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NEW BIOMATERIALS FOR THE DESIGN OF NANOMEDICINES THAT MODULATE GLIOBLASTOMA STEM CELLS

Carla García Mazás

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NOVOS BIOMATERIAIS PARA O DESEÑO DE NANOMEDICINAS QUE MODULAN AS CÉLULAS NAI DO GLIOBLASTOMA

Carla García Mazás

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New biomaterials for the design of nanomedicines that modulate glioblastoma stem cell

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Conflict of interest

I declare that some of the authors of this work, including myself, have a patent related to the synthesis of new cationic polyphosphazenes developed in the context of this thesis:

C. García Mazás, N. Csaba, M. García Fuentes; "Polímeros para terapia génica", P202031074, Spain, October 27th, 2020

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A mi familia,

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“No juzgues cada día por la cosecha que recoges, sino por las semillas que plantas", Robert Louis Stevenson

“Experiencia es el nombre que damos a nuestras equivocaciones.” Oscar Wilde

“Nunca choveu que non escampara”

“Hakuna Matata” El rey León

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Abstract

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Abstract

Gene therapy emerged in the 70s as an alternative to small drugs and proteins in the treatment of a large variety of diseases. However, the administration of nucleic acids still remains a challenge due to the biological barriers that need to be overcome before reaching the target cells. Indeed, polynucleotides are very sensitive to degradation and cannot cross cell membranes. To overcome these obstacles, nucleic acids are often included in viral, lipid or polymeric particles. Polymeric gene nanocarriers offer chemical flexibility and good protection for the therapeutic genes, but the materials used still need to be optimized to achieve improved efficiency in the gene delivery process.

Considering this background, the objective of the thesis has been the development of new prototypes of polymeric nanoparticles for their use in gene therapy and to test their potential for the treatment of glioblastoma. For this, a variety of commercially available and synthetic cationic polymers have been combined with plasmid DNA or with both plasmid and an endosomolytic polymer. The nanoparticles were characterized for their physicochemical properties, for their toxicity and transfection efficiency in cell cultures. Toxicity of selected prototypes were also tested in 3D spheroid cultures and in zebrafish embryos. The polymer having primary amines and hydrophobic side groups, combined with the endosomolytic polymer provided some of the best results regarding their transfection/toxicity ratio. This advanced prototype was used with a therapeutic plasmid encoding Bone Morphogenic Protein 4 (BMP-4) as a potential treatment against glioblastoma. These therapeutic nanoparticles showed the capacity to suppress glioblastoma growth in a murine xenograft model when combined with Temozolomide, due to the synergistic effect between those two treatments administered together.

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Resumen

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Resumen

La terapia génica surgió en los años 70 como una alternativa al uso de fármacos y proteínas en el tratamiento de una gran variedad de enfermedades. Sin embargo, la administración de ácidos nucleicos implica un gran desafío debido a las barreras que tienen que superar antes de llegar a las células diana. De hecho, estas moléculas son muy sensibles a la degradación y no pueden atravesar las membranas celulares. Para superar estos obstáculos, los ácidos nucleicos se incluyen con frecuencia en partículas virales, lipídicas o poliméricas. Los vectores poliméricos ofrecen flexibilidad química y buena protección para los genes terapéuticos, pero los materiales utilizados aún tienen que ser optimizados para lograr una eficiencia aceptable en el transporte de genes.

Teniendo en cuenta estos antecedentes, el objetivo de la tesis ha sido el desarrollo de nuevos prototipos de nanopartículas poliméricas para su uso en terapia génica y probar su potencial en el tratamiento del glioblastoma. Para ello, se han combinado una variedad de polímeros catiónicos sintetizados en el laboratorio y comerciales con ADN plasmídico o con una combinación de plásmido y un polímero endosomolítico. Las nanopartículas se caracterizaron por sus propiedades fisicoquímicas, por su toxicidad y transfección *in vitro*. La toxicidad de algunos de estos prototipos también se testó en cultivos de esferoides 3D y en embriones de pez zebra. El polímero que tiene aminas primarias y cadenas laterales hidrofóbicas, combinado con el polímero endosomolítico, proporcionó algunos de los mejores resultados con respecto a la relación transfección / toxicidad. Este prototipo se utilizó con un plásmido terapéutico que codifica la proteína morfogénica ósea 4 (BMP-4) como posible tratamiento contra el glioblastoma. Estas nanopartículas terapéuticas son capaces de suprimir el crecimiento tumoral en un modelo de xenoinjerto murino de glioblastoma cuando se combinaron con Temozolomida, debido al efecto sinérgico de la administración conjunta de estos dos tratamientos.

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Resumo *in extenso*

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Na maioría dos tumores existe unha subpoboación celular que presenta características de células nai, coñecida co nome de células nai tumorais (CSCs). Estas células caracterízanse por atoparse nun estado desdiferenciado, ser capaces de autorrexenerarse, presentar resistencia á quimioterapia e pola súa alta capacidade de migración e invasividade. Por iso, as CSCs son consideradas as principais responsables das recidivas e a súa supresión é fundamental para a loita contra o cancro. Existen numerosas estratexias para modular as CSCs, tanto actuando especificamente sobre estas células, como sobre o nicho tumoral onde se atopan situadas e que contribúe ao seu mantemento e protección [1,2].

Hoxe en día, a terapia xénica postulouse como alternativa á utilización de pequenos fármacos e proteínas no tratamento de enfermidades con base xenética como o cancro. Aínda así, a liberación de xenes entraña unha serie de problemas biofarmacéuticos como a fácil degradación dos ácidos nucleicos nos medios biolóxicos e as barreiras que deben atravesar ata chegar ao lugar onde desenvolven o seu efecto [3,4]. Inicialmente para evitar estas limitacións dos ácidos nucleicos, estas moléculas incluíronse en vectores virais, sistemas que presntan alta eficacia, pero que poden presentar problemas de seguridade [5]. A nanotecnoloxía permite o deseño de vectores non virais con capacidade para mellorar a expresión xénica *in vitro* e *in vivo*, tendo un mellor perfil de bioseguridade. Estes nanovehículos pódense modificar químicamente para dirixir o ácido nucleico especificamente ao tecido diana e incrementar a súa internalización [6].

Tendo en conta estes antecedentes, o obxectivo da tese foi o desenvolvemento de novos prototipos de nanopartículas poliméricas para o seu uso en terapia xénica como tratamentos contra as CSCs. Por unha banda, investigouse que grupos catiónicos dos polímeros empregados en terapia xénica, xeran unha transfección máis eficiente. Nunha primeira etapa desta tese, estudáronse combinacións de diferentes polímeros comerciais usados en terapia xénica cun polímero endosomolítico, co fin de determinar qué tipo de estruturas e combinacións dan lugar aos sistemas máis eficientes. O polímero endosomolítico escollido (6 MHA- PPZ) desenvolveuse previamente no noso laboratorio [7], demostrando resultados prometedores na redución da toxicidade e mellorando a transfección de nanopartículas preparadas a base de polímeros catiónicos.

Nunha segunda parte da tese, sintetizáronse un pequeno grupo de polifosfacenos catiónicos con diferentes funcionalidades químicas. O obxectivo foi determinar qué tipo de materiais e cales son as funcionalidades que dan lugar aos sistemas con mellor cociente de eficacia/toxicidade, tanto por si sós como en combinación co polímero endosomolítico 6 MHA-PPZ. Na terceira parte desta tese, o prototipo de nanopartículas baseadas en polifosfacenos que presentou mellores propiedades como vehículo de terapia xénica no capitulo anterior, foi empregado para encapsular unha secuencia supresora de CSCs, e foi avaliado en modelos *in vitro* e *in vivo* de glioblastoma.

1. **Estudo de cómo afecta a estructura química dos polímeros catiónicos e a súa asociación con un polifosfaceno aniónico á toxicidade e eficacia dos nanosistemas.**

Polietilenimina (PEI), protamina e quitosano son polímeros moi empregados no desenvolvemento de novas formulacións para terapia xénica, pola súa capacidade de asociar os ácidos nucleicos [8]. Estes polímeros presentan diferentes grupos catiónicos encargados de asociar o ácido nucleico e que afectan as propiedades fisicoquímicas, toxicidade e transfección dos nanosistemas. Na súa estrutura o PEI presenta aminas secundarias e terciarias, a protamina conten grupos guanidino presentes no aminoácido arginina e o quitosano ten aminas primarias.

Todos os prototipos presentaron unhas propiedades fisicoquímicas similares, en canto a tamaño, carga superficial e capacidade de asociación do ácido nucleico, sendo en todos os casos adecuadas para o seu uso en terapia xénica (Figura 1.a). Os grupos amino do PEI demostraron resultar máis tóxicos para as células que os grupos guanidina presentes na protamina (Figura 1. b), pero doutra banda estes grupos amino aumentaron notablemente a capacidade de transfección respecto a protamina (Figura 1. c). Os nosos resultados están en concordancia cos traballos anteriores que demostraron que tanto as aminas secundarias e terciarias [9,10] coma os grupos guanidino [11,12] evitan a degradación endosomal do ácido nucleico, pero no noso traballo observóuse que as aminas secundarias e terciarias teñen un efecto moito máis marcado mellorando a transfección de forma máis notable.

A asociación do 6 MHA- PPZ a estes polímeros catiónicos mellorou a eficacia de transfección tanto do PEI como da protamina, sen afectar as súas propiedades fisicoquímicas

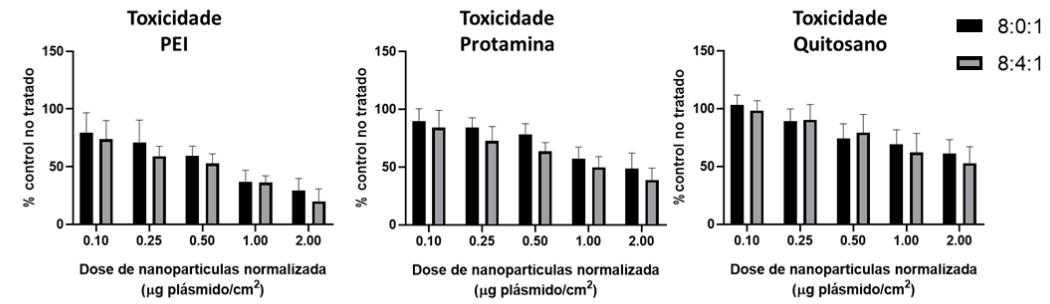
nin á súa toxicidade (Figura 1. b e c). No caso das nanopartículas (NPs) de PEI/6 MHA- PPZ alcanzouse unha eficacia de transfección 5 veces superior ao PEI só e á formulación de polifosfacenos previamente desenvolvida polo grupo [7]. Esta eficacia foi ademais 20 veces superior á obtida co control positivo do estudo (Lipofectamine 2000) (Figura 1.d).

En conclusión, o polímero que conten aminas secundarias e terciarias demostrou mellores propiedades para o seu uso en terapia xénica. Además, a súa asociación có 6MHA-PPZ produciu un incremento notable da transfección polas propiedades endosomolíticas de este polímero aniónico, sen afectar ás propiedades fisicoquímicas das NPs.

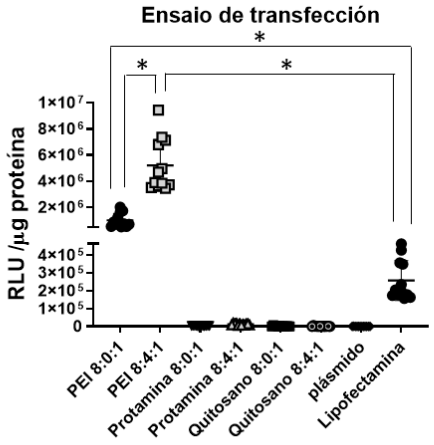
A

	Tamaño (nm)	PDI	Carga superficial (mV)
PEI 8.0.1	106±5	0.2	31±4
PEI 8.4.1	119±6	0.1	36±5
Protamina 8.0.1	130±5	0.2	24±3
Protamina 8.4.1	125±2	0.1	23±1
Quitosano 8.0.1	142±7	0.2	22±4
Quitosano 8.4.1	128±5	0.2	23±3

B



C



D

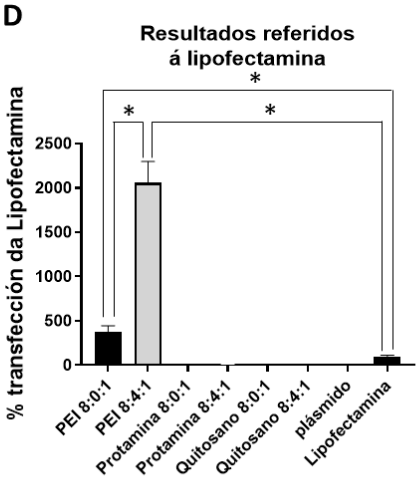


Figura 1. Propiedades fisicoquímicas, toxicidade e transfección das nanopartículas que combinan os polímeros catiónicos comerciais có polifosfaceno aniónico. A. Características do tamaño, polidispersión e carga superficial. B. Toxicidade *in vitro* a diferentes concentracións expresadas como dose normalizada de nanopartículas (µg plásmido/cm^2). C. Ensaio de transfección determinado mediante a medida da luminiscencia emitida polas células transfectadas e correxida pola cantidade de proteína. D. Eficacia de transfección, sendo o 100% o valor da Lipofectamina: 2.5 x 10^5 RLU/µg proteína. RLU: Unidades relativas de luminiscencia. *Analise estatístico $p < 0.05$.

2. Síntese e caracterización de nanopartículas baseadas en polifosfacenos catiónicos para o seu uso en terapia xénica

Tras os resultados acadados no apartado anterior, planteouse o desenvolvemento de sistemas baseados en combinacións de novos polifosfacenos catiónicos e 6MHA-PPZ, empregados como alternativas biodegradables ao PEI. Para iso, utilizóuse a plataforma previamente desenvolvida no grupo, que permite derivar polifosfacenos a través de reaccións "click" de tiol-eno [7]. Considerando os estudos anteriores do grupo e deste traballo (capítulo 2), propúxose o desenvolvemento de heteropolímeros con aminas primarias e con outros sustituyentes variables: un grupo anfifílico cun hidroxilo, unha amina secundaria, ou un grupo alifático. En todos os ensaios incluíuse como referencia o homopolímero catiónico substituído unicamente con aminas primarias, xa publicado previamente [7].

Tanto o precursor como os polímeros caracterizáronse estruturalmente mediante resonancia magnética nuclear (RMN ^{31}P, ^{1}H, COSY e HSQC) (Figura 2) e tamén se determinou o seu peso molecular, que está comprendido entre 50-100 KDa. Deseñáronse varios prototipos de nanopartículas por condensación cun plásmido modelo que expresa os marcadores da proteína fluorescente verde e a luciferasa (pEGFLuc). As nanopartículas preparáronse co mesmo cociente de carga (N: C: P) sendo N o número de aminas primarias do polímero catiónico, C o número de grupos carboxilo do 6 MHA- PPZ e P o número de grupos fosfato das bases do ADN. Seleccionáronse os cocientes 8:0:1 para as NPs sen 6 MHA- PPZ e 8:4:1 para as NPs con 6 MHA-PPZ.

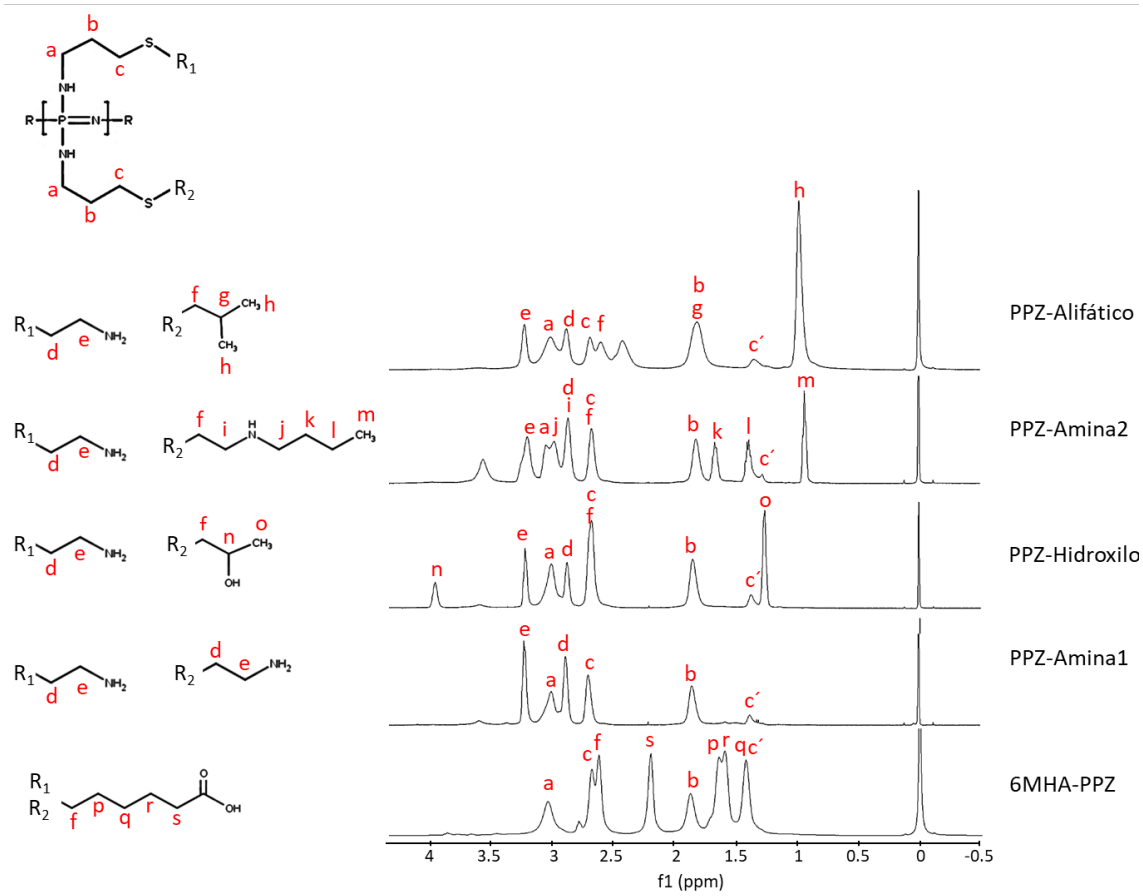


Figura 2. Estructura e caracterización dos polifosfacenos mediante resonancia magnética nuclear de protón (^{1}H-RMN).

Todas as formulacións presentan propiedades fisicoquímicas adecuadas para o seu uso en terapia xénica (Figura 3), sendo nanopartículas de forma esférica cun tamaño inferior a 200 nm, carga superficial positiva e cunha asociación reversible do plásmido [13]. As nanopartículas que conteñen o polímero aniónico tiveron unhas características similares ás formadas polo polímero catiónico só e o pDNA, excepto porque presentaron unha concentración de partículas moi superior, demostrando un mellor rendemento durante a súa preparación.

	Tamaño (nm)	**PDI**	**Carga superficial** (mV)	**Concentración** (partículas/mL)
PPZ_Alifático 8.0.1	150±4	0.2	+38±3	5.45±0.1 x 10^{10}
PPZ_Alifático 8.4.1	143±4	0.2	+32±3	1.69±0.05 x 10^{11}
PPZ_Amina2 8.0.1	126±4	0.2	+37±4	6.94±0.3 x 10^{10}
PPZ_Amina2 8.4.1	129±3	0.1	+31±3	1.55±0.04 x 10^{11}
PPZ_Hidroxilo 8.0.1	111±3	0.1	+38±2	7.22±0.3 x 10^{10}
PPZ_Hidroxilo 8.4.1	135±3	0.1	+39±3	1.71±0.05 x 10^{11}
PPZ_Amina1 8.0.1	119±2	0.2	+36±2	5.29±0.2 x 10^{10}
PPZ_Amina1 8.4.1	122±2	0.1	+35±4	1.62±0.08 x 10^{11}

Figura 3. Caracterización fisicoquímica das nanopartículas baseandose no seu tamaño, carga superficial e concentración. PDI: índice de polidispersión.

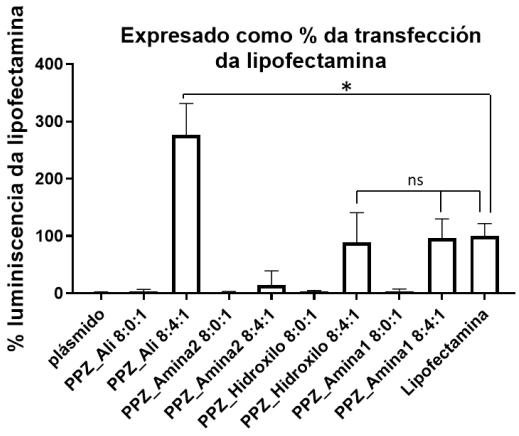
Neste caso, a adicción do 6 MHA- PPZ provocou unha redución significativa da toxicidade (Figura 4.a) na maioría dos nanosistemas, esta redución foi validada tamén en ensaios *in vivo* en peixes zebra. O prototipo que contén o polifosfaceno co radical alifático (PPZ_ Alifático) foi o que demostrou menor toxicidade. En canto á transfección, as partículas que combinan os polímeros catiónicos e o 6 MHA- PPZ mostraron un incremento notable da transfección (>100 veces) respecto a os prototipos sen 6 MHA- PPZ (Figura 4. c). En particular, o prototipo que combina PPZ_ Alifático e 6 MHA- PPZ supera en case 3 veces a transfección da referencia comercial (Lipofectamine 2000) (Figura 4. b). Polo tanto, os estudos indican que os materiais que combinan cadeas con grupos amino terminais e cadeas hidrofóbicas teñen unha mellor transfección, probablemente porque ambas as cadeas evitan a degradación endosómica, e ademais as cadeas hidrofóbicas facilitan a internalización celular das nanopartículas, como xa se observou tras a modificación doutros polímeros catiónicos con grupos hidrofóbicos [14–16].

A

IC50 das nanopartículas expresado como µg de plásmido/cm²

Polímero catiónico	Ratio 8.0.1	Ratio 8.4.1
PPZ_Alifático	0.999	1.46
PPZ_Amina2	0.78	1.48
PPZ_Hidroxilo	0.338	0.86
PPZ_Amina1	0.742	1.24

B



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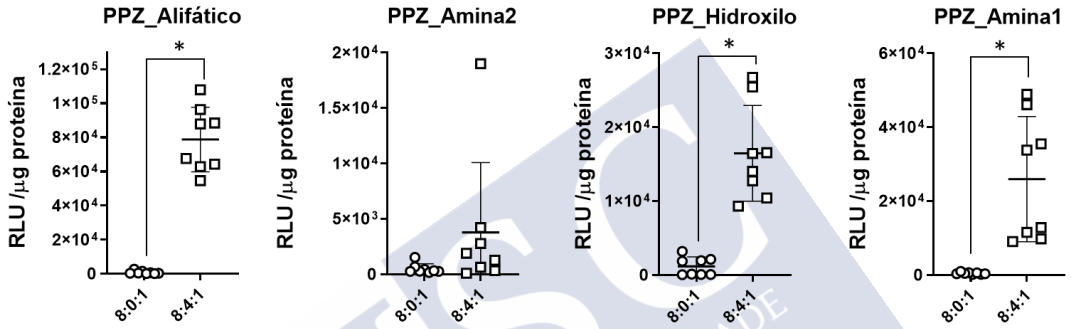

Figura 4. Toxicidade e transfección dos nanosistemas *in vitro* nunha liña celular de glioblastoma cerebral humano (U87MG). A. Toxicidade dos nanosistemas expresada como dose letal para o 50% da poboación celular (LC50), as concentracións das nanoparticulas están referidas á cantidade de plásmido para facilitar a comparación entre os diferentes nanosistemas. B. Comparación da transfección para cada nanosistema con e sen polímero aniónico. C. Transfección dos nanosistemas a unha concentración de 0.5µg de plásmido/cm², a determinación da transfección realizouse ao medir a luminiscencia das células transfectadas e os resultados están referidos á porcentaxe de transfección da Lipofectamina 2000, considerando o 100% de transfección 2.5 x 10^4 RLU/ µg proteína. RLU: Unidades relativas de luminiscencia * Análise estatístico $p < 0.05$.

Tras os resultados acadados *in vitro* co prototipo composto pola asociación do PPZ_Alifático e 6MHA-PPZ, estas nanopartículas foron seleccionadas para avaliar a súa eficacia cun plásmido terapéutico *in vitro* e *in vivo*.

3. Eficacia *in vitro* e *in vivo* de nanoparticulas baseadas en polifosfacenos nun modelo de glioblastoma

Unha das estratexias terapéuticas empregadas na loita contra as CSCs e a inducción da súa diferenciación cara a un fenotipo menos maligno e sensible á quimioterapia convencional [17].

As proteínas morfoxénicas do óso (BMPs) están implicadas na diferenciación celular e demostraron ser eficaces no tratamento de numerosos tumores, ao ser capaces de inhibir a vía de señalización do TGF-β, altamente implicada no mantemento das CSCs [18]. O maior problema que presenta a administración de estas proteínas é a súa rápida degradación no organismo, que impide unha activación continuada da vía de señalización. Neste traballo, proponse a inclusión dun plásmido que codifica BMP nas nanopartículas poliméricas para a súa liberación controlada tras a administración intratumoral. Como plásmido terapéutico seleccionouse un que codifica BMP-4 (pBMP4) [19,20], o plásmido asocióuse as nanopartículas compostas polos polímeros PPZ_Alifático e 6MHA-PPZ, que xa habían demostrado a mellor ratio toxicidad/transfección nos estudos anteriores (capítulo 3).

O cambio de plásmido respecto ao empregado no capítulo 3 non afectou nin o tamaño nin á capacidade de asociación do ácido nucleico, sendo estas nanopartículas adecuadas para os ensaios *in vitro* e *in vivo*. A eficacia terapéutica das nanopartículas-pBMP4 (NPs-pBMP4) estudouse como monoterapia ou tras a súa asociación coa Temozolomida (Tz), sendo este o fármaco de elección empregado no tratamento do glioblastoma cerebral. O ensaio *in vitro* realizouse en dúas liñas celulares de glioblastoma humano (U87MG y U251) e baseouse na evaluación da clonoxenicidade celular. Tras a administración das NPs-pBMP4 observouse un descenso na capacidade clonoxénica das células tumorais, e dito efecto foi máis marcado tras a coadministración das nanoparticulas coa Tz (Figura 5.a). De feito, unha evaluación detallada dos datos confirmou que a combinación das NPs-pBMP4 e Tz ten un efecto sinérxico.

Finalmente, a capacidade antitumoral das nanopartículas foi avaliada nun modelo murino de xenoinxerto de glioblastoma. Neste modelo observouse que a administración das NPs-pBMP4 por sí mesma non reducía o tamaño do tumor. Este resultado foi contrario ó esperado e podese explicar porque a poboación de CSCs na liña tumoral (U87MG) empregada na xeración do modelo murino, é inferior ó 1%. Doutra banda, a asociación das NPs-pBMP4 e temozolomida, produciu unha reducción moi significativa na progresión tumoral (Figura 5.b), quedando o volumen prácticamente en estasis. Tamén se observou un claro aumento na supervivencia dos ratos que foron tratados coa coterapia.

Finalmente, os tumores foron diseccionados e analizáronse os niveis de expresión xénica do BMP-4 e doutros marcadores de malignidade (Figura 5.c). Observouse que a expresión de BMP4 foi máis de 1000 veces maior nos grupos tratados con NPs-pBMP4 que no grupo control, o cal indica unha transfección eficiente e duradeira do xene terapéutico. Con todo e iso, fora dunhas pequenas modificacións que acadaron resultados significativos en xenes relacionados coa malignidade das celulas tumorais (Sox2 e Nanog) [21], o tratamento con pBMP4 non pareceu modificar significativamente o fenotipo CSC dos tumores. Esto pode ser debido a falta de sensibilidade ao BMP-4 da liña celular empregada (U87MG) que presenta unha población de CSCs inferior ao 1%. Ademáis, observouse que o tratamento dos tumores con Tz produciu unha inducción da bomba de fluxo MDR, este xene está implicado na resistencia a quimioterapia convencional [22], acadando niveis 3 veces superiores a os obtidos nas células non tratadas. A asociación da Tz as NPs-pBMP4 normalizou a expresión de MDR a niveis basais, sendo esta unha posible explicación ao efecto sinérxico encontrado entre ambos tratamentos.

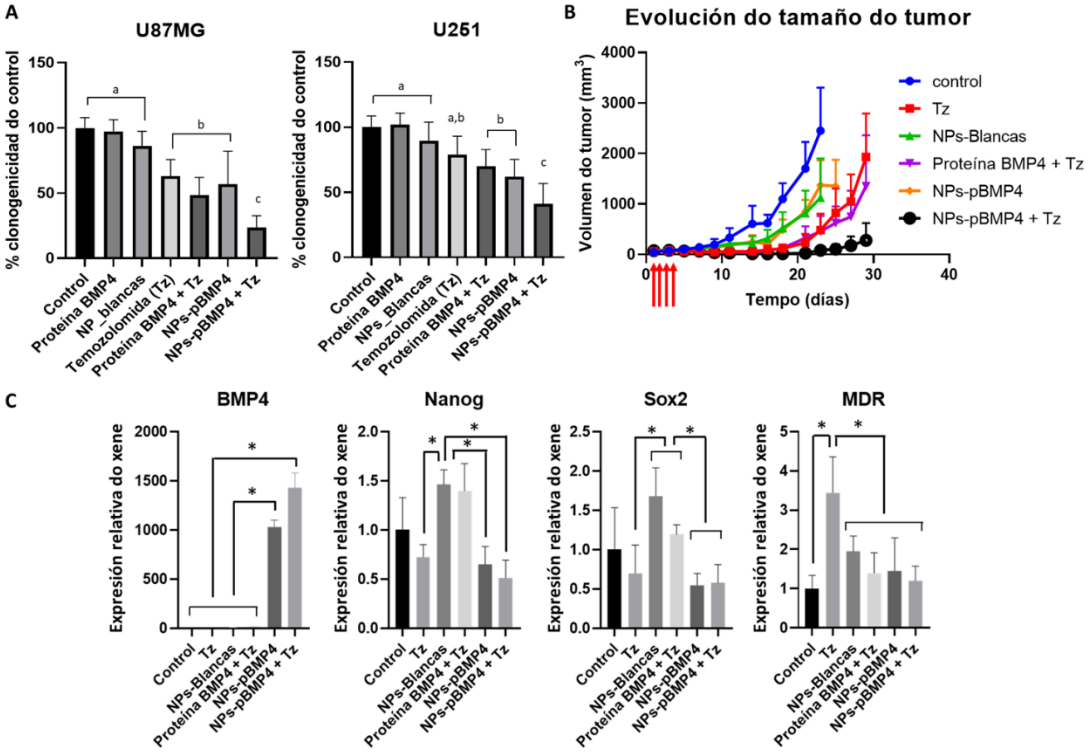

Figura 5. Ensaio de eficacia antitumoral das nanopartículas *in vitro* e *in vivo* nun modelo de xenoinxerto murino de glioblastoma humano. A. Ensaio de clonoxenicidade das nanopartículas terapéuticas solas ou en combinación con Tz en dúas liñas celulares de glioblastoma humano (U87MG y U251), os tratamentos significativamente homoxéneos nomearonse coa mesma letra. B. Evolución do tamaño do tumor tras o tratamento coas nanopartículas terapéuticas soas ou en combinación con Tz, as frechas vermellas fan referencia á pauta de administración. C. Expresión relativa dos xenes implicados no mantemento das células nai tumorais. Tz: temozolomida; BMP-4: Proteína Morfoxénica do óso 4; NPs-pBMP4: Nanopartículas que conteñen o plásmido que codifica BMP-4. *Analise estatístico $p < 0.05$.

Como conclusión, nesta tese demostrouse o potencial de novos nanovehículos poliméricos baseados en polifosfacenos catiónicos optimizados e na súa combinación co polímero endosomolítico 6MHA-PPZ para a liberación controlada de xenes. Os sistemas desenvolvidos son biodegradables e mostran ratios de eficacia/toxicidade punteros. Os resultados acadados mostran o gran potencial da plataforma de nanopartículas para diversas aplicaciones de terapia xénica, e en concreto para a aproximación desenvolvida neste traballo baseada na asociación da temozolomida cun plásmido codificante da proteína BMP4.

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Resumen *in extenso*

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En la mayoría de los tumores existe una subpoblación celular que presenta características de células madre, conocida como células madre tumorales (CSCs). Estas células se caracterizan por su desdiferenciación, son capaces de autorregenerarse, presentan resistencia a la quimioterapia y alta capacidad de migración e invasividad. Por ello, las CSCs son consideradas las principales responsables de las recidivas y su supresión es fundamental para la lucha contra el cáncer. Existen numerosas estrategias para modular las CSCs, tanto actuando específicamente sobre estas células, como sobre el nicho tumoral donde se encuentran ubicadas y que contribuye a su mantenimiento y protección [1,2].

Hoy en día, la terapia génica se ha postulado como alternativa a la utilización de pequeños fármacos y proteínas en el tratamiento de enfermedades con base genética como el cáncer. Aun así, la liberación de genes entraña una serie de problemas biofarmacéuticos como la fácil degradación de los ácidos nucleicos en los medios biológicos y las barreras que deben atravesar hasta llegar al lugar donde desarrollan su efecto [3,4]. Inicialmente para evitar estas limitaciones de los ácidos nucleicos se incluyeron en vectores virales, sistemas que con alta eficacia, pero que pueden presentar problemas de seguridad [5]. La nanotecnología permite el diseño de vectores no virales con capacidad para mejorar la expresión génica *in vitro* e *in vivo*, teniendo un mejor perfil de bioseguridad. Estos nanovehículos se pueden modificar químicamente para dirigir el ácido nucleico específicamente al tejido diana e incrementar su internalización [6].

Teniendo en cuenta estos antecedentes, el objetivo de la tesis ha sido el desarrollo de nuevos prototipos de nanopartículas poliméricas para su uso en terapia génica como tratamientos contra las CSCs. Por un lado, se ha investigado qué grupos catiónicos de los polímeros empleados en terapia génica, generan una transfección más eficiente. En una primera etapa de esta tesis, se han estudiado combinaciones de diferentes polímeros comerciales usados en terapia génica con un polímero endosomolítico, con el fin de determinar qué tipo de estructuras y combinaciones dan lugar a los sistemas más eficientes. El polímero endosomolítico escogido (6MHA-PPZ) se ha desarrollado previamente en nuestro laboratorio [7], demostrando resultados prometedores en la reducción de la toxicidad y mejorando la transfección de nanopartículas preparadas a base de polímeros catiónicos.

En una segunda parte de la tesis, se sintetizaron un pequeño grupo de polifosfacenos catiónicos con diferentes funcionalidades químicas. El objetivo fue determinar qué tipo de materiales y que funcionalidades dan lugar a los sistemas con mejor ratio de eficacia/toxicidad, tanto por sí solos como en combinación con el polímero endosomolítico 6MHA-PPZ. En la tercera parte de esta tesis, el prototipo de nanopartículas de polifosfacenos con mejores propiedades como vehículo de terapia génica, fue utilizado para encapsular una secuencia supresora de CSCs, y evaluada en modelos *in vitro* e *in vivo* de glioblastoma.

1. **Estudio de cómo afecta la estructura química de los polímeros catiónicos y su asociación a un polifosfaceno aniónico a la toxicidad y eficacia del nanosistema.**

Polietilenimina (PEI), protamina y quitosano son polímeros muy empleados en el desarrollo de nuevas formulaciones para terapia génica, por su capacidad de asociar los ácidos nucleicos [8]. Estos polímeros presentan diferentes grupos catiónicos encargados de asociar el ácido nucleico y que afectan a las propiedades fisicoquímicas, toxicidad y transfección de los nanosistemas. En su estructura el PEI presenta aminas secundarias y terciarias, la protamina los grupos guanidino del aminoácido arginina y el quitosano aminas primarias.

Todos los prototipos presentaron unas propiedades fisicoquímicas similares, en cuanto a tamaño, carga superficial y capacidad de asociación del ácido nucleico, siendo en todos los casos adecuadas para su uso en terapia génica (Figura 1.a). Los grupos amino del PEI han demostrado resultar más tóxicos para las células que los grupos guanidina presentes en la protamina (Figura 1.b), pero por otro lado estos grupos amino aumentaron notablemente la capacidad de transfección respecto a la protamina (Figura 1.c). Nuestros resultados están en concordancia con trabajos anteriores que han demostrado que tanto las aminas secundarias y terciarias [9,10] como los grupos guanidino [11,12] evitan la degradación endosomal del ácido nucleico, pero en nuestro trabajo se ha observado que las aminas secundarias y terciarias tiene un efecto mucho más marcado.

La asociación del 6MHA-PPZ a estos polímeros catiónicos mejoró la eficacia de transfección tanto del PEI como de la protamina, sin afectar a sus propiedades fisicoquímicas ni a su toxicidad (Figura 1.b y c). En el caso de las nanopartículas (NPs) de PEI/6MHA-PPZ se alcanzó una eficacia de transfección 5 veces superior al PEI sólo y a la formulación de polifosfacenos

previamente desarrollada por el grupo [7]. Esta eficacia fue además 20 veces superior a la obtenida con el control positivo del estudio (Lipofectamine 2000) (Figura 1.d).

En conclusión, el polímero con aminas secundarias y terciarias ha demostrado mejores propiedades para su uso en terapia génica. Además, su asociación con el 6MHA-PPZ produce un incremento notable de la transfección por las propiedades endosomolíticas de este polímero aniónico, sin afectar a las propiedades fisicoquímicas de las NPs.

