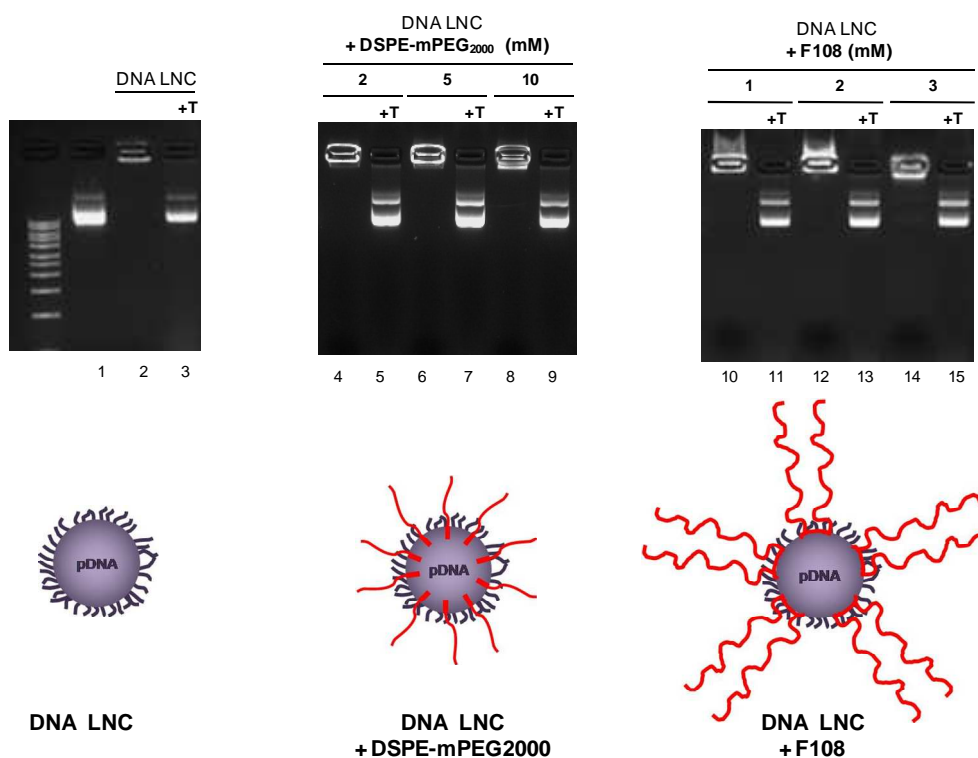


Figure 1. Encapsulation efficiency of DNA in LNCs and schematic representation of the different DNA LNCs. The influence of coating on encapsulation efficiency was tested for all types of DNA LNC suspensions: DSPE-mPEG₂₀₀₀-coated (lanes 4 to 9) and F108-coated DNA LNCs (lanes 10 to 15). No migration of DNA into the gel indicates an efficient encapsulation. DNA molecules can not migrate once encapsulated in nanocapsules (lane 2), contrary to free DNA (pCMVluc) (lane 1). The incubation of nanocapsules with Triton® X100 (+T) led to the release of DNA molecules that migrated into the gel (lanes 3,5,7,9,11,13 and 15).

3.2 - Macrophage uptake

Free lipoplexes, empty LNCs or encapsulated lipoplexes (DNA LNCs) were firstly tested for their cytotoxicity against THP-1 cells (Figure 2). Free DNA was clearly non-toxic to THP-1 cells whatever the concentration from 0.46 to 44.6µg/ml. Empty LNCs and DNA LNCs were not toxic at concentrations under 4.46µg/ml. By contrast, at this concentration, free lipoplexes induced significant cell death (45% of cell survival versus 100% for LNCs encapsulating the same DNA concentration). The encapsulation of lipoplexes in LNCs thus provided an efficient loss of toxicity.

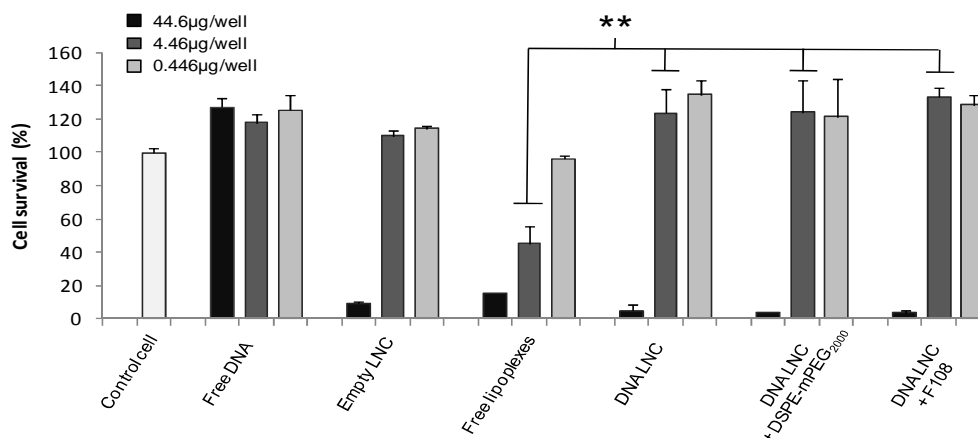


Figure 2. Particle cytotoxicity assessed by the MTT test. THP-1 cells were confronted with different formulations : free DNA, free lipoplexes, DNA LNCs, 5mM DSPE-mPEG₂₀₀₀ and 2mM F108-coated DNA LNCs, at different concentrations of pDNA: 44.6µg, 4.46 µg, and 0.46µg per well. Cells without treatment were taken as the reference (100%). As a control, empty LNCs were added at the same concentration (mg of LNC components per ml) of DNA LNCs. Results are expressed as the percent of the optical density of the cells alone, as the mean ± SD of 3 wells in 2 independent experiments. **: P< 0.01 (Dunnnett test).

The influence of PEG concentration and chain length in empty and DNA-LNCs on macrophage uptake was then studied. For this purpose, fluorescent LNCs were synthesized allowing the tracking of these vectors inside macrophage cells (THP-1 lineage) by fluorescent confocal microscopy (Figure 3A). DNA LNCs were loaded with both Dil fluorochrome (associated to the lipid core) and lipoplexes. The cells were treated with a dose of 1.5mg/ml of nanocapsules (representing 4.46µg of DNA per ml). DNA LNCs and 2mM F108-coated DNA LNCs were internalised within 20 minutes at 37°C, mostly inside vesicles. Empty LNCs and 5mM DSPE-mPEG₂₀₀₀-coated DNA LNCs did not give any signal within the cells, suggesting the absence of particle uptake. This was confirmed by quantitative analysis exposing the number of dots per cell (Figure 3B) and showing that DSPE-mPEG₂₀₀₀-coated

DNA LNCs have the same behavior as empty LNCs. Same data were obtained after 24 hours of incubation at 37°C (data not shown).

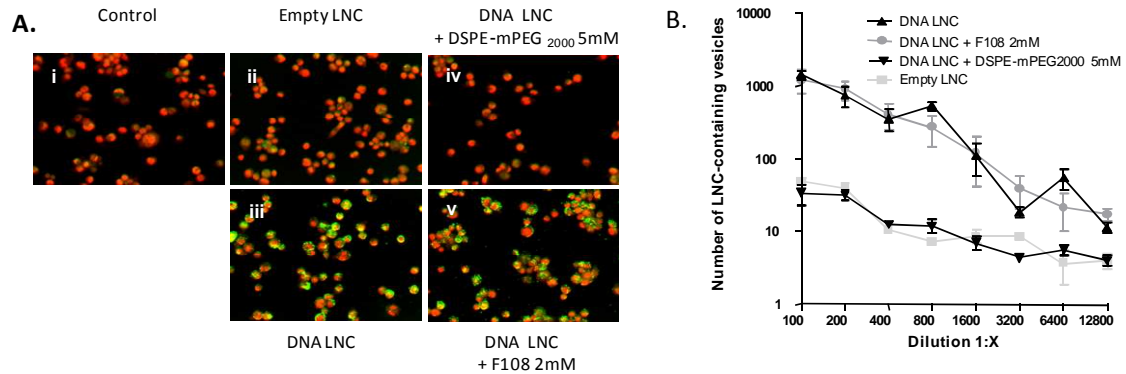


Figure 3. Quantitative fluorescent confocal microscopy of living THP-1 cells exposed to fluorescent Dil-labelled blank LNCs (i), DNA LNCs(ii) and coated DNA LNCs with DSPE-mPEG₂₀₀₀ (iv) and F108 (v). The different Dil-labelled suspensions were incubated with differentiated THP-1 macrophages for 20min at 37°C. After extensive washes, cells were labelled with Syto60 red stain and images were acquired using an automated, confocal, fluorescence microscope. Representative pictures are shown in panel A. Dil-labelled vesicles can be seen in green. Note the important internalisation observed with non-coated DNA LNCs (iii) and F108-coated DNA LNCs (iv) whereas really small amounts of DNA LNCs coated with DSPE-PEG 5mM are detected. Images span 0.450x0.340 mm². Image-based quantification of the number of LNC-containing vesicles is displayed for serial 2-fold dilutions of each LNC type (panel B).

3.3 - Complement consumption

Complement consumption was evaluated as the lytic capacity of the serum towards 50% of antibody-sensitized sheep erythrocytes (CH50 units) after exposure to free lipoplexes, empty LNCs, DNA LNCs and DNA LNCs coated with different concentrations of DSPE-mPEG₂₀₀₀ (Figure 4A) and F108 (Figure 4B).

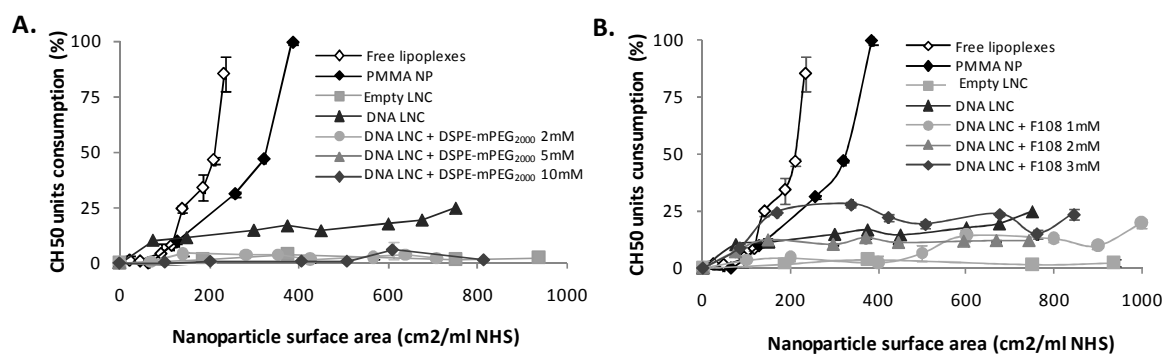


Figure 4. Influence of DSPE-mPEG₂₀₀₀ (A) and F108 (B) on complement consumption by DNA LNCs. The CH50 consumption was represented as a function of the nanoparticle surface area (cm²) representing an increase in nanocapsule concentration. Suspensions of nanoparticles were incubated for 60min at 37°C in human serum diluted ¼ (v/v) in VBS²⁺. Complement consumption was evaluated as the lytic capacity of the serum (amount of CH50 units) towards antibody-sensitised sheep erythrocytes after exposure to blank LNCs, DNA LNCs and DNA LNCs coated with different concentrations of DSPE-mPEG₂₀₀₀ (A) and F108 (B). Each datum point represents the group mean ± s.d. of the CH50 unit consumption.

PMMA nanoparticles, already described as strong complement activators [14], exposed a CH50 consumption of 100% at low concentrations (expressed in nanoparticle surface area). Free lipoplexes, which are positively charged, were even stronger activators than PMMA particles, with 100% of CH50 unit consumption for a lower concentration. The consumption of CH50 units reached a maximum of 5% for empty LNCs, whereas DNA LNCs led to 25% of consumption with the same quantity of nanoparticles. Nevertheless, for the same DNA concentration, the consumption unit of free lipoplexes was much stronger than lipoplexes encapsulated in LNCs (DNA LNCs). When coated with DSPE-mPEG₂₀₀₀, DNA LNCs were weaker activators than non-covered DNA LNCs (Figure 4A), because less than 10% of CH50 unit consumption was obtained, whatever the quantity of particles and the polymer concentration (2, 5 and 10mM). In the case of F108 (Figure 4B), complement activation obtained with this coating reached 20% of CH50 unit consumption for 1 and 2mM.

The nanocapsules coated with F108 at a concentration of 3mM showed an even higher activation level than non-coated DNA LNCs.

3.5 - Blood distribution of LNCs in Swiss mice

With the goal of following the blood half-lives of LNCs in Swiss mice, we used fluorescent-labeled LNCs injected in the tail vein at a DNA concentration of 3.35mg/kg of animal weight. Thereafter blood samples were collected from one minute (expressed as the 100% of the injected dose) to 24 hours, and the plasma was dosed for its fluorescent content.

In parallel, blood samples were harvested for the dosage of transaminases ALAT (alanine aminotransferase) and ASAT (aspartate aminotransferase) (data not shown). In comparison of these mice before administration of the LNCs (ALAT = 40.83 ± 30.88 ; ASAT = 23.04 ± 5.45), an increase of these two enzymes in the plasma 24h after the injection of non coated DNA LNCs (ALAT = 410.18UI/l, ASAT = 507.41UI/l) was noted whereas no or lowest increase was detected for F108 (ALAT = 62.90UI/l, ASAT = 69.90UI/l) and DSPE-mPEG₂₀₀₀-coated DNA LNCs (ALAT = 193.18UI/l, ASAT = 208.67UI/l) respectively.

As seen in Figure 5, DNA LNCs were quite rapidly cleared from the circulation with 50% of fluorescence detected in the plasma at 0.3h post injection (versus 0.7h for empty LNCs). DNA LNCs coated with 2mM DSPE-mPEG₂₀₀₀ exhibited a $t_{1/2}$ of distribution increase from 1.4h to 1.6h (Table 2), and the AUC raised from 194 to 325% of the injected dose per hour (Table 2). When adding 5mM and 10mM of DSPE-mPEG₂₀₀₀, respectively, to the surface of DNA LNCs, 47% and 56% of the injected dose was still circulating 4 hours after

injection (Figure 5A), to reach a $t_{1/2}$ of distribution of 7.1 hours with 10mM of DSPE-mPEG₂₀₀₀. The AUCs of these formulations were 539 and 773% of the injected dose per hour respectively (Table 2), which represents a huge increase compared to DNA LNCs (194%). The mean residence time (MRT) was of 2.3h and 2.4h for DNA LNCs coated with DSPE-mPEG₂₀₀₀ 5mM and 10mM (versus 1.5h for DNA LNCs) and the $t_{1/2}$ of elimination reached 8.6 hours with DSPE-PEG 10mM (versus 5.5h with DNA LNCs).

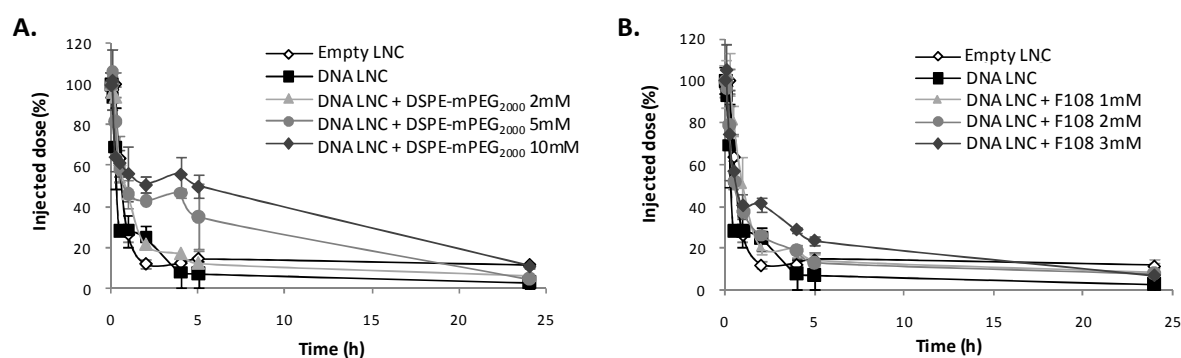


Figure 5. Kinetic blood profiles of LNCs coated with various concentrations of DSPE-mPEG₂₀₀₀ (A) or F108 (B) following systemic injection in Swiss mice. The percentage of injected dose (3.35mg of DNA/kg of animal weight) remaining in plasma following a single bolus injection is displayed as a function of time. Administration of empty LNCs and DNA LNCs are shown as a control. Each datum point represents the group mean \pm s.d. of the percent injected dose.

When adding 1, 2 and 3mM of F108, the $t_{1/2}$ of distribution were of 1.7, 1.9 and 2.7h, respectively. The $t_{1/2}$ of elimination attained 7 hours whatever the concentration of F108 at the LNC surface. Although the coating of DNA LNCs with F108 showed a weak improvement in $t_{1/2}$ of distribution, the AUC [1-24h] was increased (with 366, 355, and 453% of the injected dose per hour, for 1, 2 and 3mM, respectively). The MRT reached 2h with these block copolymers at the surface of DNA LNCs.

Formulation	$t_{1/2}$ distribution [0-5h] (h)	$t_{1/2}$ elimination [0-24h] (h)	MRT [0-5h] (h)	AUC [0-24h] (% injected dose/h)
Empty LNC	1.5 ± 0.1	4.9 ± 0.2	1.6 ± 0.4	260 ± 4.3
DNA LNC	1.4 ± 0.1	5.5 ± 0.3	1.5 ± 1.1	194 ± 3.2
DSPE-PEG 2mM	1.6 ± 0.2	7.1 ± 1.8	1.6 ± 1.7	325 ± 5.4
DSPE-PEG 5mM	3.9 ± 1.7	6.1 ± 0.1	2.3 ± 0.2	539 ± 8.9
DSPE-PEG 10mM	7.1 ± 0.1	8.6 ± 1.1	2.4 ± 0.8	773 ± 12.9
F108 1mM	1.7 ± 0.2	7.5 ± 0.4	1.7 ± 2.8	366 ± 6.1
F108 2mM	1.9 ± 0.1	7.6 ± 1.1	1.8 ± 1.3	355 ± 5.9
F108 3mM	2.7 ± 0.1	7.0 ± 0.9	2.1 ± 0.8	453 ± 7.5

Table 2. The main pharmacokinetic characteristics of various formulations of LNCs after a single i.v. injection in Swiss mice. Plasma clearance of LNCs was measured over a 24h period in animals treated with 3.345mg of DNA/kg of mouse weight. The half lives were calculated as follows: $t_{1/2} = \text{Log}(2)/L_z$. The L_z was determined from linear regression using defined intervals (respectively 5h and 24h, for $t_{1/2}$ distribution [0-5h], and $t_{1/2}$ elimination [0-24h]). The AUC was calculated following the trapezoidal rule during the whole experimental period (1min to 24h) without extrapolation. The mean residence time was calculated from 1min to 5h, from the following equation: $\text{MRT [0-5h]} = \text{AUMC [0-5h]} / \text{AUC [0-5h]}$. Each datum point represents the group mean ± s.d.