A

	Tamaño (nm)	PDI	Carga superficial (mV)
PEI 8.0.1	106±5	0.2	31±4
PEI 8.4.1	119±6	0.1	36±5
Protamina 8.0.1	130±5	0.2	24±3
Protamina 8.4.1	125±2	0.1	23±1
Quitosano 8.0.1	142±7	0.2	22±4
Quitosano 8.4.1	128±5	0.2	23±3

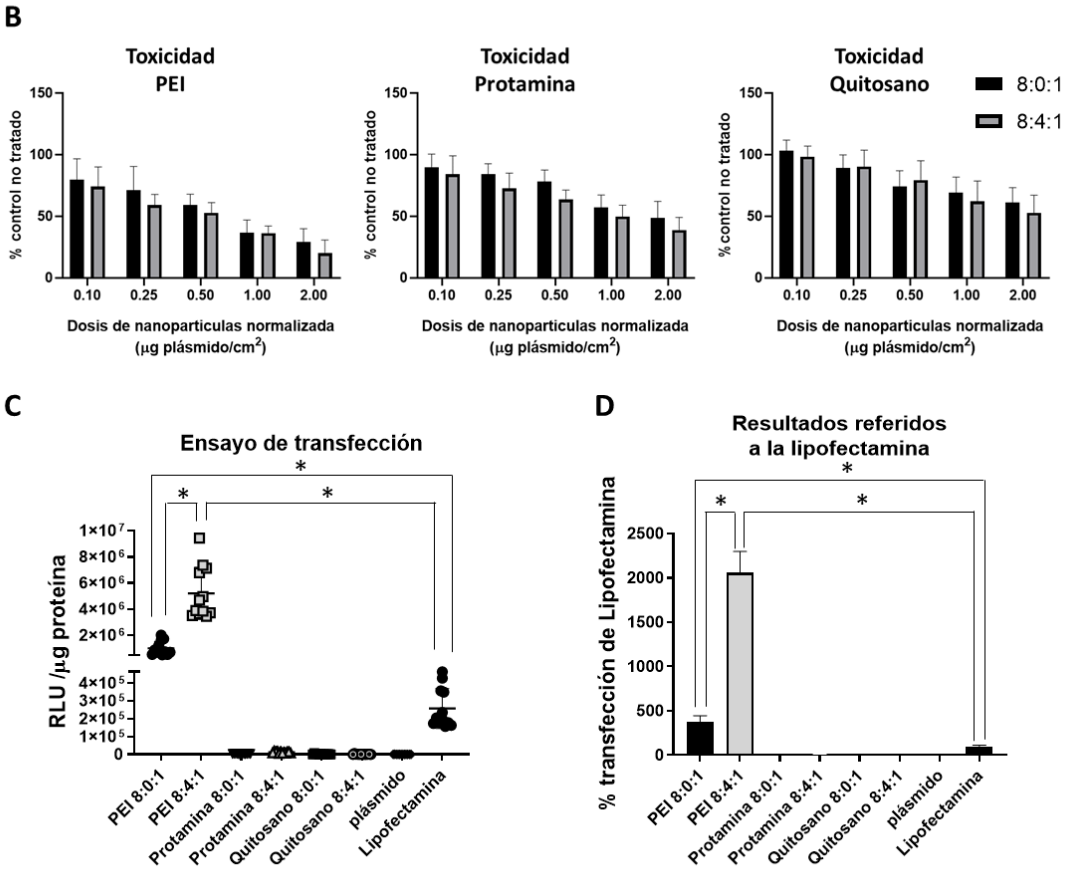


Figura 1. Propiedades fisicoquímicas, toxicidad y transfección de nanopartículas que combinan los polímeros catiónicos comerciales con el polifosfaceno aniónico. A. Características de tamaño, polidispersión y carga superficial. B. Toxicidad *in vitro* a diferentes concentraciones expresadas como dosis normalizada de nanopartículas (µg plásmido/cm2). C. Ensayo de transfección determinado mediante la medida de la luminiscencia emitida por las células transfectadas y corregida por la cantidad de proteína. D. Eficacia de transfección, siendo el 100% el valor de la Lipofectamina: 2.5 x 105 RLU/µg proteína. RLU: Unidades relativas de luminiscencia. *Analisis estadistico $p < 0.05$.

2. Síntesis y caracterización de nanopartículas basadas en polifosfacenos catiónicos para uso en terapia génica

Tras los resultados obtenidos en el apartado anterior, se planificó el desarrollo de sistemas basados en combinaciones de 6MHA-PPZ y nuevos polifosfacenos catiónicos, utilizados como alternativas biodegradables al PEI. Para ello, se utilizó la plataforma previamente desarrollada en el grupo, que permite derivar polifosfacenos a través de reacciones "click" de tiol-eno [7]. Considerando los estudios anteriores del grupo y de este trabajo (capítulo 2), se propuso el desarrollo de heteropolímeros con aminas primarias y con otros sustituyentes: un grupo anfifílico con un hidroxilo, una amina secundaria, o un grupo alifático. En todos los ensayos se incluyó como referencia el homopolímero catiónico sustituido únicamente con aminas primarias, ya publicado previamente [7].

Tanto el precursor como los polímeros se caracterizaron estructuralmente mediante resonancia magnética nuclear (RMN ^{31}P, ^{1}H, COSY y HSQC) (Figura 2) y también se determinó su peso molecular, que está comprendido entre 50-100 KDa. Se diseñaron varios prototipos de nanopartículas por condensación con un plásmido modelo que expresa los marcadores de la proteína fluorescente verde y la luciferasa (pEGFLuc). Las nanopartículas se prepararon a la misma ratio de carga (N:C:P) siendo N el número de aminas primarias del polímero catiónico, C el número de grupos carboxilo del 6MHA-PPZ y P el número de grupos fosfato de las bases del ADN. Se seleccionaron las ratios 8:0:1 para las NPs sin 6MHA-PPZ y 8:4:1 para las NPs con 6MHA-PPZ.

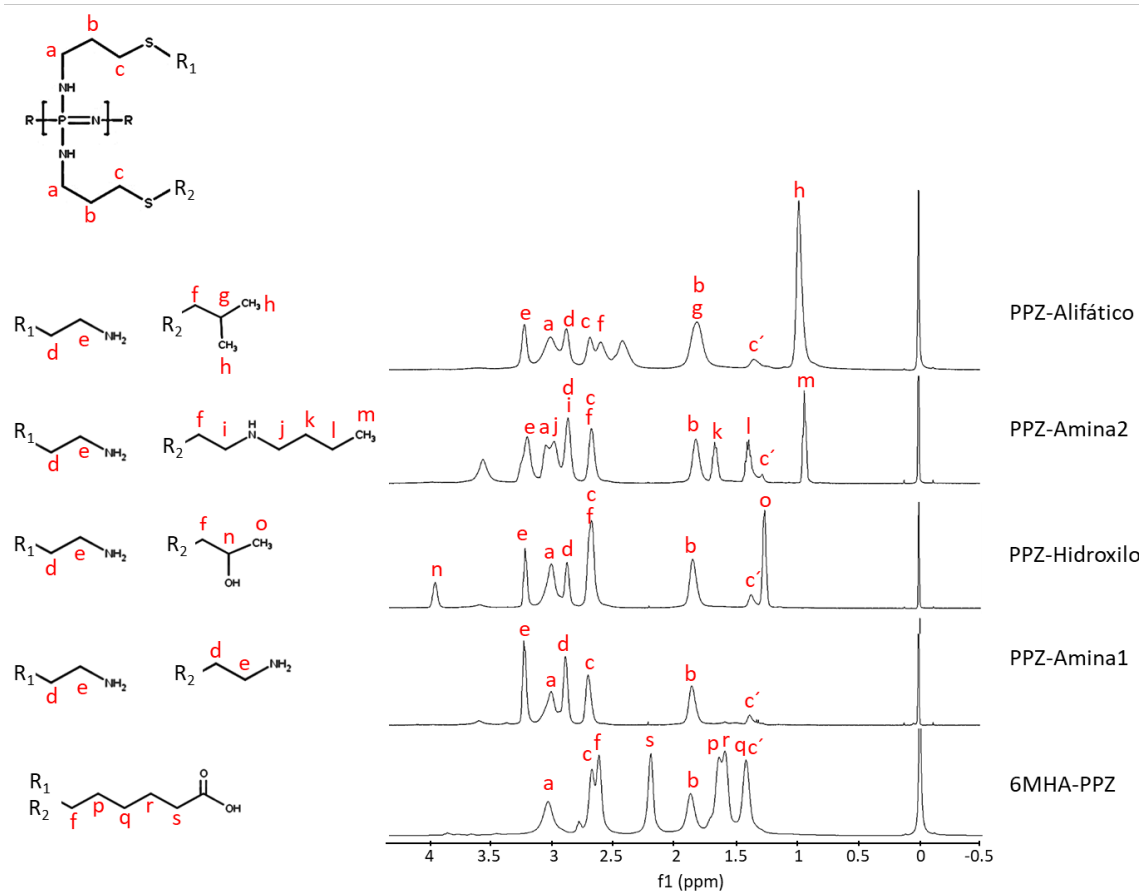


Figura 2. Estructura y caracterización de los polifosfacenos mediante resonancia magnética nuclear de protón (^{1}H-RMN).

Todas las formulaciones presentan propiedades fisicoquímicas adecuadas para su uso en terapia génica (Figura 3), siendo nanopartículas esféricas con un tamaño inferior a 200 nm, carga superficial positiva y con una asociación reversible del plásmido [13]. Las nanopartículas que contienen el polímero aniónico tuvieron unas características similares a las formadas por el polímero catiónico sólo y el pDNA, excepto porque presentaron una concentración de partículas muy superior, demostrando un mejor rendimiento durante su preparación.

	Tamaño (nm)	**PDI**	**Carga superficial** (mV)	**Concentración** (partículas/mL)
PPZ_Alifático 8.0.1	150±4	0.2	+38±3	5.45±0.1 x 10^{10}
PPZ_Alifático 8.4.1	143±4	0.2	+32±3	1.69±0.05 x 10^{11}
PPZ_Amina2 8.0.1	126±4	0.2	+37±4	6.94±0.3 x 10^{10}
PPZ_Amina2 8.4.1	129±3	0.1	+31±3	1.55±0.04 x 10^{11}
PPZ_Hidroxilo 8.0.1	111±3	0.1	+38±2	7.22±0.3 x 10^{10}
PPZ_Hidroxilo 8.4.1	135±3	0.1	+39±3	1.71±0.05 x 10^{11}
PPZ_Amina1 8.0.1	119±2	0.2	+36±2	5.29±0.2 x 10^{10}
PPZ_Amina1 8.4.1	122±2	0.1	+35±4	1.62±0.08 x 10^{11}

Figura 3. Caracterización fisicoquímica de las nanopartículas en base a su tamaño, carga superficial y concentración. PDI: índice de polidispersión.

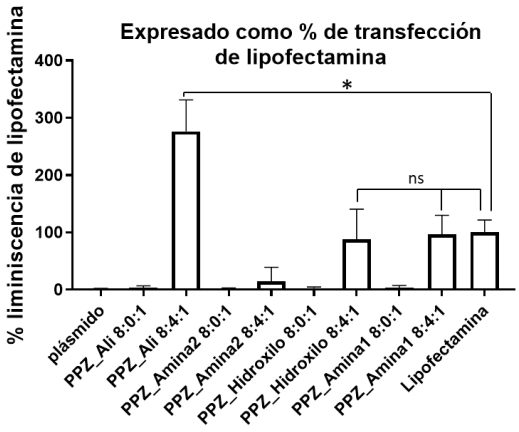
En este caso, la adicción del 6MHA-PPZ provocó una reducción significativa de la toxicidad (Figura 4.a) en la mayoría de los nanosistemas, esta reducción fue validada también en ensayos *in vivo* en peces zebra. El prototipo que contiene el polifosfaceno con el radical alifático (PPZ_Alifático) fue el que demostró menor toxicidad. En cuanto a la transfección, las partículas que combinan los polímeros catiónicos y el 6MHA-PPZ mostraron un incremento notable de la transfección (>100 veces) respecto a los prototipos sin 6MHA-PPZ (Figura 4.c). En particular, el prototipo que combina PPZ_Alifático y 6MHA-PPZ supera en casi 3 veces la transfección de la referencia comercial (Lipofectamine 2000) (Figura 4.b). Por lo tanto, los estudios indican que los materiales que combinan cadenas con grupos amino terminales y cadenas hidrofóbicas tienen una mejor transfección, probablemente porque ambas cadenas evitan la degradación endosómica, y además las cadenas hidrofóbicas facilitan la internalización celular de las nanopartículas, como ya se había observado tras la modificación de otros polímeros catiónicos con grupos hidrofóbicos [14–16].

A

IC50 de las nanopartículas expresado como µg de plásmido/cm²

Polímero catiónico	Ratio 8.0.1	Ratio 8.4.1
PPZ_Alifático	0.999	1.46
PPZ_Amina2	0.78	1.48
PPZ_Hidroxilo	0.338	0.86
PPZ_Amina1	0.742	1.24

B



C

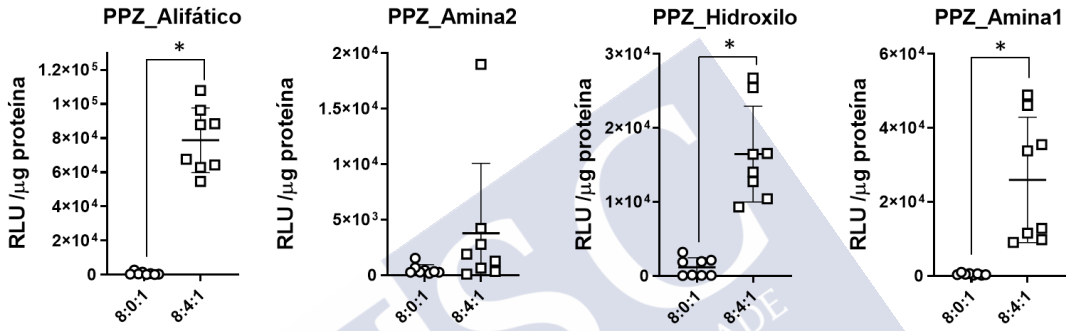


Figura 4. Toxicidad y transfección de los nanosistemas *in vitro* en una línea celular de glioblastoma cerebral humano (U87MG). A. Toxicidad de los nanosistemas expresada como dosis letal para el 50% de la población celular (LC50), las concentraciones de las nanoparticulas están referidas a la cantidad de plásmido para facilitar la comparación entre los diferentes nanosistemas. B. Comparación de la transfección para cada nanosistema con y sin polímero aniónico. C. Transfección de los nanosistemas a una concentración de 0.5µg de plásmido/cm^2, la determinación de la transfección se realizó al medir la luminiscencia de las células transfectadas y los resultados están referidos al porcentaje de transfección de la Lipofectamina 2000, considerando el 100% de transfección 2.5 x 104 RLU/ µg proteína. RLU: Unidades relativas de luminiscencia * Análisis estadístico $p < 0.05$.

Tras los resultados obtenidos *in vitro* con el prototipo compuesto por la asociación del PPZ_Alifático y 6MHA-PPZ, estas nanopartículas se seleccionaron para testar su eficacia con un plásmido terapéutico *in vitro* e *in vivo*.

3. Eficacia *in vitro* e *in vivo* de nanoparticulas basadas en polifosfacenos en un modelo de glioblastoma

Una de las estrategias terapéuticas empleadas en la lucha contra las CSCs es inducir su diferenciación hacia una fenotipo menos maligno y sensible a la quimioterapia convencional

[17]. Las proteínas morfogénicas del hueso (BMPs) están implicadas en la diferenciación celular y han demostrado ser eficaces en el tratamiento de numerosos tumores, al ser capaces de inhibir la vía del TGF-β, altamente implicada en el mantenimiento de las CSCs [18]. El mayor problema que presenta la administración de estas proteínas es su rápida degradación en el organismo, que impide una activación continuada de la vía de señalización. En este trabajo, se propone la inclusión de un plásmido que codifica BMP en las nanopartículas poliméricas para su liberación controlada tras administración intratumoral. Como plásmido terapéutico se ha seleccionado uno que codifica BMP-4 (pBMP4) [19,20], y este ha sido asociado a las nanopartículas compuestas por PPZ_Alifático y 6MHA-PPZ, que habían demostrado la mejor ratio toxicidad/transfección en estudios anteriores (capítulo 3).

El cambio de plásmido respecto al utilizado en el capítulo 3 no afectó ni al tamaño ni a la capacidad de asociación del ácido nucleico, siendo estas nanopartículas adecuadas para testarlas *in vitro* e *in vivo*. La eficacia terapéutica de las nanopartículas-pBMP4 (NPs-pBMP4) se estudió como monoterapia o tras su asociación con Temozolomida (Tz), el fármaco de elección utilizado en el tratamiento del glioblastoma. El ensayo *in vitro* se realizó en dos líneas celulares de glioblastoma humano (U87MG y U251) y se basó en la evaluación de la clonogenicidad celular. Tras la administración de las NPs-pBMP4 se observó un descenso en la capacidad clonogénica de las células tumorales, y dicho efecto fue más marcado cuando las nanoparticulas se co-administraron con Tz (Figura 5.a). De hecho, una evaluación detallada de los datos confirmó que la combinación de NPs-pBMP4 y Tz tiene un efecto sinérgico.

Finalmente, la capacidad antitumoral de las nanopartículas fue testada en un modelo murino de xenoinjerto de glioblastoma. En este modelo se observó que la administración de las NPs-pBMP4 por sí misma no reducía el tamaño del tumor. Este resultado fue contrario al esperado y se puede explicar porque la población de CSCs en la línea tumoral (U87MG) utilizada en la generación del modelo murino, es inferior al 1%. Por otro lado, la asociación de NPs-pBMP4 y temozolomida, produjo una reducción muy significativa en la progresión tumoral (Figura 5.b), quedando el volumen prácticamente en estasis. También se observó un claro aumento en la supervivencia de los ratones que fueron tratados con la co-terapia.

Finalmente, los tumores fueron diseccionados y se analizaron los niveles de expresión génica de BMP-4 y de marcadores de malignidad (Figura 5.c). Se observó que la expresión de BMP4 fue más de 1000 veces mayor en los grupos tratados con NPs-pBMP4 que en el control, lo cual indica una transfección eficiente y duradera del gen terapéutico. Sin embargo, fuera de unas pequeñas modificaciones que alcanzaron resultados significativos en genes de malignidad (Sox2 y Nanog) [21], el tratamiento con pBMP4 no pareció modificar significativamente el fenotipo CSC de los tumores. Esto puede ser debido a la falta de sensibilidad al BMP-4 de la línea celular empleada (U87MG) con una población de CSCs inferior al 1%. Además, se observó que el tratamiento de los tumores con Tz producía una inducción de la bomba de eflujo MDR, un gen implicado en la resistencia a quimioterapia convencional [22], alcanzando niveles 3 veces superiores a las células no tratadas. La asociación de la Tz a las NPs-pBMP4 normalizaba la expresión de MDR a niveles basales, siendo esta una posible explicación al efecto sinérgico encontrado entre ambos tratamientos.

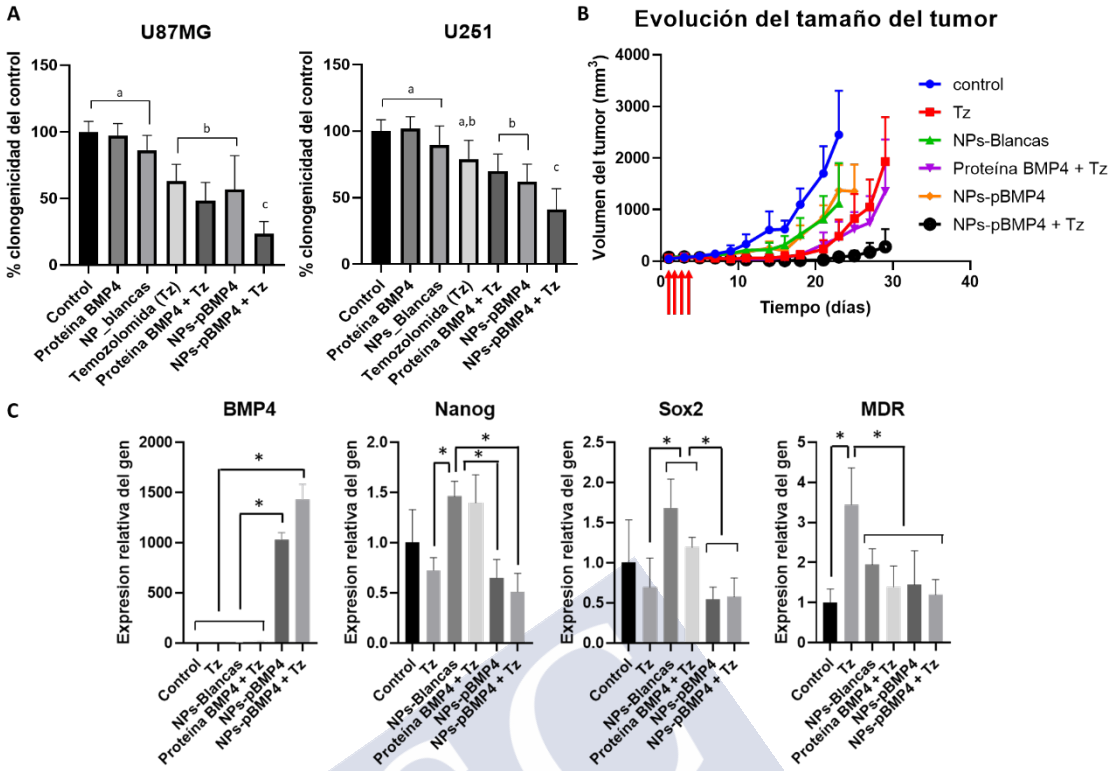


Figura 5. Ensayo de eficacia antitumoral de las nanopartículas *in vitro* e *in vivo* en un modelo de xenoinjerto murino de glioblastoma humano. A. Ensayo de clonogenicidad de las nanopartículas terapéuticas solas o en combinación con Tz en dos líneas celulares de glioblastoma humano (U87MG y U251), los tratamientos significativamente homogéneos se han nombrado con la misma letra. B. Evolución del tamaño del tumor tras el tratamiento con las nanopartículas terapéuticas solas o en combinación con Tz, las flechas rojas hacen referencia a las pautas de administración. C. Expresión relativa de los genes implicados en el mantenimiento de las células madre tumorales. Tz: temozolomida; BMP-4: Proteína Morfogénica del hueso 4; NPs-pBMP4: Nanopartículas que contienen el plásmido que codifica BMP-4. * Analisis estadistico p <0.05

Como conclusión, en esta tesis hemos demostrado el potencial de nuevos nanovehículos poliméricos basados en polifosfacenos catiónicos optimizados y en el polímero endosomolítico 6MHA-PPZ para la liberación controlada de genes. Los sistemas desarrollados son biodegradables y muestran ratios de eficacia/toxicidad punteros. Los resultados muestran el gran potencial de la plataforma de nanopartículas para diversas aplicaciones de terapia génica, y en concreto para la aproximación desarrollada en este trabajo basada en la asociación de temozolomida con un plásmido codificante de la proteína BMP4.

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Introduction

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Challenges and strategies of gene delivery systems

Many non-infectious diseases are caused by errors or defects in the expression of genes responsible for correct cell functioning. A possible solution is the silencing of the abnormal gene, its replacement for a healthy copy or the induction of the expression of a new modified gene to the cell (Figure 1) [1]. Although gene therapy is not yet widely used at the clinical level, it has been studied for the treatment of many diseases such as cancer, autoimmune diseases, tissue engineering and nervous system diseases [2].

Gene therapy emerged in the 70s, but it was not until two decades ago when clinical trials began to test its effectiveness. At that time, this form of treatment was conceived as a revolution in medicine, because instead of treating the symptoms, this technique sought to address the underlying disease mechanism. At first, results were not as encouraging as expected, mainly because the vectors of the nucleic acids were not optimal and did not allow the efficient release of the polynucleotides at the target site [3]. After more than 50 years and 2000 clinical trials studying gene therapy, there have been considerable advances towards the design of safe, specific and efficient vectors for gene therapy and some treatments based on this pharmaceutical strategy have even been commercialized (Glybera®, Spinraza®, Onpattro®, Waylivra®) [4].

Nucleic acids can be administered by different routes, being intravenous and local injections the most frequent. In addition, in some cases the transfection can be performed *ex vivo*; this is the case of the Vigil platform (Gradalis, Inc.), already in clinical trials, where the cells are extracted from the patient, transfected with different molecules to stimulate the immune system, and reimplanted (Figure 1).

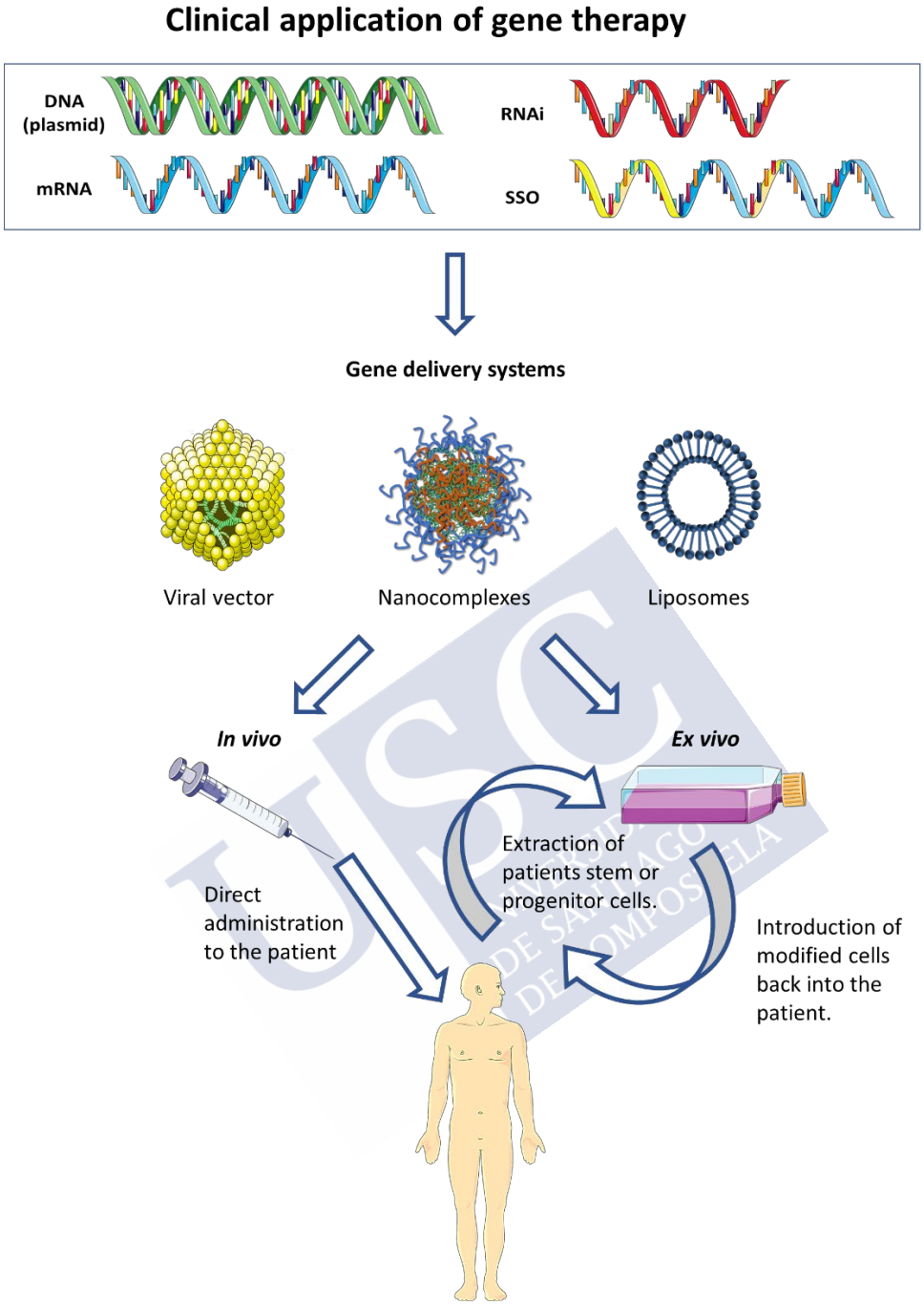

Figure 1. Squeme of the clinical application of gene therapy.

1. Therapeutic nucleic acids

Therapeutic nucleic acids can act on the different steps of gene expression process, either promoting or silencing a gene. When silencing, different polynucleotides can either block the transcription of the gene or its translation into proteins. Exogenous nucleic acids can be

divided as DNA, messenger RNA (mRNA), interference RNA (iRNA) or splice-switching oligoncleotides (SSO) (Figure 1).

Plasmid DNA (pDNA) is a double-stranded nucleic acid construct frequently used to deliver genes of interest. After its internalization and nuclear translocation, it can be transcribed to generate: (I) a mRNA, which is translated into a therapeutic protein, or (II) interference RNA or oligonucleotides that act directly on the expression of other genes. Some advantages of the use of pDNA in gene therapy are its higher stability, the possibility to include several genes in the same plasmid, and the possible integration into the genome of the host cell, which would allow the gene to be conserved after cell division. However, plasmids also present some inconveniences like the difficulty to cross the cell membrane, due to their large size (1.5-20 kilobase pairs, kbp) and hydrophilicity [5]. Besides, the same capacity for integration can also be considered a disadvantage since if it happpens in an uncontrolled manner, it could lead to insertional mutations in the cell genome. Some examples of therapeutic plasmids encode tumor suppressors [6], growth factors [7], cytokines, and antigens for immunization [8].

Messenger RNA (*mRNA)* is a single-stranded RNA molecule that can be delivered to the cytoplasm for direct translation into a protein. This strategy improves the therapeutic efficiency compared to the plasmids due to its smaller size (1-15 kb; kilo-bases) and because mRNA has its target site in the cytosol instead of the nucleus. Other advantages of mRNA are that it lacks any risk of mutagenesis and is effective in non-dividing cells. To make it more effective for *in vivo* administration, mRNA has to be chemically modified to improve its stability and reduce cellular immune responses that might block effective translation. There are many therapeutic mRNAs described in the literature, and some of them have reached clinical trials: BNT111, BNT113 and BNT 122 (BioNTech RNA Pharmaceuticals GmbH), AZD8601 (Moderna, Inc.) and Rocapuldencel-T (Argos Therapeutics, Inc.) [9].

Interference RNA (RNAi) is a noncoding single or double-stranded RNA with a length around 20-25 nucleotides (nt) [10]. RNAi are present in the cells as endogenous regulators of gene expression and are generated by fragmentation of longer precursors. This small RNA targets a complementary mRNA and inhibits the translation or induces its degradation by RISC [11]. We can classify them in two groups: small interfering RNA (siRNA) and microRNA (miRNA).

siRNA is double-stranded (21-22 nt), where one of the strands is complementary to the mRNA and is incorporated in a protein complex called RISC (RNA-induced silencing complex) responsible of mRNA degradation. On the other hand, miRNAs are single-stranded (19-25 nt) and also complementary to mRNAs but has a hairpin structure that avoids the translation into protein [12]. Some examples of iRNAs-based medicines in clinical trials are: QPI-1002 (Quark Pharmaceuticals, Inc.), ARO-AAT, ARO-APOC3 and ARO-ANG (Arrowhead Pharmaceuticals, Inc.). Onpattro® (Alnylam Pharmaceuticals, Inc.) is the first iRNA therapy to achieve FDA approval in 2018.

Splice-switching oligonucleotides (SSOs) are synthetic, antisense, double-stranded nucleic acids (15–25 nt) complementary to pre-mRNA. SSOs block the normal splicing of the transcript by blocking the interactions RNA–RNA or protein–RNA, triggering an alternative splicing to the next exon [13,14]. Some of these biopharmaceuticals are in clinical trials or commercialized as Vitravene (Ionis Pharmaceuticals, Inc. and Novartis), Tegsedi®, Waylivra® (Akcea Therapeutics and Ionis Pharmaceuticals, Inc.) or Spinraza® (Biogen and Ionis Pharmaceutical, Inc.).

2. Main obstacles in gene therapy

Nucleic acids, in general, present several problems that makes difficult their direct administration.

a) Polynucleotide degradation

Efficient DNA transfection has been demonstrated in some tissues when it is administered under very specific conditions. Nucleic acids are normally easily degraded before reaching their target site [15]. Systemic administration typically shows low transfection because of the instability of nucleic acids in biological fluids, mainly due to their degradation by the presence of nucleases, and by the low concentrations reached at the therapeutic target [16]. Local administration is more effective, since higher concentrations of nucleic acid can reach the cells. Even so, this route of administration is limited to a few tissues.

The inclusion of these nucleic acids in vectors can protect them from degradation and deliver them inside the cell. With some specific ligand conjugation technologies, these vectors can recognize target cells and use their receptors to achieve efficient internalization [17,18].

b) Polynucleotide penetration and intracellular trafficking

Cell membrane is mainly composed of amphipathic lipids and only small and neutral molecules can cross it passively. The rest of the molecules enter the cells by active transport mediated by transporter proteins or endocytosis. Nucleic acids, due to their size and negative charge, do not cross the membrane passively [17,19]. Moreover, some nucleic acid therapeutics have their action site in the nucleus, and thus, they must also cross the nuclear membrane. The nuclear pore complex (NPC) allows the passage of molecules smaller than 250 nt passively [20]. In addition, the presence of some active mechanisms of nuclear uptake have also been described [21]. It has also been observed that dividing cells are easier to transfect than non-dividing cells due to nucleus fragmentation [22].

There are several degradation mechanisms for intracellular polynucleotides, such as the presence of cytoplasmic nucleases that degrade DNA in less than 90 minutes [23,24]. Still, the most important degradation route is the endosomal pathway. Upon uptake of polynucleotides, the early endosome is acidified due to an ATPase-proton pump, which activates several enzymes that completely degrade nucleic acids. Some studies have shown that less than 2% of the administered nucleic acid escapes endosomal degradation [24]. There are some strategies based on the introduction of the nucleic acid into a delivery vector. These vehicles have a composition that facilitates endosomal escape and several molecules are involved (Figure 2).

Cytoplasm is a viscous medium due to the presence of a high number of molecules and microtubules [19]. This property might restrict the mobility of the nucleic acids once released to the cytosol, reducing their capacity to reach their target at the nucleus or the ribosomes.

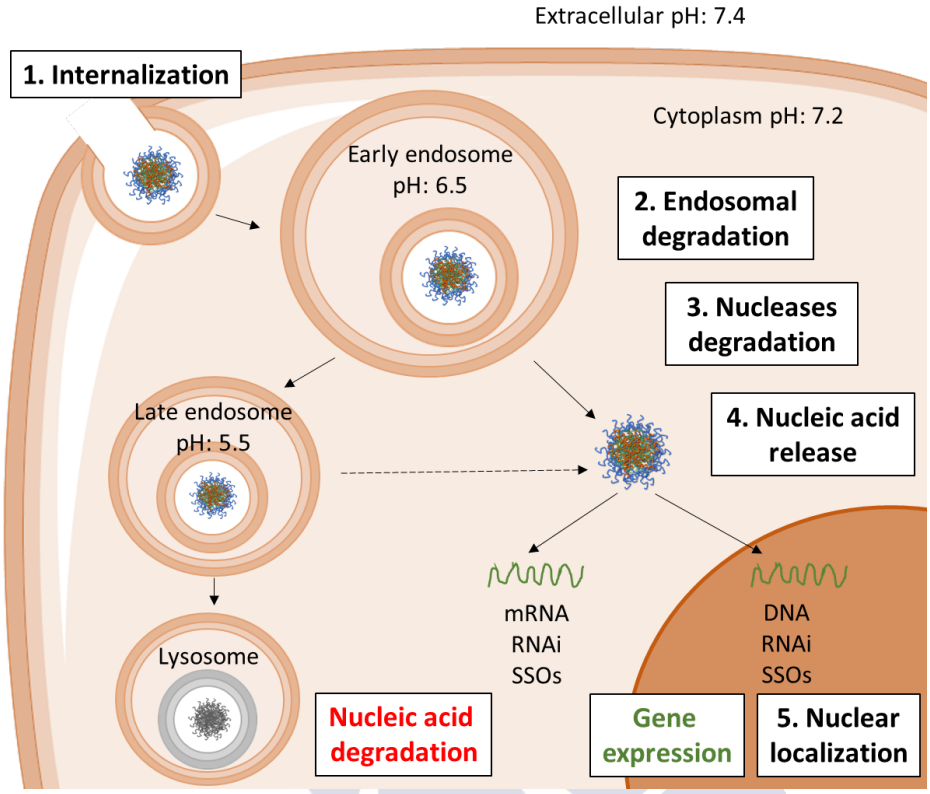

Figure 2. Intracellular barriers to overcome for the nucleic acid.

c) Immunogenicity

The administration of some free nucleic acids produces the activation of the immune system, as they are recognized and phagocytosed by immune cells and induce the release of pro-inflammatory cytokines. This immune reaction is especially marked in nucleic acids that have an unmethylated CpG dinucleotide located between two purines and two pyrimidines, but their inclusion in a delivery vehicle reduces these reactions [25]. The presence of uridine has also been shown to be immunogenic *in vitro* and *in vivo* [26].

3. Strategies to improve gene transfection

There are several strategies to improve gene transfection such as physical techniques, viral and non-viral vectors. This section is focused mainly on lipid and polymer-based carriers.

a) Viral vectors

Due to their inherent infective capacity, engineered viruses have been widely used as gene delivery vectors. Their transfectability depends on the type of virus used, i.e. retrovirus,

adenovirus, adeno-associated virus, or lentivirus. Viruses have to be modified before their use to eliminate the genes involved in their replication in the host cells. Some types of viruses carry the nucleic acid to the nucleus and mediate their integration into the genome. The transport capacity of these vectors also varies between 8-38 kb.