3.6 - Tumor accumulation of coated DNA LNCs

To estimate time dependant excretion profile and tumor accumulation of the polymer coated LNCs exposing the greatest residence time in bloodstream (10mM DSPE-mPEG₂₀₀₀), these suspensions were intravenously injected in the tail vein of tumor bearing mice and compared to non coated DNA LNCs. Tissue distribution was evaluated thanks to NIR biofluorescence imaging (BFI) system. First of all, the early fluorescence signals were much more intense after injection of coated DNA LNCs than after the administration of uncoated particles. When regarding non covered DNA LNCs, the fluorescence intensity increased in the liver area from 3h after injection up to 24h, whereas no accumulation in this anatomical area was observed with 10mM DSPE-mPEG₂₀₀₀-coated DNA LNCs at any time (Figure 6). In parallel, a fluorescence emission was observed 3h, 5h, 24h, and 48h after DNA LNCs injection on the kidney area, which could therefore let think to an elimination of DNA LNCs

via urinary system. At 24h and 48h after injection, DSPE-mPEG₂₀₀₀-covered DNA LNCs displayed stronger fluorescence intensity in the tumor and in its vicinity, compared to non coated DNA LNCs.

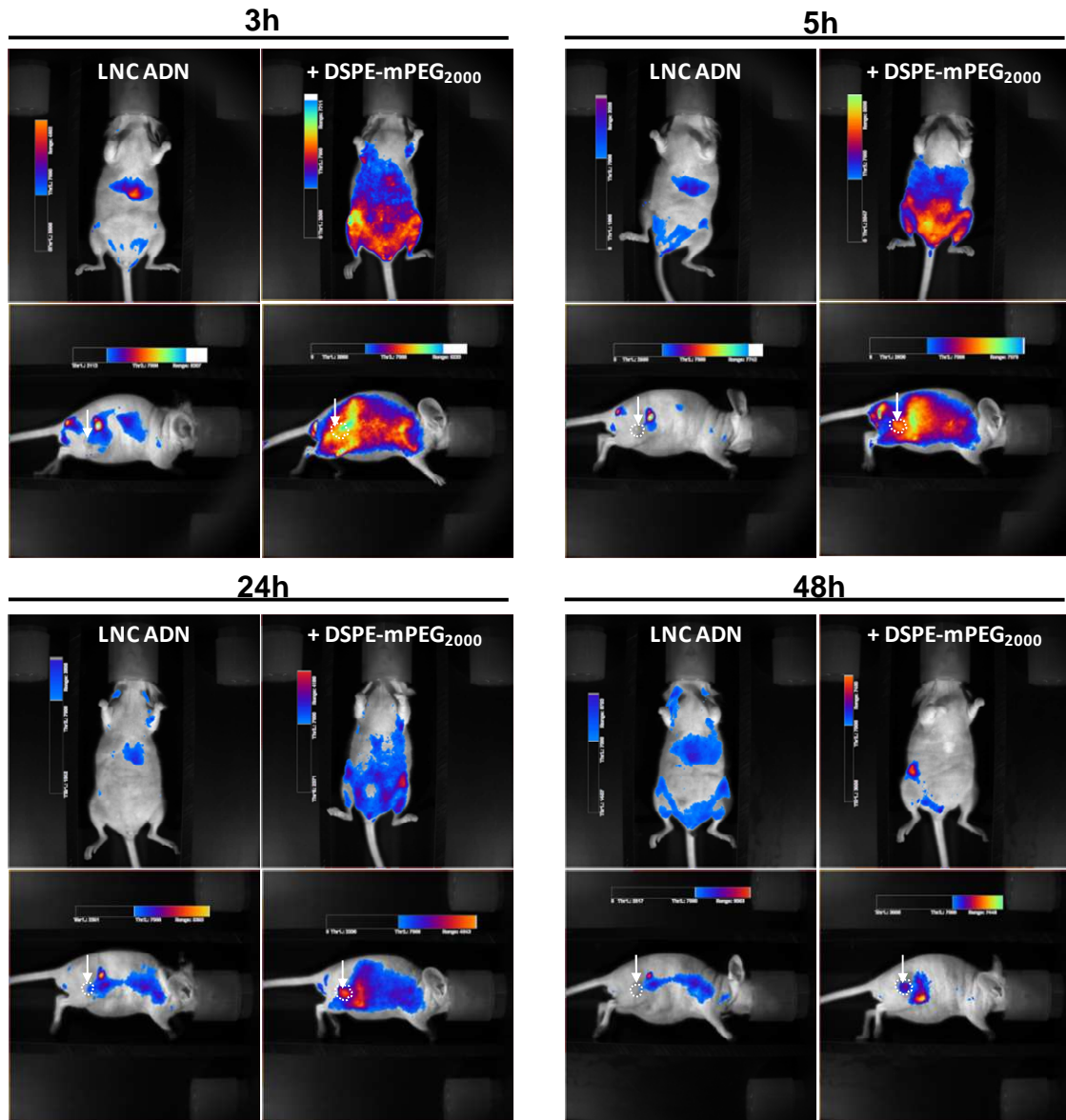


Figure. 6 *In vivo* fluorescence imaging of athymic nude mice bearing U87MG tumors after intravenous injection of DNA LNCs or DSPE-mPEG₂₀₀₀ coated DNA LNCs. Optical images of nude mice with 152mg/ml tail vein injection of DNA LNCs or DSPE -mPEG₂₀₀₀ coated DNA LNCs (representing 46µg of pDNA per mice). Coloured bar on the left or upper part of the picture indicates the signal efficient of the fluorescence emission coming out from the animal. The tumor location is specified with a white arrow.

4 - Discussion

The formulation process led to the creation of empty LNCs or DNA LNCs with very different size and surface charge properties (Table 1) predicting a difference of behavior when confronted with a biological environment. By coating DNA LNCs with amphiphilic polymers, our aim was to improve their circulation time in order to give them the adequate features for *in vivo* injection and tumor accumulation.

PEG lipid derivatives DSPE-mPEG₂₀₀₀ and block copolymers F108 were associated to pre-formed nanocapsules by the post-insertion method, usually used to create stealth liposomes, and recently applied to LNCs [18, 19]. The centrifugation (15,000g, 20min., 20°C) of coated DNA LNCs revealed a good level of stability of all the particles. Nevertheless, high concentrations of F108 (>3mM) led to demixing (data not shown), whereas this was never observed with DSPE-mPEG₂₀₀₀ at any concentration. This could be explained by steric overcrowding, due to the high molecular weight of F108 chains (14,600Da) that induced a weaker reachable density of F108 molecules at the surface of the particles (Table 3). The theoretical calculations of several characteristics of the coating exposed in Table 3 were based on the mean diameter measurements ($A=4\pi r^2$), the molar concentration of the post-inserted polymers at the surface of DNA LNCs and on the assumption that there was a full binding of the polymers [20, 21].

Agarose gel electrophoresis experiments showed no migration of DNA molecules after nanocapsule formulation followed by post-insertion, indicating that (a) coated DNA LNCs were stable, (b) most of the lipoplexes were encapsulated, and (c) DNA molecules were not degraded by the coating process. Cytotoxicity studies confirmed the biocompatibility of empty LNCs and DNA LNCs compared to free lipoplexes (Figure 2) This is a significant

advantage of DNA LNCs when confronting lipoplexes, certainly due to charge dissimulation [1].

	Non coated DNA LNC Solutol®* PEG ₆₆₀ -HS	DSPE-mPEG ₂₀₀₀ (mM)			F108 (mM)		
		2	5	10	1	2	3
Number of molecules per nanocapsule	14,046	1,297	3,254	6,509	648	1,297	1945
Surface density (molecules/nm ²)	0.44	0.04	0.10	0.20	0.02	0.04	0.06
Surface area (nm ² /molecule)	2.3	25	10	5	50	25	16
Distance between two molecules (nm)	1.5	5	3.16	2.23	7.07	5	4

Table 3. Theoretical calculation of coating characteristics of nanocapsules as a function of DSPE-mPEG₂₀₀₀ and F108 polymer concentration at their surface.

As already described [5, 6, 14], free lipoplexes and PMMA NP strongly activated the proteins of the complement system as assessed *in vitro* by the CH50 test (Figure 4). The high cationic and anionic charges at lipoplexe and PMMA NP surfaces respectively, govern interactions with plasma-complement proteins via the alternative pathway and also interactions with cells membranes [22, 23]. In comparison with empty LNCs that present no complement activation and no macrophage uptake (Figures 3 and 4), DNA LNCs showed weak complement activity, but more pronounced macrophage uptake (Figure 4). While the coating of DNA LNCs with F108 led to the same activation as with non-coated DNA LNCs, DSPE-mPEG₂₀₀₀ led to the inhibition of complement activation down to the empty LNC level. As expected, the addition of DSPE-mPEG₂₀₀₀ improved macrophage escape (Figure 3). The high charge of DNA LNCs (+30mV) was dissimulated thanks to the use of DSPE-mPEG₂₀₀₀ polymers, to reach a still positive (+22mV) or negative (-12mV or -41mV) surface charge. These differences of zeta potentials are linked to the fact that DSPE-PEG chains can form

negative dipoles that are able to diminish the surface charge proportionally to their concentration [24]. This negative charge, close to that of empty LNCs (-14mV) prevented their removal via non-specific interactions with receptors by electrostatic attraction at the macrophage surface [25, 26], mainly observed with positive charges. By contrast, F108-coated DNA LNCs were largely taken up; this could be linked to their positive charge (+22mV) or to a dissociation of F108 copolymers and DNA LNCs which resulted in their rapid uptake by the MPS. One hypothesis is also that the PPO hydrophobic moieties present on F108 could be accessible to opsonins and consequently provide more association to cells compared to the hydrophobic moieties of DSPE-mPEG₂₀₀₀ which are anchored in the nanocapsule core [18, 27]. It is now well established that a dissociation of the PEG chains from the particles is required to interact with cell membranes [28] and, in this case, the disadvantage of F108 can become an advantage. Indeed, DSPE-mPEG₂₀₀₀ coated DNA LNCs were less efficient than F108 covered ones in *in vitro* HeLa cell transfection as previously described [29]. Anyway, there is, *in vivo*, a need of finding equilibrium between transfection and pharmacokinetic behavior [28].

As our final aim was to obtain long-circulating vectors for systemic gene delivery, we then investigated the plasma clearance of fluorescent LNCs in Swiss mice. It is well known that the low circulation time of free lipoplexes explains their poor efficiency for gene delivery *in vivo*, with only 1% detected in the blood 5min after injection in mice [30]. By contrast, DNA LNCs exposed 93% of the injected dose at the same time (Figure 5).

In order to enhance their circulation time we chose to protect the DNA LNC surface with DSPE-mPEG₂₀₀₀ or F108 poloxamers. The half-lives and the mean residence time for all the DSPE-mPEG₂₀₀₀ and F108-coated DNA LNCs were higher than for DNA LNCs. However,

F108 poloxamers did not efficiently improve circulation properties, probably because the surfactant is displaced on dilution in the blood and enters into competition with opsonins. This confirms that the anchorage of PEG chains in DNA LNCs is essential for prolonging the *in vivo* residence time [31]. By contrast, DSPE-mPEG₂₀₀₀-coated DNA LNCs had extended half-lives, and the blood circulation time increased with the density of DSPE-mPEG₂₀₀₀ chains. The distance between two PEG chains from 5 to 2.33nm (Table 3) and their density is in good agreement with other long-circulating systems such as PLA-PEG nanocapsules [31].

The excretion profile of DNA LNCs and 10mM DSPE-mPEG₂₀₀₀ DNA LNCs in live tumor bearing mice was then tested by monitoring real time NIR fluorescence intensity in the whole body, as this last formulation was the more promising in terms of circulation time. Near-infrared (NIR)-absorbing dyes represent an intriguing way for extracting biological information from living subjects since they can be monitored with safe, non-invasive optical imaging/contrasting techniques. While light in the visible range is routinely used for microscopy, imaging deeper tissues (>500 μm to cm) required the use of near-infrared light, as hemoglobin and water, the major absorbers of visible and infrared light have their lowest coefficient in the NIR section (650-900 nm). The advantages of imaging in the NIR region are numerous: the significant reduction of background absorption, fluorescence and light scattering along with high sensitivity, the availability of low-cost sources of excitation and the versatility of different reporter probes.

Consistently with blood kinetic profiles (Figure 5 and Table 2), DNA LNCs were rapidly localized in the liver and in the kidney suggesting a removal by the MPS. By contrast, DSPE-mPEG₂₀₀₀ coated DNA LNCs were able to accumulate in the tumor and its

neighborhood by passive targeting thanks to a sufficient circulation time in blood. The low hepatotoxic impact of these coated particles was also a positive point.

This newly formed vector can advantageously be compared to other gene therapy systems reported in the literature. Actually, as exposed in the study of Cui *et al.*, 30 min. after tail-vein injection in mice, only 40% of the pDNA entrapped in nanoparticles synthesized from emulsion remained in the circulating blood [32]. Even the clinically relevant systemic RNAi-mediated gene silencing in non-human primates developed by Zimmermann *et al.* [33], exposed a half-life in mice of 38 minutes. However, the circulation time of stabilized plasmid lipid particles (SPLP) in the blood can vary from 1h to 16h, depending on the PEG lipid anchor used. Nevertheless this study also showed that the PEG lipid anchor has to be disassociated from the particle surface in order to transform the complex from a stable particle to a transfection-competent entity [34-36], which occurs with one of the shortest lipid anchors (C₁₄) and a distribution $t_{1/2}$ of 2h.

5 - Conclusion

The DSPE-mPEG₂₀₀₀-coated DNA LNCs developed here are able to circulate in the bloodstream without being degraded or captured by the cellular defense mechanisms, and to accumulate in the tumor area. One hurdle, the extracellular one, is therefore crossed, but numerous barriers still exists at the cellular level, and efforts have to be made to still improve this vector. Nevertheless, this DNA delivery system seem to be an excellent candidate for an efficient *in vivo* transfection, either by the enhanced and permeability retention effect (EPR effect) [7] or by active targeting thanks to the grafting of specific molecules to the extremity of the longest PEG chains.

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Publication n°3

Les LNC galactosylées comme vecteurs d'ADN pour un ciblage actif des hépatocytes

Améliorer le ciblage spécifique des vecteurs non viraux est un des challenges du transfert de gène. Les vecteurs synthétiques cationiques non modifiés interagissent avec les cellules de façon électrostatique et non-spécifique. Ce mode d'interaction particulièrement efficace en transfection *in vitro* avec les lipoplexes et polyplexes devient, comme décrit précédemment (revue bibliographique et publication n°2), un inconvénient majeur *in vivo*. Une des possibilités pour améliorer la transfection *in vivo* est d'utiliser des vecteurs proches de la neutralité et présentant des ligands de ciblage afin d'améliorer la transfection spécifique d'un type cellulaire visé. Dans cet objectif, nous avons cherché à voir si les LNC ADN, recouvertes ou non de longues chaînes de polymères, étaient efficaces en transfection. Le dosage de l'expression du transgène luciférase a été réalisé, dans un premier temps, sur des cellules de lignée. Dans un second temps, le greffage de galactose à l'extrémité des longues chaînes de PEG a été testé afin d'observer un ciblage actif et spécifique des récepteurs aux asialoglycoprotéines surexprimés à la surface des cellules épithéliales du foie : les hépatocytes.

« Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting »

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GALACTOSYLATED DNA LIPID NANOCAPSULES FOR EFFICIENT HEPATOCYTE TARGETING

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Abstract

The main objective of gene therapy via a systemic pathway is the development of a stable and non-toxic gene vector that can encapsulate and deliver foreign genetic materials into specific cell types with the transfection efficiency of viral vectors. With this objective, DNA complexed with cationic lipids of DOTAP/DOPE was encapsulated into lipid nanocapsules (LNCs) forming nanocarriers (DNA LNCs) with a size suitable for systemic injection ($109\pm 6\text{nm}$). With the goal of increasing systemic delivery, LNCs were stabilised with long chains of poly (ethylene glycol) (PEG), either from a PEG lipid derivative (DSPE-mPEG₂₀₀₀) or from an amphiphilic block copolymer (F108). In order to overcome internalization difficulties encountered with PEG shield, a specific ligand (galactose) was covalently added at the distal end of the PEG chains, in order to provide active targeting of the asialoglycoprotein receptor present on hepatocytes. This study showed that DNA LNCs were as efficient as positively charged DOTAP/DOPE lipoplexes for transfection. In primary hepatocytes, when non galactosylated, the 2 polymers significantly decreased the transfection, probably by creating a barrier around the DNA LNCs. Interestingly, galactosylated F108 coated DNA LNCs led to a 18-fold increase in luciferase expression compared to non-galactosylated ones.

1 – Introduction

In the field of gene delivery, the most common lipid-based carriers of polynucleotides are lipid-DNA complexes, also called lipoplexes, obtained by mixing cationic liposomes with DNA at a precise +/- charge ratio. Whereas many cell types are transfected *in vitro* with cationic lipids (thanks to electrostatic interactions between negatively-charged membranes and positively-charged systems), the excess of cationic lipids used to complex DNA can lead to high cytotoxicity. Dose-dependent toxicity of different kinds of lipoplexes [GL-67 [1] (*N*4-spermine cholesterylcarbamate) and GL-62 [1] (*N*1-spermine cholesterylcarbamate), DMRIE (1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethylammonium bromide), DOTMA:DOPE] has been observed, for instance, after their injection into mice leading to hair erection and lethargy [2]. Clinical studies have also shown dose-dependent haematological and serological changes typified by profound leukopaenia, thrombocytopaenia, and elevated serum transaminase levels, indicative of hepatocellular necrosis. Moreover, another obstacle in the use of lipoplexes via systemic delivery is their aggregation, instability and propensity to be captured by the mononuclear phagocyte system (MPS). Indeed, positively-charged particles can be opsonised with plasma proteins such as immunoglobulin M, complement C3, and proteins from the coagulation cascade [3] leading to their rapid clearance by MPS phagocyte cells in the liver, spleen, lungs and bone marrow [4].

In this context, numerous efforts have been carried out to obtain effective, stable and long-circulating gene delivery systems, mostly using the dissimulation of the positive charges by 'shielding' the vector surface with hydrophilic and flexible polymers such as poly (ethylene glycol) (PEG). The use of this surface modification (also called pegylation) of vectors destined for systemic injection has drawn considerable interest [5-7].

In parallel to the studies based on lipoplexes, nanoparticle-based systems have been developed. With the aim of use for systemic injection, dissimulating DNA from blood nucleases by encapsulation in nanocapsules seems to be the best alternative to keep the integrity of nucleic acids [8]. Heurtault *et al.* [9] developed lipid nanocapsules synthesised by a solvent-free method and covered by PEG₆₆₀ at a high density, allowing really weak complement activation and low macrophage uptake [10]. In a previous work, the formulation of these nanocapsules was adapted to obtain DNA nanocapsules (DNA LNC) [11]. The lipid core allowed the entrapment of plasmid DNA molecules after the formation of lipoplexes. The DNA LNCs were small ($109 \pm 6\text{nm}$), suitable for intravenous injection, but *in vivo* stability and plasmatic half-life remained low and ill-adapted to efficient *in vivo* transfection. For these reasons we chose to modify the surface of this gene-delivery system, inserting longer PEG chains to the surface of DNA LNCs between the already existing dense PEG₆₆₀ chains from the nanoencapsulation process. This was carried out by using two kinds of amphiphilic and flexible polymers. The first one was F108, a block copolymer, consisting of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure (EO₁₃₂-PO₅₀-EO₁₃₂). The second one was a lipid PEG derivative, the 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀). We demonstrated a significant increase of *in vivo* circulation time in mice, especially for the DSPE-mPEG₂₀₀₀ coating, with a $t_{1/2}$ of elimination of about 7h, i.e. around 5-fold more than for non coated DNA LNCs (submitted results).

Nevertheless, coating vectors with PEG presents some drawbacks: although the PEG coating enhances plasma circulation time and consequently leaves time for the objects to reach their targets [3, 12, 13], it also represents a major barrier for internalisation and

endosomal escape [14, 15]. This is the paradox of PEG use: this polymer has to protect the vector in blood circulation, but once at its target site, the carrier has to uncoat itself to allow its internalisation in the targeted cells, endosomal escape, and finally transport into the nuclei [8]. Therefore, a compromise has to be found between sufficient circulation time in the blood and efficient transfection. A solution to overcome these difficulties and to avoid potential problems of non-specific interactions, is to attach a specific ligand to the gene delivery system, resulting in active targeting and receptor-mediated endocytosis [16-18]. Among the cellular targets for such ligands, the asialoglycoprotein-receptor (ASGP-R) is expressed exclusively on hepatocytes [19]: ASGP-R naturally binds and internalises the terminal galactose-binding asialoglycoprotein. Thus, galactose molecules grafted at the surface of a carrier could provide an active targeting. This can be useful for efficient targeting of the liver, and a consequent secretion of therapeutic gene products into systemic circulation.

We first chose in this study to test the influence of F108 and DSPE-mPEG₂₀₀₀ on the transfection efficiency of DNA LNC on cancer cell lines. In a second time, the two kinds of polymers used for the coating were galactosylated (DSPE-PEG-gal and F108-gal), following different synthesis pathways, and the so formed galactosylated DNA LNCs were tested for their *in vitro* transfection efficiency on primary hepatocytes, with the goal to evidence a specific transfection of these vectors.

2. Materials and methods

2.1 - Preparation of carriers

2.1.1 - Liposome / lipoplexe preparation

DOTAP (1,2-DiOleoyl-3-TrimethylAmmonium-Propane) and DOPE (1,2-DiOleoyl-sn-glycero-3-PhosphoEthanolamine) (Avanti Polar Lipids, Inc, Alabaster, USA) were first dissolved in chloroform (Sigma, Saint-Quentin Fallavier, France) and then dried by evaporation under vacuum. The formed lipid film was hydrated with deionized water. Then liposomes were sonicated in a bath for 20 minutes. Lipoplexes were prepared by mixing DOTAP/DOPE (1/1, M/M) liposomes at a positive charge concentration of 25mM with 660µg of luciferase encoding plasmid [20] (gWIZ-luc, 6732 bps, amplified and purified for research grade, by GENEART, Regensburg, Germany) at a charge ratio (+/-) of 5 per 150mM NaCl.

2.1.2 - DNA-loaded lipid nanocapsules (DNA LNCs)

The formulation of LNCs was based on a phase-inversion process described by Heurtault *et al.* [21]. LNCs were made with lipophilic Labrafac® WL 1349 (caprylic-capric acid triglycerides, European Pharmacopia, IVth, 2002) and oleic Plurol® (Polyglyceryl-6 dioleate) which were kindly provided by Gattefossé S.A. (Saint-Priest, France) and Solutol® HS-15 (70% of PEG 660 hydroxystearate (HS-PEG) and 30% of free PEG 660 Dalton, European Pharmacopia, IVth, 2002) which was a gift from BASF (Ludwigshafen, Germany). Briefly, 3.9 % of oleic Plurol® (w/w), 5.9 % of Solutol® (w/w), 9.9 % of Labrafac® (w/w), 78.9 % of water (w/w) and 1.4 % of NaCl, were mixed together under magnetic stirring. Previously formed DOTAP/DOPE lipoplexes were introduced in the water phase of the emulsion to form DNA

LNC [11]. Six temperature cycles were applied to reach phase inversion, between 20 and 60°C, from an oil-in-water to a water-in-oil emulsion. Thereafter, the mixture underwent a fast cooling-dilution process with water at 0°C, leading to the formation of LNCs in water.

2.1.3 - Galactosylation of polymers

The synthesis of galactosylated F108 was performed by enzymatic galactosylation as already described [7]. The synthesis of DSPE-PEG-gal was performed by chemical galactosylation via a reductive amination which required lactose use. Under nitrogen atmosphere, lactose (245mg, 716 μ mol, 40 equiv) was added at room temperature in 10 ml of anhydrous dichloromethane/methanol (1:1, v/v) and mixed with 50 mg of DSPE-PEG (18 μ mol, 1 equiv). The reaction mixture was stirred for 2 h at 50°C. Then 28.2 mg of sodium cyanoborohydride (4.47 mmol, 25 equiv) in methanol (200 μ l) was added and the mixture was again heated at 50°C for 4h. The solution was concentrated under reduced pressure, diluted with phosphate buffer (pH 7, 0.12 M) and purified by dialysis (Cellu•Sep® H1 dialysis membrane 2,000 MCWO) against distilled water at 4°C, followed by lyophilisation to afford DSPE-PEG-gal as a white solid (70 mg, up to 25% of galactose incorporation).

2.1.4 - Preparation of coated and galactosylated DNA LNCs by the post-insertion method

Two kinds of polymers were used for post-insertion: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG₂₀₀₀) (MW = 2,805g/mol) (Avanti Polar Lipids, Inc, Alabaster, USA) and Pluronic® F108 (Poly(ethyleneoxide)₁₃₂-poly(propyleneoxide)₅₀-poly(ethyleneoxide)₁₃₂) (MW = 14,600g/mol)

kindly provided by BASF, galactosylated or not. These polymers were added to a fixed quantity of DNA LNCs by the post-insertion method [22, 23] in order to obtain a final concentration of 2, 5 and 10mM (DSPE-mPEG₂₀₀₀ or DSPE-PEG₂₀₀₀-gal) and 1, 2, 3mM (F108 or F108-gal). Prior to post-insertion, the LNCs were purified thanks to the use of PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France) and then concentrated by ultrafiltration with Millipore Amicon® Ultra-15 centrifugal filter devices (Millipore, St Quentin-Yvelines, France). Preformed DNA LNCs were co-incubated for 4h at 30°C with DSPE-mPEG₂₀₀₀ or F108 with or without galactose to form the so-called coated DNA LNCs or galactosylated DNA LNCs. The mixture was vortexed every 15 minutes and then quenched in an ice bath for 1 minute. To provide controls, the same thermal treatment was applied to LNC suspensions without polymers.

2.2 - Nanoparticle characterisation

2.2.1 - Physico-chemical characteristics of coated DNA LNCs and galactosylated DNA LNCs

The average hydrodynamic diameter, the polydispersity index (PI) and the zeta potential of DNA LNCs were determined by dynamic light scattering (DLS), using a Malvern Zetasizer® (Nano Serie DTS 1060, Malvern Instruments S.A., Worcestershire, UK). DNA LNCs were diluted 1:100 (v/v) in deionised water at 25°C in order to assure convenient scatter intensity on the detector.

2.2.2 - DNA stability study

The stability of nanocapsule suspensions during storage at 4°C was assessed by measuring the size distribution. The stability was also tested after centrifugation at 15,000G at room temperature for 20min in order to visualise separation among all the components.

The stability of encapsulation and the integrity of DNA molecules after the process of nanocapsule formulation and post-insertion were evaluated by electrophoresis. A volume of LNC or lipoplex suspension equivalent to 0.2µg of DNA before and after treatment with Triton® 100X (Sigma, Saint-Quentin Fallavier, France) was mixed with a gel-loading solution (Sigma, Saint-Quentin Fallavier, France) and deposited in each well of agarose gel 1% containing ethidium bromide (Sigma, Saint-Quentin Fallavier, France). Controls were constituted by 0.2µg of free DNA in solution or associated to cationic lipids. Samples were left to migrate for 30 minutes at 100V in Tris- EDTA buffer.

2.2.3 - Galactose accessibility

Soybean lectin from *Glycine max* (Sigma, Saint-Quentin Fallavier, France) also called Soybean agglutinin (SBA) (1mg/ml in PBS) was added to an equal volume of DNA LNCs, coated DNA LNCs (DNA LNCs + DSPE-mPEG₂₀₀₀ 4mM or F108 2mM) or galactosylated DNA LNCs (DNA LNCs + DSPE-PEG₂₀₀₀-galactose 4mM or F108-galactose 2mM). These concentrations of polymers were chosen to have the same number of galactose (648 galactose moieties) per nanocapsule whatever the tested polymer. Agglutination induced by SBA was monitored by measuring the turbidity of the solution at 450nm using a spectrophotometer UV, Uvikon 922, (Kontron Instruments, Montigny Le Bretonneux, France). To saturate galactose binding sites on SBA, SBA was pre-incubated with D-galactose (100µM) and an additional absorbance scan was performed after addition of the nanocapsules.

2.3 - *In vitro* transfection studies

2.3.1 - *Cell line culture*

The HeLa human cervical cancer cell line and H1299 lung cancer cell line were grown in high glucose DMEM (Invitrogen) (4.5g/l). Cell culture media were supplemented with 10% FBS, 2mM L-glutamine, 10µg/ml streptomycin, 100u/ml penicillin at 37°C in humid conditions with 5% CO₂. Cells were plated at a density of 35,000/cm² 24h prior to transfection in the same medium.

2.3.2 - *Primary culture of hepatocytes*

Hepatocytes were isolated from the liver of fed, male rats or mice by the collagenase method [24] and modified as described elsewhere [25]. Briefly, their livers were perfused with Hank's balanced salt solution (HBSS) and washed at a rate of 5ml/min using the inferior *veina cava* before collagenase was added (0.025%). Dead cells were eliminated through a density gradient using Percoll®, and viable cells were plated at a density of 75,000/cm² on collagen-coated plates. Cells were given a time span of 2h to attach themselves to William's medium E with Glutamax® (Invitrogen), 10% fetal bovine serum (FBS), 10µg/ml streptomycin, 100u/ml penicillin, 100nM dexamethasone and 100nM insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark).

2.3.3 – *Transfection*

Cells were transfected with DOTAP/DOPE-DNA lipoplexes formulated at DOTAP/DOPE-DNA +/- charge ratio of 5 as a control. DNA LNCs containing 2µg of plasmid-encoding luciferase were added to each well in the presence of F108, F108-gal, DSPE-

mPEG₂₀₀₀ or DSPE-PEG₂₀₀₀-gal at their surface. Cells were cultured for 24h in cell culture media supplemented with 10% FBS before gene expression was determined.

2.4 - Luciferase assay

Luciferase activity was measured using the Promega luciferase assay system (Madison, WI, USA). Cells were rinsed twice with 500µl of phosphate-buffered saline (PBS) and lysed with 200µl of reporter lysis buffer (Roche Diagnostics, Mannheim, Germany) supplemented with a protease-inhibitor cocktail (Roche Diagnostics). Hepatocyte cells were then subjected to 4 freeze/thaw cycles. The cells were then centrifuged at 10,000G for 5min at 4°C before being assayed for luciferase activity. Each datum point represents the triplicate mean and is normalised to protein content. 20µl of cellular homogenate supernatant was mixed with 100µl luciferase assay buffer (Promega luciferase assay system, Madison, WI, USA). The luciferase activity was assayed by measuring light emission with a VICTOR² multilabel counter (Perkin Elmer, Les Ulis, France) and the relative light units of each sample were counted for 10s. Protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, Brebières, France).

3 - Results

3.1 - Galactosylation of polymers

In regards with the method used for synthesized F108-gal [7], the enzymatic galactosylation was not adapted to DSPE-aminoPEG₂₀₀₀. Indeed, this kind of galactosylation uses the transglycosylation activity of the galactoside hydrolase from *Aspergillus oryzae*, and

it utilizes the transfer of a glycosidically bound sugar to another hydroxyl group. Therefore, the synthesis of DSPE-PEG₂₀₀₀-gal was performed by chemical galactosylation (Figure 1) via a reductive amination which required lactose use: during the functionalisation, the first saccharose unit was opened providing galactose as the only targeting moiety. As the galactosylation was not complete, the average number of galactose units grafted onto steric stabilisers was determined by the ratio of the anomeric signal of galactose (1H, H1 gal, 4.30ppm, d, $J = 7.8\text{Hz}$) and the chemical shifts of non-modified terminal methylenes CH₂-O-H ($\delta = 3.75\text{-}3.80\text{ ppm}$) of F108 or the non-modified terminal methylenes CH₂-NH₂ ($\delta = 4.01\text{-}3.90\text{ ppm}$) of DSPE-aminoPEG₂₀₀₀. This calculation indicated that around 25% of the terminal groups of the F108 (2 groups per molecule) and DSPE-aminoPEG₂₀₀₀ (1 group per molecule) polymers were linked to galactose.

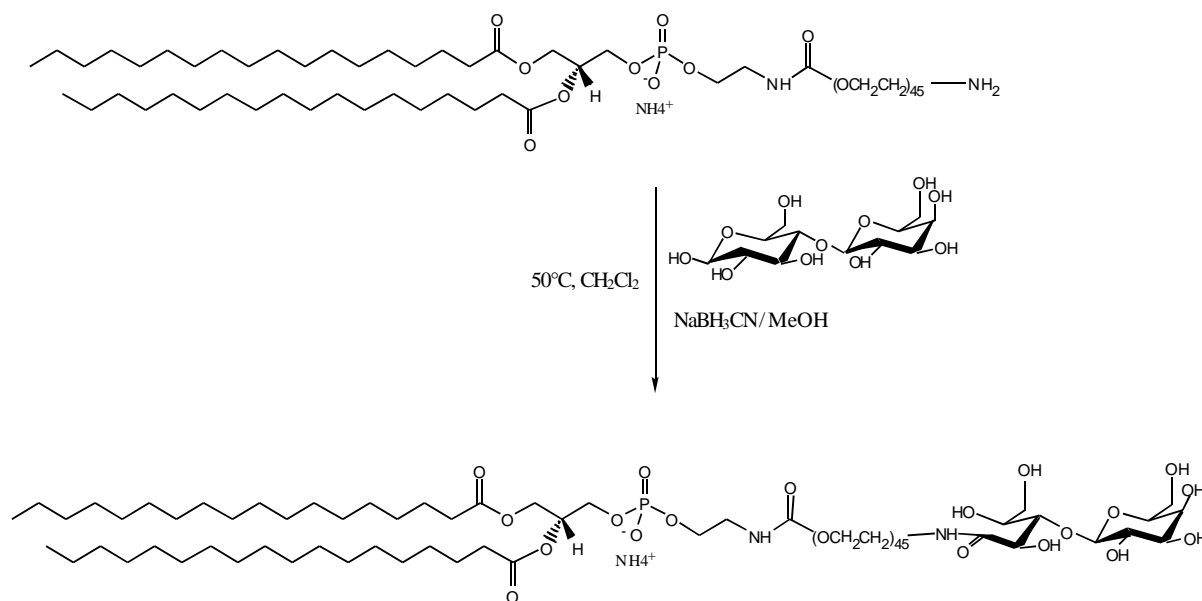


Figure 1. Chemical synthesis of galactosylated DSPE-PEG₂₀₀₀ via a reductive amination reaction. The synthesis of DSPE-PEG₂₀₀₀-gal required lactose use providing the formation of lactosylated distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino polyethyleneglycol-2000) (DSPE-PEG-Lac). The glucose unit of the lactose was opened providing galactose as the only targeting moiety.

3.2 - Formation of DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs

As already described, lipid nanocapsules were slightly modified to encapsulate lipoplexes of DOTAP/DOPE (1/1 molar ratio) at a +/- charge ratio of 5 in their lipid core [11]. The thus-synthesized vector was called DNA LNCs. With the aim to synthesize coated DNA LNCs, PEG lipid derivatives DSPE-mPEG₂₀₀₀ and F108 block copolymers were associated to pre-formed DNA LNCs by the post-insertion method, usually used to create stealth liposomes and recently applied to LNCs [22, 26]. The galactosylated DNA LNCs were synthesized following the same method but using galactosylated DSPE-PEG₂₀₀₀ or galactosylated F108 (Figure 2). The polymers were added at different concentrations (2, 5 and 10mM for DSPE-PEG₂₀₀₀ and 1, 2 and 3mM of F108) to a constant concentration of pre-formed DNA LNCs.