Although important advances have been made, viral vectors continue to present problems such as: (a) immunogenicity by causing inflammation of the treated tissues or even systemic effects and immune responses can limit also their transduction efficiency; (b) alterations in the correct functioning of the cells, due to possible mutations when the nucleic acid is inserted in the genome; (c) expression level and duration of effect are still limited [27–30]. Besides, viral vector manufacturing and security levels make them complicated structures for pharmaceutical translation. Viral vectors have been used in the treatment of diseases such as cancer [31], autoimmune [32] or endocrine diseases [33].

b) Non-viral vectors

Synthetic vectors should be specifically designed to overcome all the barriers that exist in gene therapy treatment. The ideal vehicle for the efficient transport of nucleic acids must protect polynucleotides during their transport and release them at their site of action.

Non-viral vectors are easily produced, economic and reproducible. These vehicles allow the packaging of high amounts of nucleic acids, although their transfection efficiency is still limited. Successful vehicles are designed to avoid endosomal degradation by compromising the integrity of the endosome membrane. While this is essential for successful transfection, endosomal disruption can cause cytotoxicity by interfering with the cytoplasmic or mitochondrial membranes. Another potential problem of non-viral vectors is a highly stable polynucleotide complexation that can limit nucleic acid release at the target site. To overcome these problems, vectors can be designed to be biodegradable, biocompatible as well as sensitive to environmental stimuli through changes in the chemical structure of their components [16].

For example, the variations in pH at different points of the nucleic acid delivery pathway, can be used as a stimulus for their release. The pH of the blood is 7.4, in the cytoplasm 7.2, in the early endosomes 6.5 and in the late endosomes 5.5. Thus, synthetic carriers can

incorporate polymers and/or lipids containing low-pKa amines, which are deprotonated in the cytosol and protonated in the endosome. These cationic groups improve endosomal escape by a mechanism known as the "proton-sponge effect". This mechanism postulates that these amines act as buffering agents during endosome acidification. Since protons enter the endosome together with their counterions, their accumulation induce membrane rupture by an increase in the osmotic pressure [34]. The “proton sponge effect” is highly questioned and another hypothesis have been put forward. One of those postulates the membrane permeabilization by electrostatic binding between cationic polymers/lipids and the cellular membrane anionic lipids, causing their destabilization and inducing pore formation. Nucleic acids escape the endosome through these pores [35,36]. In any case, it is clear that this pH-sensitive behaviour facilitates nucleic acid escape to the cytosol, and as a result, prevents partially polynucleotide degradation [37,38]. Other possible strategy to avoid the endosomal degradation is the use of fusogenic peptides (listeriolysin O). These molecules are activated at low pH and produce pore formation in the endosome membrane, facilitating the release of the nucleic acid [39,40]

Another example exploits the differences in redox potentials in cell compartments. The presence of glutathione and glutathione reductase within cells generates a more reductive potential as compared to extracellular space. Carriers with disulphide linkages between the nucleic acid and the vehicle have shown a selective release and more efficient transfection [41].

- Lipidic based nanocarries

Nanocarriers used for gene delivery can also be formed by lipids or phospholipids, since these molecules interact naturally with the cell membrane facilitating their internalization. There are numerous lipid-based nanocarriers in clinical trials and even some commercialized, which combine gene therapy in the treatment of numerous diseases, including cancer. These formulations can be classified into liposomes, lipid nanoparticles or emulsions. The main differences are that liposomes are vesicles composed of a phospholipid bilayer and an aqueous core, which facilitates the encapsulation of hydrophilic molecules such as nucleic acids. For its versatility, easy of preparation and modification, liposomes are the most widely

used carriers in gene therapy [42,43]. Solid lipid nanoparticles, by contrast, have a lipophilic core that makes the nucleic acid encapsulation very difficult. These nanoparticles are characterized by the presence of solid lipids at room temperature, some of the advantages of this type of formulation are stability and the scalability [42,44]. Finally, nanoemulsions are less stable systems formed by the mixture of two immiscible phases and in some cases surfactants [42]. An important advantage of using lipid nanosystems over polymeric nanosystems is that they allow the co-encapsulation of nucleic acids and hydrophobic drugs in the same system, being very useful in the treatment of diseases such as cancer [45].

Some liposomes have been commercialized as reagents for *in vitro* transfection: e.g. Lipofectin, Lipofectamine. Their cationic lipid components have a common structure: a hydrocarbon backbone linked to a cationic head. The cationic charge efficiently associates the nucleic acid by electrostatic interactions that, depending on the preparation method, gives rise to liposomes or lipoplexes. The most used cationic lipids are di-octadecenyl-trimethylammonium propane (DOTMA), dioleoyl-trimethylammonium propane (DOTAP) and zwitterionic lipids as phosphatidylethanolamine (PE), phosphatidylcholine (PC) and their derivatives (Figure 3). These cationic or zwitterionic lipids undergo structural modifications when they are at low pH, such as inside the endosome, generating pores allowing for the release of the nucleic acid, avoiding its degradation and improving transfection [46–48]. Cationic lipids can be toxic, since upon their internalization they act as a detergent, causing alterations in the integrity of cell membrane and triggering immune reactions [49,50]. Often, these formulations contain other lipids as cholesterol (Ch) to control membrane fluidity and lipidic surfactants to improve the stability of the nanosystem.

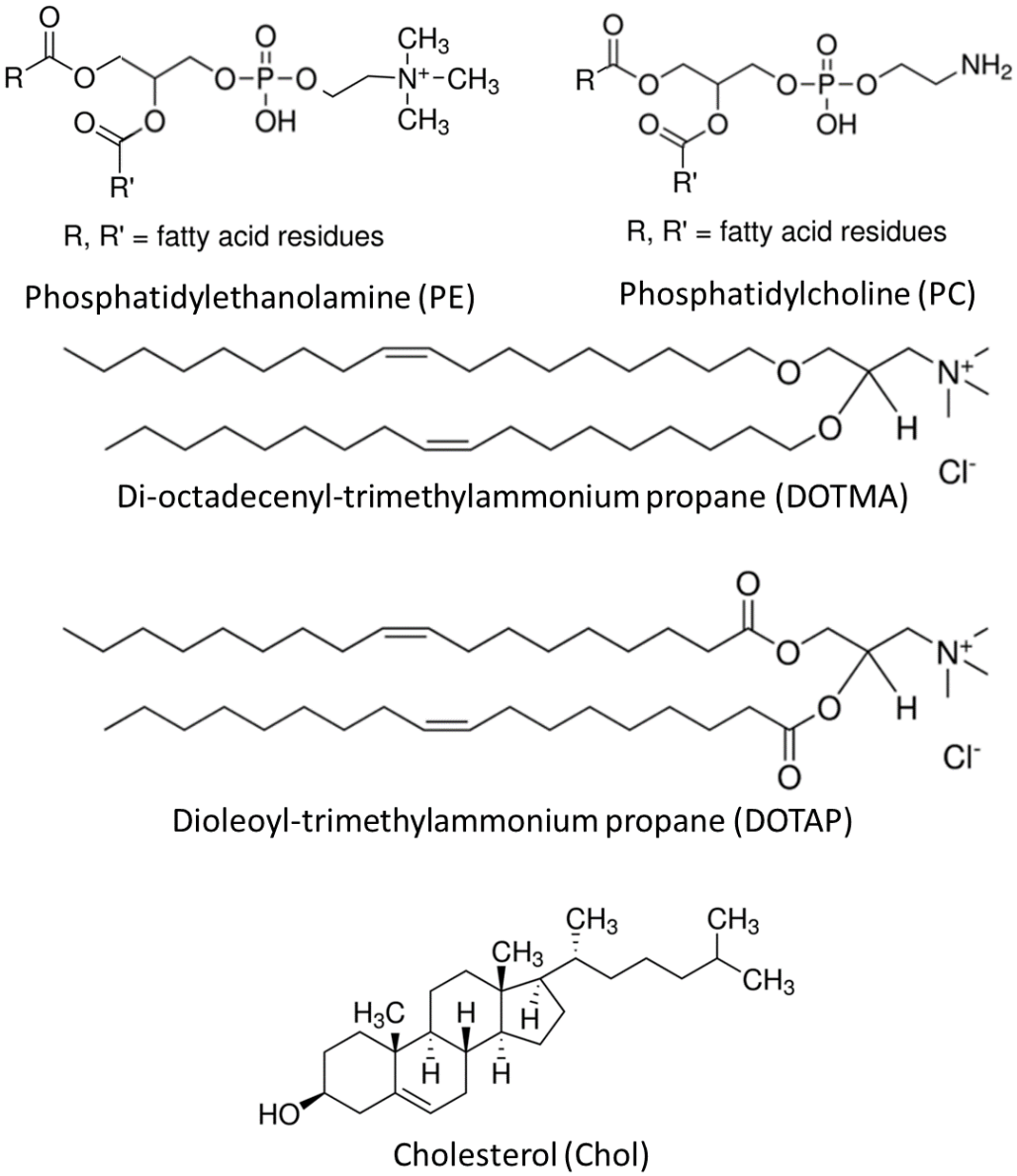

Figure 3. Chemical structure of the most used lipids in the preparation of synthetic gene delivery vectors.

Generally, mixtures of these above lipids have demonstrated better physicochemical properties and performance than the nanosystems formed only by a single cationic lipid. Liposomes containing DOTAP have demonstrated efficient nucleic acid transfection *in vivo* [51,52]. Cationic lipids can also be modified to improve their tolerance and transfection efficiency. For instance, the modification of DOTAP with small peptides has shown improvements in the transfection *in vitro* and *in vivo* [53]. Nanoemulsions have also been

developed with promising results, such as the vaccine developed by Novartis based on a self-amplifying mRNA. This vaccine carrier composed of DOTAP and other stabilizing lipids has already been tested in humans, showing activation of the immune system by amplifying the signal of molecules that act as adjuvants in immunity [54]. DOTMA-lipoplexes also showed a selectively accumulation *in vivo*, in addition to a more efficient transfection than the commercial reference system. These carriers are widely used in the development of vaccines and as cancer nanomedicines [55,56].

Liposomes based on the phosphatidylcholine derivative 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) are widely used for the delivery of combined siRNAs/chemotherapeutics treatments. These liposomes have shown to be effective in different xenograft and orthotopic mouse tumor models, resulting in: reduced tumor size [57], invasiveness [58,59], and angiogenesis together with increased tumor cell apoptosis [60].

The decoration of lipid complexes with polyethylene glycol (PEG), poloxamer or poloxamine improves water solubility, decreases cytotoxicity and increases plasma circulation time by controlling the process of adsorption of blood components upon injection [45]. However, PEGylation can counteract endosomal escape and reduce transfection due to steric hindrance. This limits nucleic acid delivery and therefore a compromise needs to be achieved for PEGylation degree [61]. PEG-liposomes might be transported passively to inflamed or tumor tissue, but these vectors can also be further functionalized with antibodies or ligands to improve their selective transport to the target cells, or with fusogenic molecules such as GALA peptide that promotes protonation and release of therapeutic molecules [46].

- Cationic polyplexes

Some of the most used polymers in the preparation of polymeric complexes in gene therapy are polyethylenimine (PEI), poly(L-lysine) (PLL), protamine, chitosan (CS) and poly(amidoamine) (PAMAM) (Figure 4). All these polymers can be found with various molecular weights (Mw) and other modifications. Molecular weight is an important factor to take into account since the Mw can affect both the stability, toxicity and transfection of the nanosystem [62–64].

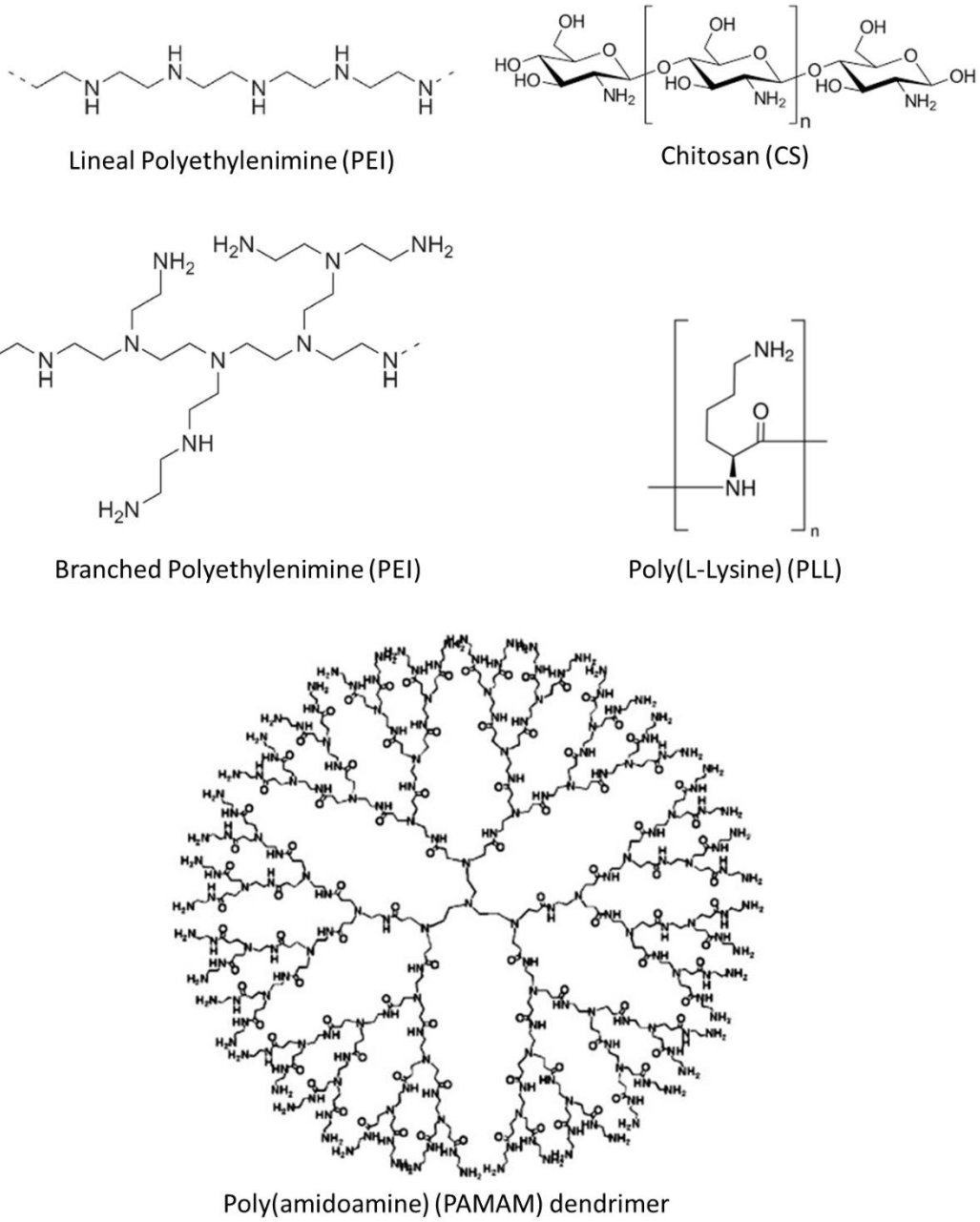

Figure 4. Chemical structure of the most used cationic polymers in the preparation of synthetic gene delivery vectors.

PEI is the most used polymer for gene therapy, due to its ability to associate a high amounts of nucleic acid, efficiently protect of DNA from degradation by DNases and for its ability to prevent endosomal degradation [65,66]. PEI amines with low pKa are critical to avoid endosomal degradation of nucleic acids but also contribute to its cytotoxicity. Some modifications have been made to reduce PEI toxicity without compromising transfection

capacity [37]. Many authors have reported on possible modifications to reduce PEI toxicity, for example by the association with pullulan and ascorbic acid. These modifications may in addition improve the stability, internalization and transfection efficiency of the nanosystems [67]. Another strategy relies on the elimination of some primary amines from PEI through the addition of polyethylene glycol (PEG), which also decreases toxicity and increases the accumulation and gene expression in solid tumors *in vivo* [68]. Hydrophobic modifications have also been made through association with a hydrophobic polypeptide, and these modifications also resulted in an enhancement in gene delivery in a tumor model [69]. Finally, other strategies to reduce PEI toxicity are based on coating with anionic polymers such as hyaluronic acid [70]. Moreover, PEI association with chitosan reduced nanoparticle toxicity 2-fold, while transfection capacity was found to be 2.5-fold higher than pure PEI vector [71].

Cationic polypeptides such as poly(L-lysine) (PLL) or protamine are also widely used in the preparation of non-viral vectors. There is the hypothesis that cationic polypeptides can facilitate entry into the nucleus through nuclear pores, by mimicking nuclear localization signals (NLS) [72]. PLL is a homopolymer approved by the FDA as antimicrobial agent [73] and a modification of PLL is also approved as skin test to detect penicillin allergy [74]. PLL is widely used in cancer immunology because contains a high number of hydrophilic amine groups, which allows the conjugation with several molecules at the same time. For example, the conjugation of PLL with immune control point inhibitors and photosensitive immune adjuvants in the same formulation improves anticancer efficiency and photoimmune response *in situ* [75]. It is normally associated with other components such as chloroquine, to avoid the degradation in the endosome [76]. Askarian et al. modified PPL with alkyl and PEI substituents and these complexes showed lower cytotoxicity and higher nucleic acid release than unmodified polymers. The formulation was able to reduce the expression of tumor markers in lung cancer [77]. The same effect was observed upon the formation of a triblock copolymer of PEG-PLL and poly (L-Leucine) containing the nucleic acid and an antitumoral drug as adjuvant [78]. The formulation showed high stability, biocompatibility and passive tumor-targeting and the co-association of the drugs showed a synergistic antitumoral effect in breast cancer cells (MCF-7)[78]. PLL can also be conjugated with cyclodextrins to reduce toxicity and improve blood compatibility, and *in vivo* efficacy [79].

Protamine is a polypeptide of natural origin, which is approved by FDA as drug (heparin antagonist) and as excipient (NPH insulin), a sign indicating its safety and low toxicity. Protamine efficiently condenses nucleic acids and shows higher transfection capacity than PLL. Still, protamine might have important changes in activity depending on the production batch due to its natural origin [80,81]. Protamine has positive charges due to the presence of 21 arginine residues, which are important for tight polynucleotide condensation and for its capacity to protect DNA from degradation by nucleases [72]. In addition to its use in the formation of polymeric nanoparticles, protamine is also used as a coating polymer for nanocapsules, where it has shown promising results by increasing the expression of an oncosuppressor gene [82]. Low-Mw protamine peptide has also been modified with neutral lipids, such as cholesterol, that generates amphiphilic conjugates with a higher transfection than PEI [83].

Polysaccharides such as chitosan (CS) are also used for gene therapy. This natural polysaccharide is obtained by deacetylation of chitin from the exoskeleton of crustaceans. This linear polymer is biocompatible, biodegradable, and used in the production of multiple drug delivery formulations for ocular diseases [84], cancer [85] or in the preparation of vaccines [86]. The molecular weight of CS affects its physicochemical properties and its effectiveness. Nanocomplexes formed by chitosan and nucleic acids in general present low stability, specificity, and transfection, and for this reason they are often modified or associated with other polymers to achieve more efficient vectors. For example, chemical modification with targeting moieties such as galactose, transferrin, folate or mannose [87] to specifically target a cell population and facilitate its uptake. The modification with alkyl groups increases nucleic acid packaging, cellular uptake and improves transfection [88]. Stability of chitosan complexes in serum can also be enhanced by PEGylation, although depending on the substitution degree, these modifications can decrease transfection efficiency by reducing uptake and endosomal leakage [89].

Another promising approach for polymeric gene delivery, are dendrimers. These are synthetic polymers formed by controlled repetitions of cascade branches. These structures have a high number of secondary and tertiary amines which makes them very effective in gene therapy [15,90]. One of the polymers most used in its preparation is poly(amidoamine)

(PAMAM) which is commercially available (Superfect^{TM}) [91]. Dendrimers are monodispersed by definition, and their size and number of functional groups can be easily controlled for system optimization. Their main disadvantage is their production because requires multiple stages, which implies cost and time [76]. Partial degradation or removal of the core of dendrimers can even improve their transfectability [92]. For example, *in vivo* tests with dendrimers containing an antitumoral nucleic acids showed a reduction in tumor growth and angiogenesis, and improved the survival of the animals in a carcinoma murine model [93,94].

Cationic polymers can be combined with pH-sensitive polymers containing zwitterionic or carboxylic groups in their structure to improve transfection capacity. These groups present a pKa comprised between the ensodosomal (pH 5) and the physiological pH (pH 7.4). Because of that, these polymers are negatively charged at physiological pH, but lose this charge at endosomal pH. As they lose their negative charge, these polymers became more hydrophobic and increase their interaction with lipid membranes, which improves transfection of the nanocarriers; these materials are called endosomolytic polymers [95,96]. Their pH-sensitive ability can be studied by comparing their haemolysis at physiological and endosomal pH [97–99]. In addition, these polymers have the ability to compensate the cationic charge excess of lipo- and polyplexes at physiological pH, which improves its internalization and reduces the cytotoxicity [100]. Some of the polymers with endosomolytic capacity are poly (propyl acrylic acid)[96,99,101–103], malic acid [100,104–106], oligomeric sulfonamides [95] and some examples of zwitterionic polymers [107–109].

4. Future directions

Given that natural polymers offer a limited array of functionalities, the synthesis or modification of polymers and lipids with specific functionalities is becoming more and more frequent. These synthetic processes adapt the carrier to the requirements of the therapeutic molecule to arrive intact to the action site, maximizing its therapeutic effect.

For example, lipo-oligomers are characterized by chemically precise covalent unions of oligoaminoacids and the hydrophobic domains of the fatty acids. The integration of different structures allows the integration of specific functionalities for efficient transport and nucleic acid protection, including uptake and endosomal escape domains. Dual conjugates of lipo-

oligomers can target specific cell populations and generate combined antitumoral effects through the conjugation of several therapeutic molecules [110,111].

Another example, polyphosphazenes, are synthetic polymers, characterized by the inorganic backbone based on phosphorus chemistry. These polymers were first synthesized in the mid-1960s [112] and are characterized by a huge number of possibilities for substitution with different radicals (PEG, polypeptides, prodrugs, polysaccharides, hydrophobic side chains). This structural flexibility, together with their biocompatibility, and the low toxicity of their degradation products, makes them very interesting polymers for medical applications [113]. Indeed, a few polyphosphazene derivatives have been used for gene delivery [114–117], and recently, Hsu et al. reported the facile generation of small library of compounds based on polyphosphazenes, generated towards optimizing cell transfection [97].

Overall, these new specifically designed materials are expected to significantly improve gene delivery in the multiple steps involved in this complex process. This improved performance is expected to be translated in a future generation of new gene delivery products.

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Chapter 1

Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

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Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

This chapter has been adapted/extracted from our previous published review [1].

Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

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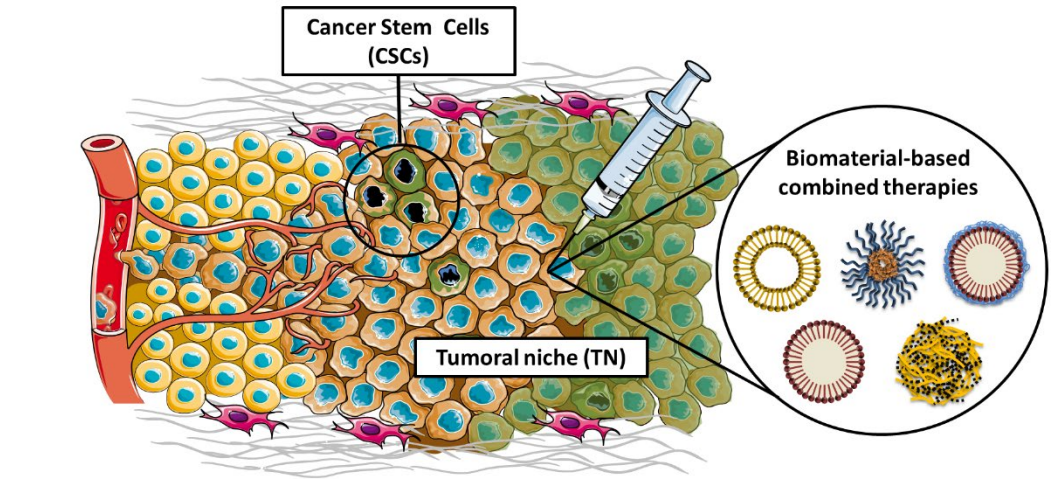
Abstract

Lack of improvement in the treatment options of several types of cancer can largely be attributed to the presence of a subpopulation of cancer cells with stem cell signatures and to the tumoral niche that supports and protects these cells. This review analyses the main strategies that specifically modulate or suppress cancer stem cells (CSCs) and the tumoral niche (TN), focusing on the role of biomaterials (i.e. implants, nanomedicines, etc.) in these therapies. In the case of CSCs, we discuss differentiation therapies and the disruption of critical signalling networks. For the TN, we analyze diverse strategies to modulate tumor hypervascularization and hypoxia, tumor extracellular matrix, and the inflammatory and tumor immunosuppressive environment. Due to their capacity to control drug disposition and integrate diverse functionalities, biomaterial-based therapies can provide important benefits in these strategies. We illustrate this by providing case studies where biomaterial-based therapies either show CSC suppression and TN disruption or improved delivery of major modulators of these features. Finally, we discuss the future of these technologies in the framework of these emerging therapeutic concepts.

Graphical abstract



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Introduction

Conventional cancer treatment is based on two premises: first, that cancer cells are a homogeneous population that displays a distinct phenotype as compared to healthy cells, and that medicines can take advantage of these differences to eliminate the disease [2]. The second premise is that the tumoral niche is a clinically advantageous feature, at least for nanomedicine-based therapies, since its enhances permeability to macromolecules and nanocarriers and promotes their accumulation in the tumor [3]. Nowadays, there is growing evidence demonstrating that these two premises are incorrect or at least incomplete.

Tumor cell heterogeneity is now a widely accepted feature of cancer and can be discussed at the genetic and developmental levels, being both of these tightly connected. At the genetic level, tumor cells present intrinsic genetic variability, which results in several cancer subclones that evolve following Darwinian processes in an attempt to adapt towards the environment. This process leads to an enrichment of cells presenting advantageous mutations and more aggressive phenotype [4]. At the developmental level, it has been confirmed that tumor initiation and relapse is driven by a selected tumor cell subpopulation that has high resistance towards conventional therapies and that takes advantage of stem cell-specific features [5,6]. Antitumorals are designed to target rapidly cycling cells such as those from the tumor bulk, but will spare the quiescent (but deadly) cancer stem cells (CSCs) that will generate tumor relapse and metastasis [2].

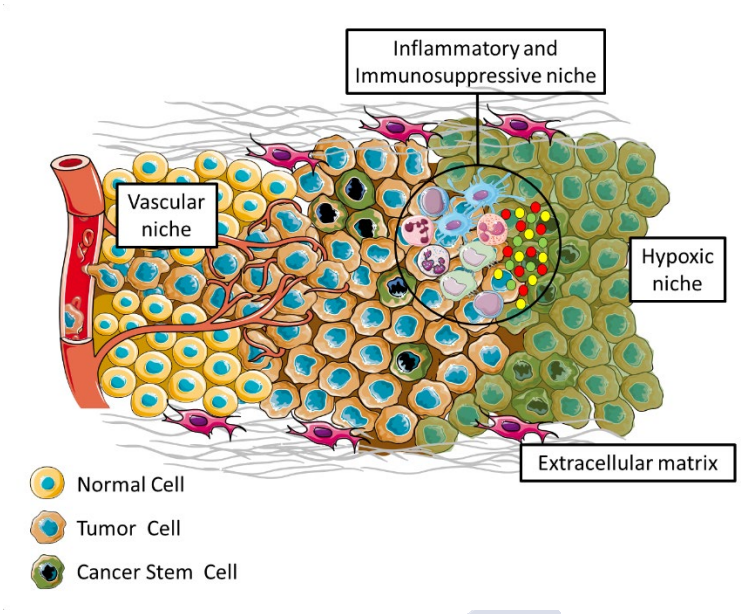

Figure 1.1. General overview of the organization of cancer stem cells (CSCs) and their tumor niche (TN). CSCs dwell in complex colonies together with differentiated cancer cells and other non-tumoral cell types. The tumoral niche presents several features that are critical for CSC physiology and relevant for the design of new therapies. These are: (i) a disorganized and hypertrophic vascular niche, (ii) a highly dynamic, remodeled extracellular matrix, (iii) the formation of hypoxic regions and (iv) the generation of an inflammatory microenvironment.

Tumor niche (TN) refers to the microenvironment that interacts with tumor cells and regulates their fate. TN has been revealed as a critical barrier for cancer treatment and it is analyzed in this manuscript through four different features: the vascular niche, the inflammatory and immunosuppresive niche, the hypoxic niche and the extracellular matrix, all of which are closely related among themselves, with the CSC phenotype (Figure 1.1). The tumor microenvironment was mostly seen as a potential advantage in the past since it enhances the permeability and retention of the nano-sized drugs in the tumor (i.e. the EPR effect). However, tumor vasculature is highly irregular and could be tight in some regions, while being leaky in others. This irregular growth of tumor vasculature also generates non-functional branches, leading to poorly irrigated regions that cannot be easily accessed with chemotherapy [7]. Besides, the accumulation of stroma in the tumor and the high intratumoral pressure also prevent drug transport to the inner regions. A recent survey of the literature has indicated that only a 0.7% of the nanocarrier dose is delivered to solid tumors [8], a result that suggests the failure of the overall concept of passive targeting as it is understood nowadays.

Besides its barrier effect to drug delivery, the tumor niche also provides important signalling, often related to the cancer stem cell phenotype, that promotes tumor spreading and protection. CSCs and their niche have been recognized as critical features of cancer progression in the last years, and are currently in the focus of intense programs for drug development. Indeed, some prototypes have been developed to the stage of clinical implementation or are in advanced clinical trials (Figure 1.2). Most of the programs, however, are still focusing on separate aspects of CSCs and the TN, and as it will be illustrated in this review, those features are tightly interconnected (Figure 1.1) and might not be effectively addressed separately.

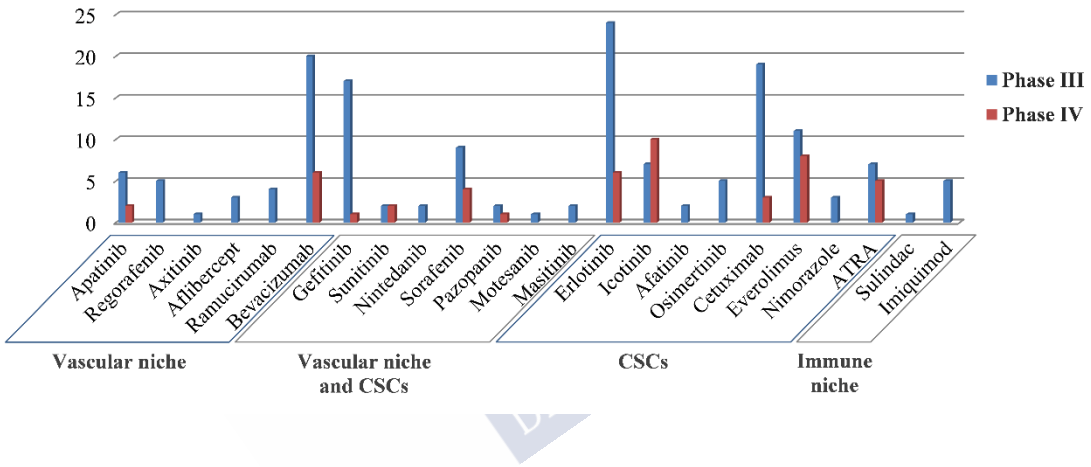

Figure 1.2. Advanced clinical trials (phase III or higher) of therapies against CSCs and/or their niche (only years 2011-2016). Further information on Supplementary Information (Table S1). Source: ClinicalTrials.gov (www.clinicaltrials.gov).

The field of biomaterials and drug delivery, mostly in tissue engineering, has focused on pulsing important cell signalling routes, particularly those related to stem cell development, and understanding and mimicking the biological substrate (the "niche"). Concretely, scaffolds and other tissue engineering devices are frequently used to: (i) induce stem differentiation [9], (ii) deliver cell-cycle modulators [10] , (iii) modulate the inflammatory niche [11], (iv) modulate tissue vasculature [12] and (v) induce extracellular matrix remodeling [13]. This spectrum of biological activity fits perfectly the requirements of a new generation of antitumorals capable of modulating CSCs and their niche.

The objective of this review is to analyze the properties and implications of the CSC phenotype and the TN, and to cover the main therapies designed to address these characteristics, focusing on the potential role of biomaterial-based technologies (i.e. implants, nanomedicines, etc.) in such therapies.

1.1 Cancer stem cells

Cancer stem cells (CSCs) have been defined as a cell subpopulation in the tumor bulk that possesses stem cell capacities. CSCs may be derived from adult stem cells or progenitor cells, but also from terminally differentiated cells that undergo epigenetic changes [6,14]. In any case, malignant cells take advantage of stem cell-specific signalling to drive tumor development.

CSCs were isolated for the first time in the 1990´s in acute myeloid leukemia and were named “tumor initiating cells” (TICs) because they were able to start by themselves a tumor. Later, CSCs were isolated in several types of solid tumors (colon, glioma, pancreatic, lung, breast etc.). The fundamental traits of CSCs can be listed as: (i) ability for self-renewal and tumor reactivation, even in the absence of growth signals; (ii) evasion of apoptosis by secreted factors; (iii) increased activity of drug efflux transporters that enhances their resistance to chemotherapy and radiation; (iv) quiescence; (v) capacity to differentiate into any cell of the tumor population; (vi) ability to migrate and metastasize to other tissues, and (vii) increased capacity for DNA repair [15,16]. From a molecular biology perspective, CSC traits are driven by the activation of specific signalling pathways (Table 1.1), many of them present also on non-pathological stem cells.

Table 1.1. Important cell signalling pathways implicated in the CSCs phenotype.

Pathway	Mechanism	Reference
Hedgedog (Hh)	Cell proliferation, differentiation, survival, self-renewal, CSCs maintenance, epithelial mesenchymal transition (EMT).	[17,18]
Wnt	Cell proliferation, differentiation, self-renewal and migration.	[19,20]
Notch	Cell proliferation, differentiation, self-renewal, communication cell-to-cell and apoptosis.	[21,22]
NF-κB	Cell proliferation, migration and apoptosis	[23,24]

The key implication of CSCs is that a reduced number of these cells have the capacity to regenerate the tumor. Therefore, any therapy that aims at successfully increasing survival needs to be effective in fully eliminating these cells, which are more resistant to conventional cytotoxic drugs. A corollary to this is that tumor reduction is only informative on the capacity of the drug to eliminate the bulk tumor cells and might not correlate with medium or long-term survival. There are, however, some drugs that treat specifically CSCs, as described in seminal works in oncology [20, 25-30]. Although some overlapping is admitted, for clarity, we classify these CSCs-specific therapies by two action mechanisms: (i) CSC differentiation and (ii) targeting CSC signalling pathways. The most studied drugs that act by these two mechanisms are presented in the following sections, together with biomaterial-based systems that have shown the capacity to enhance their activity in cancer models or at least improve their delivery profile.

1.1.1 Differentiation therapy

1.1.1.1 Retinoid derivatives

Since cancer stem cells take advantage of specific cell programs to boost their malignancy, the CSC pool can be depleted by inducing differentiation towards a mature phenotype (Figure 1.3). The use of differentiation therapies is intrinsically linked with the discovery of CSCs in hematopoietic cancers, where the most studied drugs have been retinoid derivatives. The

mechanism of action of retinoid derivatives is related to ALDH, an enzyme that oxidizes intracellular aldehydes and retinol to retinoic acid, which induces cell differentiation.

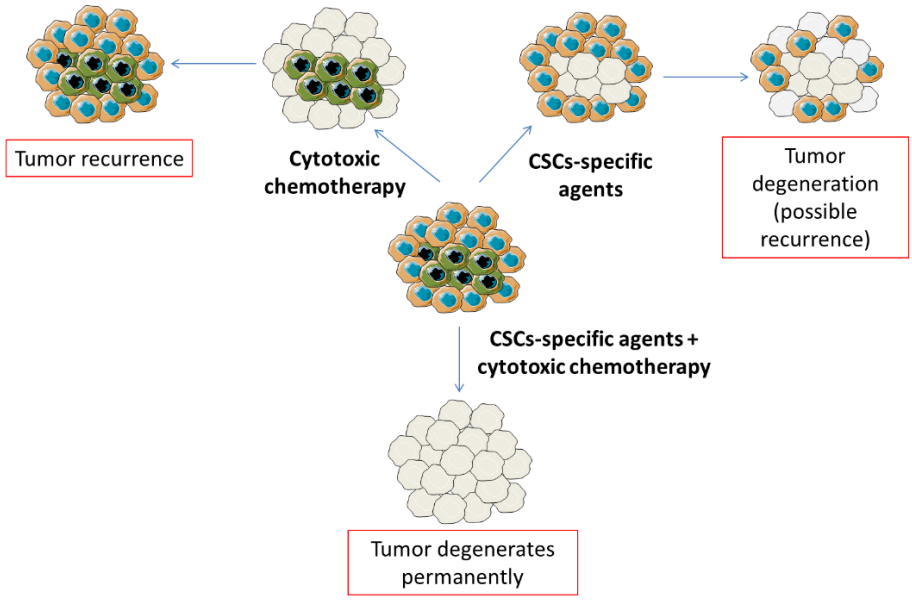

Figure 1.3. The significance of treating cancer stem cells (CSCs). The figure illustrates the outcome of conventional cytotoxic therapies that eliminate the bulk tumour (orange cells) and spare CSCs (green cells) vs. the outcome of therapies specifically directed against CSCs. Tumors depleted from CSCs might degenerate temporarily, but if some cells from the tumor bulk dedifferentiate cancer might recur. The combination of both strategies could lead to a more efficient elimination of the tumor.

Acute promyelocytic leukemia (APL) has benefited the most from treatments based on inducing cell differentiation. All-trans retinoic acid (ATRA) has dramatically turned APL therapy, and has further benefited from the introduction of another differentiation and pro-apoptotic agent, arsenic trioxide, that is given as a combined therapy with ATRA [36]. Traditionally considered a lethal disease, APL is nowadays one of the most treatable cancers, with the ATRA/arsenic trioxide combination achieving around 90% remissions. Other cancers where retinoid-based differentiation could be useful are melanoma, teratocarcinoma, squamous cell carcinoma, neuroblastomas and colon carcinoma, although the clinical efficacy of these treatments is still under investigation [37,38].