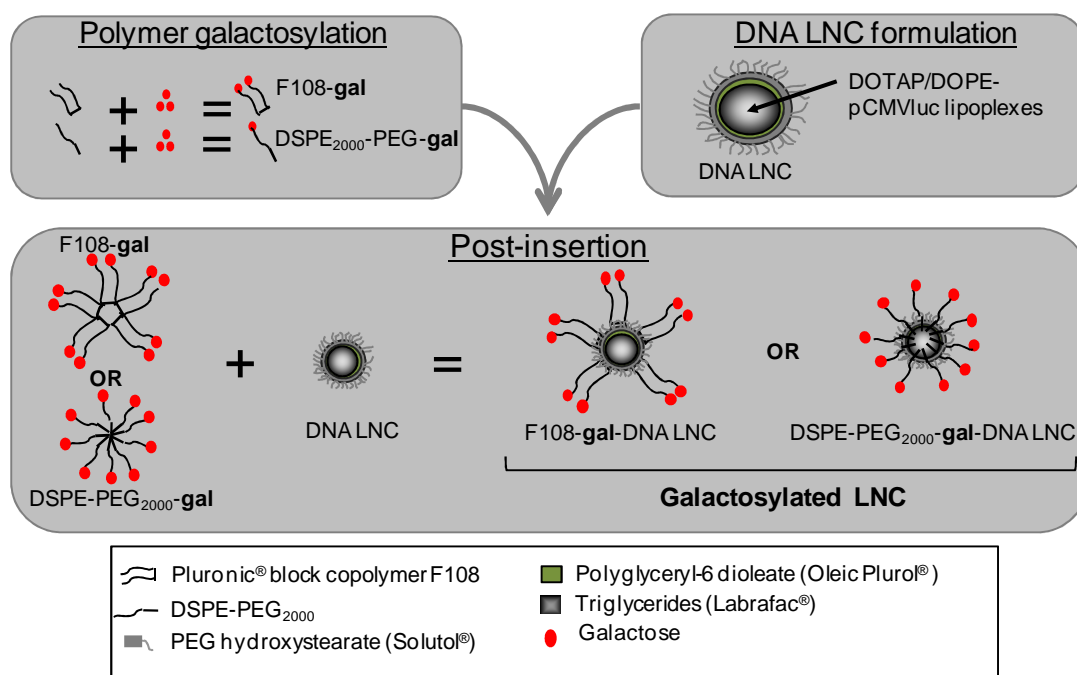


Figure 2. Schematic representation of the formulation of galactosylated DNA LNCs. DSPE-PEG₂₀₀₀ and F108 were galactosylated, to provide DSPE-PEG-gal and F108-gal. The hydrophobic moieties of the polymers (DSPE anchor of DSPE-PEG₂₀₀₀gal and poly(propyleneoxide) (PPO) part of F108-gal) were associated to the lipid nanocapsules, forming the so-called galactosylated DNA LNCs.

3.3 - Physical characteristics of DNA LNCs, coated DNA LNC and galactosylated DNA LNCs

DSPE-PEG₂₀₀₀-gal and F108-gal densities at the surface of DNA LNCs were calculated as previously described [27, 28] using mean diameter measurements ($A = \frac{4}{3}\pi r^3$) and the molar concentration of the post-inserted polymers (Table 1). DSPE-mPEG₂₀₀₀ concentrations from 2 to 10mM (representing approximately 1,297 to 6,509 PEG chains per nanocapsule) and F108 concentrations from 1 to 3mM (representing 1,297 to 3,890 PEG chains per nanocapsule) were used. As the chemo-enzymatic and chemical galactosylations were not total (25% of the terminal groups of F108 and DSPE-PEG₂₀₀₀ were linked to galactose), we estimated that the number of galactose molecules per nanocapsule was estimated between 324 and 1,627 for DSPE-PEG₂₀₀₀-gal coated DNA LNCs, and between 324 and 972 for F108-gal coated DNA LNCs (Table 1).

	DSPE-PEG ₂₀₀₀ -gal (mM)			F108-gal (mM)		
	2	5	10	1	2	3
Surface density of PEG chains (PEG/nm ²)	0.04	0.10	0.20	0.04	0.08	0.12
Number of PEG chains per nanocapsule	1,297	3,254	6,509	1,297	2,592	3,890
Number of galactose per nanocapsule	324	813	1,627	324	648	972

Table 1. Theoretical calculation of the number of coating molecules at the DNA LNC surface

The physico-chemical properties (Table 1 and Table 2) and the DNA encapsulation ability (Figure 3) of DNA LNCs were examined before and after the post-insertion of DSPE-mPEG₂₀₀₀, DSPE-PEG₂₀₀₀-gal, F108 or F108-gal. The coupling of DSPE-PEG₂₀₀₀, DSPE-PEG₂₀₀₀-gal, F108 or F108-gal slightly increased the size (Table 2), but this still resulted in vectors with a size inferior to 200nm. Non coated DNA LNCs exposed a size of 109 ± 06 nm

and a zeta potential of +30mV. The DSPE-mPEG₂₀₀₀ coating led to the formation of nanoparticles with surface charge of +23, -12, -41mV for 2, 5, 10mM of DSPE-mPEG₂₀₀₀ respectively. When coated with F108, the nanocapsules exposed a surface charge from +14 to +22mV. The addition of galactose at the extremity of DSPE-mPEG₂₀₀₀ and F108 led to DNA LNCs with surface charges from +22 to +26mV for DSPE-PEG₂₀₀₀-gal and from +5 to +13mV for F108-gal (Table 2).

	Size (nm) ± SD	Pdl	Zeta (mV) ± SD
DNA LNC	109 ± 06	0.257	+31 ± 02
DSPE-mPEG ₂₀₀₀ 2mM	131 ± 10	0.230	+23 ± 08
DSPE-mPEG ₂₀₀₀ 5mM	139 ± 19	0.374	-12 ± 03
DSPE-mPEG ₂₀₀₀ 10mM	142 ± 20	0.250	-41 ± 11
DSPE-PEG ₂₀₀₀ -gal 2mM	132 ± 19	0.274	+23 ± 10
DSPE-PEG ₂₀₀₀ -gal 5mM	136 ± 29	0.305	+22 ± 12
DSPE-PEG ₂₀₀₀ -gal 10mM	172 ± 07	0.376	+26 ± 05
F108 1mM	117 ± 06	0.276	+14 ± 05
F108 2mM	139 ± 15	0.261	+17 ± 04
F108 3mM	138 ± 05	0.388	+22 ± 08
F108-gal 1mM	129 ± 03	0.289	+05 ± 03
F108-gal 2mM	138 ± 12	0.329	+13 ± 08
F108-gal 3mM	182 ± 21	0.426	+11 ± 09

Table 2. Influence of the incorporation of DSPE-mPEG₂₀₀₀ and F108 at the surface of DNA LNCs on size, polydispersity and zeta potential. DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs were analyzed for dynamic light scattering after the formulation and/or post-insertion process. Results show the mean ± SD of at least 4 independent formulations and 3 measurements per sample.

Agarose gel electrophoresis experiments showed that DNA molecules did not migrate after the galactosylated nanocapsule formulation process (Figure 3). By contrast, incubation of nanocapsules with Triton® X100 led to the release of DNA molecules that migrated into the gel. These results clearly indicated that the addition of galactosylated polymers at the surface of DNA LNCs did not disturb the encapsulation of DNA (lanes 16 to 27) and that DNA

molecules were still well protected inside the nanocapsules, as already observed for the post-insertion of non-galactosylated polymers (lane 4 to 15).

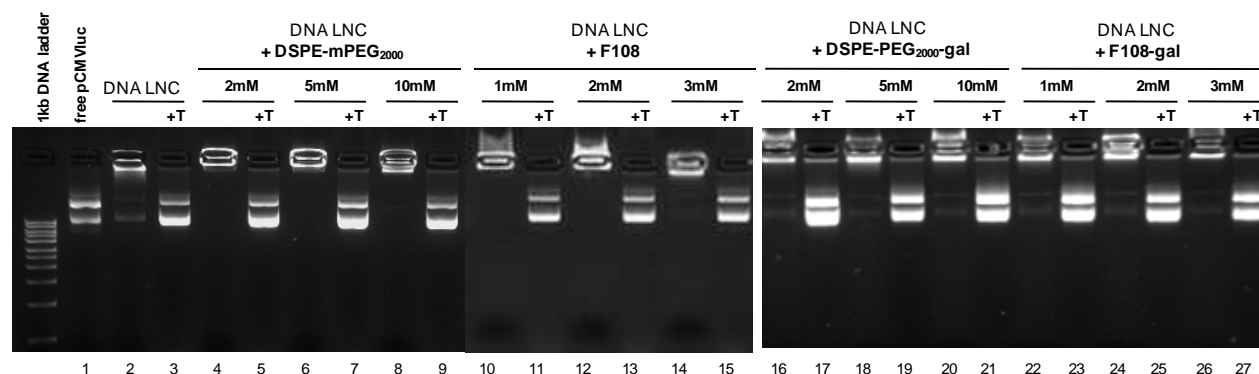


Figure 3. Agarose gel electrophoresis. The influence of coating on the encapsulation efficiency was tested for all the types of DNA LNC suspensions: coated DNA LNCs (lanes 4 to 15), galactosylated DNA LNCs (lanes 16 to 27). DNA molecules can not migrate once encapsulated in nanocapsules (lane 2,4,6,8,10,12,14,16,18,20,22,24,26), contrary to free DNA (pCMVluc) (lane1). The incubation of nanocapsules with Triton X100 (+T) led to the release of DNA molecules that migrated into the gel (lane 3,5,7,9,11,13,15,17,19,21,23,25,27).

3.4 - Galactose accessibility

To assess the accessibility of the galactose residues at the DNA LNCs surface, we monitored the binding of DNA LNCs to a galactose specific lectin, the soybean agglutinin (SBA) by measuring the absorbance of the suspension at 450nm: when a specific agglutination occurred between nanoparticles and SBA, these aggregates induced an increase in absorbance. As shown in Figure 4, the turbidity was not significantly different between nanoparticles incubated with SBA alone and nanoparticles incubated with SBA + D-galactose for DNA LNCs and coated DNA LNCs with the 2 polymers. This tends to prove that there was no specific targeting of SBA with these formulations. A slight but significant increase in absorbance was observed for DNA LNCs coated with DSPE-PEG₂₀₀₀-gal

compared to DNA LNCs coated with non-galactosylated DSPE-PEG₂₀₀₀ (0.175 vs. 0.135). The presence of F108-gal at the surface of DNA LNCs induced a strong increase in absorbance (0.637 vs. 0.202 with non galactosylated F108 coated DNA LNCs) implying a specific agglutination with SBA. This association was not observed when SBA was pre-incubated with D-galactose, confirming that the interaction between complexes and the lectin is specific, and occurred through the galactose binding sites of SBA. Moreover, a non specific interaction exists between F108 and SBA (0.385 with SBA galactose binding sites blocked with an excess of galactose). Nevertheless, these results demonstrated that galactose residues were well-displayed at the surface of DNA LNCs coated with F108-gal.

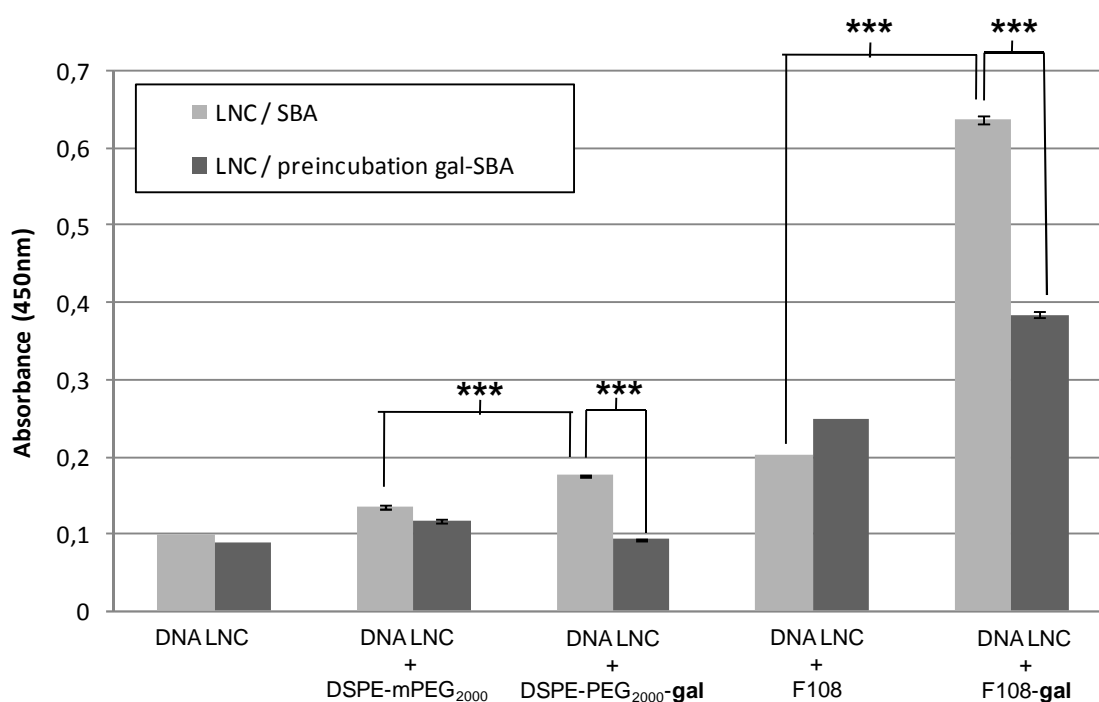


Figure 4. Observation of lectin-induced agglutination of DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs. The absorbance at 450nm of a solution containing the nanoparticles incubated with Sobaean agglutinin (SBA) was measured (grey). As a control, SBA was pre-incubated with an excess of free D-galactose, to saturate all the galactose binding sites present on SBA, and nanoparticles were added in a second time (dark grey) before measuring the absorbance. **: P<0.01 (Dunnett test)

3.5 - Transfection efficiency

The transfection ability of DNA LNCs and polymer-coated DNA LNCs, encapsulating pCMVluciferase as a reporter gene, was investigated in HeLa human cervical and H1299 lung cancer cell lines used as model cells by measuring luciferase activity (Figure 5a). Since these cells are not expressing ASPG-R, the galactosylated nanocapsules were not tested here. Lipoplexes were used as positive controls. Firstly, DNA LNC transfection efficiency was found to be at the same scale as this control (around 200ng of luciferase/protein mg). Significantly higher transfection efficiency was observed with F108-coated DNA LNCs, whatever the concentration, both in HeLa and H1299 cells, with a maximum of luciferase expression for 2mM F108 (223ng luciferase/protein mg for H1299 and 353ng luciferase/protein mg for HeLa). As observed here, the transfection efficiency of F108-coated DNA LNCs in HeLa was slightly superior to those of H1299. By contrast, DSPE-mPEG₂₀₀₀ DNA LNCs did not lead to significant luciferase expression, with a maximum of only 4ng luciferase/protein mg expressed in H1299 cells, and 32ng luciferase/protein mg in HeLa cells.

We next investigated the influence of active *in vitro* targeting using galactosylated DNA LNCs on primary hepatocyte cells (Figure 5b), which have numerous ASPG-Rs expressed at the surface. To prove receptor-mediated transfection efficiency, the difference of behaviour between non-galactosylated and galactosylated DNA LNCs was compared. DNA LNCs without coating exposed a naturally good tendency to transfect primary hepatocytes (2,240ng luciferase/protein mg), almost equivalent to that of DOTAP/DOPE lipoplexes (2,672ng luciferase/protein mg). This efficiency was also found with the galactosylated F108 coating at the concentration of 2mM (2,247ng luciferase/protein mg), with an 18-fold increase in

luciferase expression compared to non-galactosylated 2mM F108 DNA LNCs. By contrast, the attachment of galactose at the extremity of DSPE-PEG₂₀₀₀ did not show any increase in transfection efficiency compared to non galactosylated DSPE-PEG₂₀₀₀. Indeed, with or without galactose, the luciferase expression did not exceed 10ng of luciferase per mg of proteins for this kind of coating.

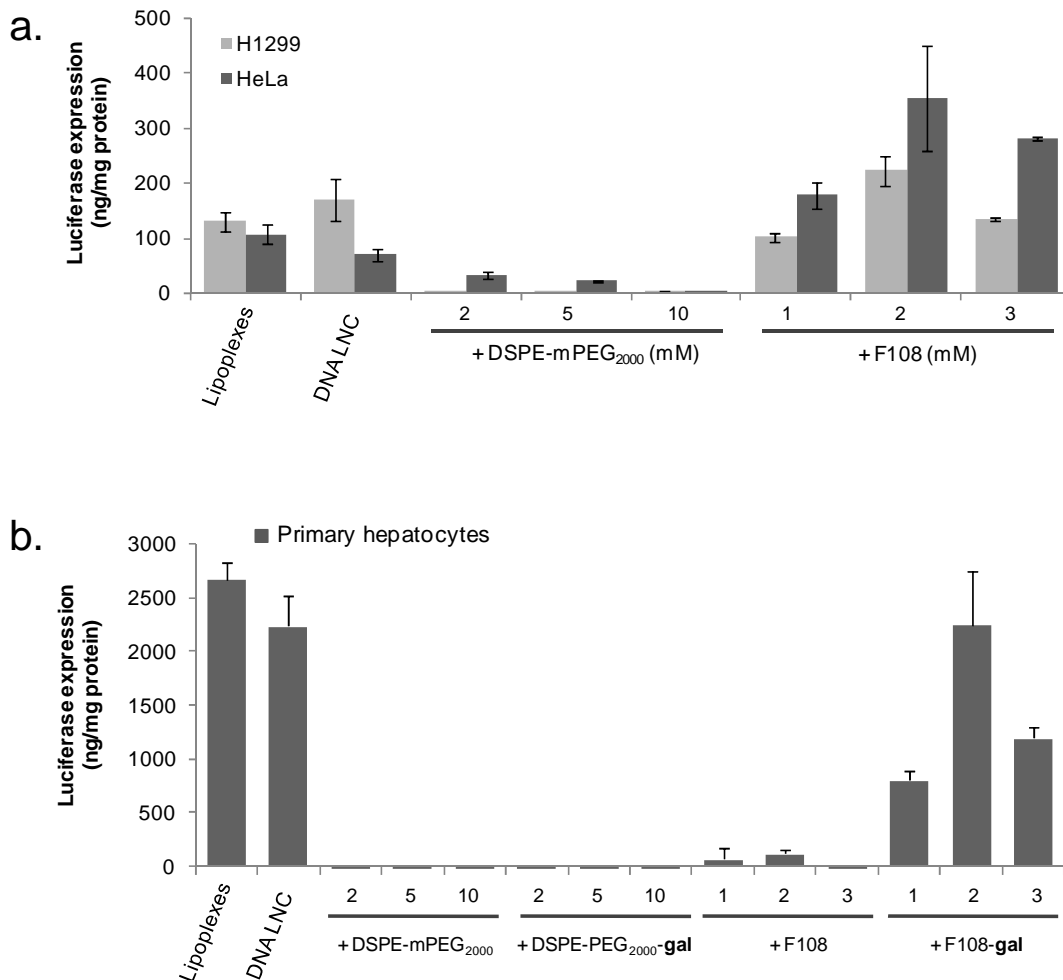


Figure 5. *In vitro* transfection activity of DNA LNCs (a) in tumour cell lines. HeLa and H1299 cells were incubated with DNA LNCs and DSPE-mPEG₂₀₀₀ or F108-coated DNA LNCs, and DOTAP/DOPE lipoplexes were tested as a positive control. The results are given in ng of luciferase per protein mg. Values are shown as the mean \pm s.d. (n=3). **(b) in primary hepatocytes.** Cells were incubated with DNA LNCs, coated DNA LNCs, or galactosylated DNA LNCs. DOTAP/DOPE lipoplexes were tested as a positive control. The results are given in ng of luciferase per protein mg. Values are shown as the mean \pm s.d. (n=3).

4 - Discussion

The specific delivery of a therapeutic gene to the liver is of great importance since hepatocytes (i.e parenchymal liver cells) are key targets for the secretion of gene products in the systemic circulation system. However, systemic targeting remains a real challenge and a compromise has to be found between sufficient circulation time, non-toxicity and transfection efficiency on targeted cells. The significant increase in plasmatic circulation time in mice due to the shield created by PEG around DNA LNCs via DSPE-mPEG₂₀₀₀ or F108 polymers (submitted results) could be sufficient to reach the desired organs, especially the endothelial fenestrae in the liver which constitute an open communication lane for circulating gene transfer vectors to the Disse space and hence provide subsequent access and uptake in hepatocytes [29]. The chance of gene expression in these cells could therefore be facilitated [30, 31].

Zeta potential measurements revealed that DNA LNCs was able to mask partially the positive charge due to the presence of the lipoplexes (Table 2). The negative values obtained with the use of DSPE-mPEG₂₀₀₀ could be attributed to the presence of negative PEG dipoles that can form a mushroom/brush intermediate PEG conformation as already described by Vonarbourg *et al.* [32]. When galactose molecules were added at the extremity of post-inserted polymers, the dipole effect was cancelled in case of DSPE-PEG₂₀₀₀ proving that galactose can interact with the PEG chains. In a different way, the shielding of lipoplexe charges was accentuated in F108 case, which is in favour of the presence of galactose at the extremity of the polymer chains (Table 2).

To check if the galactose displayed at the DNA LNC surface was accessible and recognizable by a galactose receptor, galactosylated DNA LNCs were confronted to soybean

lectin, a tetrameric glycoprotein containing 4 galactose binding sites [33]. This study confirmed that galactose moieties were accessible at the surface of galactosylated DNA LNCs coated with F108. This effect was much less pronounced with galactosylated DNA LNCs coated by DSPE-PEG₂₀₀₀. This difference in galactose exposition between the two polymers could be explained by a difference in PEG conformation and PEG length. Indeed, the PEG chain density as calculated at the surface of DNA LNCs could lead to a spatial conformation called mushroom/brush intermediate or mushroom like [32]. Therefore, if the DSPE-PEG₂₀₀₀ (45 PEG units) chains are in such a folded conformation, their distal end, bearing the galactose moiety, will be hidden inside the short PEG₆₆₀ chains already present at the surface of DNA LNC (15 PEG units). By contrast, the length of each PEG chains on F108 is more important (135 PEG unit per chain), and even if these PEG chains are submitted to a mushroom like conformation, the galactose will be more distant from the highly PEGylated environment present at the surface of DNA LNCs, and will remain more accessible to its receptor.

Then, the ability of *in vitro* transfection of non-galactosylated long-circulating carriers was tested on HeLa and H1299 cell line models (Figure 5a). It was interesting to note that comparable *in vitro* transfection was observed for DOTAP/DOPE lipoplexes (well known as efficient *in vitro* transfection reagents) and non-coated DNA LNCs. As DNA LNCs exposed positive surface charge, the interaction with the cell membrane could be facilitated [34, 35]. Moreover, this positive surface charge could help the endosomal escape thanks to a physical disruption of the negatively charged endosomal membrane occurring on direct interaction with the positively charged cationic vector, as suggested for both PAMAM dendrimers and Poly(L-lysine) [36]. Regarding the sterically stabilised DNA LNCs with DSPE-mPEG₂₀₀₀,

transfection efficiency failed in comparison to non-coated DNA LNCs (by 42-fold in H1299 and by 2-fold in HeLa cells). In HeLa cells, this decrease was in correlation with the increase in DSPE-mPEG₂₀₀₀ coating concentration (Figure 3). This result is in good agreement with the well-known drawback of PEG, and it is probably due to the prevention of the association of DSPE-mPEG₂₀₀₀ coated DNA LNCs with cell membranes and / or to PEG-inhibition of endosomal escape linked to their lower surface charge (compared to non-coated DNA LNCs) [14, 37]. Indeed, the strong association between DNA LNCs and DSPE-PEG₂₀₀₀ due to lipid anchoring in the LNC core [22] and the negative surface charge (for DSPE-mPEG₂₀₀₀ 5 and 10mM) could result in an impossible dissociation of endosomes and a subsequent degradation of the vector in lysosomes. By contrast, F108 block copolymer coating led to a significant increase in luciferase expression, with an optimal concentration of 2mM representing 1,297 F108 molecules per nanocapsule. This transfection efficiency could be explained by the presence of poly (propylene oxide) (PPO) segments which can provide improved interaction of these molecules with biological membranes [38]. Indeed, block copolymers have recently been seen to have considerable promise for the delivery of pDNA, thanks to their proven *in vivo* transfection efficiency [20, 39-42].

With the goal of evaluating if steric hindrance induced by PEG could be overcome by galactose attachment, we tested galactosylated DNA LNCs on primary hepatocytes. As observed for cell line transfection studies (Figure 5a), DNA LNCs were as efficient in transfecting hepatocytes as DOTAP/DOPE lipoplexes (Figure 5b). The luciferase expression was significantly more important in these primary cultured cells compared to the cancer cell lines (i.e. luciferase expression 32-fold more important in primary hepatocytes compared to HeLa cell line for non coated DNA LNCs). The transfection efficiency of DNA LNCs seems

therefore to be cell-dependant, as observed for other gene delivery systems, i.e. chitosan DNA nanoparticles [43]. However, with the goal of systemic delivery, the coating of DNA LNCs is required *in vivo*, as the $t_{1/2}$ of elimination of DNA LNCs was too low in mice (data not shown), mainly due to their positive charge ($31\pm 2\text{mV}$). As expected in regards with the degree of accessibility of galactose when attached to F108 (Figure 4), the F108-gal coating was found to strongly improve gene delivery in primary hepatocytes (2,247ng luciferase/protein mg), compared to F108 without galactose which exposed low transfection efficiency (121ng luciferase/protein mg). Nevertheless, the grafting of galactose to DSPE-PEG₂₀₀₀ molecules did not improve transfection efficiency, probably due to a non-sufficient accessibility of galactose when attached to this polymer (Figure 4). Indeed, as described in the literature, it seems important to place ligands several nanometers away from the surface of the particle in order to provide effective binding to cell surface receptors [44] and the DSPE-PEG₂₀₀₀ chains are probably in an inadequate conformation to present their distal end to the ASPG-R. The presence of free DSPE-PEG₂₀₀₀-gal could also provide, by a competitive process for the ASPG-R, a decrease in transfection efficiency.

We have demonstrated here that DNA LNCs can be used to achieve targeted-gene expression based on a cell-specific, receptor-mediated, endocytosis process, when coated with F108-gal with an optimal concentration at 2mM. This kind of nanoparticles could therefore allow gene targeting to hepatocytes by systemic injection thanks to their circulating properties, and provide a promising systemic gene delivery system.

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DICUSSION GÉNÉRALE ET PERSPECTIVE

A ce jour, l'utilisation de la voie systémique est indispensable pour traiter les organes et tumeurs inaccessibles par voie percutanée ou les sites tumoraux multi-localisés tels que les métastases. Dans le cadre d'une thérapie génique, il serait intéressant de développer un vecteur stable et non toxique pouvant encapsuler et délivrer son matériel génétique au sein des cellules visées, et ceci avec l'efficacité des vecteurs viraux.

Les premiers vecteurs développés pour la vectorisation d'acides nucléiques, tels que les liposomes et les polymères cationiques [1, 2], ont l'inconvénient d'être très rapidement pris en charge par le système immunitaire après une injection systémique. En effet, en raison de leur forte charge positive, ces systèmes particuliers induisent une forte toxicité [3, 4] et sont rapidement éliminés par les organes du système des phagocytes mononucléés (SPM), tels que le foie et la rate. Afin de modifier leur distribution tissulaire, la surface des particules peut être modifiée par des polymères hydrophiles et flexibles comme le polyéthylène glycol (PEG), créant ainsi des vecteurs dits de « seconde génération », (Cf. Revue bibliographique, Tableau 2) [5-8]. Ce recouvrement hydrophile génère autour de la surface une barrière stérique empêchant l'adsorption d'opsonines, protéines plasmatiques reconnues par des récepteurs spécifiques situés sur les macrophages, et augmentant par conséquent le temps de résidence vasculaire des vecteurs [9]. Ces nanovecteurs dits furtifs sont ainsi capables de tirer profit de l'effet « enhanced permeability and retention » (EPR), qui favorise l'accumulation des nano-objets et leur rétention au niveau des tissus cancéreux [10, 11]. Ce type de vectorisation est nommé vectorisation passive.

Par la suite, s'inscrivant dans une stratégie de ciblage actif, une troisième génération de vecteurs de médicament a été développée. Ces systèmes particuliers intelligents ont été mis au point dans le but d'administrer de manière spécifique au sein de l'organisme les

principes actifs sélectionnés. Pour ce faire, des biomolécules, reconnaissant des antigènes surexprimés sur la membrane de cellules cibles, ont été greffées à la surface de vecteurs furtifs (Cf. revue bibliographique, Tableau 2) [12-15].

Dans ce contexte, nous avons cherché, au cours de ce travail de thèse, à obtenir des vecteurs de seconde et troisième génération, à partir d'un système déjà existant, les nanocapsules lipidiques (LNC) [16]. Ce système nanoparticulaire a récemment été modifié au laboratoire pour permettre l'encapsulation d'ADN plasmidique (pCMV luciférase) dans le cœur lipophile des LNC, grâce à sa complexation avec des liposomes cationiques de DOTAP/DOPE. Un système stable, et de taille appropriée pour une injection systémique, nommé LNC ADN a donc été créé [17]. Cependant, malgré la présence de molécules de PEG (660Da) à haute densité à leur surface, le temps de circulation des LNC ADN est insuffisant pour obtenir une activité efficace *in vivo*.

1 - Modification du système existant : vers un vecteur furtif

1.1 - Formulation des LNC ADN PEGylées

Le polymère hydrophile le plus couramment utilisé pour modifier la charge de surface des vecteurs est le PEG. Dans le cas des LNC, comportant déjà des chaînes de PEG de petite taille (660 Da), des chaînes de polymères plus longues, pour prodiguer plus de répulsion stérique, peuvent être associées à la surface, selon deux cas de figure :

- substitution de l'hydroxystéarate de PEG (HS-PEG₆₆₀) utilisé dans la formulation des LNC par des chaînes d'hydroxystéarate comportant plus d'unités de PEG, **pendant la formulation** [18].
- ajout des chaînes de PEG plus longues une fois les LNC synthétisées, par une méthode utilisée avec les liposomes et adaptée aux LNC, appelée méthode de **post-insertion** [19].

L'incorporation de DSPE-mPEG₂₀₀₀ ou de F108 comme surfactants au cours de la formulation des LNC ADN s'est révélée infructueuse, quelque soit la concentration en polymère testée. Aucune zone d'inversion de phase n'est apparue détectable lors des cycles de température. Ainsi, les objets formés, d'une taille de l'ordre du μm et de polydispersité trop élevée, n'ont pas permis l'encapsulation d'ADN. Ceci s'explique par le fait que le concept de la méthode d'inversion de phase est en relation avec le nombre d'unités d'éthylène glycol (EG) que comporte le surfactant choisi [20]. En effet, plus le nombre d'unités est élevé, plus la température d'inversion de phase (TIP) est élevée, et donc difficile à atteindre. Ce problème est amplifié par le fait que les LNC ADN, modifiées par ajout de Plurol[®] oléique dans leur formulation [17], ont déjà une température d'inversion de phase plus faible comparativement aux LNC classiques (35°C par rapport à 80°C). Cette faible température permet d'éviter une dénaturation de l'ADN.

La méthode de post-insertion s'est donc imposée. Cette technique est composée d'une étape de co-incubation des LNC préformées en présence d'un dérivé amphiphile de PEG, suivie d'une étape de refroidissement rapide qui va figer le système. L'étape de co-incubation doit être menée à une température très légèrement plus forte que celle de la

transition gel/liquide du surfactant utilisé (température de transition de phase des constituants du Solutol[®] = 16.6 et 27.9°C [16]) pour permettre la fluidité de la couronne des LNC, mais plus basse que la TIP des LNC dans le but d'éviter une désorganisation du système possible pendant les inversions de phase. Dans le cas des LNC classiques, une incubation d'1h30 à 60°C est réalisée, avec agitation toutes les 15 min [19]. A la fin de cette période d'incubation, les préparations sont immergées dans un bain de glace durant 1min pour figer le système et bloquer de ce fait les molécules de PEG insérées dans la coque des LNC. Une température de co-incubation plus faible a été envisagée pour post-insérer les LNC ADN, en raison de la plus faible TIP caractérisant ces objets (30°C) [17]. Selon Hoarau *et al.* [19] il est possible de mener une co-incubation à des températures plus faibles, comme dans le cas de l'utilisation de PEG fonctionnalisés avec des groupes thermosensibles tels que des protéines ou des oligopeptides, mais sur des temps d'incubation plus importants. Nous avons donc testé différentes températures de co-incubation, de 60°C à 25°C, en augmentant les temps d'incubation. C'est une période d'incubation de 4h à 30°C qui a été choisie comme la plus adaptée pour obtenir une post-insertion stable, tout en gardant les propriétés d'encapsulation des LNC ADN (Cf. Chapitre 2, Figure 1), indispensables à notre objectif de protection des acides nucléiques après injection intraveineuse.

1.2 - Caractérisation physicochimique de la surface

Différentes concentrations de polymères ont été testées, de 1 à 10mM de DSPE-mPEG₂₀₀₀ et de 1 à 5mM de F108. En effet, la concentration de LNC ADN restant constante dans la suspension, l'addition de différentes concentration de polymères nous a permis de

faire varier la densité de chaînes présentes à la surface des LNC, facteur important permettant de créer une barrière stérique efficace à la surface du vecteur [21]. Les molécules de F108 étant de taille beaucoup plus importante que celle de DSPE-mPEG₂₀₀₀ (14 600 g.mol⁻¹ par rapport à 2805 g.mol⁻¹), une plus faible quantité de copolymère à bloc a dû être envisagée. En effet, au dessus de 3mM, un déphasage était observé, traduisant un excès de copolymère à bloc dans la solution. En se basant sur une association de 100% des polymères sur les LNC ADN, nous avons calculé la valeur théorique de densité de chaînes présente à la surface des LNC, en notant que chaque molécule de F108 était composée de 2 chaînes de PEG (Tableau 1). La densité de chaîne obtenue est comparable à celle des vecteurs décrits dans la littérature, tels que les nanocapsules de PLA-PEG [22].

	DSPE-PEG ₂₀₀₀ -gal (mM)			F108-gal (mM)		
	2	5	10	1	2	3
Densité de surface des chaînes de PEG (PEG/nm ²)	0.04	0.10	0.20	0.04	0.08	0.12
Nombre de chaînes de PEG par nanocapsule	1,297	3,254	6,509	1,297	2,592	3,890

Tableau 1. Calcul théorique de la densité de chaînes présentes à la surface des LNC ADN en fonction de la concentration de polymère utilisé.

L'addition de PEG à la surface d'un vecteur lui confère de nouvelles propriétés physico-chimiques. En plus de la densité, la flexibilité et la longueur des chaînes de PEG sont des facteurs importants qui régissent les interactions avec les protéines plasmatiques [21]. Pour cette raison, l'influence de l'association du DSPE-mPEG₂₀₀₀ ainsi que du F108 sur les propriétés de surface des LNC ADN nouvellement recouvertes a été étudiée (Publication n°1), dans le but de caractériser la conformation des chaînes de polymères. En effet, il existe

différentes conformations spatiales possibles lorsque des molécules de PEG sont associées à la surface d'un vecteur, comme décrit dans la figure 1 :

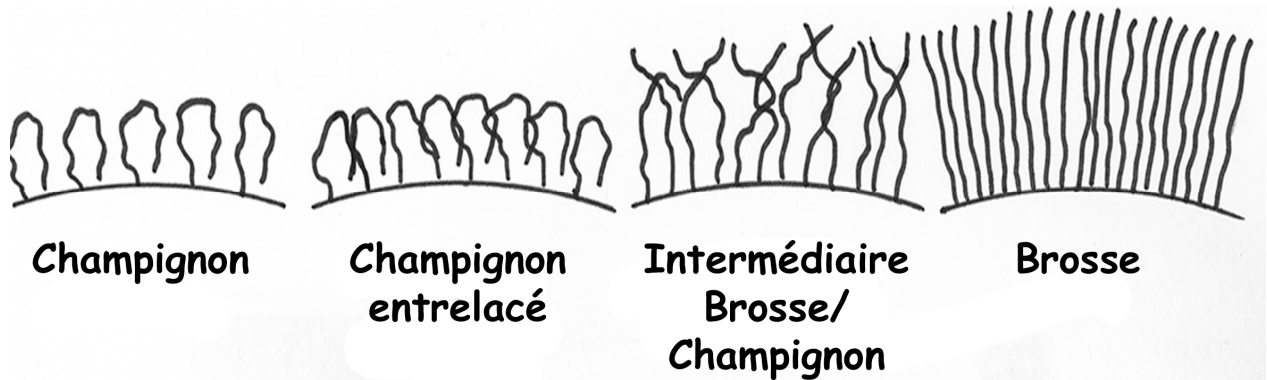


Figure 1. Différentes conformations possibles des chaînes de PEG à la surface des LNC, d'après Vonarbourg *et al.* [21].