ATRA has been encapsulated in several advanced formulations to address its low aqueous solubility and improve its stability, to enable its controlled release, to reduce hematological toxicity, and to improve its pharmacokinetic profile *in vivo*. These formulations include microemulsions, nanoparticles and liposomes. Liposomal formulations have reached clinical

trials (phase I and II) for the treatment of solid tumors (clinical trial references NCT00195156, NCT00005969 and NCT00003656). A microemulsion encapsulating ATRA was developed using PEGylated-phospholipids as surfactants, providing improved stability and solubility for the drug [39]. Similarly, different polymeric nanoparticle compositions have been tested for ATRA delivery, most based on PEGylated polymers that prolong circulation time and optimize tumor targeting. These nanoparticles offer more opportunities for controlling drug release; for example poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG) nanoparticles showed controlled release of ATRA, and the pharmacokinetic profile of the formulation could be modified through changes in the polymer molecular weight, drug loading and polymer concentration [40]. Encapsulation of ATRA in nanocarriers is also beneficial to prevent the hemolysis produced by the free drug [41], and to improve the antitumoral activity *in vivo*, an effect probably associated to the improved pharmacokinetic and biodistribution profile [39,40,42,43].

Because ATRA only induces the differentiation of the CSCs, it is necessary to combine it with other drugs as it is done in the clinical setting to get total relapse of the tumor. Nanocarriers are ideal platforms to host multiple drugs, and poly-lactic acid -poly(ethylene glycol) (PLA-PEG) nanoparticles have been loaded with ATRA and doxorubicin to get a synergistic effect that targets both CSCs and the bulk tumor cells. This effect was found to be stronger than that achieved by the co-administration PLA-PEG nanoparticles encapsulating both drugs separately, and markedly better than the monotherapies formulated in nanoparticles [44].

1.2.1.2. TGF-β superfamily modulators

Other strategy to induce differentiation is based on the premise that CSCs are sensitive to developmental signalling such as bone morphogenetic proteins (BMP) and transforming growth factor-*beta* (TGF- *beta*) inhibitors. The actions of BMP-signalling are complex and cell-specific, often resulting in contradictory outcomes in different cancers [45]. However, for some tumors BMP-signalling shows marked capacity to stop tumor proliferation and to drive CSCs towards more benign phenotypes, potentially treatable by conventional chemotherapy [46]. Another member of the same signals superfamily, TGF-β, has been unveiled as an

important inductor of CSCs stemness, as a regulator of the epithelial-mesenchymal transition (EMT) and as a promoter of CSC self-renewal. Treatments with TGF-β inhibitors result in reduced CSC markers in the tumor and in cell migration [30,47].

Advanced formulations for BMPs have been designed for managing glioblastoma stem cells. The therapeutic concept here was the generation of a local reservoir for the controlled release of the CSC suppressor BMP-7, which could be implanted at the time of primary tumor resection. The controlled release properties of this formulation are essential due to the physiological half-life of BMP-7 that is limited to a few minutes. A poly(lactic-co-glycolic acid) (PLGA) microsphere formulation was optimized towards this aim, where BMP-7 was encapsulated in the form of a nanocomplex with heparin and poloxamine. The formulation achieved over 90% encapsulation, minimal burst and sustained BMP-7 release for over two months in bioactive form [48]. Further *in vivo* studies using human primary glioblastoma stem cell lines confirmed that the implantation of this formulation was able to activate the BMP-canonical pathway in the tumor for over two months, and that this activation results in reduced tumor development and downregulation of malignancy markers related to the CSC phenotype (Figure 1.4) [49].

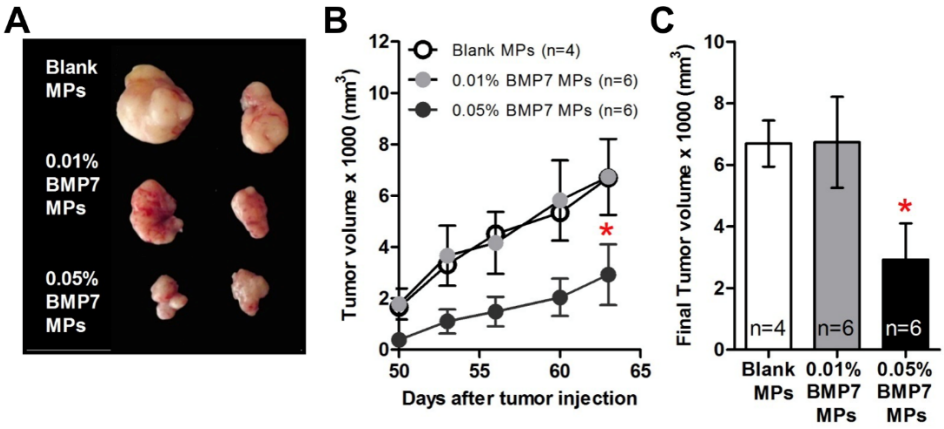

Figure 1.4. BMP-7 microspheres decreased tumor growth in a primary human glioblastoma stem cell xenograft model. Three groups were compared, blank microspheres (Blank MPs), and microspheres loaded either with 0.01% or 0.05% (w/w) BMP-7 (0.01% BMP7 MPs and 0.05% BMP7 MPs, respectively). (A) Representative picture of the tumors on the last day of the experiment. (B) Tumor volume measured at different times. (C) Final tumor volume measurements. *Statistical analysis at $p < 0.05$. Reproduced from [49] under a Creative Commons Attribution License.

1.2.2 Targeting CSC signalling pathways

In general, CSCs are considered as hyper-resistant to chemotherapy. However, due to their intrinsic signalling pathways, some molecules might have specific effects on CSCs. These molecules generally have broad effects in cell function, but most converge towards an apoptotic effect in the CSCs. We will cover here the most relevant examples of these drugs, which include Hedgehog, Wnt, NF- kB and PI3K/Akt/mTOR inhibitors.

1.2.2.1 Hedgehog pathway inhibitors

The Hedgehog pathway inhibitor HPI-1 has shown promising activity for suppressing CSCs [50], but its clinical use is limited by low aqueous solubility and bioavailability. To overcome these problems, HPI-1 has been encapsulated in PLGA-PEG nanoparticles (NanoHHI) [51]. NanoHHI showed 3 to 4-fold higher oral and intraperitoneal bioavailability than the same drug in a conventional formulation (i.e. parent compound). NanoHHI showed marked suppression of tumor growth *in vivo* and the attenuation of metastasis. This advanced formulation showed a remarkable reduction of the CD133+ cell subpopulation, which is identified as the CSC pool [52]. Besides, NanoHHI has also been combined with gemcitabine, and this association was able to inhibit tumor growth without systemic toxicity in a pancreatic xenograft model [51].

1.2.2.2 Wnt inhibitors

Wnt signalling regulates the proliferation, differentiation and survival of cancer cells, and can be divided into two pathways: canonical and non-canonical. The canonical pathway, also known as WNT-β-catenin pathway, is correlated with poor prognosis in cancer, and its inhibition reduces tumor progression through the downregulation of cell cycle proteins cyclin D1, c-Myc and c-jun [53,54]. Inhibitors of the Wnt/β-catenin signalling pathway include antioxidants (quercetin, resveratrol, curcumin and EGCg), anti-inflammatories (tetrandine, Sulindac) and other synthetic compounds (PRI-724, OMP-18R5), some of them currently in clinical trials [54,55].

Quercetin, resveratrol and tetrandrine are three validated Wnt inhibitors that share some biopharmaceutical and therapeutic limitations: low aqueous solubility, important toxicity and low oral bioavailability. Because of that, nanoformulations have been proposed with two aims: (i) to improve their biodistribution following i.v. administration and (ii) to improve their oral bioavailability.

PEGylated nanocarriers have been the most used systems to improve the biodistribution of these molecules, although other carriers such as magnetic nanoparticles have also been investigated [56]. Among PEGylated nanocarriers, the most investigated formulations have been liposomes and nanoparticles based on biodegradable polyesters. PEGylated liposomes have been used to encapsulate quercetin and resveratrol, and these formulations have shown better pharmacokinetics, higher tumor accumulation, improved antitumoral effect and reduced toxicity in healthy tissues [57,58]. In order to get a synergistic cytotoxic effect, a second drug can be loaded in the liposomes. The combination of quercetin and vincristine produces higher effect than the monotherapy with either drug alone, and requires lower drug doses and has lower systemic toxicity [59,60]. Resveratrol and curcumin were included in a liposomal formulation targeted with an antibody against HER-2, and the system showed remarkable efficacy while decreasing the toxicity of the drugs [61].

Other important nanoformulations used to improve the biodistribution of Wnt inhibitors are core-shell type nanoparticles that have been used to deliver resveratrol and tetrandrine. Studies of resveratrol encapsulated in mPEG-PCL or PCL-PLGA-PEG nanoparticles and studies

of tetrandrine encapsulated in polyvinylpyrrolidone-block-poly(ε-caprolactone) (PVP-b-PCL) nanoparticles indicate that these nanoformulations improve the intracellular transport of these drugs and increase their therapeutic efficacy [62-65]. *In vivo* studies, performed in a ectopic human ovarian tumor xenograft, with resveratrol–bovine serum albumin nanoparticles have shown higher accumulation of the drug in tumor, liver and kidney, and an increased pro-apoptotic effect as compared with the reference formulation [66]. Nanoparticles can also be used to deliver drug combinations such as tetradrine with paclitaxel loaded in mPEG-PCL nanoparticles. This formulation enhanced reactive oxygen species (ROS) accumulation in the tumor and produced a higher pro-apoptotic effect *in vitro* [67].

Another objective of nanoformulations is improving oral bioavailability. A nanomicellar formulation of quercetin based on 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol (DSPE-PEG) has been developed, and it showed improved drug solubilization, protection of the drug from intestinal enzymes and enhanced drug permeability through the intestinal barrier. An *in vivo* assay in a lung tumor xenograft mouse model showed significantly increased anti-tumor efficacy for quercetin nanomicelles compared to quercetin suspensions when administered orally [68].

1.2.2.3 NF-kB inhibitors

NF-kB signalling regulates the apoptosis, adhesion and migration of cancer cells, through a balance in the expression of pro-apoptotic proteins (FLICE-like inhibitory protein) and inhibitors of apoptosis (anti-apoptotic Bcl-2 family). Moreover, it also participates in the immune response against the tumor [23,69]. Some inhibitors of this pathway are curcumin and disulfiram [23,70] and both have been formulated in nanocarriers, but with different objectives.

Curcumin is an inhibitor of NF-kB, but also transduces its effect through other networks such as Wnt, covered before. Curcumin has a plethora of interesting properties for cancer since it is pro-apoptotic, antiangiogenic, anti-inflammatory, immunomodulatory and antimitogenic. Curcumin is also poorly soluble in water and this conditions its oral bioavailability, a limitation that has been addressed by its incorporation into mucoadhesive systems, concretely polyacrylic acid-based nanoparticles [71] and stearic acid-g-chitosan

micelles [72]. These systems have shown the capacity to improve curcumin solubilization and bioavailability. Importantly, they are also able to improve the pro-apoptotic, antitumoral effect of the drug following oral administration while reducing systemic side effects. *In vivo* studies also confirmed a specific effect on the CSC subpopulation [72].

In the case of disulfiram, its effects are dependent on the presence of copper in the medium [73], and for this reason, their co-encapsulation is advantageous. A liposomal formulation of disulfiram and copper in liposomes has been developed and has shown extended plasma half-life as compared to the free drug. The liposomal disulfiram/copper formulation also had antitumoral effect in mice breast cancer xenografts and minor systemic toxicity *in vivo* [74].

1.2.2.4 PI3K/Akt/mTOR inhibitors

The phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) network is an essential pathway for cell proliferation, differentiation and survival, and is considered critical for CSC function [75]. Rapamycin is the classical inhibitor of this pathway, although currently there are several new and more specific compounds in clinical trials [76]. Rapamycin has low water solubility, low specificity for tumor cells and important side effects. For these reasons, several nanoformulations for this drug have been tested, mostly based on PEGylated polyester nanoparticles and liposomes.

PEGylated polyester micelles based on PLA or PCL have been developed for rapamycin delivery. In general, rapamycin in PEGylated micelles has better solubility, intracellular uptake and anti-proliferative effect [77]. Rapamycin delivered in PEGylated micelles also enhances the efficacy of concomitant radiotherapy [78] and paclitaxel administration [79,80]. *In vivo*, PEG-PCL micelles have demonstrated higher accumulation ratio of both drugs in the tumor compared to the accumulation in liver and spleen. Rapamycin and paclitaxel encapsulated in PEG-PCL micelles can suppress tumor growth completely in a breast cancer murine model by acting specifically through the mTOR pathway [81].

Rapamycin has been encapsulated in PEGylated liposomes in combination with the cytotoxic drug paclitaxel. This formulation was more effective *in vitro* than any or the combination of the free drugs. *In vivo* results confirmed that the nanoformulation was effective in controlling tumor growth in a breast cancer murine model [82].

Another interesting drug association is that of rapamycin and perifosine, integrated in albumin-bound nanoparticles. An *in vivo* study in a multiple myeloma murine model indicated mutual suppression of the PI3K/Akt/mTOR pathway by rapamycin and perifosine, inducing synergistic tumor cell elimination in multiple myeloma xenograft mouse model [83]. Rapamycin biodistribution can also be improved through the active targeting of the nanocarriers. PLGA nanoparticles targeted with a EGFR-antibody on their surface showed better cellular uptake and a superior anti-proliferative activity compared to the free drug or to non-conjugated nanoparticles [84].

1.2 Tumoral niche

1.2.1 Aberrant Vasculature

The formation of abnormal blood and lymphatic vessels is one of the critical hallmarks of solid tumors, which is driven by the secretion of high concentrations of angiogenic factors by the tumor cells. On one hand, the role of this abnormal vasculature is the nutrition and oxygenation of the tumor, but it also has a regulatory role in the secretion of growth factors and cytokines that spur tumor growth. Tumor-induced angiogenesis results in different vessels from those in normal vasculature as they are more tortuous, irregular and highly permeable, even to large proteins [85]. The irregularity of the vessels generates sometimes non-functional branches [86], while in other cases, the interstitial pressure strangles functional vascular branches. This leads to areas with poor irrigation within the tumors that explains the resistance to radio- and chemotherapy [7]. Besides, these low perfusion areas result in hypoxic microenvironments that will be the subject of a further section in this manuscript (section 1.3.3). There are several molecules implicated in the regulation of angiogenesis. The most prominent are: the vascular endothelial growth factor family (VEGF), pigment epithelium-derived factor (PEDF) and basic fibroblast growth factor (b-FGF) [87].

Targeting the vascular niche is advantageous because: (i) tumor endothelial cells are homogenous across different tumors, and they are less prone to genetic instability than cancer cells, and (ii) the tumor vascular endothelium is accessible to systemically administered drugs and thus, does not require drug transport through the tumor tissue. Because of these advantages, it is in principle more straightforward to design biological-based therapies (e.g.

monoclonal antibodies) towards these targets, and reduce the cytotoxic side effects associated to conventional chemotherapy that is directed against the tumor [88].

Traditionally, antiangiogenic therapies were aimed at completely destroying the tumoral vascular niche to deprive tumors from the oxygen and nutrients required for its growth. In the last decade, this concept has coexisted with that of "vascular normalization", where the active agents ''normalize'' the abnormal vasculature to make it more efficient for oxygen and drug delivery, avoiding drug resistance and the formation of the hypoxic niche [7] (Figure 1.5). Even though these concepts have different implications in overall therapy, they largely use the same active compounds and both mediate their effect by sabotaging the vascular niche. Therefore, within the particular scope of this review, we do not consider further this distinction in antiangiogenic therapies.

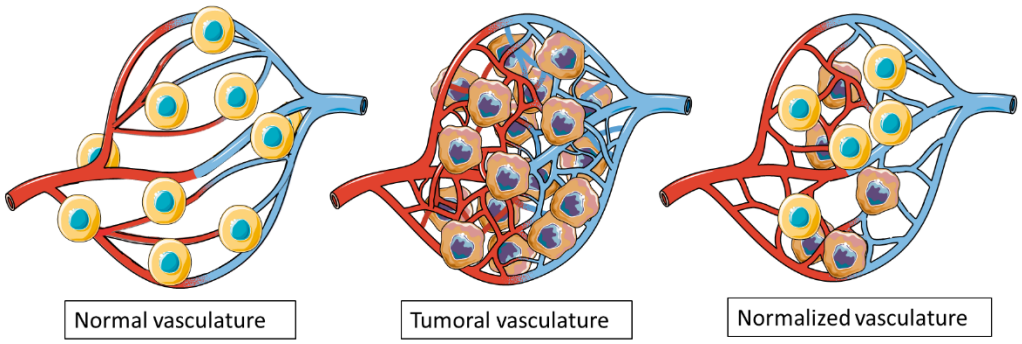

Figure 1.5. The tumor vascular niche. Compared to physiologically organized vascular branches (left), tumor vasculature is chaotic and hyperthrophic (middle). This lack of organization leads to non-homogeneous oxygen supply, and the formation of hypoxic regions. Anti-angiogenic therapies can eliminate tumor vasculature, but in excess, they can promote hypoxia and the acquisition of a CSC phenotype. At controlled doses, these compounds can "normalize" tumor vasculature (right), resetting physiological organization, improving drug delivery and destroying the nurturing features of the perivascular niche for CSCs.

Targeting the vascular niche can be achieved through two possible strategies: (i) interfering with pro-angiogenic molecules, their receptors or downstream signalling, or (ii) upregulating or releasing endogenous inhibitors. The conventional therapy used in the clinic for this purpose is based on anti-VEGF monoclonal antibodies (bevacizumab), tyrosine kinase inhibitors such as imatinib, sorafenib, sunitinib, or combinations of them [89]. Some of these molecules have already reached extended clinical use that is expected to expand even further

as the results from many clinical trials validate their potential in many other indications (Figure 1.2).

Biomaterial-based therapies against the vascular niche have also been investigated based on the same two strategies outlined above. As compared to other therapies, these systems try to provide added value fundamentally through their improved targeting. As mentioned before, molecular targets have a particularly high exposure in the tumor endothelium, which makes actively targeted nanocarriers especially useful. Most drugs used for modulating tumor vasculature are non-selective by nature, and therefore, could benefit from encapsulation in an actively-targeted nanocarrier that increases the local concentration of the antiangiogenic agent, prolongs its release and decreases drug concentration at off-target sites.

Even integrating active compounds that could be considered targeted such as antibodies, nanocarriers can provide some distinct advantages derived from the fact that many ligands increase cooperatively or synergistically their affinity when binding at several sites or by several receptors ("clustering"). This effect has spurred the interest on investigating multivalent carriers where ligand spacing is optimized for ligand-receptor interaction [90]. In summary, actively-targeted nanocarriers could have the main benefit of concentrating both specific ligands and a high drug payload in distinct carriers.

Another region of interest in the solid tumor vascular system are lymphatic vessels, whose defective function contribute to the enhanced tumor interstitial pressure [91], but also constitutes a possible route of metastatic cell spreading [92,93]. To maximize lymphatic drainage of drug nanocarriers after subcutaneous administration, it is important to optimize their physicochemical properties, which should comprise a highly passivated surface and small particle size [94]. Moreover, Abellán-Pose et al. have studied the biodistribution of poly-glutamic acid-poly(ethylene glycol) (PGA-PEG) nanocapsules loaded with docetaxel upon i.v. administration in a mice model, and observed that maximum extravasation to the lymphatic vessel was obtained with particles below 150 nm [95]. This prototype has been administered in a metastatic lung cancer murine model and has shown higher effect than the commercial formulation Taxotere® both in the primary tumor and in the lymphatic metastasis [96].

Nanocarriers can also be actively targeted to the lymphatics by conjugation with VEGFR-3, podoplanin, and the hyaluronan receptor LYVE-1 [92,93].

1.3.1.1 Nanocarriers that interfere with pro-angiogenic signalling

VEGF is the main factor that promotes angiogenesis, thus blocking this signalling pathway is an attractive strategy to modulate tumor vasculature. Gold and silver nanoparticles have intrinsic antiangiogenic properties because of their interaction with endothelial heparin-binding glycoproteins associated to the VEGF receptor that inhibits their activity [97-98]. Moreover, gold nanoparticles bind vascular permeability factor and b-FGF, which are two other angiogenic mediators. These mechanisms result in inhibited endothelial and fibroblast proliferation [98]. Other metallic nanoparticles with antiangiogenic properties are silver and cerium oxide nanoparticles. These nanoparticles have the capacity to modify ROS intracellular levels, inhibiting the PI3K/Akt/mTOR pathway. The inhibition of this pathway reduces the production of pro-angiogenic growth factors, including VEGF and FGF, ultimately reducing cell invasion and migration [97,99,100].

Gene therapy is another option for the design of antiangiogenic therapies, and several groups have designed nanocarriers for silencing the gene expression of VEGF. For instance, He et al. designed an efficient calcium carbonate nanocarrier for abrogating VEGF expression in colon cancer [101,102]. In another study, a siRNA against a VEGF receptor was included in alginate-modified polyethyleneimine (PEI) nanoparticles. These nanoparticles were designed to profit from the complexing and endosomal escape properties of PEI, but improving the efficacy/toxicity ratio of carriers through mixing with alginate [103]. Sakurai et al. have also designed liposomes to silence VEGFR2, composed by a pH sensitive cationic lipid, known as YSK05, and conjugated with an integrin ligand αvβ3 (cRGD). *In vivo* assays showed a selective action in tumor endothelial cells, but no effect in normal endothelial cells. This formulation also produced a reduction in the tumor volume in a renal cell carcinoma mouse model [104].

In recent decades, the antiangiogenic effect of glucocorticoids (GCs) has also been described. GCs are implicated in transcriptional responses to the majority of inflammatory, angiogenic, immunomodulatory and apoptotic genes, either by binding directly to DNA or to transcription factors involved in gene regulation. The antiangiogenic effect of GCs is mainly

achieved by downregulating the secretion of pro-angiogenic factors. To reduce the toxicity of GCs, these molecules have been encapsulated in PEGylated-liposomes. Concretely, four different GCs were encapsulated in this system: budesonide, dexamethasone, methylprednisolone, and prednisolone. Liposomes efficiently delivered the GCs to the tumor, reduced the system side effects, and prolonged tumor growth inhibition. Budesonide encapsulated in liposomes resulted the most potent regarding their antiangiogenic effects. Liposome PEGylation was critical to realize this prolonged drug circulation and to achieve enhanced drug extravasation to the tumor in a melanoma murine model [105,106].

1.3.1.2 Nanocarriers that enhance antiangiogenic signalling

Pigment epithelium-derived factor (PEDF) is a protein that has antiangiogenic, anti-inflammatory, antitumoral and antioxidant effects [107-109]. Therefore, several works in the literature have attempted to deliver this protein, frequently in the form of gene therapy. Dass et al. described the first delivery system for a cancer therapy based on a PEDF, which was based on chitosan microparticles loaded with a PEDF-encoding plasmid. Animals treated with this therapy showed a reduction in bone lysis and in tumor progression, as observed in an osteosarcoma mouse model. Moreover, it also showed a reduction in the number of lung metastases, probably due to reduced invasiveness of the cells [110]. After this first report, other nanocarriers were also developed from PLGA, PEI and their PEGylated analogues [111-113]. Targeting moieties can also be added on these nanoparticles to improve selective accumulation at the tumor site. For example, PEG–PEI nanoparticles conjugated to the integrin ligand αvβ3 (cRGD) showed enhanced transfection efficiency in comparison with non-targeted PEG–PEI nanoparticles, despite of a worse capacity to complex DNA [112].

A combined therapy based on paclitaxel and PEDF-encoding plasmid, both loaded in PEG−PLGA nanoparticles was described by Xu et al. *In vivo* experiments indicated a superior anticancer effect for nanoparticles containing the combined therapy as compared to nanoparticles loaded with the drugs separately. Specifically, the combined therapy reduced tumor weight and improved survival in a colon adenocarcinoma mouse model. This positive effect was accompanied by a clear reduction on the tumor microvessel density [112,113].

Another molecule with antiangiogenic activity is TNP-470, a fumagillin analogue that is considered to be one of the most potent and broad-spectrum angiogenesis inhibitors [114,115]. TNP-470 has been conjugated to a N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer to prolong its plasma half-life after intravenous administration and to improve its biodistribution by passive targeting mechanisms. Indeed, conjugation to HPMA prevents drug transport across the blood brain barrier (BBB), reducing the neurotoxicity typical of the free drug in glioblastoma, melanoma, pancreatic adenocarcinoma and breast cancer mouse models [114-116]. TNP-470 has also been encapsulated in mPEG-PLA micelles to improve its solubility and to enhance its intestinal absorption. An *in vivo* study in a murine melanoma model showed that the micellar formulation achieved good oral bioavailability and sustained plasma levels of the drug [115].

1.3.2 Aberrant Extracellular Matrix

The extracellular matrix (ECM) is responsible for maintaining the architecture and homeostasis in normal tissue and it serves as support for tissue specific cells, immune cells, capillaries and fibroblasts [117]. It is composed by proteins (i.e. collagen, elastin and fibronectin) and polysaccharides such as hyaluronic acid and proteoglycans (i.e. perlecan, agrin, syndecans and glypicans) [118,119]. Cells bind to the ECM through functional structures, integrins, and the mechanical forces transduced can have important signalling roles [120]. The ECM also has polysulfated regions to bind growth factors and other signalling proteins, and acts as reservoir of these molecules whose release is triggered by ECM degradation [121]. Globally, both growth factor binding, release and cell-ECM interactions have critical roles in cancer development. In cancer, the ECM is deregulated, disorganized and enriched in pro-tumoral molecules (Figure 1.6) [122,123]. From a therapeutic standpoint, the ECM in the CSC niche raises two issues. First, ECM components in conjunction with enhanced intratumoral pressure hinder drug movement and protect CSCs from chemotherapy. The second issue is that the highly dynamic ECM environment promotes tumor development through increased signalling and could favor cancer cell migration [119,123]. Biomaterial-based technologies to modulate both of these properties are currently under investigation.

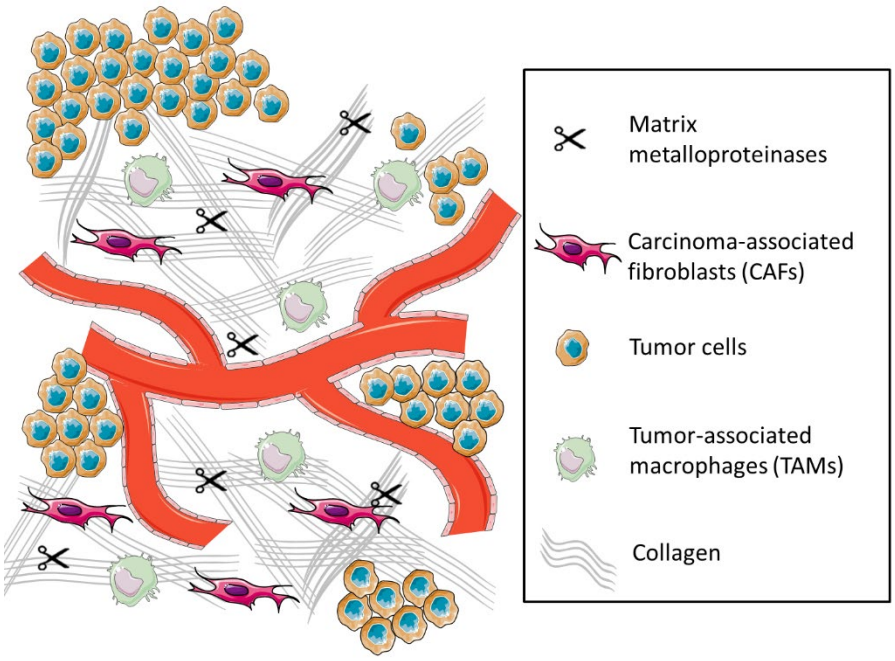

Figure 1.6. Tumoral extracellular matrix (ECM) has distinct features that contribute to tumor development. These include disorganized collagen fibers and high concentrations of ECM-degrading enzymes (metalloproteinases) that trigger the release of growth factors from cryptic ECM-sites. The ECM hosts other non-tumoral cells such as tumor-associated macrophages (TAMs) and carcinoma-associated fibroblasts (CAFs) that contribute to the properties of the TN.

1.3.2.1 Biomaterials that modulate ECM permeability

Collagen IV, one of the principal constitutes of ECM, interacts anomalously with proteoglycans in cancer. Proteoglycan concentration is also higher in tumor than in normal tissue, and this feature is directly correlated with tumor aggressiveness [124]. These characteristics result in a compact ECM that constitutes a physical barrier for drug transport, and particularly for macromolecules [125]. For regional delivery routes, the hindrance to drug transport can be reduced by co-administration of ECM degrading enzymes (e.g. collagenase or hyaluronidase). Unfortunately, this strategy cannot be easily implemented for intravenous administration, since the enzymes would be distributed throughout the body. Alternatively, these enzymes could be integrated onto the surface of nanoparticles. As a proof-of-concept, Goodman et al. have reported the bioconjugation of collagenase on polystyrene nanoparticles. The 100 nm polystyrene nanoparticles with collagenase showed four times higher transport through the ECM than 100 nm albumin-coated nanoparticles *in vitro*, in a

multicellular spheroid culture of human cervical carcinoma [126]. The concept is pending of *in vivo* validation and of implementation in more pharmaceutically acceptable materials.

Lysyl oxidase (LOX) is an enzyme that increases collagen cross-linking, thereby enhancing ECM rigidity. It is overexpressed in cancer [127], and therefore, its inhibition is a potential strategy to enhance the ECM-penetration. LOX inhibiting antibodies have been conjugated onto PLA-PEG nanoparticles. *In vivo* studies, in an orthotopic breast cancer mouse model, revealed that antibody-nanoparticle conjugates have a higher enzyme inhibitory effect and lower toxicity than the free antibody. In addition, the tumor treated with the antibody-nanoparticle conjugates exhibited lower stiffness than those treated with the free antibody. The enhancement in efficacy observed for the nanoparticle conjugate allowed a 15-fold reduction of the administered dose, and is likely a consequence of ligand clustering effect [128].

1.3.2.2 Biomaterial-based devices to suppress ECM-mediated protumoral signals

Matrix metalloproteinases are enzymes that degrade the ECM, and are involved in cell proliferation, migration, and tumor development. These enzymes need the presence of Cu or Zn ions to preserve their catalytic properties. Treatments to prevent tumoral ECM remodelling are based on matrix metalloprotease inhibitors such as chelating agents capable of sequestering the metal ions from the enzymes active-sites. Some investigated metalloprotease inhibitors include β-aminopropionitrile [122], marimastat [129,130] and batimastat [131]. Another example is doxycycline, an antibiotic that non-selectively inhibits metalloproteinases by an unknown mechanism. Administration of these agents is limited by significant side effects, because metalloproteases are also mediators of platelet and endothelial function, and participate in other processes such as scarring, inflammation and atherosclerosis. Considering the severe side effects of metalloproteinase inhibitors, the benefits of integrating these agents in selective delivery systems is clear. For instance, liposomes and Eudragit-based nanoparticles encapsulating doxycycline showed no toxicity, but were able to induce clear metalloprotease inhibition *in vitro* [132].

1.3.3 Hypoxic foci

While tumors are characterized by extensive vascularization, the presence of aberrant, non-functional vascular branches and poor blood flow derived from the high intratumoral pressure often generate hypoxic foci inside the tumor (Figure 1.7). This feature is important because presence of tumor hypoxia is linked to poor clinical prognosis [133]. Indeed, several studies have shown that activation of hypoxia-related intracellular signalling results in higher expression of efflux pumps, reduced pro-apoptotic signalling, and higher tendency towards quiescence [134]. Tumor cells in hypoxic regions are more resistant to radiotherapy since the generation of ROS that mediates its effect is hindered due to the reductive environment [135,136]. Tumor cells under hypoxia are also more aggressive and have higher tendency for metastasis. All these unfavourable features are related to the acquisition of an undifferentiated, stem cell-like phenotype with a gene expression pattern that is a direct target of the intracellular transduction signals of hypoxia [137].

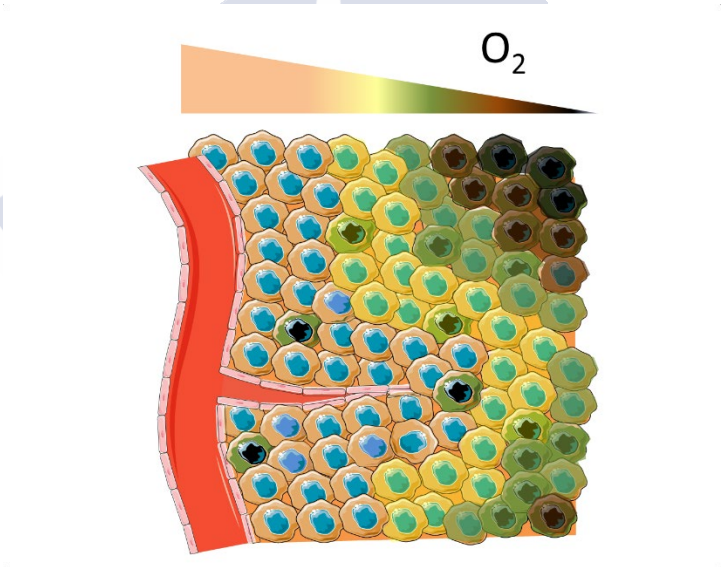

Figure 1.7. The hypoxic niche. Irregular or non-functional branches generate regions characterized by low oxygen tension (hypoxia) that promote a CSCs phenotype. CSCs are also hosted in the perivascular space but are mainly enriched in these hypoxic foci with restrictive therapeutic access.

The cellular response to hypoxia is mediated by hypoxia inducible factors (HIFs). There are two types of HIFs, HIF-1 and HIF-2, and they are composed by 2 subunits: α (catalytic subunit) and β (constitutively expressed subunit). The function of HIF-1α is to regulate the cellular adaptation to low oxygen levels, including the survival of tumor cells under hypoxic conditions. HIF-1α activates the transcription of genes involved in angiogenesis, glycolytic

metabolism, reduced oxygen consumption, cell migration, and tumor cell invasion [138]. Under normoxic conditions HIF-1α is hydroxylated and interacts with the tumor suppressor Von Hippel–Lindau (VHL) protein, which produces the degradation of the factor inside the proteasome. When cells are in hypoxic conditions, non-hydroxylated HIF-1α translocates to the nucleus where it activates the transcription of numerous target genes [139,140]. HIF-2α participates in the regulation of stem cell self-renewal and multipotency, but it is expressed only in some specific cell types [135]. HIF-2α acts through Oct4, the key transcription factor regulating cell "stemness" [141,142].

Two major therapeutic approaches are being investigated to counteract hypoxic effects. From a clinical standpoint, the more advanced option is the use of vascular normalization agents (see Section 1.3.1), which are supposed to improve perfusion homogeneity under controlled conditions. Vascular normalization has demonstrated to improve vessel lining and its maturation, resulting in enhanced tumor perfusion and oxygenation, together with inhibited tumor cell invasion, intravasation, and metastasis. Furthermore, the good oxygenation of the tumor improves its sensitivity to the chemotherapy and radiation [143].

The second strategy relies on the selective inhibition of HIFs. Since these are intracellular proteins, interfering RNA strategies based on siRNAs or miRNAs have been the most frequently investigated[136,144]. The major problem of RNA interference is the delivery of the therapeutic sequence to the cell cytosol, and due to this reason, nanocarriers have a major role in these therapeutic strategies [145]. Liu et al. designed cationic micellar nanocarriers composed by a combination of amino-phosphate poly(ε-caprolactone)-block-poly(2-aminoethylethylene phosphate) (PCL-b-PPEEA) and poly(ε-caprolactone)-block-poly(ethylene glycol) (PCL-b-PEG), and used these materials to complex siRNA against HIF-1α. *In vitro* studies showed that these micellar systems inhibit HIF-1α, and by this mechanism, they reduce the secretion of proangiogenic factors, tumor growth and cell migration. A synergic antitumoral effect was observed *in vivo*, when these micelles were administered in combination with doxorubicin in a prostate tumor xenograft model [140,146]. SiRNA targeting HIF-1α has also been formulated in a new biodegradable copolymer, D-α-tocopheryl polyethylene glycol 1000 succinate-b-poly (ε-caprolactone-ran-glycolide). *In vivo* experiments in a xenograft nasopharyngeal carcinoma murine model confirmed that the nanoparticles did not produce a

cytotoxic effect per se, but were able to decrease 2-fold the expression of HIF-1α in 24 hours. This reduction in HIF-1α resulted in a proportional reduction in tumor volume [147].

1.3.4 Inflammatory and immunosuppressive environment

The tumor microenvironment contains heterogeneous non-tumoral cell populations that include both innate and adaptive immune cells [148]. These immune cells have an important role in tumor development since they produce growth factors, cytokines, chemokines, prostaglandins and ROS [149]. Tumor-promoting agents, including CXCR4/SDF1α signalling and Gremlin-1-expressing mesenchymal stem cells (MSCs) that stimulate the recruitment of cells implicated in inflammation. Among these, immature myeloid cells and carcinoma-associated fibroblasts positive for α-smooth-muscle-actin are of special interest [150]. Tumor associated-macrophages (TAMs) are also present in the tumoral niche during all the stages of tumor progression. Through the secretion of growth factors, chemokines, interleukins, enzymes and other mediators, these cells enhance the proliferation and invasion of cancer cells, promote angiogenesis, and trigger immunosuppressive effects that prevent the attack of natural killer and T-cells [151].

The tumor microenvironment is particularly rich in pro-inflammatory molecules, mainly cytokines. Cytokine receptors are present both bound to the cell membrane and in soluble form. They are transduced through G-proteins and regulate cell behaviour including chemotaxis, growth, differentiation, and immune stimulation or immune suppression. Depending on the type of cytokine, they can promote or inhibit cancer progression and metastasis [152]. Chemokines are group of over 50 chemotactic cytokines with only 18 chemokine receptors, implying overlapping in ligand-receptor specificity. For cancer, the most important is CXCR4, a receptor with low expression in most healthy tissues, but overexpressed in many highly metastasizing tumors [153,154]. The interaction of the CXCR4 ligand (CXCL-12) produces the activation of migratory, proliferative and survival signalling pathways [15]. Preclinical studies in several murine cancer models have shown that blocking these receptors reduces metastasis and tumor invasion [155,156].

Other important cytokines present in the tumor niche are interleukins (IL) [152], tumor necrosis factor-α (TNF-α) and interferons (IFNs) [148,157]. These proteins have very diverse

functionalities that result in pro- or antitumoral effects, depending on the specific cytokine and the concomitant signalling activated.

Disconnecting the tumor immunosuppressive environment is a critical step to rescue immune responses against tumors, and is now considered a major strategy to fight cancer including metastasis. The main drug delivery strategies to reactivate immunity in the tumor are described below and are classified in: (i) biomaterials as "danger signals", (ii) cytokine delivery systems, (iii) biomaterials that inhibit chemotaxis and (iv) integrative systems.

1.3.4.1 Biomaterials as "danger signals"

The integration of "danger signals" in biomaterials, i.e. toll-like receptor (TLR) ligands, is a method to stimulate the immune system. The presentation of such ligands can revert TAM polarization to an immunostimulatory phenotype and provide effective antitumoral cytotoxic responses through CD8+ T-cell activation. The most used "danger signal" is lipopolysaccharide (LPS) but others such as PolyI:C or CpG are also widely reported [158,159].

PLGA and polyurethan-urea nanoparticles have been used to integrate TLR ligands tyrosine related protein 2 (TRP2), 7-acyl lipid A or LPS. The administration of these formulations *in vivo*, in a melanoma mouse model, resulted in antitumoral immune responses with recruitment of dendritic cells and increased secretion of pro-inflammatory cytokines [160-161]. To produce a more localized immunostimulation with reduced systemic side effects, these particles can be targeted with antibodies against adhesion molecules present in inflamed endothelial cells (VCAM-1 and ICAM-1) [162]. If a potent, direct antitumoral effect is desired, a drug such as paclitaxel can be co-encapsulated with a LPS to obtain a synergistic effect. *In vivo* studies in a melanoma mouse model showed that treatment with nanoparticles with this combined therapy achieved a 40% reduction in tumor size compared to mice treated only with Taxol®. A clear improvement in animal survival was also observed [163].

Cationic polymers such as cationic dextran and PEI are also capable of interacting with TLRs and shifting TAMs polarization, resulting in higher IL-12 expression. This effect reduces angiogenesis, produces immunoactivation, inhibits tumor progression, and ultimately, increases survival in murine cancer models [164].

1.3.4.2 Cytokine delivery systems

Cytokines have very short half-lifes *in vivo*, and therefore, it is critical to provide sustained levels to achieve a therapeutic effect. The two major ways to achieve these sustained levels are to use controlled release devices and/or to use gene therapies encoding these cytokines.

IL-12 is a cytokine that can produce tumor regression by enhancing natural killer and cytotoxic T-lymphocyte activity. Due to this potent activity, IL-12 has been encapsulated in controlled release systems. Liu et al. designed implantable biodegradable gelatine hydrogels for subcutaneous delivery. This formulation showed controlled release of IL-12 over 12 days and efficient suppression of colon carcinoma growth in mice [165]. Chitosan and cholesterol-bearing pullulan nanoparticles have also been used for the controlled release of IL-12. These formulations showed low toxicity, capacity to induce antitumoral immunity, and the capacity to target metastasis in colon carcinoma murine model [166,167].

IL-12 delivery is also interesting for combination therapy. For example, PLA microspheres loaded with IL-12, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) were used as antitumoral therapy in a murine breast cancer model. The microspheres were administered by intratumoral injection, where they initiated a major infiltration of polymorphonuclear cells and CD8+ cytotoxic T-cells that resulted in increased the number of tumor free mice at the end of the study. This therapy also resulted in specific memory T-cells that could prevent tumor relapse in a murine model of breast cancer [168].

In another combined therapy case, Park el al. designed a liposome-type carrier loaded with IL-2 and the TGF-β antagonist SB505124. The concept was to combine IL-2 immunostimulation and SB505124 blockage of TGF-β mediated immunosuppression. These carriers were based on an innovative concept where both drugs were encapsulated in a photopolymerized nanogel, and this was subsequently coated by a PEGylated lipid bilayer. The liposome-type formulation was able to increase the cytokines´ half-life in circulation, to induce innate immunity and to inhibit tumor growth in melanoma-bearing mice [169].

Nanoparticles are also frequently used for gene therapies aimed at inducing local cytokine expression due to their capacity to stabilize plasmids and improve their intracellular delivery. Examples of polymeric biomaterials used to improve the transfection of IL-12 encoding

plasmids are the amphiphilic block copolymers poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) [170] and poly(N-methyldietheneamine sebacate) (PMDS) [171]. The general idea was validated by Maheshwari et al. who encapsulated IL-12 coding plasmids in PAGA nanoparticles. The formulation showed activation of host immunity and an antitumoral effect in a colon adenocarcinoma mouse model, an effect which could not be observed with the naked plasmid [170]. In another study, PDMS-nanoparticles were used for combined therapy comprising an IL-12 encoding plasmid and paclitaxel. The results indicated a synergistic effect of the active agents, where enhanced tumor sensitivity to paclitaxel was achieved after a reduced number of administrations [171].

Another cytokine of interest for T-cell activation and cancer treatment is IL-18, and a nanoemulsion-based gene delivery strategy for sustained expression of this cytokine has already been reported. This nanoemulsion formulation was composed of the standard cationic lipids DOPE and DOTAP, the PEGylated surfactant Tween 80 and different oils. In a lung tumor mouse model, this formulation generated a stable system with higher transfection activity and higher capacity for T-cell activation than the commercial agent Lipofectamine [172].

1.3.4.3 Biomaterials that inhibit chemotaxis

Another important strategy to treat the inflammatory niche is to inhibit chemokine activity. Due to its relevance in many tumors, silencing CXCR4 expression is a particularly interesting idea where interfering RNA strategies can play an important role. Abedini et al. associated CXCR4-siRNA to dextran-spermine nanoparticle. This formulation was selected for its beneficial efficacy/toxicity ratio as compared to other reference materials (i.e. PEI and DOTAP/cholesterol) tested in the same study [173]. A *in vivo* test performed in a colon carcinoma mouse model showed that these siRNA nanoparticles were able to produce improved CXCR4 inhibition as compared to the naked siRNA [174,175].

Another approach to inhibit CXCR4 is to use the synthetic drug antagonist perixaflor. Misra et al. designed PLGA-acrylate nanoparticles for the controlled release of this drug. The formulation has shown effective receptor inhibition *in vitro*, with a better dose/response curve than the free molecule [176].

1.3.4.4 Integrative systems

Particularly powerful devices can be designed when biomaterials are used to integrate some of the elements described in the previous sections in a rational manner. An early example of this integration was provided by Hori et al. who included activated dendritic cells and IL-15 in an alginate gel for peritumoral injection in a mouse melanoma model. The matrix was able to reduce tumor size and improve survival [177].

Some of the best devices for integrative immunomodulation, often involve the combined presence of “danger signals”, cytokine release and antigen presentation, all integrated in scaffolds that provide a cellular context. Ali et al. prepared PLGA-scaffolds loaded with GM-CSF, tumor cell lysates and CpG-ODN. This scaffold produced an inmunostimulatory response that increased recruitment of CD8+ T-cells and production of proinflammatory cytokines in the tumor and dendritic cells at the vaccination site. The device achieved a complete regression of the tumors and enhanced survival of mice bearing established melanomas. Finally, this study confirmed the synergic effect of the three elements of the immunomodulatory material [178]. Afterwards, similar results were obtained with another prototype where CpG-ODN was changed for poly(I:C). In this study, the effect of the integrated system was compared with the intratumoral injection and the injection of the free components in a rat glioma model. Only the immunomodulatory material achieved complete tumor remission and improved mice survival. This result clearly indicates that the scaffold is not a passive substrate, but rather a part of immunomodulatory microenvironment that can surveyed by immune cells [179].

1.4 Outlook and conclusions

While there have been considerable advances in oncology over the last decades, several types of cancer still present very low survival rates. Many of these are the cancers where a tumor initiating cell subpopulation with stem cell-like has been reported. Despite the existence of this population, therapies that where under clinical development in the last decade failed to recognize the importance of cancer stem cells (CSCs), while they also neglected the importance of the main traits of the CSC niche including abnormal vasculature, hypoxia, ECM-dysregulation and inflammation.

In the last years, the interest on CSCs has translated from the molecular biology laboratories to pharmaceutical industry and drug delivery science. Together with this change, there has been a wider recognition of the importance of the tumor niche that supports these CSC features. This has resulted in a very high number of therapies in clinical trials that are directed to CSCs for solid tumors, and even some therapies in clinical practice for leukemia. Many of these therapies, however, might face important challenges before they can advance towards medical use. Their main limitations are due to their sub-optimal delivery characteristics and the unmet necessity to be integrated in combined therapies, together with regulatory and industrial challenges.

Delivery issues for these of therapies are important because many dysregulate signalling pathways or environmental features that, although critical for CSCs and their niche, can also affect other important populations such as non-cancer stem cells. Thus, side-effects for these therapies are almost inevitable, and delivery systems capable of improving selective drug biodistribution towards tumoral cells are a must. Additionally, many of these therapies combine this fine biodistribution requirements with fast degradation times. Such characteristics call for controlled release systems with active targeting, or if cell spreading can be neglected, to regional delivery using controlled release devices. This last concept has been the basis in CSC-specific strategies such as the use of controlled release microspheres for the regional delivery of BMP-7 in the treatment of glioblastoma [48]. On the other hand, gene therapy could be important to manipulate CSCs signalling pathways (Hedgehog, Wnt, etc.) or effectors of environmental cues such as HIF. Gene therapy in the context of CSC treatment shares most of its challenges with other *in vivo* applications, with the provision that the nanotherapeutics need to reach the CSCs in their niche. Considering that CSCs are a minor part of the tumor, and that they are often in its most inaccessible regions, this becomes and additional difficulty. In general terms, any therapy that is not capable of being transported through the tumor ECM or that binds tumor cells non-selectively, will either be retained at the periphery of the tumor or will be absorbed by terminally differentiated cancer cells acting as sacrificial barriers.

Another future challenge of future therapies against CSCs and their niche is their necessity to be integrated in combined therapies to have substantial effects. Since cellular processes

are typically redundant, any effect on a targeted pathway might be compensated by additional activation of other pathways. For drug combinations to be effective, however, it is critical that there is mechanistic cooperation or synergism between the two agents. Acute promyelocytic leukemia provides a clinically successful example of how a very potent differentiation agent (ATRA) would be ineffective without a pro-apoptotic inducing agent, since the cells would become resistant to this medication in a short time span. When combined with a proapoptotic agent, the cells differentiated by ATRA are easily removed before any resistance is generated. Such roles of combination with ATRA were traditionally provided by anthracycline-based agents, and since 2013, by arsenic trioxide that combines a pro-apoptotic with a further pro-differentiation effect.

Because of their intrinsic flexibility, biomaterial-based devices are ideal platforms for combined therapies. For instance, Sun et al. have taken advantage of a rationally selected drug combination (ATRA and doxorubicin) and a suitable delivery platform to deliver this combined therapy [44]. A more sophisticated example of the use of biomaterials to maximize the therapeutic value of drug combinations is provided by the work of Mooney´s group, which combines anticancer vaccines, TLR agonists and controlled release of cytokines to design potent immunomodulatory materials. However, a critical aspect of these technologies is that the diverse elements are integrated within the context of a polymeric scaffold that helps to generate a regional environment. The effects of these elements are thereby focalized while providing a mechanical and spatial context for the recruited immune cells [158]. Because of their focus on stem cell differentiation, modulation of stem cell signalling and mimicking the cell microenvironment, tissue engineering devices can find surprising new applications in cancer suppression as it has been illustrated through some examples in this chapter.

A final challenge for therapies directed to CSCs and their niche could come from regulatory authorities and industry. For these actors, an important issue would be identifying experimental outcomes during screening and preclinical experimentation that correlate with positive clinical endpoints. Cytotoxicity in cell panels and reduction of tumor volume are routinely used for anticancer drug screening, but are not necessarily indicative of CSC elimination and survival. Also, tumor niche modulation might not be able to eliminate the tumor by itself, but could have instrumental effects in well-chosen combination therapies.

Identifying and measuring the effects of these combinations at early stages and selecting combined therapies with great potential from less interesting ones, could prove to be difficult.

Addressing cancer by suppressing the CSCs and their supporting niche is still far from being a validated general strategy. In the next years, there will be more data available from clinical trials of drug candidates aimed at addressing some of these relatively new aspects of tumor biology. We expect, however, that higher benefit will be achieved with combined therapies that address simultaneously the CSC phenotype and the niche, and probably, in the presence of another drug capable of eliminating differentiated tumor cells. Despite the open questions, it is already clear that the CSC phenotype and the tumor niche are essential features of cancer, and thus, it is reasonable to expect that addressing them will be essential for future therapies. In this sense, the design of devices capable of integrating these strategies in a coordinated and synergistic fashion could be critical to achieve maximum benefits.

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Supplementary Material Chapter 1

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Table S1: Overview of the drugs in clinical trials against CSCs and/or their niche. Only trials from years 2011-2016 and in phase III or higher are presented. Source: ClinicalTrials.gov (www.clinicaltrials.gov).

Intervention	Clinical phase	Condition	Mechanism of action	Number
Apatinib	Phase III	Non-squamous non-small-cell lung cancer	VEGFR inhibitor	NCT 02824458 NCT 02332512 NCT 02875457
		Small cell lung cancer		NCT 02329860
		Hepatocellular carcinoma		NCT 01512745
		Gastric cancer		NCT 02537171
	Phase IV			NCT 02426034
		Gastric cancer		NCT02776527
Regorafenib	Phase III	Colorectal cancer	VEGFR inhibitor	NCT 02664077 NCT 01584830 NCT 01853319 NCT 01939223
		Hepatocellular carcinoma		NCT 01774344
Axitinib	Phase III	Clear cell renal carcinoma	VEGFR inhibitor	NCT 01599754
Aflibercept	Phase III	Colorectal carcinoma	VEGFR inhibitor	NCT 01661270 NCT 01571284 NCT 01670721
Ramucirumab	Phase III	Hepatocellular carcinoma Gastric and esophageal adenocarcinoma Non-small cell lung cancer Urothelial carcinoma	VEGFR inhibitor	NCT 02435433 NCT 02314117 NCT 02411448 NCT 02426125
Bevacizumab	Phase III	Glioblastoma	Monoclonal antibody against VEGF	NCT 01290939
		Breast cancer		NCT 02017717 NCT 01663727
		Non-small-cell lung cancer		NCT 02272413 NCT 01661790 NCT 01966003
		Non-squamous non-small-cell lung cancer		NCT 01364012 NCT 02633189 NCT 01763671 NCT 02366143 NCT 01351415 NCT 01878422
		Colorectal carcinoma		NCT 02563002 NCT 02394834 NCT 02314182 NCT 02162563 NCT 02934529 NCT 01462890
		Ovarian cancer		NCT 01802749 NCT 02420821
	Phase IV	Renal cancer		NCT 02316327
		Non-small-cell lung cancer		NCT 02582970
		Colorectal carcinoma		NCT 01972490 NCT 01695772

		Ovarian cancer Solid tumors		NCT 01706120 NCT 01588184
Gefitinib	Phase III	Advanced pulmonary adenocarcinoma Non-small cell lung cancer Non-squamous non-small cell lung cancer Lung neoplasms	VEGFR and EGFR inhibitors	NCT 02929693 NCT 02889692 NCT 02893332 NCT 02882984 NCT 02859077 NCT 01405079 NCT 01774721 NCT 00322452 NCT 02588261 NCT 01404260 NCT 01544179 NCT 02714010 NCT 00322452 NCT 02296125 NCT 02824458 NCT 02518802 NCT 01024413
	Phase IV	Thoracic neoplasm Non-small cell lung cancer		NCT 02031601
Sunitinib	Phase III	Renal cell carcinoma	VEGFR and EGFR inhibitors	NCT 02535351 NCT 02231749
	Phase IV	Renal cell carcinoma Pancreatic neuroendocrine tumor		NCT 02555748 NCT 01525550
Nintedanib	Phase III	Non-small-cell lung carcinoma Colorectal carcinoma	VEGFR, PDGFR and FGFR inhibitor	NCT 02231164 NCT 02149108
Sorafenib	Phase III	Hepatocellular carcinoma Renal cell carcinoma Leukemia cutis and myeloid sarcoma Hepatocellular carcinoma Acute myeloid leukemia Renal carcinoma	VEGFR and PDGFR inhibitor	NCT 01482442 NCT 02576509 NCT 01761266 NCT 02187081 NCT 01887717 NCT 02436902 NCT 01613846 NCT 02627963 NCT 01371981 NCT 02504983 NCT 02474290 NCT 01339962 NCT 01728948
	Phase IV			
Pazopanib	Phase III	Renal Cell Carcinoma Sarcoma	VEGFR and PDGFR inhibitor	NCT 01575548 NCT 02049905
	Phase IV	Renal Cell Carcinoma		NCT 01521715
Motesanib	Phase III	Non-small cell lung	VEGFR and PDGFR inhibitor	NCT 02629848
Masitinib	Phase III	Gastro-intestinal stromal	VEGFR and PDGFR	NCT 02009423

		tumour Colorectal cancer	inhibitor	NCT 02605044
Erlotinib	Phase III	Non small-cell lung cancer	EGFR inhibitor	NCT 01652469 NCT 00874419 NCT 01360554 NCT 01342965 NCT 02411448 NCT 01523587 NCT 02178397 NCT 02031744 NCT 01487174 NCT 02134015 NCT 02140333 NCT 01887795 NCT 02352948 NCT 02152631 NCT 02193282 NCT 02588261 NCT 02296125 NCT 02588261
		Non squamous non-small-cell lung cancer		NCT 01328951 NCT 01351415 NCT 00883779 NCT 02633189 NCT 01456325 NCT 01887886
	Phase IV	Non small-cell lung cancer		NCT 02031601 NCT 01402089 NCT 01287754 NCT 02000531
		Non squamous non-small-cell lung cancer Lung adenocarcinoma		NCT 01609543 NCT 02399566
Icotinib	Phase III	Non-small-cell lung-cancer	EGFR inhibitor	NCT 02714010 NCT 01719536 NCT 02448797 NCT 02486354 NCT 01724801 NCT 02125240
		Lung adenocarcinoma		NCT 01996098
	Phase IV	Non-small-cell lung-cancer		NCT 01926171 NCT 01465243 NCT 02778893 NCT 01665417 NCT 02404675 NCT 01646450 NCT 02031601 NCT 02103257 NCT 02194556

		Lung adenocarcinoma		NCT 02283424
Afatinib	Phase III	Non-small-cell lung carcinoma	EGFR inhibitor	NCT 01121393 NCT 00949650
Osimertinib	Phase III	Non-small cell lung carcinoma	EGFR inhibitor	NCT 02511106 NCT 02474355 NCT 02474355 NCT 02151981 NCT 02454933
Cetuximab	Phase III	Colorectal cancer	Monoclonal antibody against EGFR	NCT 01309126 NCT 02934529 NCT 02484833 NCT 01878422 NCT 02563002 NCT 01910610
		Squamous Cell Head and Neck Cancer		NCT 01810913 NCT 02383966 NCT 02551159 NCT 02236936 NCT 02105636 NCT 01884623 NCT 01969877 NCT 02252042 NCT 02358031
		Oropharyngeal Squamous Cell Carcinoma		NCT 01855451
		Nasopharyngeal Carcinoma		NCT 01302834 NCT 01874171
		Colorectal cancer		NCT 02633176
	Phase IV			NCT 00327093
		Squamous Cell Head and Neck Cancer		NCT 01315990 NCT 02015650
Everolimus	Phase III	Advanced neuroendocrine tumors	mTOR and HIF inhibitor	NCT 01524783 NCT 01668784
		Renal cancer		NCT 01865747 NCT 01773460
		Breast cancer		NCT 01805271 NCT 02511639 NCT 02137837 NCT 01626222 NCT 01674140 NCT 02404051
		Pancreatic tumor		NCT 02246127
	Phase IV	Renal cancer		NCT 01514448 NCT 02056587 NCT 01206764 NCT 02338570
		Breast cancer		NCT 01948960 NCT 01743560
		Neoplasms		NCT 02248571 NCT 01789281

Nimorazole	Phase III	Head and neck carcinoma	Inhibit glycolysis and the repair of radiation-induced cellular potentially lethal damage in hypoxic niche	NCT 01507467 NCT 01880359 NCT 01950689
ATRA	Phase III Phase IV	Acute myeloid leukemia Non-small cell lung carcinoma Acute promyelocytic leukemia	Expression of genes implicated in the differentiation	NCT 01237808 NCT 01067274 NCT 00151255 NCT 00599937 NCT 00146120 NCT 01226303 NCT 00482833 NCT 00504764 NCT 02200978 NCT 00504764 NCT 00465933 NCT 00408278
Sulindac	Phase III	Colorectal adenocarcinoma	Inmunomodulador	NCT 01349881
Imiquimod	Phase III	Nodular basal cell carcinoma Intraepithelial neoplasia	Hedgehog pathway inhibitor and immunostimulator	NCT 02242929 NCT 02329171 NCT 01283763 NCT 01861535 NCT 02059499

Background, Hypothesis and Objectives

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Background

Gene therapies are technologies of great potential for the treatment of many important diseases, but they are contingent on the inclusion of the nucleic acids in efficient delivery carriers [1–3]. Viruses are natural vectors widely used in gene therapy due to their ability to efficiently transfect cells, but they present immunogenicity and other safety problems that limit their translation [4]. Synthetic vectors have been developed as alternatives with a better safety profile.

Cationic polymers and lipids used in the preparation of non-viral vectors for gene therapy must be able to reversibly associate nucleic acids, protecting them from their degradation in the biological environment, and releasing them in the place where they develop their action [5]. Furthermore, these polymers must be biocompatible and biodegradable. Depending on the required properties, more and more polymers are being designed and synthesized "on demand" to optimize their delivery characteristics while maintaining a compromise with materials toxicity. Chemical approaches that lead to simple polymer modifications are ideal for identifying the most suitable characteristics required in a complex application.

Polymers of interest for gene delivery are not only cationic. Anionic endosomolytic polymers such as derivatives of acrylic and malic acid and 6-Mercaptohexanoic acid substituted poly(phosphazene) (6MHA-PPZ) can reduce the toxicity and improve the efficacy of cationic polymers by facilitating endosomal escape and reducing their charge density [6–9].

Cancer stem cells (CSCs) are a tumor subpopulation with key implications in tumor re-iniciation and therapy [10]. CSCs can be treated with differentiation factors that target key pathways as it is done with all-trans-retinoic acid in acute promyelocytic leukemia [11] and with TGF-β pathway modulators in glioblastoma and breast cancer models [12,13].

Bone Morphogenic Proteins (BMPs) have been described to differentiate glioblastoma CSCs to differentiated cancer cells [14–16]. However, BMPs have a very low half-life, so to prolong BMP exposure in the tumor area, these proteins have been included in controlled release systems capable of sustaining protein release for several weeks and resulting in an antitumoral

effect *in vivo* [17,18]. Expression of BMPs in specific regions can also be achieved by gene therapy approaches, using a plasmid or an mRNA encoding these proteins [19,20].

Hypothesis

1. The structure of the cationic polymers used in the preparation of nanoparticles influences their efficiency as carriers for gene therapy.
2. Engineered anionic polymers with pH-sensitive groups can be added to cationic gene nanocarriers to reduce their toxicity and increase their transfection capacity.
3. Specific polyphosphazene precursors can be grafted with cationic groups by click chemistry reactions to design a range of different materials of interest for gene delivery.
4. The protein Bone Morphogenic Protein 4 can be encoded in a plasmid and delivered to a tumor *in vivo* using an optimized gene nanocarrier, in order to induce the differentiation and suppression of glioblastoma cancer stem cells, either alone or in combination with standard chemotherapy.

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Objectives

Considering the previously outlined background and the hypothesis, the main objectives of this thesis were:

1. To study the influence of the cationic polymer structure and association with an anionic polyphosphazene on the physicochemical properties, toxicity, and transfection of nanosystems used in gene therapy.
2. The synthesis of new cationic polyphosphazenes with improved gene delivery characteristics either on their own or combined with the anionic polyphosphazene 6MHA-PPZ.
3. The use of these new optimized nanosystems for the treatment of glioblastoma though the delivery of a gene therapy capable of supressing glioblastoma cancer stem cells.

To achieve these objectives, the experimental part was divided in the following work phases.

Phase 1. Nanoparticles consisting of commercial cationic polymers, either alone or with the anionic polyphosphazene 6MHA-PPZ were studied regarding their toxicity and efficacy for gene delivery. This study identified the requirements for the polymers to be combined with 6MHA-PPZ towards the generation of highly efficient gene delivery systems. This data is presented in Chapter 2.

Phase 2. Synthesis and characterization of new cationic polyphosphazenes based on the criteria identified in the previous chapter and on literature. Screening of the derivatives with the best toxicity/efficacy ratios. This data is presented in Chapter 3.

Phase 3. Application of the optimized nanocarriers for the design of a new treatment directed towards the suppression of glioblastoma cancer stem cell. This treatment is based on a plasmid encoding Bone Morphogenic Protein-4 (BMP-4), and its efficacy was analyzed *in vitro* and *in vivo*. This data is presented in Chapter 4.

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Chapter 2

Study of the influence of the cationic polymer structure and their association with an anionic polyphosphazene on gene therapy

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Abstract

The anionic polyphosphazene 6MHA-PPZ has been described as material capable of reducing the toxicity and improving the gene delivery characteristics of a cationic polyplex. In this chapter we have studied how the cationic groups of the different polymers (PEI, Chitosan and Protamine) influences the physicochemical properties, toxicity, and transfection of mixed nanosystems of these polymers and 6MHA-PPZ. Systems without 6MHA-PPZ were prepared as reference. Both the cationic nanoparticles and those containing 6MHA-PPZ present similar physicochemical characteristics: particle size of 100-150 nm, positive surface charge (+25-35 mV) and reversible association of the nucleic acid. Regarding toxicity, the PEI-containing prototype presents the highest toxicity, due to the presence of secondary and tertiary amine groups that produce alterations in the cell membrane. PEI is also the most efficient inducing transfection by improving internalization and endosomal escape. Protamine, with guanidine groups, showed much lower transfection but also less toxicity, while chitosan had very low transfection capacity at this concentration. The association to 6MHA-PPZ produced a reduction of toxicity for all polymers and an improvement in transfection efficacy. The system with PEI/6MHA-PPZ showed outstanding transfection capacity exceeding by 20-fold that of the commercial gold standard Lipofectamine 2000. This data suggests that 6MHA-PPZ is a universal transfection enhancer and indicates the great potential of the PEI/6MHA-PPZ combination as gene delivery system.

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2.1. Introduction

Polyethilenimine (PEI), protamine and chitosan are some of the most widely used cationic polymers for the preparation of non-viral vectors. Polymer charge density is a crucial factor for the formation of stable nanoparticles, but at the same time increases the toxicity of the nanosystem. Thus, polymers such as PEI with high charge density produces very efficient transfection but have high cytotoxicity [1]. Protamine and chitosan have lower charge density, being less toxic, but also resulting in lower transfection [2].

The addition of anionic polymers can reduce charge density, and therefore, the toxicity of cationic nanoparticles [3–5]. Addition of the adjuvant polymer can be done either by coating the pre-formed nanoparticle [6], or by addition of the polymer to the genetic material phase to be condensed [3,4]. Charge neutralization can even change nanoparticle trafficking properties. Nanoparticles with a negative charge are internalized through the caveolae-mediated endocytosis route, as they do not interact electrostatically with the cell membrane [7].

Multiple nanosystems have been developed by the combination of cationic polymers such as PEI, protamine, and chitosan and anionic polymers such as poly(γ-glutamic acid), polyacrylic acid, dextran sulphate, gellan gum, alginate and poly(ethylene glycol) derivatives with carboxylic acid side chains. In all these cases an increase in cell uptake and a decrease in the nanoparticle interaction with serum proteins was observed. This has been shown to translate into an improvement in the transfection capacity of nanosystems both *in vitro* and *in vivo* [2,6,8–14]. Control over the amount of polyanion added is important, since it can cause destabilization of the nanosystem, premature release of nucleic acid, and therefore, loss of its function [10].

Other anionic polymers have been designed to mimic the anionic peptides present in influenzae viruses, which induce membrane disruption and contain amino acids with alkyl and carboxyl groups in their structure [15]. Some examples are poly(ethyl acrylic acid) and poly(propyl acrylic acid)[16,17]. These polymers present pH-sensitive behavior and are able to disrupt specifically the endosomal membrane due to the acid environment of this intracellular compartment [5,18]. A recent work has shown that the association of anionic

polyphosphazene (6MHA-PPZ) to cationic polyphosphazene nanoparticles, improves the transfection capacity in 2D monolayers, 3D spheroids and in a subcutaneous xenograft model, due to improved tumor transport and intracellular trafficking characteristics, showing better results than PEI complexes [3].

In this work, vectors for gene therapy were prepared based on the association of commercial cationic polymers and 6MHA-PPZ. The motivation was to study if this anionic polymer can enhance the transfection/toxicity ratio of other common cationic polymers, and to understand which structures are more affected by the combination with 6MHA-PPZ and which ones lead to the most efficient systems.

2.2. Materials and Methods

2.2.1 Materials

Polyethylenimine branched (PEI) Mw ~25 kDa, HEPES (≥99.5%), Heparin sodium salt (from porcine intestinal mucosa) and Tris-Acetate-EDTA buffer (10x) were purchased from Sigma-Aldrich. Chitosan 113 kDa Mw and deacetylation degree 70−90 % was bought from FMC Biopolymer/Novamatrix (Sandvika, Norway), Protamine sulfate (Mw 5 KDa) was purchased from Yuki Gosei Kogyo, Ltd., (Japan). The polymer 6-Mercaptohexanoic acid substituted poly(phosphazene) (6MHA-PPZ) was synthesized as previously reported [3]. DNAse/RNAse free water (Invitrogen), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), OptiMEM (Gibco), Fetal Bovine Serum (FBS) (Gibco), Penicillin-Streptomycin for culture medium (Gibco), Lipofectamine 2000 Transfection reagent (Life Technologies) and SYBR® Gold nucleic acid stain (Life Technologies) were purchased from ThermoFisher. MTS Cell Proliferation Assay Kit was purchased to BioVision (USA) and Alamar Blue were bought from Promega (Madrid, Spain). Luciferase Reporter Gene Assay was purchased from Roche. The pEGFPLuc plasmid was donated by Prof. Anxo Vidal laboratory (CiMUS, Universidad de Santiago de Compostela). All the products were used as received.

2.2.2 Amplification of the plasmid

To perform the optimization of the nanoparticles we used a plasmid (pEGFPLuc), which expresses two reporter proteins: enhanced Green Fluorescent Protein and Luciferase (Fig. 2.1).

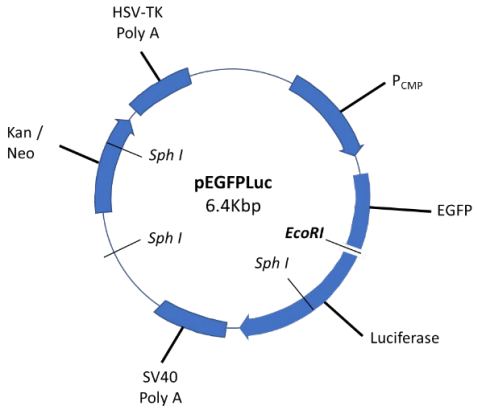


Figure 2.1 EGFPLuciferase plasmid map.

For plasmid amplification, competent E. coli DH5α bacteria were transformed with pEGFPLuc, resuspended in Luria-Bertani (LB) medium and incubated for 2 h at 37 °C with orbital agitation. Bacteria were selected through seeding on Petri dishes and supplementation of the media with the selection antibiotic (10µg/mL Kanamycin). The bacteria were incubated in this selection media at 37 °C for 24 h. An isolated colony was grown in supplemented LB in the orbital incubator and amplified until reaching an adequate number of bacteria to perform plasmid extraction.

Plasmid extraction was performed with an Invitrogen™ PureLink™ HiPure Plasmid Gigaprep Kit (Thermo Fisher, USA) following the manufacturer's instructions. Briefly, bacteria were centrifuged at 5000 rcf for 15 min, the pellet was resuspended in a RNase solution to remove the RNA and mixed with a lysis buffer to release the intracellular content. In order to remove the cellular debris, a precipitation buffer was added to the solution and filtered. Then, the pDNA was purified using the by columns provided in the kit. Finally, the plasmid was precipitated, washed, and quantified by UV absorption (Nanodrop, ThermoFisher, USA).

2.2.3 Cationic-nanoparticle formation

The nanoparticles were prepared by ionic complexation, using the model plasmid pEGFPLuc. The polymers were dissolved in HEPES 10mM (pH 5.5) and the pDNA in pure water. Nanoparticles were formed upon electrostatic interaction of pDNA (2mg/mL), with or without 6MHA-PPZ (1mg/mL), and the cationic polymer (1mg/mL); the preparation was performed under magnetic stirring (500 rpm, 1 h) (Fig. 2.2). For nanoparticles containing cationic polymer and pDNA, the ratios are based on the number of primary/ secondary amines of the polymer branches (N) and the phosphates of the pDNA (P). In this case, composition is defined as N:P ratio. In the case of nanoparticles that contain the anionic polymer 6MHA-PPZ, the amount of this material is quantified by the number of terminal carboxylic groups (C), and the composition of the nanoparticles is defined by the N:C:P ratio.

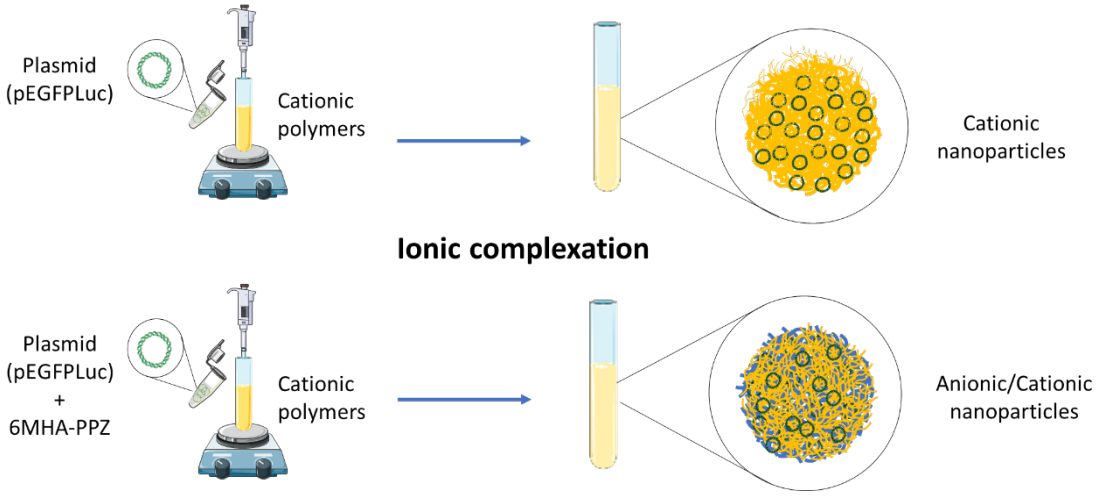


Figure 2.2. Nanoparticles preparation by ionic complexation

2.2.4 Nanoparticle characterization

2.2.4.1 Size and Zeta Potential

The size and polydispersity (PDI) of the nanoparticles were determined by Dynamic Light Scattering (DLS) and Zeta Potential was measured by laser doppler anemometry in a Nanosizer ZS (Malvern, UK). Each analysis was performed in triplicate at 25 °C, with a backscatter angle of 173°. In the case of zeta potential, the measurements were performed upon dilution 1:10 in 1 mM KCl.

2.2.4.2 Morphological analysis of the nanoparticles

Morphological analysis of nanoparticles was done by Field Emission Scanning Electron Microscopy (FESEM; Zeiss Gemini Ultra Plus, Germany) using scanning transmission electron microscopy and immersion lens detectors for sample observation.

For sample preparation, 10 µL of the nanoparticles were placed on a copper grid with carbon films and they were allowed to dry for 5 min; sample excess was removed by blotting. Then the same volume of phosphotungstic acid (2% w/v in water) was added and washed twice with water. Samples were dried overnight before observation.

2.2.4.3 Association of the pDNA

Tests were performed to verify that the nanoparticles can associate the pDNA and dissociate it under suitable conditions. A concentration of nanoparticles corresponding to 0.33

µg of plasmid was mixed with a nucleic acid visualization reagent (SYBR® Gold nucleic acid stain) and loading agent (30% glycerol and 0.25% bromophenol blue), this mixture was loaded on an agarose gel (1% w/v in Tris-EDTA 1x buffer) and allowed to run for 30 min at 100V in the electrophoresis cell (Wide Mini-Sub Cell GT Systems, BioRad, USA). For dissociation tests, the same amount of nanoparticles were incubated with heparin (20:1 w/w heparin: pDNA) for 1 h at 37 °C, and the resulting samples processed as described before.