Nous avons montré dans cette première publication, que les mesures de taille ainsi que les données de mobilité électrophorétique mettaient en évidence une conformation en brosse pour le DSPE-mPEG₂₀₀₀, et en champignon pour le F108. Ainsi, ces résultats confirment ceux de Yang *et al.* [23] décrivant que les longues chaînes de polymère ont tendance à s'enchevêtrer les unes aux autres, pour aboutir à une conformation de type champignon. Or, ces différentes configurations vont entraîner des interactions différentes avec les fluides biologiques. L'équipe de Perrachia *et al.* [24] a décrit la conformation champignon comme étant la plus efficace pour repousser les protéines. A l'inverse, de nombreuses études ont prouvé que la conformation brosse ou intermédiaire brosse-champignon était la plus adaptée pour éviter la phagocytose et l'adsorption du fragment C3 du système complément [25-27]. Dans notre cas, c'est effectivement la conformation en

brosse du PEG qui permet au vecteur d'échapper à la capture par les macrophages et à la relocalisation vers le foie après injection intraveineuse chez la souris (Publication n°1, Figures 5 et 6).

2 - Les LNC ADN PEGylées comme vecteur furtif dans le cadre d'un ciblage passif de la tumeur

Les LNC ADN non recouvertes possèdent un faible temps de demi-vie sanguine court (20min chez la souris), incompatible avec une application *in vivo* par voie systémique. Notre premier objectif a donc été d'observer si les différents recouvrements précédemment décrits amélioreraient effectivement la répulsion stérique, empêchant l'adsorption d'opsonines.

2.1 - Furtivité

Dans un premier temps, le caractère furtif des LNC ADN PEGylées a été démontré à travers plusieurs études *in vitro* de l'activation de l'immunité innée.

Malgré une activation du système complément relativement faible, les LNC ADN non recouvertes, et celles recouvertes de F108 sont fortement capturées par les macrophages (Figure 3). Etant donnée la faible de quantité de C3b associée à la surface des LNC ADN, cette internalisation est certainement aspécifique et due à la charge positive des LNC ADN capable d'induire des interactions électrostatiques avec la membrane chargée négativement [28]. En revanche, le recouvrement de DSPE-mPEG₂₀₀₀ procure une protection efficace, que

ce soit vis-à-vis de l'opsonisation des protéines du complément ou de la phagocytose par les macrophages.

De plus, après injection par voie intraveineuse des différents types de LNC ADN chez la souris, une multiplication du $t_{1/2}$ de distribution d'un facteur 5 a été mise en évidence lorsque les LNC ADN sont recouvertes de DSPE-mPEG₂₀₀₀, pour aboutir à un temps de 7.1h contre 1.4h pour les nanocapsules non recouvertes. Ce temps de circulation se rapproche de celui des liposomes furtifs synthétisés il y a une vingtaine d'années [9, 29, 30] et représentant toujours, à ce jour, les systèmes les plus performants en temps de circulation ($t_{1/2}$ de 15 à 24h chez les rongeurs et 45h chez l'homme). Ces liposomes, chargés en anticancéreux, sont largement utilisés dans le traitement des tumeurs car ils s'accumulent efficacement au niveau de ces tissus par effet EPR [31-33].

Le recouvrement par les copolymères à bloc F108 ne permet pas une augmentation de circulation aussi importante, mais le $t_{1/2}$ de distribution est tout de même doublé comparé aux LNC ADN non recouvertes ($t_{1/2}$ = 2.7h versus 1.4h). Par ailleurs, il est largement supérieur à celui des lipoplexes de DOTAP/Chol-ADN dont 1% seulement se retrouvent en circulation 5 min après injection [34]. De plus, il a été rapporté que dans le cas des « *stabilized plasmid lipid nanoparticles* » (SPLP), les systèmes les plus performants en transfection n'étaient pas forcément ceux qui possédaient un temps de circulation très long (autour de 2h) [8, 35, 36]. Enfin, le $t_{1/2}$ obtenu avec ce recouvrement de bloc copolymère est supérieur à celui obtenu avec les systèmes prometteurs, tels que les « *stable nucleic acid lipid particles* » (SNALP) ($t_{1/2}$ de 38min chez la souris) développés par Zimmerman *et al.* [37] pour le transfert de siRNA chez des primates non-humains.

La différence de comportement en terme de temps de circulation entre nos deux types de recouvrement peut s'expliquer par la différence d'association des chaînes de DSPE-mPEG₂₀₀₀ (ancrage) et de F108 (adsorption) à la surface des LNC ADN. En effet, l'adsorption est une liaison faible qui peut aboutir à une dissociation dans les milieux biologiques, comme cela a déjà été décrit [38-40]. La dissemblance de conformation des chaînes peut également être un facteur important pour expliquer cette différence d'efficacité *in vivo*. En effet, les chaînes étendues et flexibles en conformation brosse (DSPE-mPEG₂₀₀₀) sont connues pour être plus efficaces pour prévenir l'opsonisation des protéines et les interactions avec les cellules du système immunitaire, alors que la conformation super-enroulée (F108) manquera de flexibilité.

2.2 - Accumulation tumorale

Nous avons ensuite cherché à observer si les LNC ADN recouvertes de DSPE-mPEG₂₀₀₀, qui circulaient longtemps, étaient capables de s'accumuler au niveau tumoral par effet EPR. Pour ce faire, nous avons utilisé des LNC ADN marquées avec un fluorochrome lipophile émettant dans le proche infra-rouge, le DiD ($\lambda_{exc}=644\text{nm}$; $\lambda_{em}=665\text{nm}$). Le suivi des nanoparticules a pu être réalisé grâce à un système non invasif d'imagerie par fluorescence *in vivo* (Berthold France, Thoiry, France). La circulation des nanoparticules fluorescentes a été imagée sur souris porteuse de tumeur de lignée de gliome humain (U87MG) dans le flanc droit. Après injection des LNC ADN + DSPE-mPEG₂₀₀₀, nous avons observé un signal diffus sur le corps entier des animaux testés, alors que sans

recouvrement, les LNC ADN sont rapidement relocalisées vers le foie, confirmant une prise en charge par le système des phagocytes mononucléés (Figure 2).

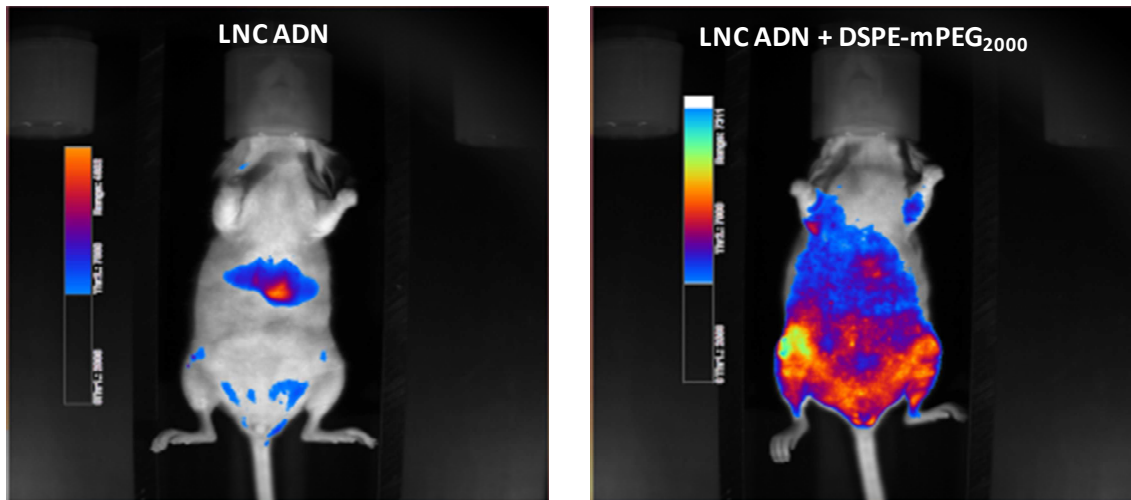


Figure 2. Echappement hépatique des LNC ADN recouvertes de DSPE-mPEG₂₀₀₀. Imagerie de fluorescence *in vivo* chez la souris *nude* porteuse de tumeur U87MG dans le flanc droit, 3h après injection intraveineuse de LNC ADN ou LNC ADN recouvertes de DSPE-mPEG₂₀₀₀.

De plus, 24h et 48h après l'injection des LNC ADN + DSPE-mPEG₂₀₀₀, une accumulation tumorale est observée (Figure 3), confirmant la capacité de ce système à passer inaperçu au sein des fluides biologiques pour atteindre plus facilement la tumeur, par effet EPR. Ainsi, nous donc avons synthétisé un système qui s'accumule au bout de 24h au niveau des tissus tumoraux, et cette accumulation est toujours visible 48h après injection, avec toutefois une diminution dans l'intensité du signal.

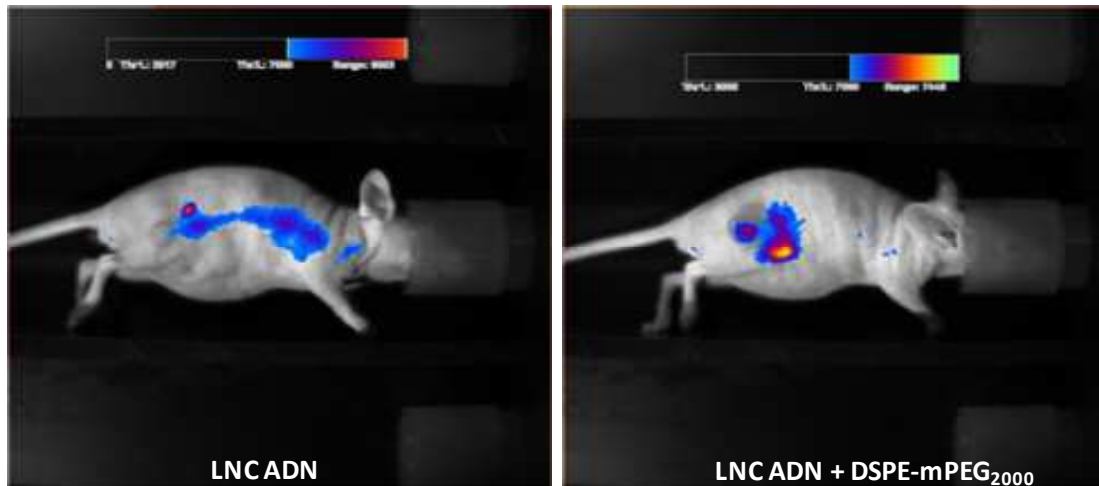


Figure 3. Accumulation tumorale des LNC ADN recouvertes de DSPE-mPEG₂₀₀₀. Imagerie de fluorescence *in vivo* chez la souris nude porteuse de tumeur U87MG dans le flanc droit, 48h après injection intraveineuse de LNC ADN ou LNC ADN recouvertes de DSPE-mPEG₂₀₀₀.

Les LNC ADN, ayant dépassé les obstacles rencontrés dans la circulation plasmatique, vont maintenant devoir franchir les nombreuses barrières intracellulaires qui les séparent du noyau où l'expression du plasmide sera alors possible grâce à la machinerie de transcription et de traduction de la cellule « hôte ».

2.3 - Transfection tumorale (RESULTATS NON PUBLIÉS)

2.3.1 - Transfection *in vitro*, choix du modèle tumoral

Dans un premier temps, nous avons testé l'efficacité de transfection des vecteurs *in vitro* par un dosage de l'activité de la luciférase, codée par notre plasmide pCMVluc, à travers le dosage de photons. Nous avons testé diverses lignées tumorales de gliomes, pathologie étudiée au laboratoire, et sélectionné la lignée U87MG comme étant la lignée la plus efficacement transfectée par les LNC ADN. Les systèmes chargés positivement, tels

que les lipoplexes de DOTAP/DOPE et les LNC ADN, se sont avérés efficaces en transfection *in vitro* (Figure 4), probablement par un phénomène non spécifique d'internalisation facilité par des interactions électrostatiques avec les membranes cellulaires négatives [41, 42]. Les LNC ADN recouvertes de F108 se sont révélées être de très bons agents transfectants, avec une concentration optimale de 2mM. Cette efficacité peut s'expliquer par des interactions favorisées entre la partie hydrophobe du F108 (PPO) et les membranes cellulaires [43, 44]. En revanche, le recouvrement de DSPE-mPEG₂₀₀₀ a induit une forte inhibition d'expression de luciférase comparativement à la transfection observée avec les LNC ADN non recouvertes. Ceci est probablement dû à une répulsion stérique trop importante des longues chaînes de PEG, leur flexibilité empêchant l'association cellulaire et/ou l'échappement endosomal [45].

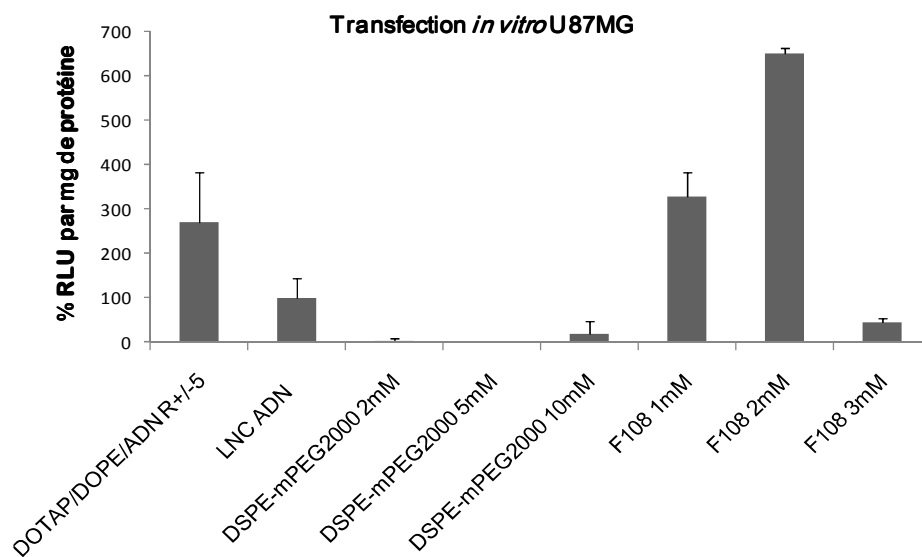


Figure 4. Activité de transfection des cellules de lignée U87MG par les LNC ADN selon leur recouvrement. Les cellules sont incubées 24h dans du milieu contenant 10% de sérum de veau fœtal (SVF) en présence de LNC ADN et LNC ADN recouvertes de DSPE-mPEG₂₀₀₀ ou F108 à différentes concentrations. Les complexes DOTAP/DOPE-ADN sont testés comme contrôles positifs. %RLU/mg protéine = pourcentage d'unité de luminescence relative par mg de protéine par rapport à l'expression de luciférase avec LNC ADN sans recouvrement.

2.3.2 - Transfection *in vivo*

Il a été largement rapporté qu'une efficacité de transfection *in vitro* ne permettait pas d'anticiper l'activité des systèmes de vectorisation après une administration *in vivo*, du fait de la différence d'environnement biologique et de l'éventuelle modification des propriétés colloïdales des vecteurs, lors de l'interaction avec les tissus biologiques. C'est pourquoi nous avons voulu évaluer la capacité de transfection *in vivo* des LNC ADN avec les deux types de recouvrement, après injection intraveineuse.

Malheureusement, malgré le phénomène d'accumulation tumorale observé en dosage de fluorescence, le dosage de l'activité luciférase dans la tumeur n'a montré aucun résultat, quelque soit la formulation injectée par voie intraveineuse (Figure 5).

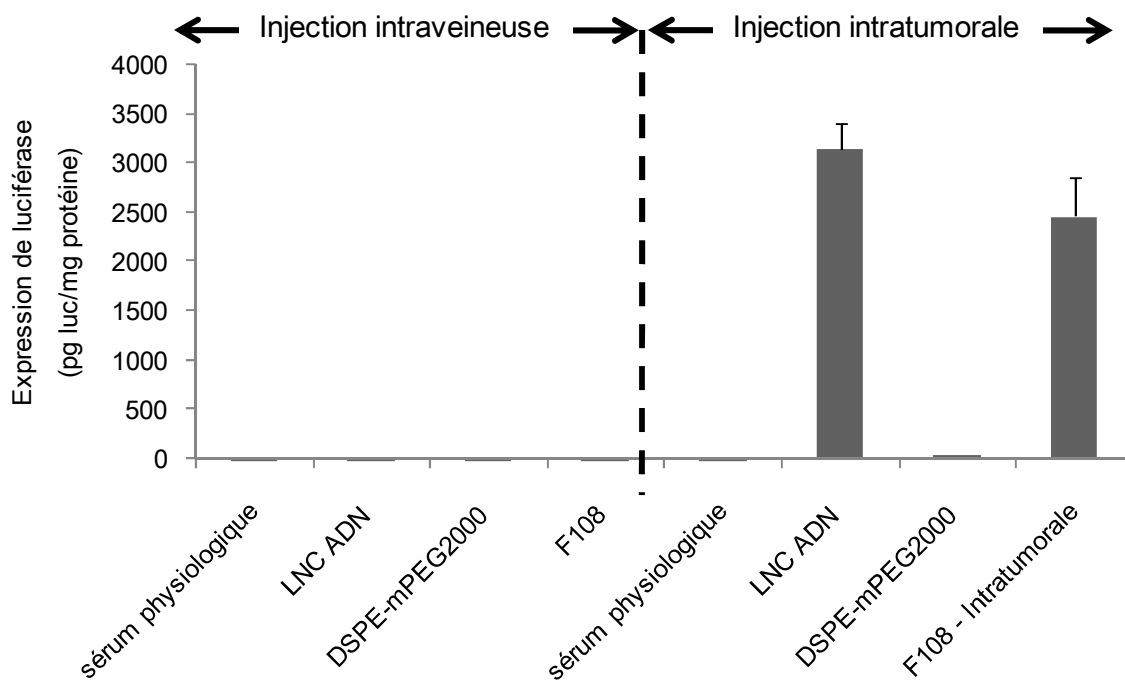


Figure 5. Efficacité de transfection *in vivo* après injection intraveineuse ou intratumorale des LNC ADN, et LNC ADN + DSPE-mPEG₂₀₀₀ ou F108. 24h après injection, les tumeurs sont récoltées et broyées à l'Ultraturax® afin de doser l'activité luciférase.