2.2.5 Cell Culture

All *in vitro* assays were performed in a U87MG glioblastoma cell model. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10 % (v/v) heat inactivated Fetal Bovine Serum (FBS) (Gibco, USA) and 1 % (v/v) Penicillin/Streptomycin (P/S) (Gibco, USA) and incubated at 37 °C (95 % relative humidity and 5% CO_2) up to 85 % confluence. At this point they were subcultured by trypsinization, dilution and plating.

2.2.6 *In vitro* toxicity

For 2D toxicity assay, 8,000 cells/well were seeded on a 96-multiwell plate and incubated for 24 h. Nanoparticles were diluted in supplement medium and incubated with the cells for 4 h at different pDNA concentrations (0.1- 2 µg pDNA/cm^2). Then, nanoparticles were removed, cells were washed with PBS and replaced with fresh medium. After 48 h recovery time, cytotoxicity was determined by MTS assay (BioVision, USA), 10 µl of MTS reagent was added per well and the absorbance was measured after 3 h of incubation in a plate reader at 495nm.

For 3D toxicity assay, neurospheres were prepared by placing 500 cells/well in a ULA 96-multiwell plate (Ultra Low Attachment) and centrifuged 30 mins at 200 rcf. After 3 days of growing, different nanoparticles concentrations (corresponding to 0.33 - 6.67µg pDNA/mL) were incubated with the neurospheres for 12 h. After this, the nanoparticles were removed and the neurospheres were washed twice and incubated for 72 h with fresh medium. Toxicity was evaluated by two parameters: the size of the neurospheres and through a resazurin reduction assay (CellTiter-Blue®, Promega, USA) performed after a 72 h recovery time. For this assay, 40µl of the reagent was added in each well and incubated for 4 h. The fluorescence was

evaluated by a plate reader set at 539 nm for excitation wavelength and at 620 nm for emission.

In all toxicity assays the negative control was HEPES 10 mM and the positive control was Triton 0.1 %.

2.2.7 *In vitro* transfection

For the transfection assay, 56,000 cells/well were seeded in a 24-multiwell plate in supplemented DMEM medium (10 % FBS and 1% P/S). After 24 h, the nanoparticles were diluted in Optimem (Gibco, USA) at 0.5 µg de pDNA/cm^2, and incubated with the cells for 4 h. Afterwards, cells were washed with PBS and incubated 48 h more in fresh DMEM supplemented medium. Transfection was measure by a Luciferase Reporter Gene Assay (Roche, Germany). Briefly, cells were washed twice with PBS and 100 µL of lysis buffer were added. After 5 mins the lysate was centrifuged at 1200 rcf for 15 s. Fifty µL of the supernatant were placed in a white plate and using an automatic injector, and 25 µL of luciferin from the commercial kit was added. The samples were measured in a luminometer (Mithras LB 940, Berthold).

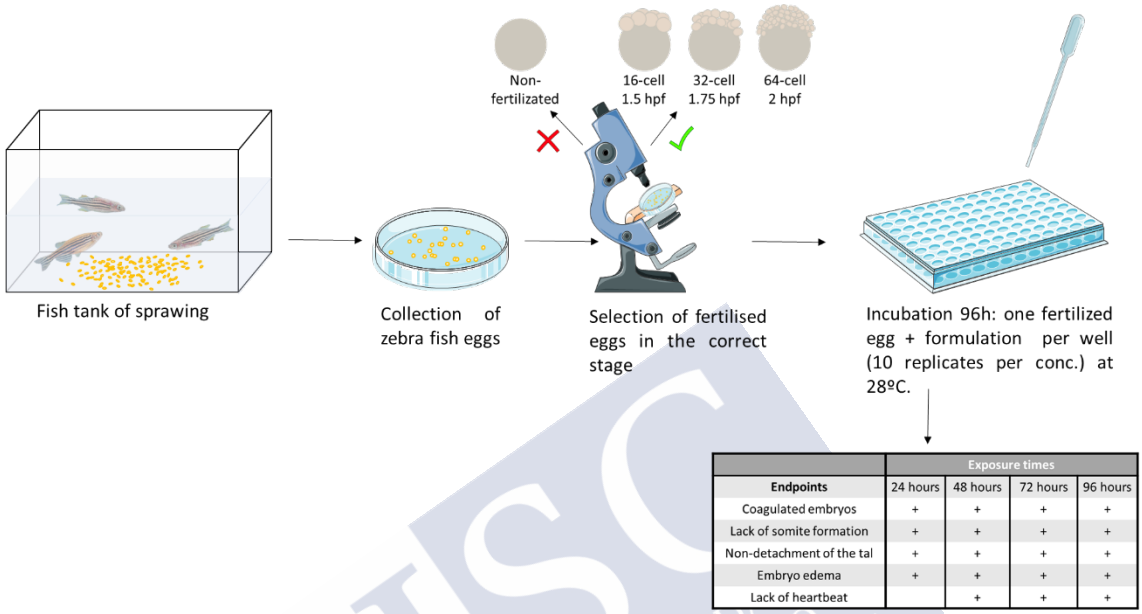
The results were corrected for protein content, quantified by a Bio-Rad Protein Assay (BioRad, USA). In this assay, 40 µL of the reagent were added to the sample and the absorbance was measured at 595nm.

2.2.8 Fish Embryo Acute Toxicity (FET) Test

This experiment was carried out in collaboration with the group of Prof. Laura Elena Sánchez Piñón. The experimental design has been carried out based on the protocol of the OECD (Organization for Economic Cooperation and Development) for the study of toxicity in fish embryos known as Fish embryo acute toxicity test [19]. Briefly, zebrafish fertilised eggs were selected 2-3 h after fertilisation, at the stages of 16-32 cell blastomeres. Ten viable fertilised eggs per group were each placed in a well of a 96-multiwell plate in reverse osmosis water with different concentrations of the nanoparticles. Embryos were incubated at 26±1 °C for 96 h and were observed on an inverted microscope every 24 hours until the end of the test, looking for toxicity signs. The observations performed to determine the toxicity include

the detection of coagulation of embryos, lack of somite formation, non-detachment of tail, lack of heartbeat (after 48 h) or edema in the embryo (Figure 2.3).

To consider the experiments valid, in the negative control there must be a mortality rate ≤ 10% with a hatching rate > 80% at the end of the test (96 hpf).



	Exposure times			
Endpoints	24 hours	48 hours	72 hours	96 hours
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tal	+	+	+	+
Embryo edema	+	+	+	+
Lack of heartbeat		+	+	+

Figure 2.3. Scheme of the zebrafish embryo acute toxicity test procedure. hpf = hours post-fertilisation.

2.2.7 Statistical analysis

Data were represented as mean ± standard deviation (SD). Statistical differences were calculated using the Student's t-test and one-way ANOVA in combination with Tukey's multiple comparisons test. The significance was set to $p < 0.05$. All the experiments were replicated three times, if not stated otherwise.

2.3 Results and discussion

2.3.1 Nanoparticle characterization

2.3.1.1 Size and Potential

The nanoparticles were prepared at charge ratios 8:0:1 and 8:4:1 (N:C:P). All the nanoparticles showed an average size around 100-150 nm and a monodisperse distribution (PDI < 0.2), being PEI nanoparticles slightly smaller in size (Figure 2.4.a). The inclusion of 6MHA-PPZ did not change importantly particle size, but in some cases, as PEI and Protamine it reduced slightly the PDI ($p < 0.05$). Regarding surface charge, all nanoparticles have a positive zeta potential, similar in the prototypes containing protamine and chitosan (+25 mV) and slightly higher for PEI prototypes (+30-40 mV) (Figure 2.4.b). The inclusion of the anionic polymer 6MHA-PPZ did not result in a modification of the zeta potential. It was also found that the derived count rate of the nanoparticles containing the anionic polymer was around 10 times higher than for the cationic nanoparticles (Figure 2.4.c). Probably the addition of the 6MHA-PPZ increases the number of particles formed, explaining also why the prototypes containing the anionic polymer do not have lower surface charge. The small size and the positive charge make these systems suitable formulations for their application in gene therapy.

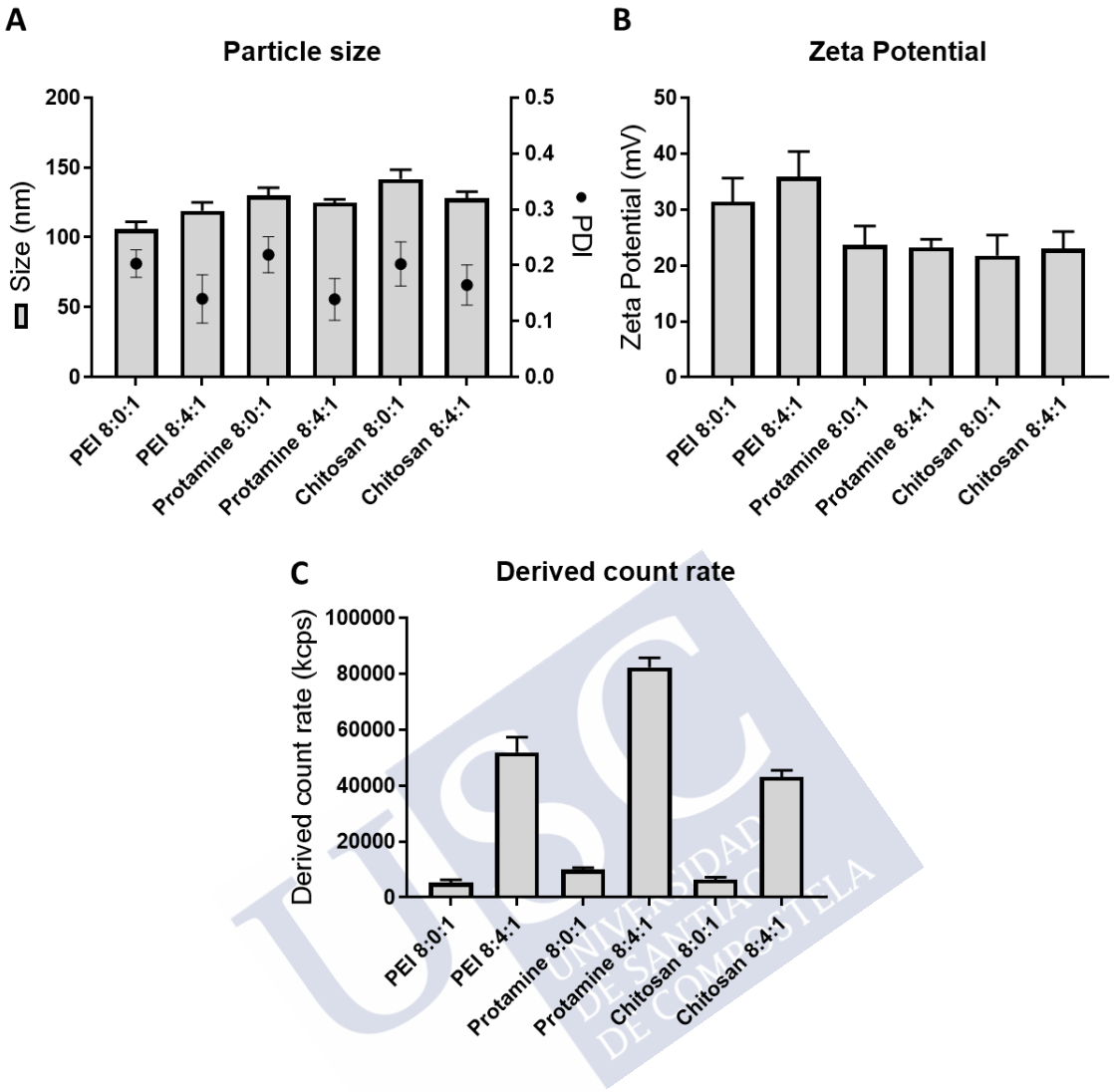


Figure 2.4 Nanoparticle characterization by size and surface potential. A. Particle size and polydispersity (PDI). B. Surface charge of the nanoparticles. C. Derived count rate. Kcps: Kilocounts per second.

2.3.1.2. Morphological analysis of the nanoparticles

The nanoparticles were also observed by electron microscopy in order to analyze their morphology and dried nanoparticle diameter. STEM images (Fig. 2.5) showed that all the particles are spherical and have a particle size in the range indicated by PCS measurements, being PEI nanoparticles the smallest. Moreover, the prototypes containing 6MHA-PPZ are more stained, which suggests a higher densitiy compare to the cationic ones.

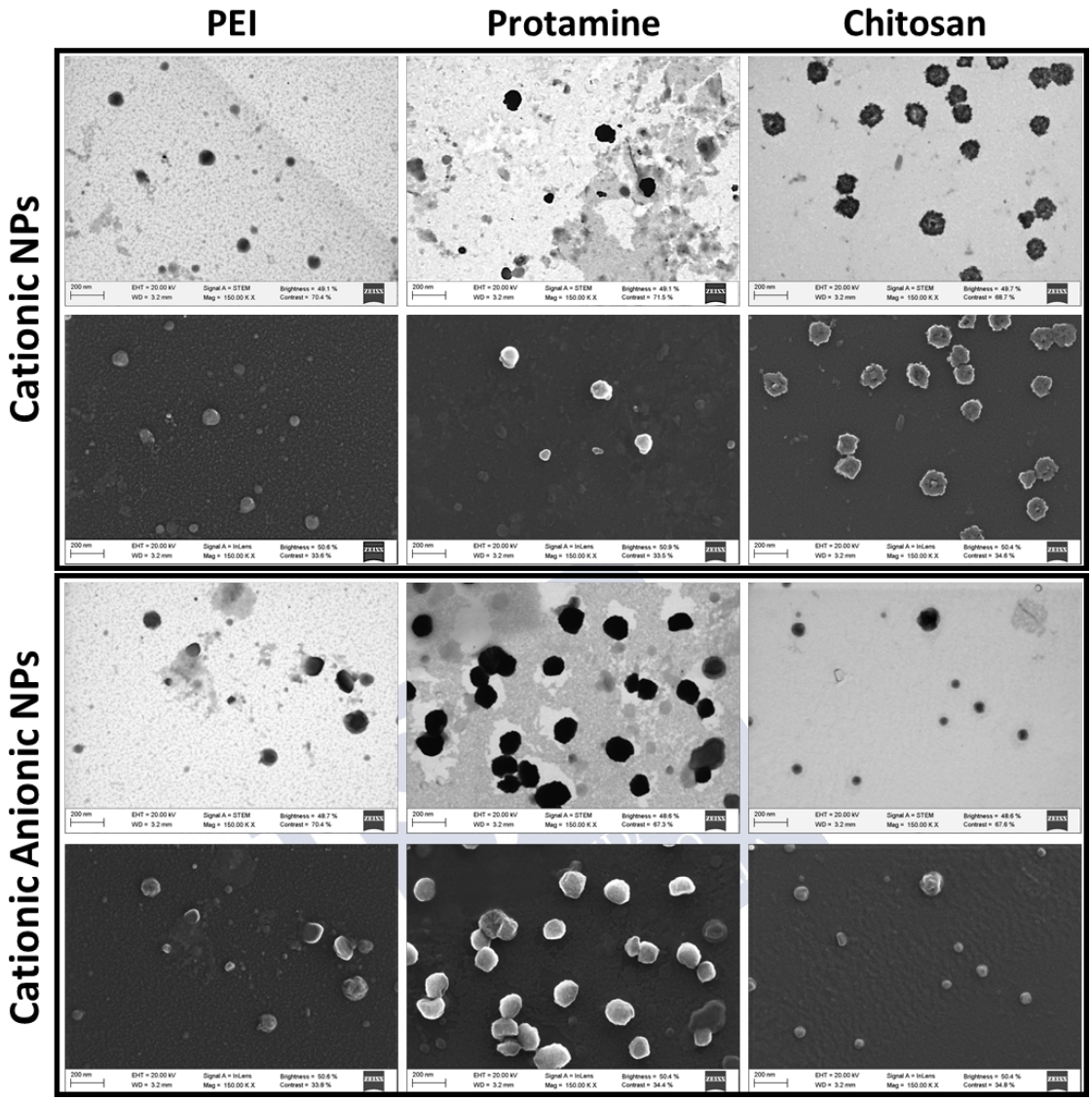


Figure 2.5 Nanoparticle morphological characterization by electronic microscopy FESEM. Transmission electron microscopy (grey images) and scanning electron microscopy (black images).

2.3.1.3 Gel retardation asay

The association capacity of the nanoparticles was determined by agarose gel electrophoresis (Figure 2.6.a). Plasmid migration was only observed in the well of the free pDNA, indicating the capacity of the nanosystems to bind eficiently the plasmid. After the incubation of the NPs with an anionic competitor (Heparin), the plasmid was released and the bands were observed in the gel, demonstrating a reversible association with the nanoparticles (Figure 2.6.b).

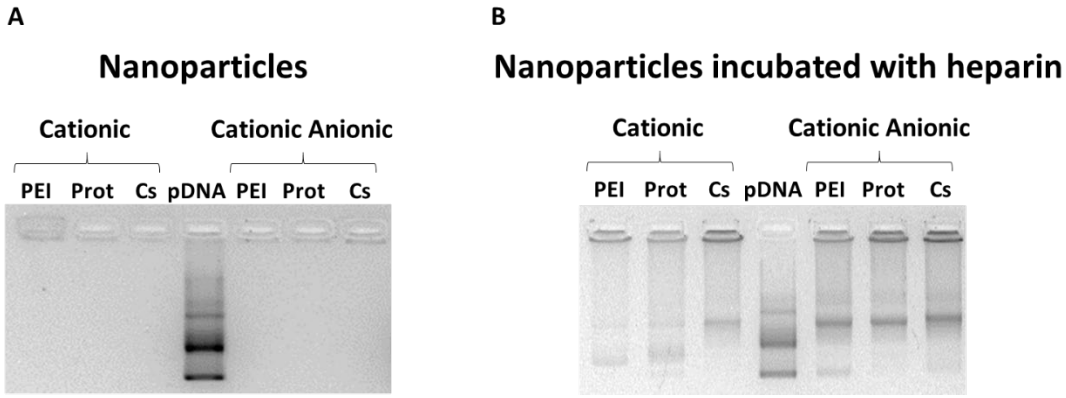


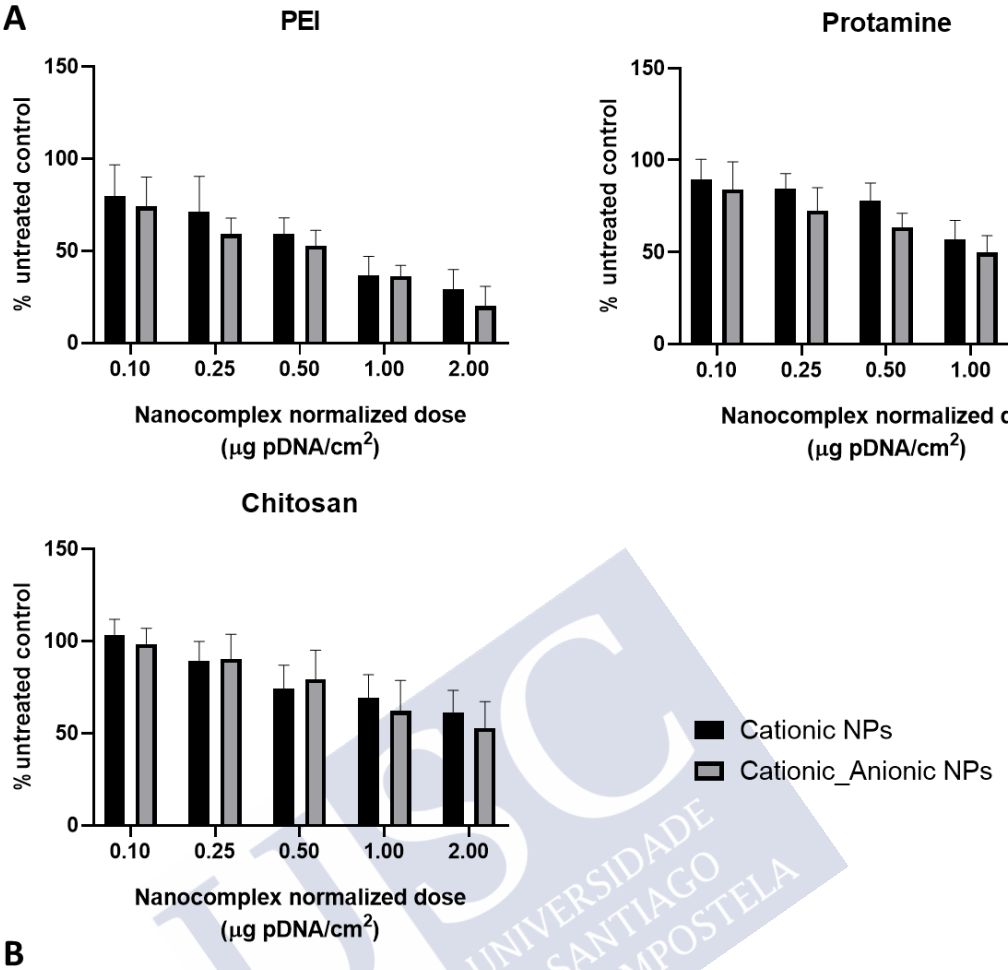
Figure 2.6 Binding efficiency of the nanosystems by gel retardation assay. A. Bands correspondent to nucleic acid association in the nanoparticles B. pDNA dissociation after the incubation with heparin as anionic competitor. Prot: protamine.

2.3.2 *In vitro* toxicity

Once prototypes had adequate physicochemical properties, the toxicity of the formulations in 2D cultures was tested. The toxicity was evaluated using a human glioblastoma cell line (U87MG), 48 h after the addition of the formulations by MTS assay. Concentrations are referred to µg of plasmid, in order to compare the toxicities of formulations with the same polynucleotide loading minding that also all of them have the same N:P or N:P:C ratios.

PEI prototypes showed higher toxicity compared with the other prototypes (Figure 2.7.a). Moreover, the association of the 6MHA-PPZ did not show an improvement in nanoparticle toxicity on any of the preparations. This result was in contrast to our previous study where 6MHA-PPZ was combined with a cationic polyphosphazene and a reduction in cytotoxicity was observed [3]. The Lethal concentration of nanoparticles for the 50 % of the cells (LC50) for the formulations was also calculated (Figure 2.7.b), demonstrating a notably difference between the different polymers: PEI nanoparticles the highest toxicity and chitosan nanoparticles the lowest.

A



B

Cationic polymer	8:0:1	8:4:1
PEI	0.6471	0.4572
Protamine	1.586	0.9320
Chitosan	2.424	1.982

Figure 2.7 Viability determination based on cell metabolic activity of human glioblastoma cell line (U87MG) treated with different concentrations of nanoparticles (ratio 8:0:1 and 8:4:1) at 48 h post-treatment. A. Representation of cell viability at different concentrations of nanoparticles. B. Lethal concentration of the nanoparticles for the 50 % of cell population (LC50), this value was calculated by extrapolation after the logarithmic representation of the normalized nanoparticle concentration.

2.3.3 *In vitro* transfection

After the determination of the nanoparticle toxicity, transfection was measured 48 h post-treatment by luminescence at the concentration of 0.5 μg pDNA / cm^2. After cell transfection cells express luciferase, and this enzyme in the presence of its substrate luciferin provides a

quantifiable result. In order to compare the results, the luminescence was corrected by the quantity of protein and was expressed as relative luminescence units (RLU) per microgram of protein.

The transfection of the different nanoparticles with and without anionic polymer was compared in the figure 2.8.a, b and c. The adittion of 6MHA-PPZ to PEI and Protamine nanoparticles produced a significant increase in the transfection ($p < 0.05$). In the case of chitosan this effect was not observed, maybe due to its colloidal instability and low buffering capacity at physiological pH [20]. Because 6MHA-PPZ has endosomolytic properties, we were expecting that this polymer would benefit the most to complexes with poor endosomal escape properties. However this was not confirmed experimentally, as a polymer known for its efficient endosomal escape (PEI) presented some of the largest improvements on transfection.

Maybe because of the reasons stated above, chitosan nanoparticles provided the lowest transfection among the tested prototypes. Protamine is a protein with high arginine content, an aminoacid with cationic guanidine side groups that has been reported to facilitates cellular uptake and endosomal escape [21,22]. Still, protamine did not showed a high transfection efficiency under these conditions. In contrast, branched PEI, which presents a structure based on primary, secondary and tertiary amino groups, has shown the best transfection capacity, maybe because of the reported capacity of this polymer to promote efficient endosomal escape [23,24].

The transfection of the nanoparticles was also compared with Lipofectamine 2000 (Figure 2.8.d), used here as a benchmark. The systems with chitosan and protamine showed a transfection efficiency much lower than Lipofectamine. Cationic PEI nanoparticles showed 3-fold higher transfection than lipofectamine, while PEI/6MHA-PPZ nanoparticles showed transfection levels 20-fold higher than Lipofectamine (Figure 2.8.e), indicating a highly efficient gene delivery system.

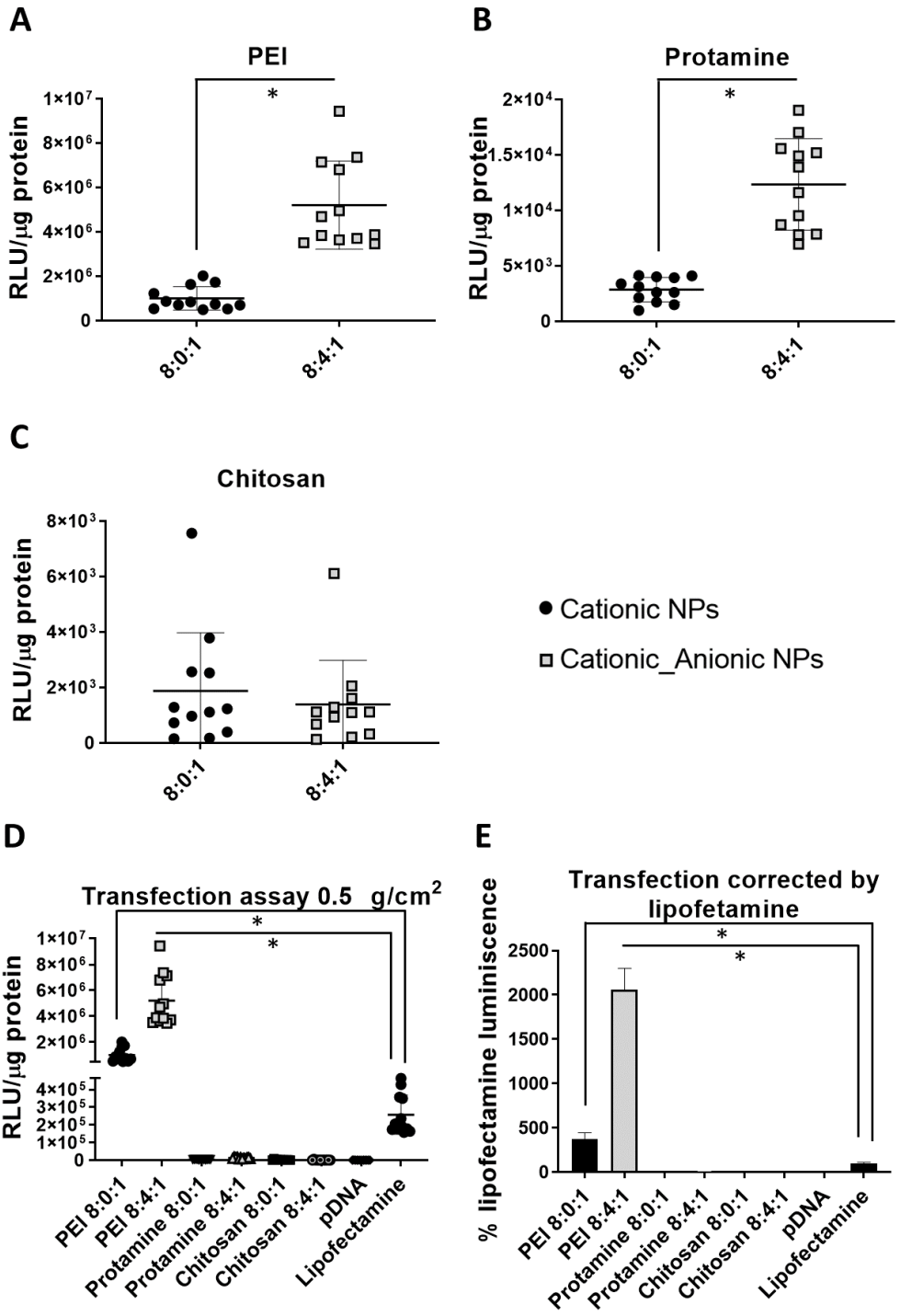


Figure 2.8 *In vitro* transfection in U87MG at 0.5 µg of pDNA / cm2. A,B,C. Transfection capacity of the different prototypes with or without 6MHA-PPZ, expressed as RLU corrected by µg protein. D. Comparison of the transfection capacity of all the prototypes with the Lipofectamine 2000 (positive control) and free plasmid (negative control). E. Transfection levels relative to Lipofectime (this group is taken a 100% luminiscence). RLU: Relative Luminescence Unit. *Statistical analysis at $p < 0.05$.

2.3.4 3D toxicity in a spheroid model

As a step closer to *in vivo* testing, protamine and PEI prototypes were selected to carry out toxicity assays in 3D neurosphere models. The chitosan prototypes were abandoned to simplify the experiments due to their lack of satisfactory transfection in the previous test. Various concentrations (0.33-6.67 µg/mL) were tested and the following parameters were studied: (I) modifications in the size and shape of the neurospheres, and (II) the metabolic activity (Resazurin assay) of the neurospheres at 72 h post- treatment.

The tumor size and morphology of the neurospheres were similar for those treated with the any of the nanoparticle prototypes and the negative control. The metabolic test supported also that the formulations are non-toxic at the studied concentrations (Figure 2.9)

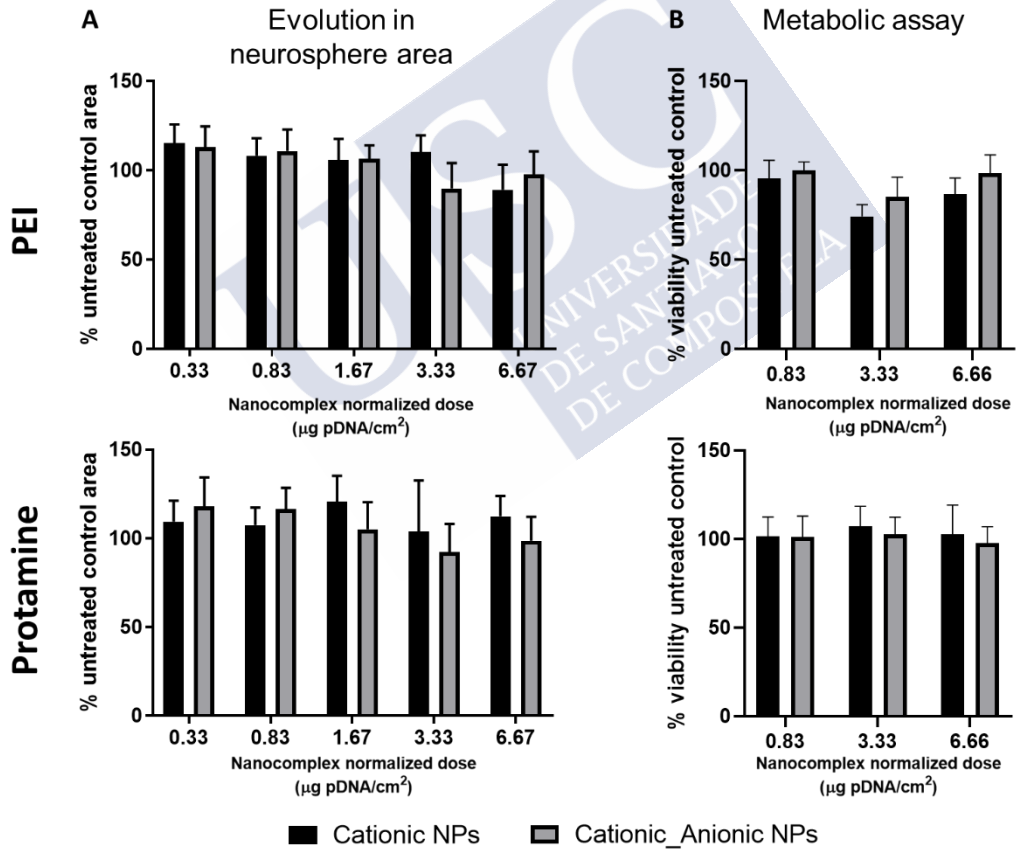


Figure 2.9 Toxicity in 3D culture models of human glioblastoma cell line (U87MG) after the treatment with the selected formulations at different nanoparticles concentrations. A. Area of neurospheres treated with different doses of nanoparticles. B. Metabolic activity (AlamarBlue assay) in neurospheres incubated with different doses of nanoparticles; measurements made 72 h post-treatment and calculated relative to the negative control (cell medium).

2.3.5 Fish Embryo Acute Toxicity (FET) Test

In this case, the formulations were diluted in reverse osmosis water at different concentrations of the plasmid, to compare the toxicity of the nanoparticles at the same loading capacity. The embryos were incubated 96 h and their evolution was studied every 24 h until the end of the study. The mortality of the negative control was less than 10% and the hatching rate was higher than 80% as required for the validity of the assay.

In this *in vivo* study, the toxicity of the formulation was dependent of the presence or absence of 6HMA-PPZ, being less toxic the formulations with this anionic polymer when tested at the same plasmid dose (Table 2.1, Figure 2.10). This was clearly seen on all parameters for chitosan and protamine prototypes but was also detected for PEI nanoparticles when considering the NOEC and LOEC parameters.

When comparing prototypes, PEI nanoparticles were the most toxic, which was expected based on the scientific literature [25,26]. On the other hand, both protamine and chitosan showed low toxicity at the studied concentrations. When combined with 6MHA-PPZ, protamine nanoparticles had a LC50 above the levels considered for the study. In the chitosan/6MHA-PPZ formulation, none of the toxicity parameters could be calculated as no toxicity events were detected at the concentrations tested.

Table 2.1. *In vivo* toxicity determined by Fish Embryo Acute Toxicity (FET) Test. Results are expressed in mg of cationic polymer/mL of osmosis water. LC50: Lethal concentration required to kill 50% of the population; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration. In some cases, the LC50 is lower than the NOEC, this is explained by the method used to calculate the NOEC, however the trends are better observed graphically.

	LC50	NOEC	LOEC
PEI 8:0:1	0.923	<0.5	<0.5
PEI 8:4:1	0.949	1.5	0.75
Protamine 8:0:1	1.925	0.75	0.5
Protamine 8:4:1	>3.5	3	2.25
Chitosan 8:0:1	2.375	2.25	1.5
Chitosan8:4:1	>3.5	>3.5	>3.5

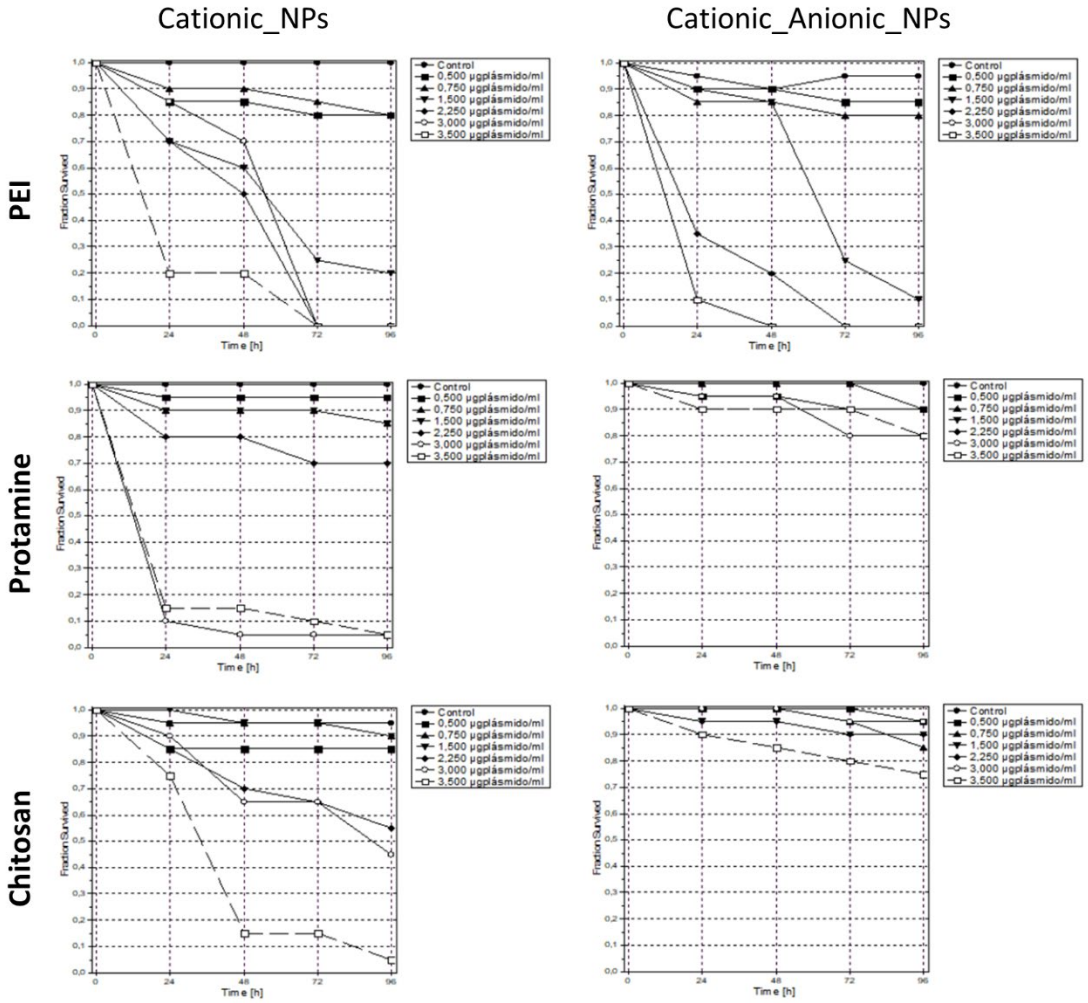


Figure 2.10. Survival of the introduced Danio rerio as observed under presence of the different prototypes.