En revanche, après une injection intra-tumorale, le même profil de transfection que celui observé *in vitro* est retrouvé, avec une plus forte efficacité de transfection pour les LNC ADN et LNC ADN recouvertes de copolymères à bloc F108. Cependant, l'inconvénient de ces formulations est d'avoir un temps de circulation insuffisant pour permettre une accumulation tumorale après injection IV. En effet, ces vecteurs sont probablement reconnus par les cellules du système immunitaire avant de pouvoir transférer les cellules tumorales.

2.3.3 - Perspectives

Cette étude permet donc de mettre en évidence le rôle ambigu du recouvrement de PEG. En effet, le recouvrement de DSPE-mPEG₂₀₀₀ confère aux LNC ADN un temps de circulation prolongé et une accumulation tumorale, mais une fois sur le site d'action, le bouclier stérique va empêcher l'entrée dans les cellules, nécessaire à la transfection du plasmide [46-48]. Dans ce sens, l'équipe de Kirpotin *et al.* [49] a comparé l'utilisation de liposomes stabilisés par des chaînes de PEG et d'immunoliposomes (liposomes associés à des anticorps ciblant de manière spécifique une onco-protéine (HER2) surexprimé sur certaines cellules tumorales). Cette étude met en évidence une accumulation identique des deux types de vecteurs au niveau tumoral, mais une efficacité beaucoup plus prononcée des immunoliposomes pour libérer la drogue encapsulée (Doxorubicine) au niveau intracellulaire. Cette étude montre également que les liposomes stabilisés s'accumulent dans le stroma des tumeurs comprenant les espaces extracellulaires et dans les macrophages infiltrant les tumeurs, contrairement aux immunoliposomes qui s'accumulent dans le cytoplasme des cellules cancéreuses, aussi bien *in vitro*, qu'*in vivo* [49]. Les LNC ADN recouvertes de

DSPE-mPEG₂₀₀₀ se trouvent donc probablement « bloquées » dans le stroma tumoral, sans trouver la « clé » de l'entrée cellulaire.

Ainsi, une thérapie génique tumorale performante *in vivo* nécessite à la fois un vecteur stable, mais également un ciblage sélectif permettant une internalisation cellulaire par association d'un ciblage passif et d'un ciblage actif. Une alternative aux problèmes d'internalisation liés au recouvrement de PEG peut être l'ajout d'un ligand ciblant de manière spécifique des récepteurs surexprimés à la surface de cellules cibles. Concernant le ciblage des tumeurs, différents types de ligands ont été ajoutés à la surface des vecteurs, tels que la transferrine [12, 50, 51], les peptides RGD [13], ou encore le folate [15, 52]. Ainsi, le greffage de peptides RGD à la surface des LNC ADN semblerait être une voie prometteuse dans le modèle tumoral U87MG, lignée cellulaire sur-exprimant de manière importante le récepteur aux intégrines $\alpha_v\beta_3$ [53].

Une seconde solution, qui peut également s'associer au concept de ciblage actif, serait d'utiliser des PEG dynamiques et amovibles. En effet, idéalement, le polymère doit rester ancré au vecteur durant le trafic sanguin, mais une fois celui-ci arrivé à son site d'action, il doit se dissocier pour permettre l'internalisation cellulaire. Ainsi, il a été établi que la longueur de l'ancre lipidique avait une importance dans l'efficacité de transfection des SPLP [35, 36]. Cette étude a prouvé qu'une ancre céramide comportant 8 carbones (CerC₈) était plus efficace en transfection que des PEG comportant une ancre CerC₁₄ ou CerC₂₀, confirmant la nécessité d'une dissociation PEG–vecteur pour permettre une transfection efficace. Cependant, ce type de dissociation ne peut pas être contrôlé, et peut subvenir

avant l'arrivée du vecteur au niveau tumoral. Une autre alternative pourrait être d'utiliser un PEG sensible à son environnement, qui pourrait se dissocier du vecteur lors de son arrivée sur le site tumoral, en exploitant par exemple l'environnement acide des tumeurs. De nombreuses équipes ont travaillé sur ce concept, en utilisant des polymères de PEG reliés à leur ancre par des liaisons pH sensibles (hydrazone [54], acétal [55, 56], vinyle éther [57], ortho ester [58]) aboutissant à des augmentations de transfection aussi bien *in vitro* [58], qu'*in vivo* [59]. De plus, la taille de la chaîne de PEG est également un facteur important : inversement aux propriétés requises pour augmenter la furtivité, plus la chaîne de PEG sera courte, plus la transfection sera efficace [36]. Dans le cas des LNC ADN, il serait donc intéressant de tester d'autres types d'ancres lipidique : des ancres plus courtes (l'ancre DSPE étant composée de 18C) et/ou associées au PEG par des liaisons pH sensibles accessibles. Parallèlement, dans le but d'améliorer le temps de circulation des LNC ADN recouvertes de copolymères à bloc, l'utilisation d'un polymère avec une partie PEG plus courte et une partie PPO plus longue, permettant une association plus forte avec les LNC ADN (tels que les copolymères à blocs P123 ou F127), pourrait être envisagée.

3 - Vers un ciblage actif du foie : les LNC ADN galactosylés

La troisième partie de ce travail de thèse a consisté en la mise au point d'un vecteur de gène pour le foie. En effet, une thérapie génique hépatique efficace pourrait permettre de

traiter de nombreuses pathologies, telles que les problèmes d'hypercholestérolémie, d'hémophilie [60] ou encore de cirrhoses [61].

Le vecteur créé, dit de troisième génération, a pour but de permettre un ciblage actif, c'est-à-dire reposant sur l'interaction spécifique d'un ligand associé à notre vecteur avec son récepteur présent sur les cellules ciblées. Le modèle que nous avons utilisé est le modèle, bien décrit [14, 62, 63], de l'utilisation d'un ligand galactose pour cibler de manière spécifique et active le récepteur aux asialoglycoprotéines (ASGPR) surexprimé par les cellules parenchymateuses du foie, les hépatocytes. Pour ce faire, nous avons cherché à obtenir des vecteurs décorés de ligands galactose que nous avons nommés LNC ADN galactosylés.

3.1 - Formulation et caractérisation des LNC ADN galactosylés

3.1.1 - Formulation

Nous avons modifié la surface des LNC ADN par post-insertion de DSPE-PEG₂₀₀₀-galactosylé (DSPE-PEG₂₀₀₀-gal) ou de F108-galactosylé (F108-gal). La fonctionnalisation des polymères a été réalisée par voie enzymatique dans le cas du F108 [63], et par voie chimique dans le cas du DSPE-PEG₂₀₀₀ [44]. Cette galactosylation a été réalisée par Emilie Bonneval au sein de l'équipe du Dr Bruno Pitard (Inserm U915, Institut du thorax, Nantes). Le pourcentage de chaînes galactosylées, compris entre 25 et 30%, nous est apparu comme étant un bon compromis entre efficacité de ciblage et efficacité de furtivité.

3.1.2 - Caractérisation

L'étude des caractéristiques physico-chimiques des LNC ADN galactosylées a montré que l'incorporation de nouveaux polymères ne modifiait pas significativement les propriétés des LNC ADN. Ainsi, les LNC ADN galactosylées ont des tailles équivalentes à celles des LNC ADN PEGylées, et possèdent les mêmes capacités d'encapsulation de l'ADN (Publication n°3, Figure 3). En revanche, le potentiel zêta est modifié comparativement à celui des LNC ADN PEGylées (Publication n°3, Table 2). En effet, la présence de galactose, même si seulement 30% des extrémités des chaînes de PEG sont galactosylées, semble dissimuler l'influence des dipôles négatifs de PEG observée pour les LNC ADN PEGylées sans galactose. Même pour des concentrations en DSPE-PEG₂₀₀₀-gal de 5 et 10mM, la charge globale des nanocapsules galactosylées reste positive (+22 et +26mV pour 5 et 10mM de DSPE-PEG₂₀₀₀-gal respectivement), alors qu'elle était négative en absence de motif galactose (-12 et -41mV). Ces modifications de charge de surface, avec une disparition des dipôles induits par les PEG, semblent démontrer une modification de conformation des chaînes de DSPE-mPEG₂₀₀₀. Cette disparition pourrait s'expliquer par un repliement des têtes de galactose suite à la formation possible de liaisons hydrogène entre le sucre et les unités d'éthylène glycol. D'autre part, un potentiel zêta plus proche de la neutralité dans le cas de l'utilisation de F108-gal atteste également d'un greffage efficace (environ 10mV pour les LNC ADN recouvertes de F108-gal contre environ 20mV pour les LNC ADN recouvertes de F108). En effet, dans ce cas, la présence des molécules neutres de galactose, induit une diminution de charge, probablement grâce à une dissimulation plus marquée du cœur positif.

3.2 - Efficacité de transfection des LNC ADN galactosylées

3.2.1 - Transfection *in vitro*

Nous avons testé la capacité de ces différentes formulations galactosylées à transférer de manière spécifique des hépatocytes primaires de rat en présence de sérum, pour être au plus proche des conditions d'injection *in vivo*.

De manière surprenante, les LNC ADN non recouvertes ont montré une forte capacité naturelle à transférer des hépatocytes. Cependant, le temps de demi-vie des LNC ADN étant faible, ces objets ne peuvent être utilisés dans le cadre d'une thérapie génique du foie par voie systémique.

En revanche, comme discuté précédemment, l'association de DSPE-mPEG₂₀₀₀ à la surface des LNC permet d'atteindre de forts temps de circulation sanguine. Même si sa capacité à transférer les cellules de lignée U87MG s'est révélée insuffisante, probablement à cause d'une trop forte répulsion stérique, la présence d'un ligand représente une alternative intéressante pour améliorer cette transfection, à travers une internalisation par endocytose médiée par les récepteurs. Malheureusement, *in vitro*, la présence de galactose associé au DSPE-PEG₂₀₀₀ n'a pas permis d'augmenter significativement cette transfection. Des tests d'agrégation des galactoses par de la lectine de Soja (Publication n°3, Figure 4), ont mis en évidence une interaction spécifique des galactoses 3 fois plus importante avec le polymère F108-gal qu'avec le DSPE-PEG₂₀₀₀-gal, montrant une différence d'accessibilité entre les deux types de vecteurs. Ce résultat, associé aux variations de potentiel zeta ainsi qu'à la faible efficacité de transfection *in vitro*, semble montrer que les chaînes galactosylées

sont repliées, dissimulant le ligand (Figure 6). En revanche, le greffage de galactose à l'extrémité des chaînes de F108 ne semble pas modifier leur conformation (Figure 6)

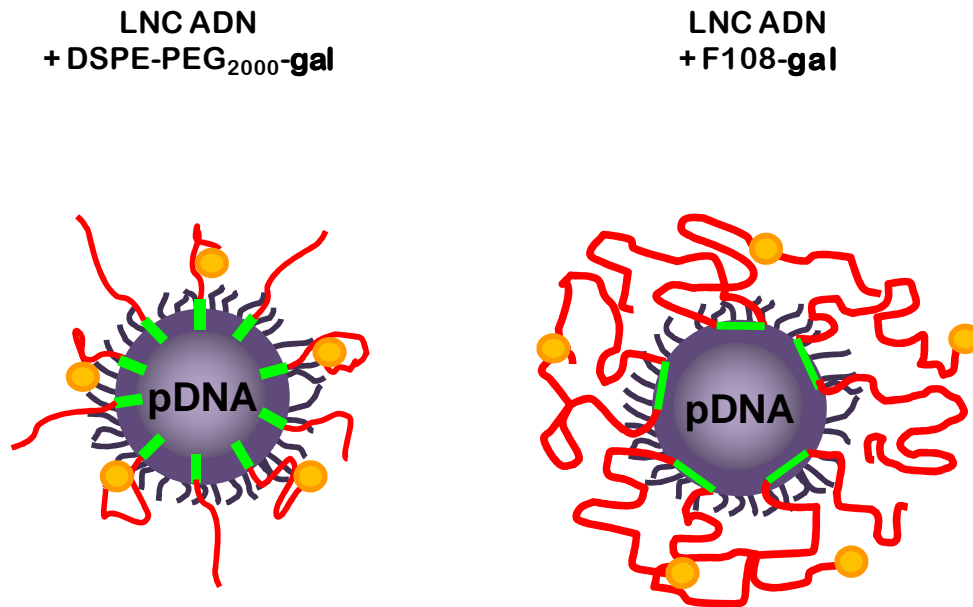


Figure 6. Représentation schématique de la configuration des chaînes de PEG à la surface des LNC ADN galactosylées.

Toujours en concordance avec les résultats d'accessibilité, les LNC recouvertes de F108 gal permettent une augmentation de la transfection 18 fois supérieure à celle des LNC ADN recouvertes de F108 non galactosylé. Ainsi, le greffage de galactose induit une transfection spécifique efficace. Cependant, en amont du phénomène d'interaction spécifique, l'association cellulaire peut également être facilitée par des interactions hydrophobes entre la partie PPO du F108 et la membrane des hépatocytes. Cette étape semble inexistante dans le cas du DSPE-mPEG₂₀₀₀, plus mobile et dont 70% des chaînes libres peuvent toujours jouer un rôle de barrière stérique. L'interaction entre le vecteur et la cellule est alors impossible, ce qui pourrait expliquer le manque d'efficacité de transfection

des LNC ADN + DSPE-PEG₂₀₀₀-gal. De plus, il a été démontré que l'orientation du ligand avait une influence sur la reconnaissance par l'ASPGR [64]. Ainsi, l'orientation du galactose en bout de chaîne n'est peut être pas optimale.

3.2.2 - *Transfection in vivo* (RESULTATS NON PUBLIÉS)

Ces nouveaux vecteurs galactosylés ont été testés *in vivo*, l'objectif étant d'obtenir un vecteur capable de cibler les cellules saines du foie après une injection intraveineuse. Pour ce faire, nous avons, comme à nouveau (publication n°2 et section 2 de la discussion) utilisé des LNC ADN marquées avec du DiD. En parallèle de la détection de fluorescence, le dosage de l'expression de luciférase *in vivo* par bioluminescence a également été réalisé. La circulation des nanoparticules fluorescentes a été imagée sur souris Swiss après injection intraveineuse dans la queue de 150 µL de LNC ADN recouvertes des différents types de polymères (DSPE-mPEG₂₀₀₀, DSPE-PEG₂₀₀₀-gal, F108 et F108-gal). Après 5h, aucune relocalisation hépatique spécifique de fluorescence n'est observée, même dans le cas des LNC ADN galactosylées (Figure 7). De plus, aucun signal n'est détecté par le dosage de l'émission de photons par bioluminescence *in vivo*.

Ces résultats confirment tout d'abord, sur un nouveau modèle *in vivo*, la furtivité déjà observée sur souris *nude* avec les LNC ADN recouvertes de DSPE-mPEG₂₀₀₀ et les LNC F108 (Publication n°1). Ils montrent, de plus, que l'ajout de galactose ne modifie pas cette furtivité *in vivo* (ici à 5h et résultats non montrés à 24h). Cependant, une accumulation dans les zones de vascularisation lacunaires et notamment au niveau des hépatocytes était attendue.

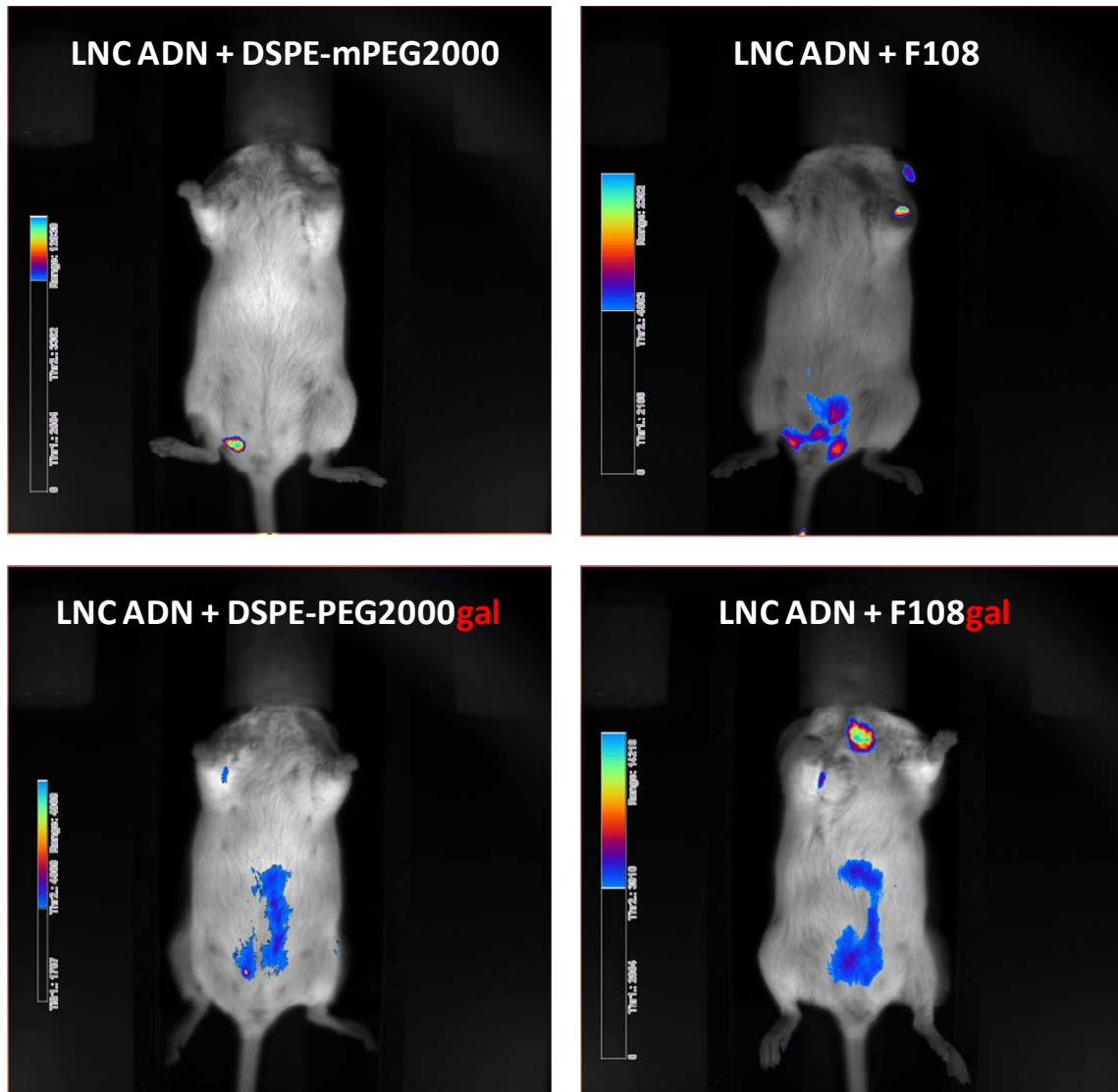


Figure 7. Biodistribution des LNC ADN galactosylées. Imagerie de fluorescence *in vivo* chez la souris *Swiss* 5h après injection intraveineuse de 150µl de LNC ADN marquées au DiD.