2.4 Conclusions

Chitosan, Protamine and PEI are materials widely used in the preparation of nanocarriers, with promising results in gene delivery applications. These polymers can be combined with the anionic polyphosphazene 6MHA-PPZ to generate new combined nanocarriers. The presence of the 6MHA-PPZ in these nanoparticles produced limited difference in their physicochemical properties but increased the nanoparticle formation yield. The presence of 6MHA-PPZ also had limited effect on the *in vitro* toxicity of the nanoparticles, although a clear toxicity reduction was observed in zebrafish tests. In general, the presence of 6MHA-PPZ increases transfection efficacy by 3 to 5-fold as compared to the nanoparticles with only the cationic polymer. PEI/6MHA-PPZ nanoparticles showed remarkable transfection levels, up to 20-fold higher than those obtained with the reference reagent Lipofectamine 2000. These results highlight the benefits of combining 6MHA-PPZ with polymers having different amine types and hydrophobic regions.

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Chapter 3

New cationic polyphosphazenes for gene therapy

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Abstract

Different polymers containing primary amines in their structure have been synthesized, in order to find polymers with the appropriate properties for the preparation of nanosystems for gene therapy. These polymers were also associated with 6-mercaptohexanoic acid substituted poly(phosphazene), and endosomolytic polymer with capacity to improve transfection/toxicity ratios for some polymer gene delivery systems. The different nanoparticles obtained had a size between 100 and 150 nm, positive surface charge (+30-40 mV) and were able to reversibly associate nucleic acid. Nanoparticles based on cationic polymers with primary amines and aliphatic grafting groups showed lower toxicity compared to the rest of the prototypes and improved transfection capacity. Moreover, the generation of nanoparticles with the cationic/anionic polymer mixture showed reduced toxicity and even higher transfection capacity, with surpassed the test benchmark Lipofectamine 2000. The efficacy/toxicity ratios observed for this new polyphosphazene polyplex composition suggest their potential as gene delivery systems for *in vivo* application.

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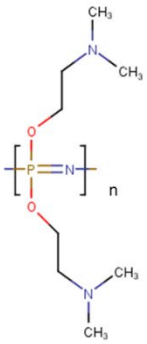
3.1 Introduction

Polyphosphazenes (PPZ) are polymers characterized by their inorganic backbone based on the alternation of phosphorus and nitrogen atoms. The phosphorous groups in these polymers have two side groups that can be modified with practically any substituent. Many PPZ are biodegradable, resulting in non-toxic metabolites, are biocompatible and low-toxic. But the most important characteristic of PPZ is their structural flexibility, since side groups with different characteristics can be easily introduced, resulting in remarkable changes in the materials properties. Recently, even some synthetic limitations in the synthesis of PPZs have started to be solved by the generation of secondary precursor polymers and grafting reactions through "click" chemistry approaches [1–3]. PPZs can be used alone or in mixtures with other polymers [4].

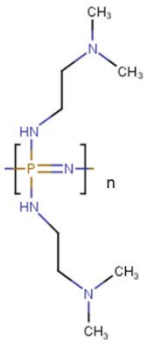
Because of the aforementioned properties, PPZ have been proposed as promising materials for the generation of drug delivery devices both for conventional [5–7] and biotechnological drugs [8], for advanced healthcare products and for tissue engineering devices [9,10]. The first FDA approved PPZ device is COBRA-PzF™, a coronary stent that contains cobalt chromium metallic backbone associated with a polyphosphazene. This platform reduces the risk of bleeding in patients with coronary heart disease, through its antithrombotic and anti-inflammatory effect, reducing the duration of the conventional antithrombotic therapy after the surgery (NCT02594501 and NCT03103620)[9].

Among these biomedical applications, PPZs have attracted much interest in gene delivery as their structural flexibility is well suited to the demanding functional requirements of this application. PPZs used in gene delivery are mostly cationic and form nanoparticles by condensation of the polynucleotides. As compared to other synthetic polymers such as polyethyleneimine (PEI), cationic PPZs have the additional advantage of being biodegradable [11,12]. Figure 3.1 shows the structure of some modified polyphosphazenes used in the preparation of vectors for gene therapy.

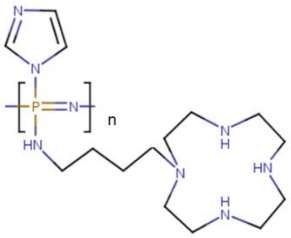
DMAE-PPZ



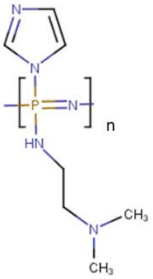
DMAEA-PPZ



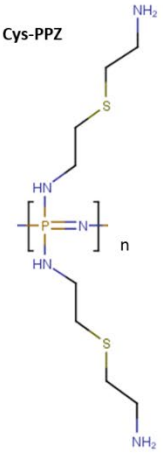
Im-PPZ-cyclen



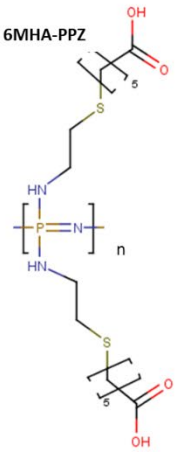
Im-PPZ-DMAEA



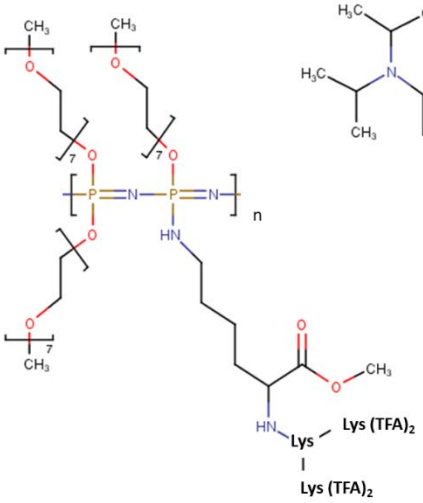
Cys-PPZ



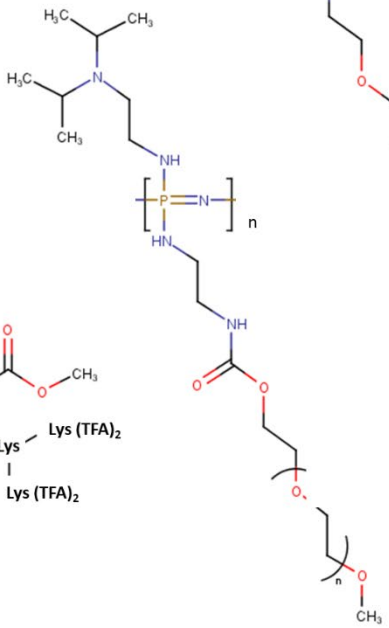
6MHA-PPZ



Tetra Lys-PPZ



PEG-PPZ-NPEA



Gal-PPZ-AEE

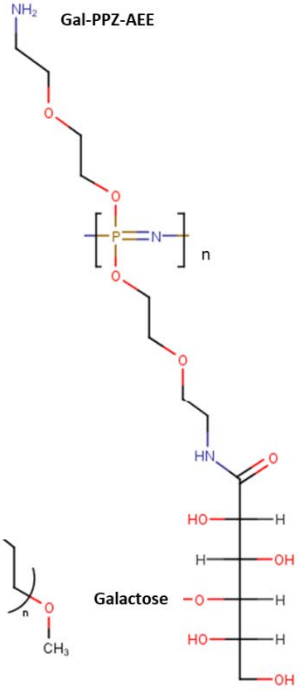


Figure 3.1 Structure of modified polyphosphazenes used in the preparation of platforms for gene therapy. DMAE-PPZ: Poly(2-di-methylaminoethanol)phosphazene; DMAEA-PPZ: Poly(2-dimethylamino ethylamino)phosphazene; Im-PPZ-cyclen): Poly(imidazole/1,4,7,10-tetraazyclodocane)phosphazene; Im-PPZ-DMAEA: Poly(imidazole/ 2-dimethylamino ethylamino)phosphazene; Cys-PPZ: Poly(2-((cysteamine)thiol)ethylamino)phosphazene; 6MHA-PPZ: Poly(2-((6-mercapto hexanoic acid)tiol)ethylamino)phosphazene; TetraLys-PPZ: Poly (tetra(L-lysine)-grafted) phosphazene; PEG-PPZ-NPEA: Poly-(Polyethilenglycol)(N,N-diisopropylethylenediamine)phosphazene; Gal-PPZ-AEE: Galactose modified Poly(2-(2-aminoethyoxy)-ethoxy)phosphazene.

The first cationic PPZs used in gene therapy was synthesized in 2003 by nucleophilic substitution of poly (dichlorophosphazene) with 2-dimethylaminoethanol (DMAE) or 2-dimethylaminoethylamine (DMAEA); both PPZs are thus grafted with tertiary amines. The nanosystems formed by these polymers showed adequate physicochemical properties and an acceptable toxicity/transfection ratio [13]. The efficacy of the polymer containing DMAEA was comparable with PEI in terms of transfection, but the polyphosphazene demonstrated an effect mainly localized in the tumor [14]. Moreover, this polyphosphazene was tested at different molecular weight and ratios to study their influence on the transfection efficiency of the nanoparticle. They showed that an increase on the molecular weight produces a reduction in the transfection capacity. Regarding the N:P ratio, the cationic polyphosphazene should be in excess to maintain the positive charge, but without reaching toxic levels [15].

To improve the efficiency of the previous prototypes, heteropolymers substituted with imidazole and another substituent such as DMAEA or cyclic polyamines were synthesized. These prototypes showed higher transfection than reference commercial agents as Lipofectamine and PEI nanoparticles, and with reduced toxicity [16,17].

By applying a click chemistry modification method, Hsu et al. was able to generate a small library of cationic and anionic PPZs for gene delivery. *In vitro* studies showed that PPZs with primary amines showed better transfection/toxicity ratio than complexes grafted with tertiary amines, and that the polymers with the best performance were those with 36 KDa of molecular weight. Moreover, analysis of the anionic PPZ library allowed the authors to identify a polymer, 6-mercaptohexanoic acid substituted poly(phosphazene) that significantly reduced toxicity and increased its transfection *in vitro* and *in vivo* [1]. Mechanistic studies

showed that this anionic polymer enhances transfection by improving endosomal escape [1]. Because of that, it could be hypothesized that this material would be particularly beneficial for nanocarriers having poor endosomal escape properties, however, studies performed by us with commercial polyplexes indicated a similar effect, independent of this characteristic, and that transfection enhancement was as robust for highly transfecting polymers (Chapter 2).

Based on this background, in this work our objective was, the synthesis of new cationic polyphosphazenes with improved gene delivery characteristics. Based on a click chemistry approach, a variety of heteropolymers were designed, which allowed us to investigate how different grafting groups can change physicochemical, toxicity and transfection characteristics. The same analysis was also performed with nanoparticles incorporating 6-mercaptohexanoic acid substituted poly(phosphazene), which were performed to optimize polymer combinations regarding their performance as gene delivery systems.

3.2. Materials and Methods

3.2.1 Materials

Aluminum chloride (99.99%), Anhydrous tetrahydrofuran (THF), Cysteamine (Cys), Chloroform-d (99.96 atom % D contains 0.03% TMS), Deuterium oxide (99.9 atom % D, contains 0.05 wt. % 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid), Heparin sodium salt (from porcine intestinal mucosa), HEPES (≥99.5%), Hexachlorocyclotriphosphazene ($(NPCl_2)_3$) (99%), Potassium chloride (BioXtra ≥99%), Triethylamine (TEA), Tris-Acetate-EDTA buffer (10x), 1-mercapto-2-propanol (MP), 2-butylamino)ethanethiol (BET), 2-(dimethylamine)ethanethiol hydrochloride (DMAES), 2,2,2-trifluoroethanol (TFE), 2,2-dimethoxy-2-phenylacetophenone (DMPA), 2-methyl-1-propanethiol (MPT), 6-mercaptohexanoic acid (6MHA) were all purchased from Sigma-Aldrich. DNAse/RNAse free water (Invitrogen), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), OptiMEM (Gibco), Fetal Bovine Serum (FBS) (Gibco), Penicillin-Streptomycin for culture medium (Gibco), Dialysis membrane (molar mass cut-off 7kDa), Lipofectamine 2000 Transfection reagent (Life Technologies), SYBR® Gold nucleic acid stain (Life Technologies) were purchased from ThermoFisher. Bio-Rad Protein Assay was provided by BioRad (CA, USA), Luciferase Reporter Gene Assay was bought from Roche (Germany), MTS Cell Proliferation Assay Kit was purchased to BioVision (USA), Alamar Blue were bought to Promega (Madrid, Spain). The pEGFPLuc plasmid was donated by Prof. Anxo Vidal laboratory (CiMUS, Universidad de Santiago de Compostela). All the products were used as received.

3.2.2 Synthesis of the precursor poly(allylamino-phosphazene) (AAPPZ)

The synthesis of the precursor was carried out following the protocol previously developed in our lab [1]. Briefly, in a previously dried flask 14.4 mmol of hexachlorocyclophosphazene ($(NPCl_2)_3$) with 7.5% aluminium chloride (w/w) (catalyst) was mixed in an inert atmosphere of nitrogen and heated at 240-250 °C for 3 hours. After the polymerization, the product was cooled to 120 °C and solubilized in diglyme to minimize crosslinking and avoid the solidification of the crude product. Then, it was centrifuged to remove the aluminum chloride (-10 °C, 7000 rcf, 5 min) and the supernatant was transferred to a flask with anhydrous THF, TEA (3 eq to chlorine) and allylamine (3 eq to chlorine). This reaction was maintained in an ice bath for one

day, and then another day at room temperature. The resulting product was filtered to remove TEA hydrochloride and subsequently precipitated with water, centrifuged (4 °C, 7000G, 10 min) and the precipitate was collected and dried under vacuum overnight. AAPPZ was characterized by ^{31}P, ^{1}H-NMR and DOSY.

3.2.3 Precursor radical modification by thiol-ene click chemistry

The side chains of AAPPZ were modified by thiol-ene click chemistry to introduce different radicals, the thiol group of the new compounds reacts with the allyl group of the AAPPZ obtaining five different polyphosphazenes (Fig. 3.2). Briefly, poly(allylamino-phosphazene) (AAPPZ) was dissolved in trifluoroethanol (TFE) and mixed with the desired substituent (3 eq to allyl group): cysteamine, 1-mercapto-2-propanol (MP), 2-(butylamine)ethanethiol (BET), 2-methyl-1-propanethiol(MPT), and 6-mercaptohexanoic acid (6MHA). The mixture was bubbled with nitrogen, and the catalyst 2,2-dimethoxy-2-phenylacetophenone (DMAES) (0.05 eq. to allyl group) was added to the reaction mixture. The reaction proceeded under magnetic stirring and UV irradiation (λ = 365 nm) for 3 hours. The resulting product was purified by dialysis (membrane molar mass cut-off 7 kDa) against HCl 2 mM for 24 hours and 48 hours against water. The dialysate was freeze-drying and then analyzed by ^{31}P and ^{1}H-NMR, COSY and HSQC.

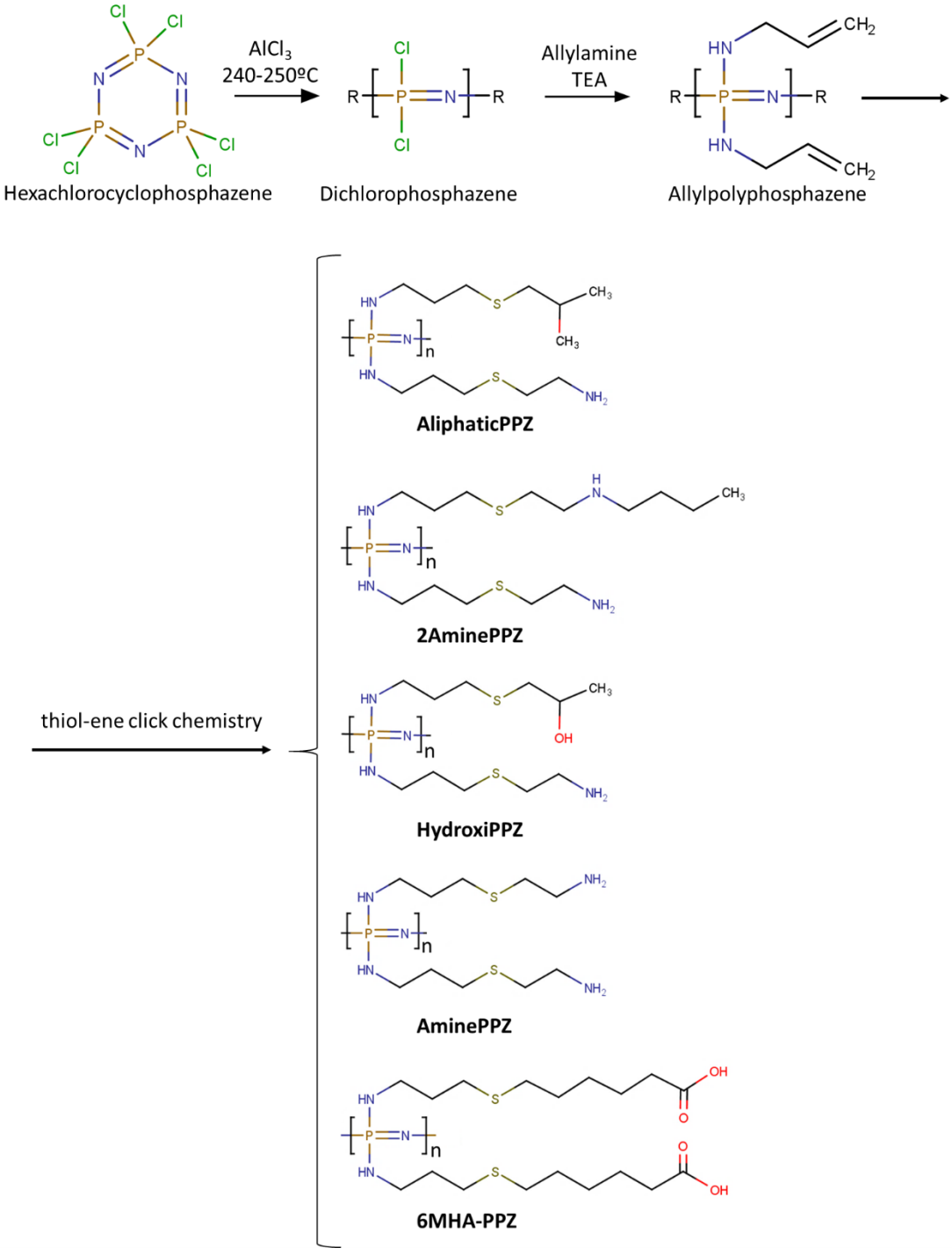


Figure 3.2. Scheme of polyphosphazene modification by thiol-ene click chemistry

3.2.4 Polymer characterization

3.2.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H and ^{31}P NMR spectra were obtained by Bruker 400 and DRX-500 spectrometers. For the bidimensional NMR (COSY and HSQC) were recorded by Varian Mercury 300 spectrometer. Solvents used for polymer dissolutions were $CDCl_3$ and D_2O and all chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) or known solvent peak positions.

3.2.4.2 Determination of polymer molecular weight

This experiment was carried out in collaboration with Dr. Federico Quatrinni. In order to determinate the molar masses, polymers were dissolved in NaCl 10mM at 5mg/mL and measured by Asymmetric Flow Field-Flow Fractionation (AF4), using an AF2000 MultiFlow FFF coupled to a Multi-Angle Light Scattering (MALS) (Postnova, Germany) detector. MALS was calibrated with BSA standard monomer (66 kDa) and QC performed with Pullulan standard (48.8 kDa) every day.

3.2.5 Amplification of the plasmid

To perform the optimization of the nanoparticles we used a plasmid (pEGFPLuc), which expresses two reporter proteins: enhanced Green Fluorescent Protein and Luciferase (Fig. 3.3).

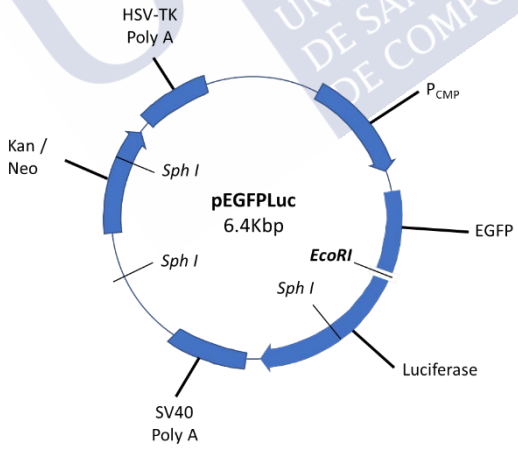


Figure 3.3 EGFPLuciferase plasmid map.

For plasmid amplification, competent E. coli DH5α bacteria were transformed with pEGFPLuc, resuspended in Luria-Bertani (LB) medium and incubated for 2 h at 37 °C with orbital agitation. Bacteria were selected through seeding on Petri dishes and supplementation of the media with the selection antibiotic (10µg/mL Kanamycin). The bacteria were incubated

in this selection media at 37 °C for 24 h. An isolated colony was grown in supplemented LB in the orbital incubator and amplified until reaching an adequate number of bacteria to perform plasmid extraction.

Plasmid extraction was performed with an Invitrogen™ PureLink™ HiPure Plasmid Gigaprep Kit (Thermo Fisher, USA) following the manufacturer's instructions. Briefly, bacteria were centrifuged at 5000 rcf for 15 min, the pellet was resuspended in a RNase solution to remove the RNA and mixed with a lysis buffer to release the intracellular content. In order to remove the cellular debris, a precipitation buffer was added to the solution and filtered. Then, the pDNA was purified using the by columns provided in the kit. Finally, the plasmid was precipitated, washed, and quantified by UV absorption (Nanodrop, ThermoFisher, USA).

3.2.6 Cationic-nanoparticle formation

The nanoparticles were prepared by ionic complexation, using the model plasmid pEGFPLuc. The polymers were dissolved in HEPES 10mM (pH 5.5) and the pDNA in pure water. Nanoparticles were formed upon electrostatic interaction of pDNA or pDNA/ anionic polymer mixtures, with the cationic polymer; the preparation was performed under magnetic stirring (500rpm, 5min) (Fig. 2.4). Different component ratios were investigated. For nanoparticles containing cationic polymer and pDNA, the ratios are based on the number of primary/ secondary amines of the polymer branches (N) and the phosphates of the pDNA (P). In this case, composition is defined as N:P ratio. In the case of nanoparticles that contain the anionic polymer 6MHA-PPZ, the amount of this material is quantified by the number of terminal carboxylic groups (C), and the composition of the nanoparticles is defined by the N:C:P ratio.

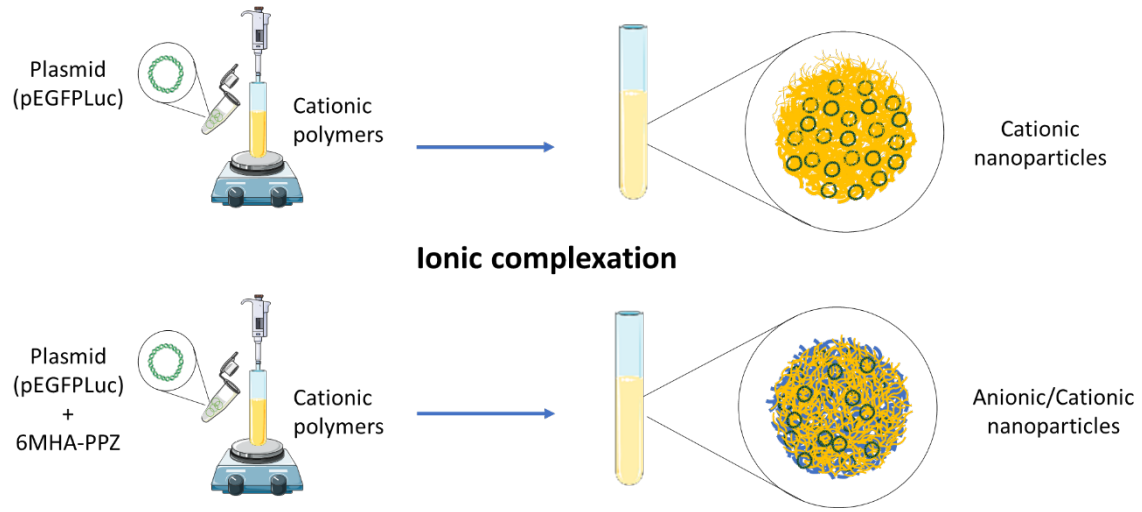


Figure 3.4. Nanoparticles preparation by ionic complexation

3.2.7 Nanoparticles characterization

3.2.7.1 Size, Zeta Potential and Concentration of Nanoparticles

The nanoparticles were characterized in terms of size, by Dynamic Light Scattering (DLS), and in terms of Zeta Potential, by laser doppler anemometry (Nanosizer ZS, Malvern, UK). Each analysis was performed in triplicate at 25 °C, with a backscatter angle of 173°. Nanoparticle size, distribution and concentration was also determined through Nanoparticle Tracking Analysis using a Nanosight NS300 system (Malvern Instruments, Worcestershire, UK) equipped with a laser operating at λ = 488 nm, after diluted the samples 1:400 in HEPES 10 mM. In the case of zeta potential, the measurements were performed upon dilution 1:10 in 1 mM KCl.

3.2.7.2 Morphological analysis of the nanoparticles

Morphological analysis of nanoparticles was done by Field Emission Scanning Electron Microscopy (FESEM) with Energy Dispersive X-ray spectroscopy (Zeiss Gemini Ultra Plus, Germany), using scanning transmission electron microscopy (STEM) and immersion lens (InLens) detectors for sample observation. For sample preparation, 10 µL of the nanoparticles were placed on a copper grid with carbon films and allowed to dry for 5 min; sample excess was removed by blotting. The sample was stained by adding the same volume of phosphotungstic acid (2% w/w in water). Afterwards the staining was washed by blotting and washed twice

with water. Once dried, the sample were observed through STEM and immersion lens (InLens) detectors.

3.2.7.3 Binding efficiency of nanoparticles

The binding efficiency of the nanoparticles was determined by gel retardation assay. The samples were loaded in an agarose gel (1% w/v in Tris-EDTA 1x buffer). Each well contained 0.33 µg of pDNA, and non-binding pDNA was used as control. For sample loading and visualization, all the samples contained 1 x SYBR® Gold nucleic acid stain and loading buffer (30% glycerol and 0.25% bromophenol blue). The dissociation assay was performed by incubating samples with an excess of an anionic competitor (20:1 w/w heparin: pDNA) for 1h at 37 °C.

3.2.8 Cell culture

All *in vitro* assays were performed in a U87MG glioblastoma cell model. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS) (Gibco, USA) and 1% (v/v) Penicillin/Streptomycin (P/S) (Gibco, USA) and incubated at 37°C (95% relative humidity and 5% CO_2) up to 85% confluence, when they were subcultured by trypsinization, dilution and plating.

3.2.9 *In vitro* toxicity

For 2D toxicity assays, 8,000 cells/well were seeded on a 96-multiwell plate and incubated 24 h before the treatment to allow cell attachment. Then, the nanoparticles were incubated in supplemented medium for 4 h at different pDNA concentrations (0.1- 2 µg pDNA/cm^2). After nanoparticle incubation, the medium was removed and replaced with fresh one, and cells were allowed to recuperate for 48 h. The cytotoxicity evaluation was performed adding 10 µl of MTS per well (BioVision, USA) and the absorbance was measured after 3 h of incubation in a plate reader at 495nm.

For a 3D toxicity assay, the nanoparticles were incubated with neurospheres. To form neurospheres, 300 U87MG cells/well were seeded on a ULA 96-multiwell plate (Ultra Low Attachment) by centrifugation (20 min, 200 rcf). After 3 days, the nanoparticles were incubated for 12 h at 2 µg pDNA/mL nanoparticle concentration. After 12 h nanoparticles were

replaced with fresh medium and the neurospheres were incubated for 72 h additional hours. Cell toxicity was quantified based on two parameters: (1) evolution on neurosphere size and shape at 0, 24, 48 and 72 h and (2) a colorimetric readout at 72 h in a resazurin reduction assay (CellTiter-Blue®, Promega, USA) that measures cell metabolism. For the CellTiter-Blue assay, 40µl of the reagent was added per well and incubated for 4 h with the cells. The fluorescence was evaluated on a plate reader at 539 nm of excitation wavelength and 620 nm of emission.

In all of the cytotoxicity tests, the negative control (no toxicity) was HEPES 10mM and the positive control (100% toxicity) was Triton 0.1%.

3.2.10 *In vitro* transfection

For the transfection assay, 56,000 U87MG cells/well were seeded on a 24-multiwell plate in DMEM medium supplemented with 10 % FBS and 1% P/S. After 24 h, nanoparticles were added at 0.5µg de pDNA / cm^2 in OptiMEM (Gibco, USA) medium and incubated for 4 h. Afterwards, the nanoparticles were washed and the medium was replaced by supplemented DMEM. The cells were allowed to grow for 48 h. To quantify transfection, we measured luciferase expression by a Luciferase Reporter Gene Assay (Roche, Germany). Briefly, the cells were washed twice with PBS and 100 µL of lysis buffer was added. After 5 min the lysate was centrifuged, and 50µL of the supernatant was collected and placed on a white plate and using an automatic injector. Then, 25 µL of luciferin from the commercial kit was added to the sample and before measurement in the luminometer (Mithras LB 940, Berthold).

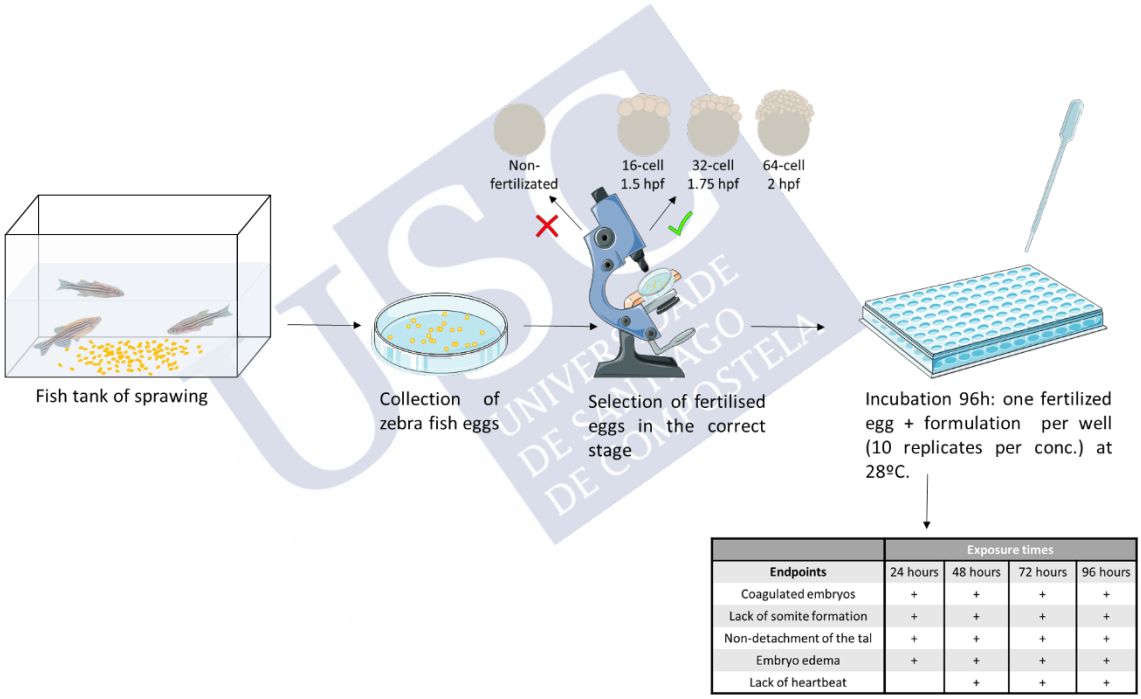
The results were corrected for protein content, quantified by a Bio-Rad Protein Assay (BioRad, USA). For this assay, 40 µL of the reagent were added to the sample and the absorbance was measured spectrophotometrically at 595nm.

3.2.11 Fish Embryo Acute Toxicity Test

This experiment was carried out in collaboration with the group of Prof. Laura Elena Sánchez Piñón. The experimental design has been carried out based on the protocol of the OECD (Organization for Economic Cooperation and Development) for the study of toxicity in fish embryos known as Fish embryo acute toxicity test [18]. Briefly, zebrafish fertilised eggs were selected 2-3 hours after fertilisation, at the stages of 16-32 cell blastomeres. Ten viable

fertilised eggs per group were each placed in a well of a 96-multiwell plate in reverse osmosis water with different concentrations of the nanoparticles. Embryos were incubated at 26±1 °C for 96 h and were observed on an inverted microscope every 24 hours until the end of the test, looking for toxicity signs. The observations performed to determine the toxicity include the detection of coagulation of embryos, lack of somite formation, non-detachment of tail, lack of heartbeat (after 48 h) or edema in the embryo (Figure 3.5).

To consider the experiments valid, in the negative control there must be a mortality rate ≤ 10% with a hatching rate> 80% at the end of the test (96 hours postfertilisation; hpf).



	Exposure times			
Endpoints	24 hours	48 hours	72 hours	96 hours
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tal	+	+	+	+
Embryo edema	+	+	+	+
Lack of heartbeat		+	+	+

Figure 3.5. Scheme of the zebrafish embryo acute toxicity test procedure. hpf = hours post-fertilisation.

3.2.11 Statistical analysis

Data were represented as mean ± standard deviation (SD). Statistical differences were calculated using the Student's t-test and one-way ANOVA in combination with Tukey's multiple comparisons test. The significance was set to $p < 0.05$. All the experiments were repeated three times, if not stated otherwise.

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3.3. Results

3.3.1 Polymer characterization

3.3.1.1 Nuclear Molecular Resonance (NMR)

New gene delivery systems were designed in this work based on homopolyphosphazenes with primary amines (PPZ- NH_2), and heteropolyphosphazenes with primary amine branches and other grafting groups of potential interest. Those structures were later used to analyze nanoparticle characteristics, their toxicity and transfection capacity.

First, the precursor poly(dichlorophophazene) was synthesized by open ring reaction after heating hexa(chlorocyclophosphazene) ($(NPCl_2)_3$) at high temperature. This method developed by Sohn et al. [19] avoids the presence of solvents used in the traditional methods of Allcock et al. [20,21]. High temperatures and the presence of a catalyst ($AlCl_3$) was used to reduce the polymerization duration and to control the molecular weight of the polymer [19,22,23]. Then the chlorine side-groups of the poly(dichlorophosphazene) were subsequently substituted with a primary amine as described Allcock in 1966 [21,24]; in this case, with allylamine [1].

The ^{31}P-NMR spectra provided proof of successful conversion of the monomer into the final allylamine substituted polyphosphazene (AAPPZ; Fig. 3.2., upper part of the figure). AAPPZ showed a ^{31}P-NMR with a unique peak at 3.6 ppm (Fig. S3.1.a). This value is in agreement with our characterization of this material [1] and with other references from the literature. It also indicates that the polymerization reaction and the substitution of poly(dichlorophosphazene) has occurred successfully, since the characteristic ^{31}P-NMR peaks of the monomer (20 ppm) and the precursor polymer (-20 ppm) could not be detected [25,26]. The side grafting groups of AAPPZ were also analyzed by ^{1}H-NMR (Figure S3.1.b). The most characteristic peak of AAPPZ were the protons at the vinyl group at 4.8 and 6 ppm (Fig. 3.3.b, groups “c” and “d”). A DOSY-NMR experiment confirmed that all groups visible in the AAPPZ spectra are part of the same molecule (Figure S3.1.b).

The next step was the substitution of the precursor with the different groups following the reaction described in the Figure 3.2. The substituted polymers were also isolated by dialysis

and characterized by NMR. ^{31}P-NMR of the modified polymers showed also single ^{31}P-NMR peaks in similar position to AAPPZ (4.4-5 ppm) (Figure S3.2), and thus, this technique did not allow us to confirm the success of the substitution reaction. In this case, ^{1}H-NMR provided much clear indications. First because it was possible to observe in all the cases that the signal from the vinyl protons (4.8-6 ppm) disappear, which was an indication of complete addition of the thiol grafting groups.

After the AAPPZ modification, two structures are generated, depending on which of the vinyl carbons is attacked during the addiction reaction. Therefore, the product results in both a majority form (≈90% addiction at the terminal carbon) and a minority form (≈10% addiction at the intermediate carbon). ^{1}H-NMR spectra of the polymers show three common peaks to all the modified polymers (a, b and c; Fig. 3.6) typical of the substituted allylamine in the majority form. A small peak at 1.4 ppm correspond to group c' (Fig. 3.6) in the minority form.

Homopolyphosphazenes; AminePPZ and 6MHA-PPZ have previously been synthetized by us. AminePPZ is a cationic polymer with capacity to condense gene material and deliver pDNA with higher efficiency than similar structures based on pendent tertiary amines. This compound is used here as benchmark to analyze whether we could improve their gene deliver characteristics. 6MHA-PPZ was used in this study because of its intrinsic capacity to reduce nanoparticles toxicity and improve the gene delivery efficiency. NMR spectra of these polymers (Fig. 3.6 and S3.3) corresponded to that previously reported by us [1].

On the other hand, AliPPZ, 2AminePPZ and HydroxiPPZ are heteropolyphosphazenes having the cysteamine group and a second grafted group. To the best of our knowledge, these compounds have never been reported in the literature. The chemical composition of the polymers was verified by analyzing the ^{1}H-NMR spectra (Fig. 3.6) or upon analysis of ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC 2D spectra (Fig. S3.3).