Plusieurs hypothèses peuvent être formulées afin d'exprimer pourquoi ce phénomène n'a pas été observées. Tout d'abord, la taille de nos objets est peut-être trop importante pour permettre de traverser les jonctions fenestrées de l'épithélium hépatique. Cependant, la taille moyenne de ces jonctions étant d'environ 200nm [63-65], il est peu probable que ces vecteurs soient bloqués à ce niveau. Par ailleurs, la sensibilité des dosages de fluorescence et bioluminescence n'est certainement pas suffisante pour permettre la détection d'une faible

quantité de particules qui se seraient effectivement accumulées sur ce site. Enfin, il est possible que, même si les particules se sont accumulées au niveau du foie, elles n'aient pas réussi à transfecter les hépatocytes, ou que cette transfection soit trop faible pour être détectée.

En parallèle et de manière surprenante, une forte intensité de fluorescence au niveau de la gorge des souris a été observée dans le cas des LNC ADN recouvertes de F108-gal (Figure 7). Ceci pourrait potentiellement représenter une accumulation de nanoparticules dans les ganglions lymphatiques, qui, comme le foie, possèdent un endothélium fenestré [65, 66]. Toutefois, cette hypothèse serait à vérifier après sacrifice des animaux et dosage de ces organes.

3.2.3 - Perspectives

Les perspectives de ce projet seraient tout d'abord, en concordance avec des études de transfection *in vitro*, de modifier la surface des vecteurs avec des résidus qui n'influencent pas significativement les temps de circulation. En effet, une certaine furtivité semble acquise et reste nécessaire pour atteindre nos objectifs. L'ajout de motifs galactose supplémentaires pourrait être une solution à envisager car une densité importante de galactose à la surface des vecteurs semble être un facteur permettant une bonne efficacité de transfection [67], les ASGPR étant placés de manière proche sur la membrane cellulaire [68].

De plus, un autre motif de ciblage pourrait être utilisé. La littérature ayant montré que l'ASGPR avait plus d'affinité pour les résidus N-acétylgalactosamine (GalNAc) que pour le galactose, l'utilisation de GalNAc en bout de chaîne pourrait donc augmenter le transfert spécifique d'ADN par les nanocapsules lipidiques [69, 70].

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CONCLUSION GÉNÉRALE

Les stratégies de ciblage d'un type cellulaire constituent des stratégies prometteuses car elles pourraient être employées aussi bien dans les systèmes de vectorisation d'ADN que d'autres médicaments, tels que les anticancéreux. Bien que peu d'essais se soient avérés positifs jusqu'à présent, ils représentent un réel espoir pour les traitements et doivent donc être améliorés afin d'obtenir un vecteur efficace. Toutefois, Il est acquis qu'il n'existe pas un système universel de transfert de gènes mais qu'il faut adapter et optimiser les caractéristiques physico-chimiques et biologiques du vecteur à chaque indication thérapeutique envisagée.

Dans ce sens, nous avons pu observer lors de ce travail, que chacun des polymères utilisé avait ses caractéristiques propres (Tableau 2), lié à sa structure, la conformation de ses chaînes de PEG, ainsi qu'à son type d'association avec les LNC ADN.

	DSPE-mPEG ₂₀₀₀	F108
Caractéristiques physico-chimiques		
- Densité de chaîne	++	-
- Flexibilité	+++	--
- Dissimulation de la charge des LNC	+++	++
Propriétés de furtivité		
- Inhibition activation du complément	+++	++
- Echappement à la capture macrophagique	+++	+
- Amélioration cinétique sanguine	+++	++
- Accumulation tumorale	++	---
Propriétés de transfection		
- Transfection lignées cellulaires	--	++
- Transfection spécifique hépatocytes (avec DSPE-PEG ₂₀₀₀ -gal et F108-gal)	---	+++
- Transfection tumorale in vivo	---	---
- Transfection hépatique in vivo	---	---

Tableau 2. Tableau récapitulatif des points forts et points faibles des différents polymères utilisés pour modifier la surface des LNC ADN.

Efficacité : +++, très bonne; ++, bonne; +, moyenne; -, faible; --, très faible; ---, nulle.

Ainsi, de manière simplifiée, nous pouvons conclure que le DSPE-mPEG₂₀₀₀ est un polymère permettant d'obtenir un vecteur furtif, alors que le copolymère à bloc F108 permettra d'obtenir un vecteur efficace en transfection. L'objectif est, à ce jour, d'obtenir un vecteur alliant ces deux propriétés. Ainsi, pour obtenir ce vecteur, l'ancrage doit être fort pour permettre un bon temps de circulation, mais faible pour permettre l'internalisation. La longueur des chaînes de polymères doit être élevée pour repousser les opsonines, mais faible pour permettre l'association avec les cellules... Il semble difficile d'imaginer qu'une seule molécule puisse répondre à tous ces impératifs. C'est pourquoi la recherche se penche à l'heure actuelle sur des systèmes multi-modulaires intelligents, mimant le mode de fonctionnement complexe des virus. En effet, la délivrance de gène par voie systémique est un processus comprenant différentes étapes (Cf revue bibliographique) nécessitant un vecteur multifonctionnel qui puisse outrepasser toutes les barrières extra- et intracellulaires rencontrées.

CURRICULUM VITAE

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Doctorante en pharmacologie expérimentale et clinique

CURSUS UNIVERSITAIRE

2006 - 2009 **Doctorat en pharmacologie expérimentale et clinique.**

Ecole doctorale d'Angers.

2005 - 2006 **Master Recherche 2^{ème} année Mention Sciences, Technologies et Organisation de la Santé (STOS).**

Spécialité Biosignalisation cellulaire et moléculaire et physiopathologies

UFR Sciences, Angers. Mention AB.

2004 - 2005 **Maîtrise de biologie cellulaire et physiologie.**

Mention génétique moléculaire et cellulaire et physiologie

UFR Sciences, Angers. Mention B.

2003 - 2004 **Licence de biologie cellulaire et physiologie.**

UFR Sciences, Angers.

EXPERIENCE PROFESSIONNELLE

11/2006 à ce jour : Doctorat de pharmacologie expérimentale et clinique.

Laboratoire Inserm U646, Ingénierie de la vectorisation particulaire,
10, rue André Boquel, 49100 Angers.

Sujet : "Thérapie génique à l'aide de nanocapsules lipidiques pegylées".

Bourse : Allocation de Recherche du ministère.

Directeurs de thèse : Dr Catherine Passirani, Pr Jean-Pierre Benoit.

Collaboration : Bruno Pitard, Inserm U915, Nantes.

Objectifs : Production d'un vecteur d'acide nucléique efficace en transfection *in vivo* par voie intraveineuse.

- Modification de surface et interaction avec le système immunitaire *in vitro* et *in vivo* après injection chez le petit animal.
- Mise au point d'un vecteur au ciblage actif pour un ciblage du foie chez l'animal sain en collaboration avec l'Inserm U533, Institut du thorax, Nantes.
- Mise au point d'un vecteur pour accumulation et transfection tumorale dans un modèle de tumeur sous cutané chez la souris nude. Comparaison avec l'action d'un agent anticancéreux encapsulé et injecté par voie IV sur la croissance tumorale.

Compétences :

- Formulation de nanocapsules lipidiques encapsulant des lipoplexes (complexes liposomes cationiques-ADN), et modification de surface par des polymères flexibles et hydrophiles par post-insertion.
- Caractérisation physico-chimique: taille, potentiel zeta, capacité d'encapsulation par électrophorèse.
- Caractérisation biologique *in vitro* : culture cellulaire de lignées (HeLa, H1299, THP-1, U87MG) et culture primaire (hepatocytes), étude de capture par les macrophages par cytométrie en flux, test de survie cellulaire MTT, transfection plasmidique et dosage luciférase, test d'activation du complément.
- Caractérisation biologique *in vivo* : cinétiques sanguine, transfection hépatique souris Swiss, modèle de tumeurs humaines sous-cutanées souris nude.

2008 : Mission dans le cadre d'un doctorat conseil au sein de la Société IN CELL ART, Nantes.

Intitulé de la mission : « **Encapsulation de siRNA et évaluation de l'efficacité biologique des nanocapsules encapsulant des siRNA** »

Compétences :

- Recherche de l'entreprise d'accueil, détermination de la mission en collaboration avec l'université et l'entreprise.
- Etablissement d'un contrat, notion de propriété scientifique.
- Mise en œuvre des connaissances acquises lors de la thèse pour élaborer un nouveau vecteur d'acide nucléique. Etude de faisabilité, expérimentation.
- Rédaction d'un bilan de fin de mission.

2006 : Stage pratique de 6 mois (Master recherche 2^{ème} année), Inserm U646, Angers

Maître de stage : Dr Emmanuel Garcion.

Projet : "Identification de cellules souches cancéreuses dans les gliomes: sensibilité à des agents anticancéreux et intérêt de leur vectorisation au travers de nanocapsules lipidiques".

Compétences : Cytométrie en flux (FACS et trieur de cellules), Western Blot, RT-PCR.

2004 : Stage pratique de 2 mois, Inserm U646, Angers. Maîtrise de biologie cellulaire

Maître de stage : Dr Emmanuel Garcion.

Projet : "Etude *in vitro* des effets de l'interleukine 18 sur le comportement des cellules de gliomes 9L et F98 de rat Fischer".

Compétences : Culture cellulaire, dosage protéique, ELISA, immunocytochimie.

2003 : Stage bibliographique, laboratoire Inserm U646, Angers. Licence de biologie cellulaire

Maître de stage : Dr Emmanuel Garcion.

Projet : "Rôle immunomodulateur de la ténascine C : application au développement des gliomes".

Compétences : Recherche bibliographique, analyse, bilan, comparaison et présentation de publications.

ACTIVITES D'ENSEIGNEMENT ET D'ENCADREMENT

ENSEIGNEMENT :

- ✓ Travaux Pratiques concernant l'utilisation de l'expérimentation animale dans un projet de recherche, dans le cadre du Master 2 STIS (Sciences, Technologie et Ingénierie de la Santé) parcourt technologies innovantes en formulation dispensé par l'ISSBA (Institut Supérieur de la Santé et des Bioproduits), 4h. Contact : Pr Frank Boury.
- ✓ Travaux pratiques en première année du DUT analyse biologique et biochimique, concernant diverse sujets, 16h. Contact : Lydie Bouvier.
 - Osmose, perméabilité membranaire, échanges d'eau, échanges de substances dissoutes
 - Dissection appareil digestif d'un omnivore (souris)
 - Histologie végétale
 - Histo-cytochimie des constituants cellulaires végétaux.

ENCADREMENT :

- ✓ Encadrement d'étudiants en 3ème année de pharmacie dans le cadre d'un stage d'initiation à la recherche (SIR), 1 mois, sur les thèmes suivants :
 - « Modification de surface de nanocapsules lipidiques encapsulant des complexes d'ADN », ².
 - « Etude de l'activation du complément par des nanocapsules lipidiques encapsulant des complexes d'ADN et influence sur leur comportement chez la souris Swiss », Elise Moutel.
- ✓ Encadrement de 2 étudiants en dernière année de DUT génie biologique, option Analyses biologiques et biochimiques, IUT Angers, 10 semaines, sur les thèmes suivants :
 - « Caractérisation de nanocapsules lipidiques pégylées encapsulant des complexes d'ADN », Alexandra Couvrant.
 - « Etude in vivo de l'accumulation tumorale de nanocapsules lipidiques fluorescentes encapsulant des complexes d'ADN », Mickael Beaufils.
- ✓ Tutorat de stage licence professionnelle BAEMOVA (Biologie Analytique et Expérimentale des Micro-Organismes, du Végétal et de l'Animal), IUT Angers.
 - Aide aux difficultés éventuelles rencontrées par l'étudiant sur son lieu de stage.
 - Participation en tant que membre du jury aux soutenances de fin de stage.

COMPETENCES COMPLEMENTAIRES

- ✓ Formation expérimentation animale Niveau 1, ENV Nantes.
- ✓ Stage Inserm : initiation gestion des risques.
- ✓ Anglais : Lu, écrit, parlé. Allemand : niveau scolaire.
- ✓ Logiciels: Word, Excel, Power Point, Adobe illustrator, Adobe photoshop, WinMDI 2.8, Kinetica 4.4, XLstat, Endnote.

Articles

“Preparation and characterization of coated DNA lipid nanocapsules: influence of the amphiphilic PEG conformation on macrophage uptake and biodistribution”

Morille M., Dufort S., Bastia G., Saulnier P., Garcion E., Coll J-L., Benoit J-P., Passirani C.
Soumis Journal of controlled released.

“Long-circulating DNA lipid nanocapsules: a new vector for systemic gene delivery.”

Morille M., Passirani C, Legras P, Brodin P, Garcion E, Pitard B, et al.
Biomaterials (2009), doi :10.1016/j.biomaterials.2009.09.044

“Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting.”

Morille M., Passirani C, Letrou-Bonneval E, Benoit J-P, Pitard B.
Int. J Pharm. 2009; 379(2):293-300.

“Progress in developing cationic vectors for non-viral systemic gene therapy against cancer.”

Morille M., Passirani C, Vonarbourg A, Clavreul A, Benoit JP.
Biomaterials 2008;29(24-25):3477-3496.

Communications orales

“Long circulating DNA lipid nanocapsules: a new vector for systemic delivery in tumors.”

Morille M., Passirani C., Montier T., Carmoy N., Eyer J., Benoit J-P.
EuroNanoMedicine, Septembre 2009, Bled, Slovénie.

“Amélioration des propriétés furtives et de l'efficacité de transfection *in vitro* de nanocapsules lipidiques d'ADN.”

Morille M., Passirani C, Garcion E, Bonneval E, Pitard B, Benoit JP
4^{ème} journée du Club des Nanomatériaux, mars 2008, Bordeaux, France.

Posters

“Long-circulating properties of DNA lipid nanocapsules improved by amphiphilic polymer coating”

Morille M., Passirani C., Brodin P., Garcion E., Legras P., Pitard B., Benoit JP.
XXIII^{èmes} Journées Scientifiques du G.T.R.V., Angers, 8-10 Décembre 2008

“Transfection evaluation of long-circulating DNA lipid nanocapsules for an efficient hepatocyte targeting via systemic pathway”

Morille M., Passirani C., Letrou-Bonneval E., Benoit JP., Pitard B.
XXIII^{èmes} Journées Scientifiques du G.T.R.V., Angers, 8-10 Décembre 2008

“Encapsulation of DNA into biomimetic lipid nanocapsules”

Vonarbourg A, Passirani C., Desigault L, Allard E, Morille M, Saulnier P, Lambert O, Benoît JP, Pitard B;

APGI – 6th world meeting on Pharmaceuticals, biopharmaceutics and pharmaceutical technology. Barcelone, 2008.

RESUME

A ce jour, l'objectif principal de la thérapie génique par voie intraveineuse est le développement de vecteurs pouvant encapsuler et délivrer des acides nucléiques au niveau de cellules cibles, avec l'efficacité de transfection des vecteurs viraux. Dans ce but, des nanocapsules lipidiques chargées en lipoplexes de DOTAP/DOPE, les LNC ADN, ont été utilisées. Ainsi, ces vecteurs ont été post-insérés avec de longues chaînes de poly (éthylène glycol) (PEG), grâce à l'utilisation de deux types de polymères amphiphiles : le DSPE-mPEG₂₀₀₀ et le copolymère F108. Une étude physico-chimique de la modification de surface a été réalisée. La présence de chaînes de DSPE-mPEG₂₀₀₀ en configuration brosse, a permis l'obtention d'un vecteur furtif aux yeux du système immunitaire capable de s'accumuler de manière significative au niveau des tissus tumoraux, grâce à un effet EPR. En parallèle, un modèle de ciblage extracellulaire du récepteur aux asialoglycoprotéines des hépatocytes a été envisagé. Le greffage de résidus galactose à l'extrémité des chaînes de PEG du copolymère F108, a permis l'expression spécifique d'un transgène au niveau des hépatocytes primaires de rat.

Mots-clés : transfection - vecteur non-viral - voie systémique - poly (éthylène glycol) - effet EPR - tumeur - galactose - hépatocytes.

ABSTRACT

The main objective of gene therapy via a systemic pathway is the development of a stable and non-toxic gene vector that can encapsulate and deliver foreign genetic materials into specific cell types with the transfection efficiency of viral vectors. In this way, lipid nanocapsules loaded with DOTAP/DOPE lipoplexes, named DNA LNCs were used. These vectors were post-inserted by with long poly (ethylene glycol) (PEG) chains, thanks to two kinds of amphiphilic polymers: DSPE-mPEG₂₀₀₀ and copolymer F108. A physico-chemical study of the surface modification was realized. The association of DSPE-mPEG₂₀₀₀ chains in a brush conformation allowed to obtain a stealth vector able to accumulate significantly in tumor tissues by EPR effect. In parallel, a model of extracellular targeting of asialoglycoprotein receptors over-expressed on hepatocytes was envisaged. The grafting of galactose residues at the extremity of F108 PEG chains allowed the specific expression of a transgene in rat primary hepatocytes.

Keywords: transfection - non-viral vector- systemic pathway - poly (ethylene glycol) - EPR effect - tumor - galactose - hepatocytes.