Integration of characteristic peaks in these heteropolyphosphazenes allowed us to investigate the real proportion of the grafting radicals (Fig. 3.6; R_1 and R_2) for each heteropolymer. For AliPPZ we found a substitution close to 50% for each substituent. In 2AminePPZ the substitution is 68% of the cysteamine radical and 32% for the 2- (butylamine)

ethanethiol radical. For HydroxiPPZ the substitution was 33% for the cysteamine group and 66% for the 1-mercapto-2-propanol radical.

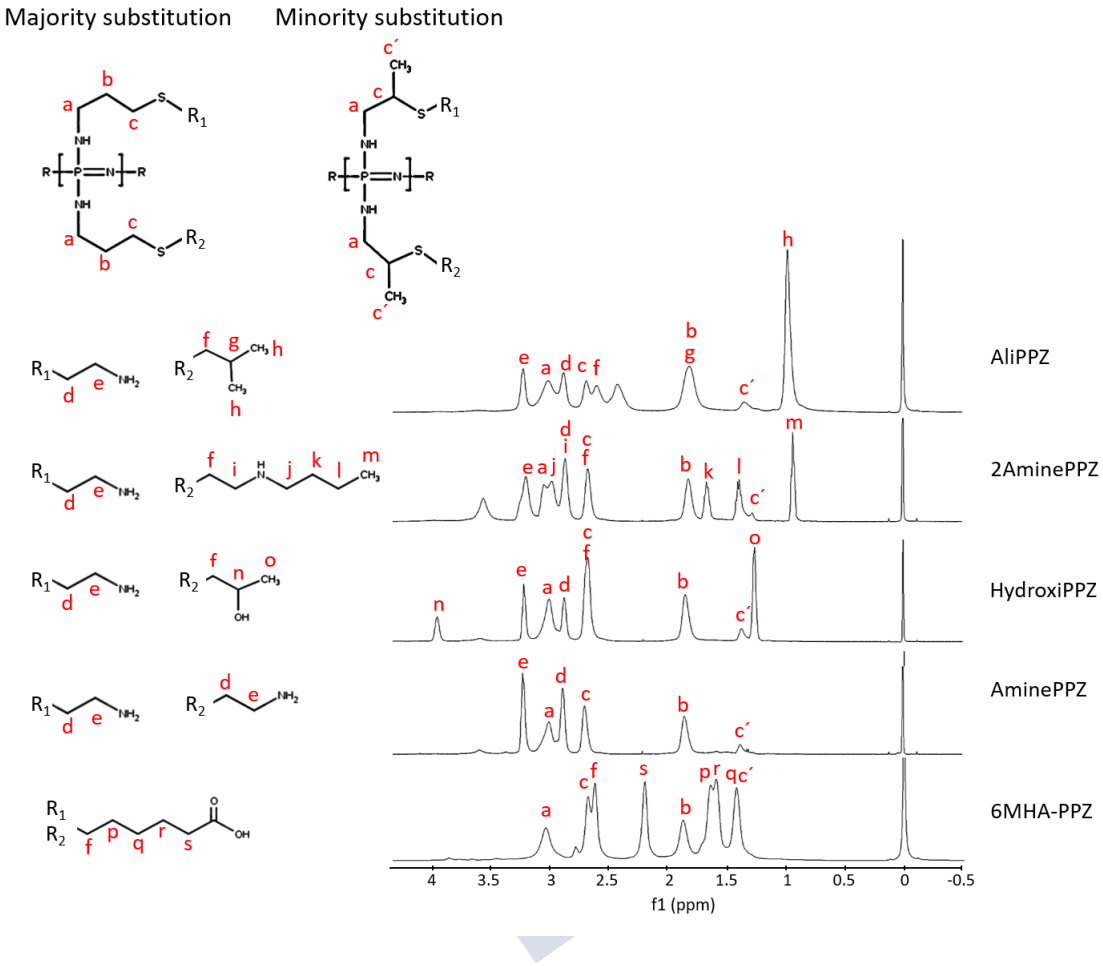


Figure 3.6 NMR-^{1}H spectra of the synthetic polymers.

3.3.1.2 Polymer molecular weight (Mw) determination

Polymer molecular mass were determined by Asymmetric Flow Field Flow Fraction (AF4). As previously described, the Mw of the polymer increases exponentially with the concentration of catalyst used. With a concentration of 2-5% aluminium chloride (w/w) the Mw obtained is in the 10^{4}-10^{5} Da range [19,27]. In this work, we used 7.5% (w/w) based on the results previously obtained by our group [1].

The molecular mass of the polymer was on the expected range for this catalysed reaction: the number-average molar mass (Mn) for all the polyphosphazenes was on the 50-100 KDa range, while their weight average molar mass (Mw) between 60 and 150 KDa (Table 3.1).

These values are also far lower than those achieved with other non-catalysed synthesis [28,29]. The molecular mass range was selected based on our previous screening with AminePPZ where this range showed the best compromise between toxicity and efficacy [1].

The polydispersity index (Đ) shows the broadness of the molecular mass distribution. In step polymerization reactions this polydispersity is around 2 [30](Table 3.1 and Figure S3.4). For the polyphosphazenes synthesized here, the polydispersity was 1.2-1.4, which corresponds to acceptable values.

Table 3.1 Polymer molecular weight and distribution measured by AF4. Mw: Molecular weight; Mn: number-average molar mass; Đ: polydispersity index.

	Mw (g/mol)	**Mn(g/mol)**	**Đ**
AliPPZ	1.48±0.01 x10^5	1.08 ±0.1 x10^5	1.37
2AminePPZ	1.10±0.1 x10^5	7.98±0.5 x10^4	1.37
HidroxyPPZ	8.18±0.1 x10^4	6.5±0.9 x10^4	1.26
AminePPZ	1.19±0.1 x10^5	8.83±1.3 x10^4	1.35
6MHA-PPZ	6.16±0.3 x10^4	5.11±0.2 x10^4	1.21

3.3.2. Characterization of polyphosphazene based polycomplexes

3.3.2.1 Size, Zeta Potential and Concentration of Nanoparticles

For the optimization of the nanoparticles, we analyzed how the Polymer:Plasmid ratio affects the size and surface charge of the nanoparticles. The ratios are expressed as the proportion of ionic charges of cationic amines (N), anionic carboxyls (C) and DNA phosphates (P). In the first step, cationic nanoparticles were prepared by mixing cationic polyphosphazenes and pDNA at different ratios and under magnetic stirring. The nanoparticles were characterized for particle size and zeta potential.

All particles, independently of the cationic polyphosphazene used showed a particle size within a small range (95-150 nm). The smallest nanoparticles were prepared with 2AminePPZ, while the largest ones were prepared with AliPZZ (Table 3.2). It was also observed that particle

size increased with higher N:P ratio. In all cases, particle size distribution was monodispersed and it only reached a PDI of 0.3 for AliPPZ nanoparticles at maximum N:P ratio tested (16:1).

Zeta potential for all the nanoparticles was clearly positive (> 30 mV) and very similar for all cationic polymers except for HydroxiPPZ; this polymer led to the systems with the lowest surface charges. Zeta potential increased with higher N:P ratio, although the differences between the values at 8:1 and 16:1 N:P ratio were small. Based on the balance between small size, low PDI and high charge we selected the 8:1 N:P ratio for further experiments.

Table 3.2 Cationic nanoparticles characterization by hydrodynamic size and zeta potential.

Cationic PPZ	Nanoparticles (N:P)	Size (nm)	PDI	Zeta Potential (mV)
AliPPZ	4:1	131±2	0.2	+46±3
	8:1	141±4	0.2	+54±1
	16:1	149±6	0.3	+54±2
2AminePPZ	4:1	96±7	0.2	+48±3
	8:1	110±5	0.1	+53±4
	16:1	122±5	0.2	+56±2
HydroxiPPZ	4:1	101±3	0.1	+31±2
	8:1	116±2	0.1	+34±4
	16:1	117±3	0.1	+41±3
AminePPZ	4:1	110±7	0.2	+46±2
	8:1	129±7	0.1	+51±1
	16:1	128±5	0.2	+54±1

For the preparation of nanoparticles containing the anionic polyphosphazenes, the plasmid was first mixed with the anionic polymer (6MHA-PPZ), and then this mixture was dropped into the cationic polyphosphazene solution under magnetic stirring. As previously stated, the N:P ratio was always maintained at 8:1.

The addition of the anionic polymer did not affect the size of the nanoparticles and was similar when analyzed by Dynamic Light Scatering (DLS) and by Nanotracking Analysis (NTA). A slight reduction in the polydispersity was observed in the nanoparticles containing anionic polymer (Figure 3.7.a and 3.7.d). Zeta potential decreased slightly for the AliphaticPPZ and 2AminePPZ 8:4:1 prototypes, although they maintain net positive values, and did not change for the prototype made with HydroxiPPZ and AminePPZ (Figure 3.7.b).

Derived count rate and concentration of 8:4:1 nanoparticles were around 3-fold higher compared to the ones containing only cationic polymers and similar between the different polymers, which implies an improvement in the nanoparticle formation yield (Figure 3.7.c and 3.7.d). The relationship between size and nanoparticle concentration was also represented in the Figure 3.7.e, particles containing the anionic polymer showed a better-defined peak and higher concentration in the mean size. When analyzing a plot of scattering intensity vs. particle size (Figure S3.5), the data also indicates higher intensity signals for nanoparticles with the 6MHA-PPZ. Perhaps the addition of the anionic polymer does not change substantially the surface charge of the particles because it increases the number of particles by allowing higher amount of the cationic material to be complexed.

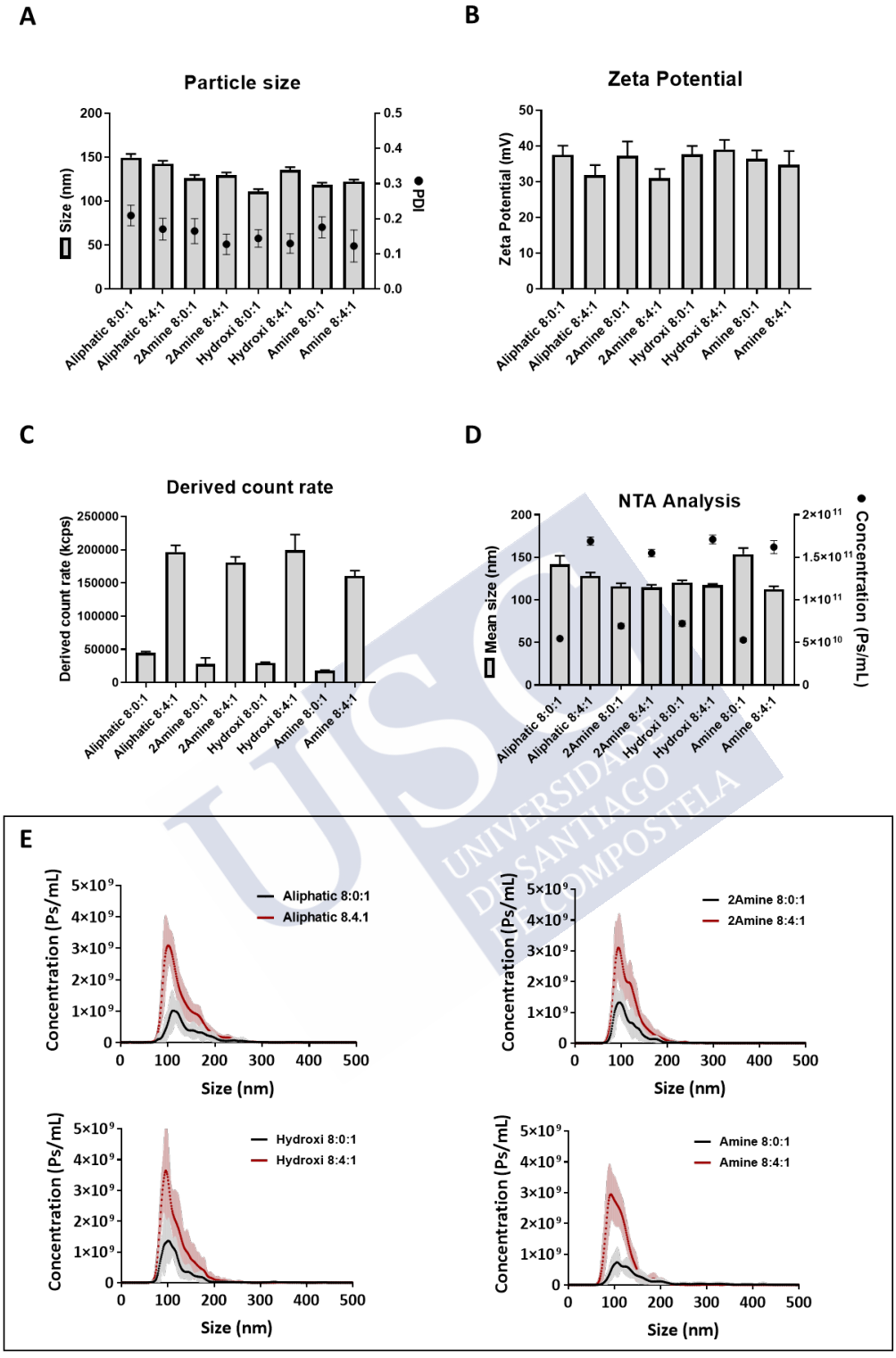


Figure 3.7. Characterization of cationic and cationic/anionic nanoparticles by Dynamic Light Scattering, Laser Doppler Anemometry and Nanoparticle Tracking Analysis. A. Particle size and polydispersity B. Surface Charge and C. Total Derived Count Rate, D. Average size of nanoparticles measured by NTA and E. nanoparticle size distribution and concentration, shaded areas correspond to the standard deviations of particle size. KCPs: kilocounts per second.

3.3.2.2 Morphological analysis of the nanoparticles

The nanoparticle prototypes were also imaged by FESEM (Field Emission Scanning Electron Microscopy) to characterize their morphology and dried particle diameter. This analysis combines scanning transmission electron microscopy (STEM) and immersion lens detectors. In all the images, the particles are spherical and the ones containing anionic polymer are more stained, which may be indicative of a higher density compare to the cationic ones. In the images it can also be observed that the particle size remains in a range similar to that obtained previously by other techniques (Figure 3.8).

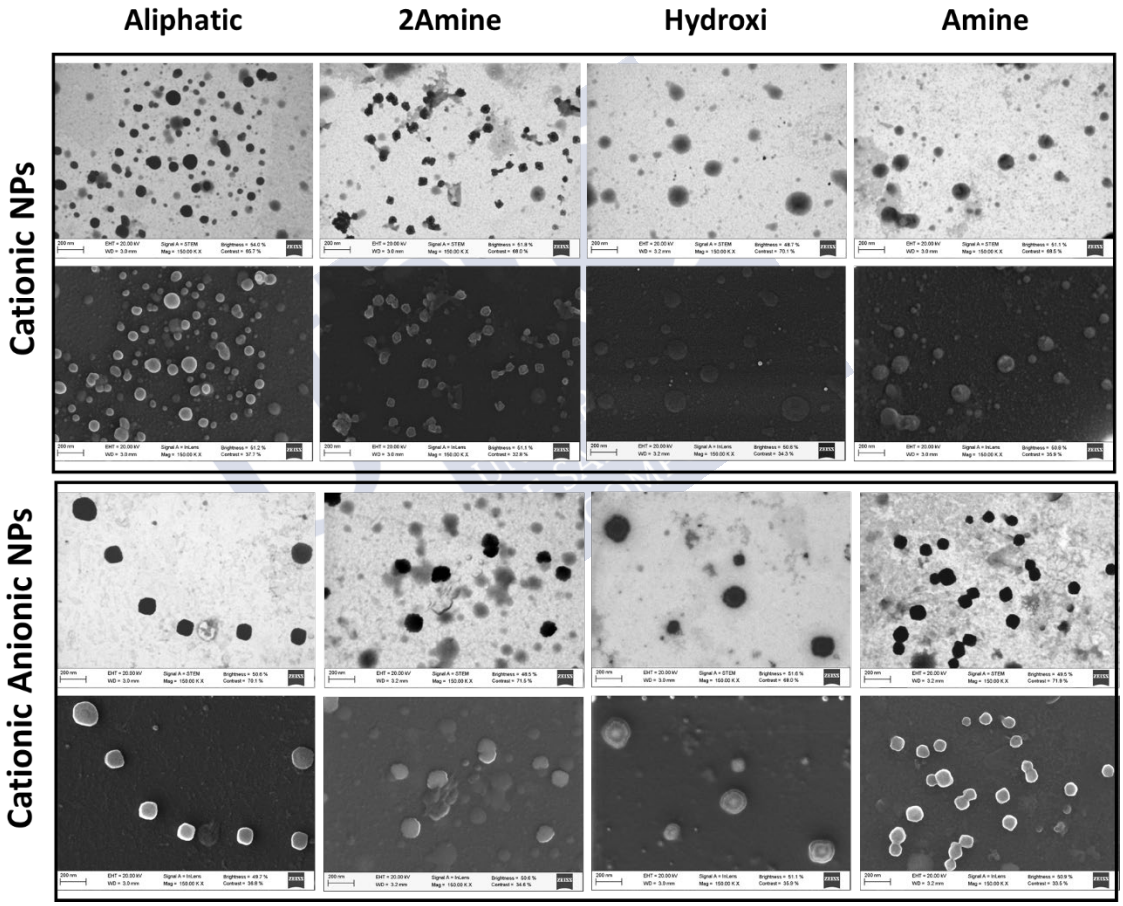


Figure 3.8. Nanoparticle morphological characterization by electronic microscopy FESEM. Transmission electron microscopy (grey images) and scanning electron microscopy (black images).

3.3.2.3 Binding efficiency of nanoparticles

In addition to the physicochemical characterization, we also verified the ability of the particles to bind efficiently the pDNA and its dissociation in the presence of a competing polyelectrolyte, which demonstrates that pDNA does not bind irreversibly to the vehicle and might be released by intracellular conditions. In the agarose gel image (no competitor) (Figure 3.9.a), no migration bands were observed, demonstrating that nanoparticles associate completely the plasmid. After the incubation of the nanoparticles for 1 h with the competitor heparin, the gel shows migration bands resulting from the dissociation of the plasmid from the polymer (Figure 3.9.b).

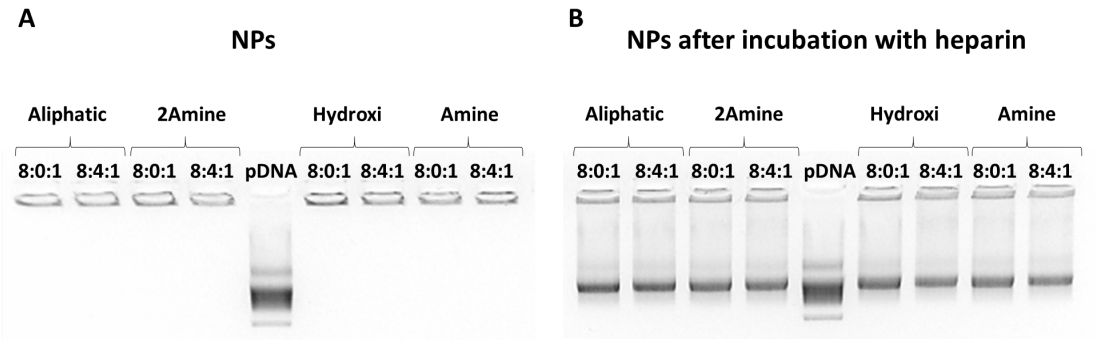


Figure 3.9. Binding efficiency of the nanosystems by gel retardation assay. A. No bands were observed in the gel corresponding to the nanoparticles, only the band of the free plasmid can be observed. B. Plasmid dissociation after the incubation of the nanoparticles with heparin as anionic competitor.

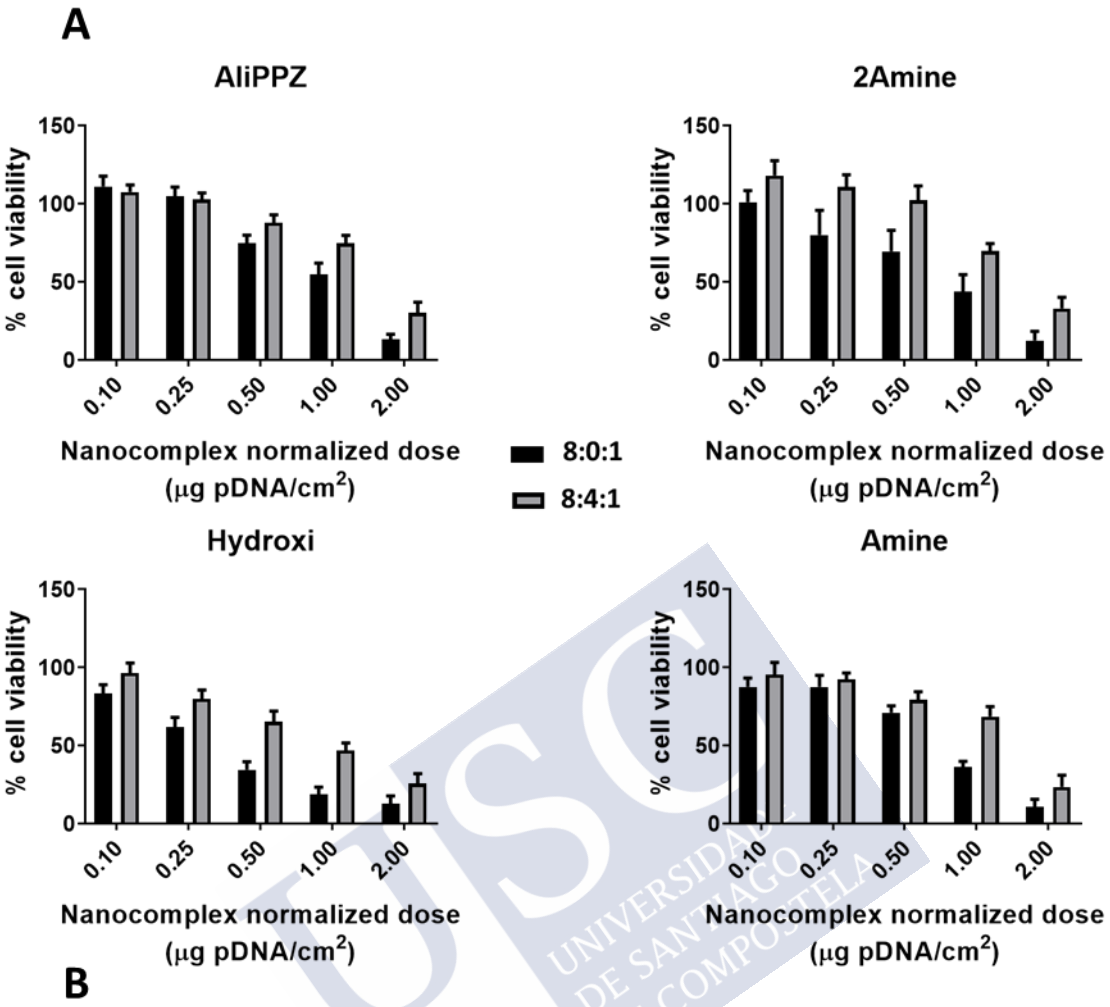
3.3.4 *In vitro* toxicity

Once several prototypes with adequate physicochemical properties had been obtained, the toxicity of the formulations in 2D cultures was tested. The toxicity was evaluated using a human glioblastoma cell line (U87MG), 48 h after the addition of the formulations and by MTS assay. Concentrations are expressed in µg of plasmid in order to compare the toxicities of formulations with the same polynucleotide loading. Note also that because all formulations were prepared at the same N:P ratio, the study was also performed with the same amount of cationic groups for the different nanoparticles.

In all cases there was a decrease in the toxicity of the nanoparticles containing the anionic polymer compared to the same formulations without this component (Figure 3.10.a). Lethal concentration 50 (LC50) was also determined to study how the addition of 6MHA-PPZ

influences in the toxicity of the nanoparticles (Figure S3.6; Figure 3.10.b). It is accepted that the addition of most anionic polymers can improve the cytotoxicity profile of nanocomplexes by reducing the charge density nanoparticles [31]. The lethal concentration in the case of nanoparticles with the 6MHA-PPZ was approximately double as compared to the same nanoparticles without this polymer. This result is in agreement with previous studies from our group performed with AminePPZs and a group of anionic PPZs [1].

When comparing the different prototypes with respect to the cationic polymer, some articles have shown that the substitution of a polymer with hydrophobic radicals reduces toxicity [32]. These considerations can explain why the prototypes with the most hydrophobic radicals (AliPPZ and 2AminePPZ) showed the highest LC50.



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Cationic PPZ	Ratio 8:0:1	Ratio 8:4:1
AliPPZ	0.999	1.46
2AminePPZ	0.78	1.48
HydroxiPPZ	0.338	0.86
AminePPZ	0.742	1.24

Figure 3.10. Viability determination based on cell metabolic activity of human glioblastoma cell line (U87MG) treated with different concentrations of nanoparticles (ratio 8:0:1 and 8:4:1) at 48 h post-treatment. A. Representation of cell viability at different concentrations of nanoparticles. B. LC50 (μg pDNA/cm^2) values of the nanoparticles calculated by extrapolation after the logarithmic representation of the normalized data.

3.3.5 *In vitro* transfection

After the determination of the nanoparticle toxicity, transfection was studied at the concentration of 0.5 μg pDNA / cm^2. When the plasmid is transfected, it co-expresses eGFP

(enhanced green fluorescent protein) that stains the cells (qualitative result), and luciferase that in the presence of its substrate luciferin provides a quantifiable result. In order to compare the results, the luminescence was corrected by the quantity of protein and was expressed as relative luminescence units (RLU) per microgram of protein.

After 48 h post-treatment, the transfection of the different particles was determined (Figure 3.11.a). It was observed that the transfection of the different cationic nanoparticles was modest and typically 100-fold lower than the benchmark (Lipofectamine 2000). With nanoparticles incorporating the 6MHA-PPZ the transfections were much higher; in fact, HydroxiPPZ and AminePPZ were statistically equal to lipofectamine even at $p < 0.05$. Two exceptions can be noted: first, 2AminePPZ nanoparticles did not show very high transfection even when mixed with the 6MHA-PPZ and were significantly below lipofectamine. Second, AliPPZ nanoparticles with the 6MHA-PPZ exceeded by almost 3-fold the transfection levels of lipofectamine ($p < 0.001$) and by a similar amount the Amine/6MHA-PPZ prototype, recently reported by us [1]. Probably due to the hydrophobic radical, this polymer is capable of interacting with the cell membranes, facilitating internalization and endosomal escape [32–34]. The differences in transfection could also be observed qualitatively by analyzing GFP expression with fluorescence microscopy (Figure 3.11.c).

When analyzing how the incorporation of the 6MHA-PPZ affected the overall transfection, we could observe that there was a significant increase ($p < 0.001$) in all cases but 2Amine nanoparticles (Figure 3.11.b). AminePPZ and HidroxyPPZ nanoparticles had a 100-fold increase when combined with the anionic polymer. The largest increase in transfection was observed for AliPPZ nanoparticles with the anionic polymer (almost 300-fold).

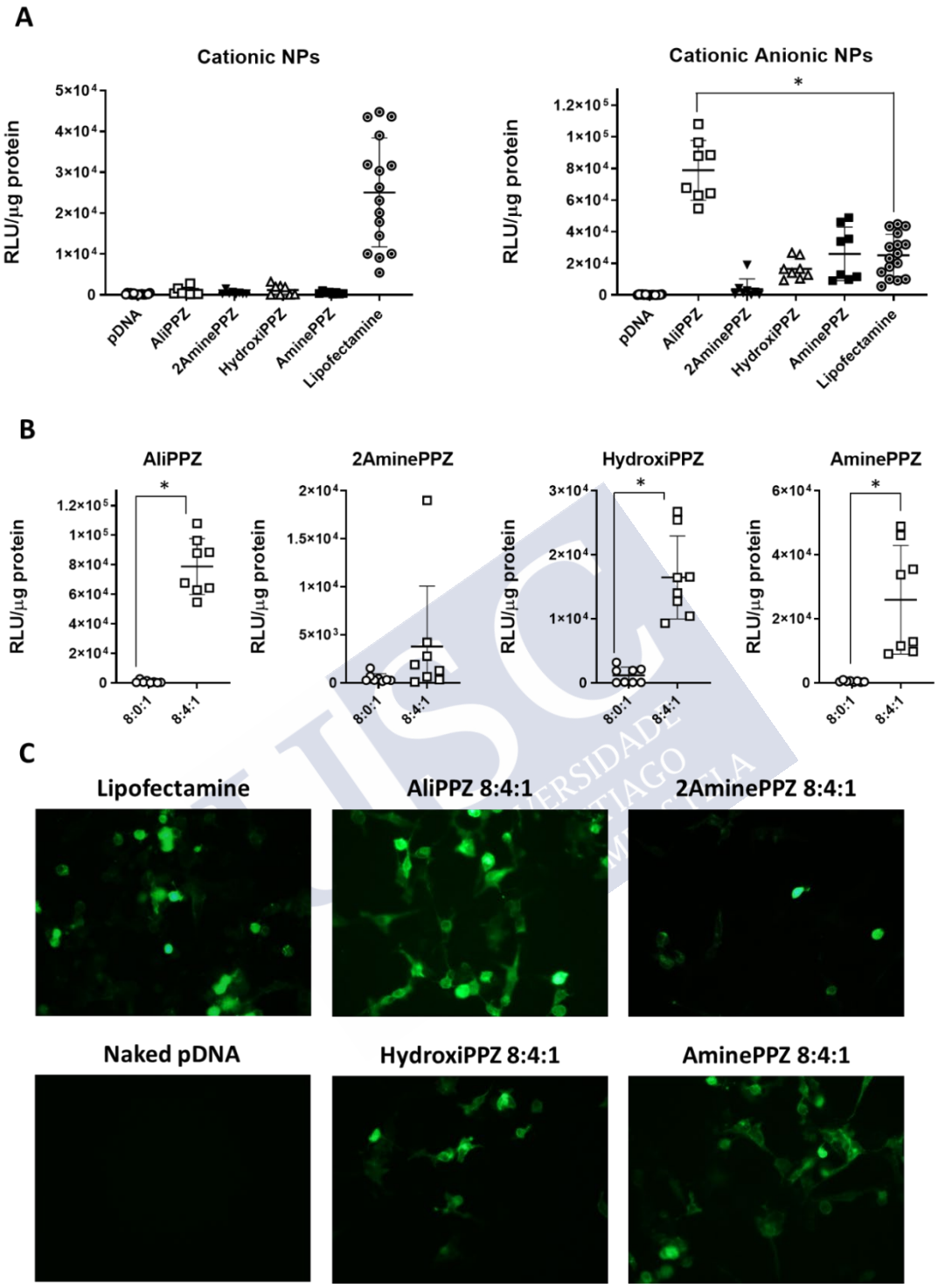

Figure 3.11. Transfection in bidimensional cultures of human glioblastoma cell line (U87MG) measured 48 h post-transfection by luminescence after the addition of luciferin substrate (A and B) and fluorescence microscopy (C). A. Comparison of transfection capacity for the different polymers in the cationic NPs and Cationic/Anionic-NPs, between them. Results are expressed as RLU corrected by quantity of protein. B. Comparison of the nanoparticles with or without the anionic polymer. C. Qualitive transfection of the Cationic/Anionic NPs by green fluorescence microscopy after 48 h pos-treatment. * Statistical analysis at $p < 0.001$. RLU: relative luminescence units.

3.3.6 Toxicity on a 3D spheroid model

As an intermediate model, closer to *in vivo*, 3D culture toxicity tests were performed. Only the prototype with the best transfection results (AliPPZ) and AminePPZ, to compare with our previous study [1], were selected to test the 3D toxicity. The concentration tested was 2 µg pDNA/mL for all of them. The parameters analyzed as indicators of toxicity were: (i) modifications in the size and shape of the neurospheres every 24 h until 72 h, and (ii) the metabolic activity of the neurospheres at 72 h.

Similar growth and morphology were observed in all neurospheres, either treated with the negative control or the formulations, indicating a lack of toxicity for the nanoparticles. On the other hand, dissociation and decrease in size was observed in the neurospheres treated with triton (positive control, Figure 3.12.a and S3.7). The metabolic test also supported the conclusion that the formulations are non-toxic at the tested concentrations (Figure 3.12.b).

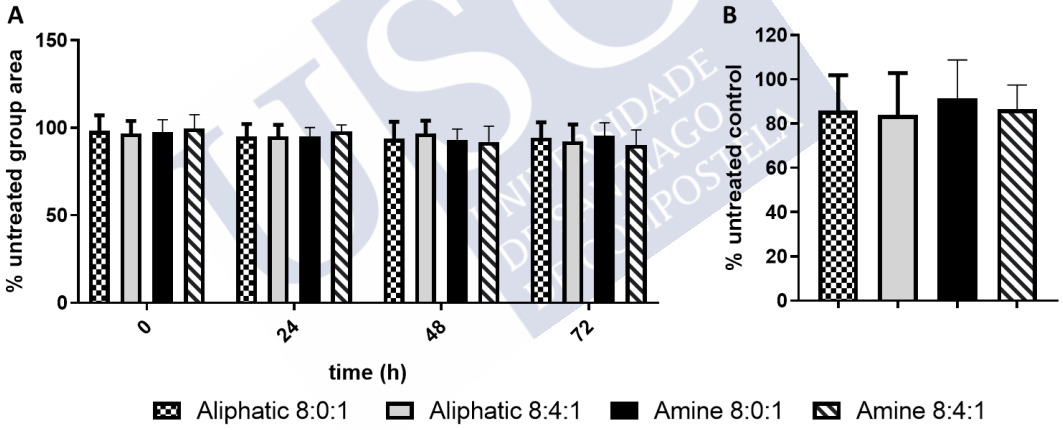

Figure 3.12. Toxicity in 3D culture models of human glioblastoma cell line (U87MG) after the treatment with the selected formulations at 2 µg pDNA / mL. A. Evolution of the area in the neurospheres treated with the nanoparticles compare with the untreated. B. Alamar Blue assay to determine the metabolic activity 72 h post-treatment compare to the untreated control.

3.3.7 Fish Embryo Acute Toxicity (FET) Test

The embryos were incubated with the nanoparticles for 96 h and their evolution was studied every 24 h until the end of the study. The mortality of the negative control was less than 10% and the hatching rate was higher than 80% as required for the validity of this test [18].

In all the cases the LC50 was higher for the nanoparticles containing the anionic polymer, results are consistent with the tendency found *in vitro*. However, the concentrations cannot be compared directly since the exposure times are higher. The results indicate that AliPPZ nanoparticles are the least toxic (Table 3.3 and Figure S3.8). However, it must be considered that due to its lower amount of charged amines the concentration of the AliphaticPPZ almost doubles that of AminePPZ and 2AminePPZ in our nanoparticles. Therefore, if considering the toxicity with respect to the number of amines, all formulations presented similar data.

Table 3.3. *In vivo* toxicity determined by Fish Embryo Acute Toxicity (FET) Test. Results are expressed in mg of cationic polymer/mL of osmosis water. LC50: Lethal concentration required to kill 50% of the population; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration.

	LC50 (mg/l)	**NOEC**	**LOEC**
Aliphatic 8:0:1	>10	<1	<1
Aliphatic 8:4:1	16.052	10	20
2Amine 8:0:1	5.854	2.5	5
2Amine 8:4:1	>10	7.5	10
Hydroxi 8:0:1	5.886	2.5	5
Hydroxi 8:4:1	9.647	5	7.5
Amine 8:0:1	5.521	1	2.5
Amine 8:4:1	9.98	10	20

3.4. Conclusions

Three new cationic heteropolymers were synthesized, having terminal primary amines and another chemical group. Nanoparticles were prepared from the complexation of these polymers and plasmid DNA, either with or without the anionic polymer 6-mercaptohexanoic acid substituted poly(phosphazene). All prototypes showed comparable properties in terms of size, surface charge, and efficiency of nucleic acid binding. The prototype with better results in terms of toxicity and transfection *in vitro* and *in vivo*, was the one composed by the heteropolymer containing an aliphatic radical (AliphaticPPZ) and complexed with the anionic homopolymer (6MHA-PPZ). This prototype showed a transfection efficiency three times higher than the one obtained with the commercial transfectant (Lipofectamine 2000). This prototype presents remarkable efficacy/toxicity ratio that indicates a promising design, suggesting the interest of additional studies with a therapeutic plasmid *in vitro* and *in vivo* models of disease.

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3.5 References

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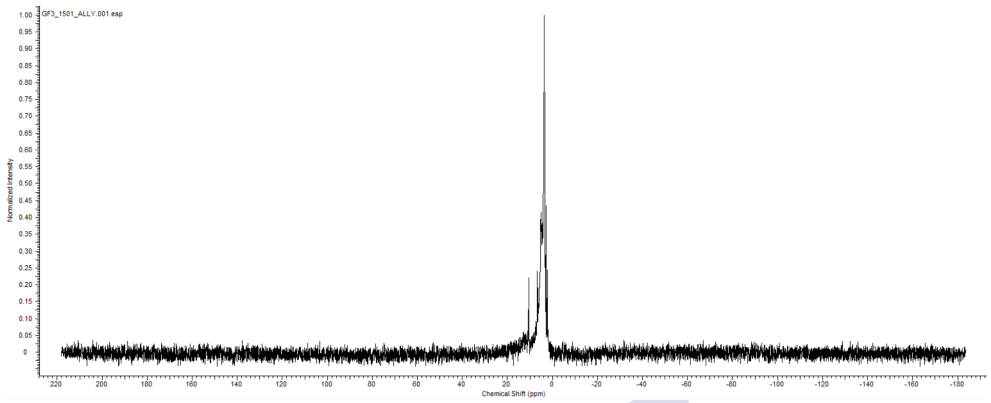
Figure S3.1. NMR characterization of the Allylamine precursor (AAPPZ). (a) ^{31}P-NMR (b) ^{1}H-NMR and DOSY spectra.

A



B

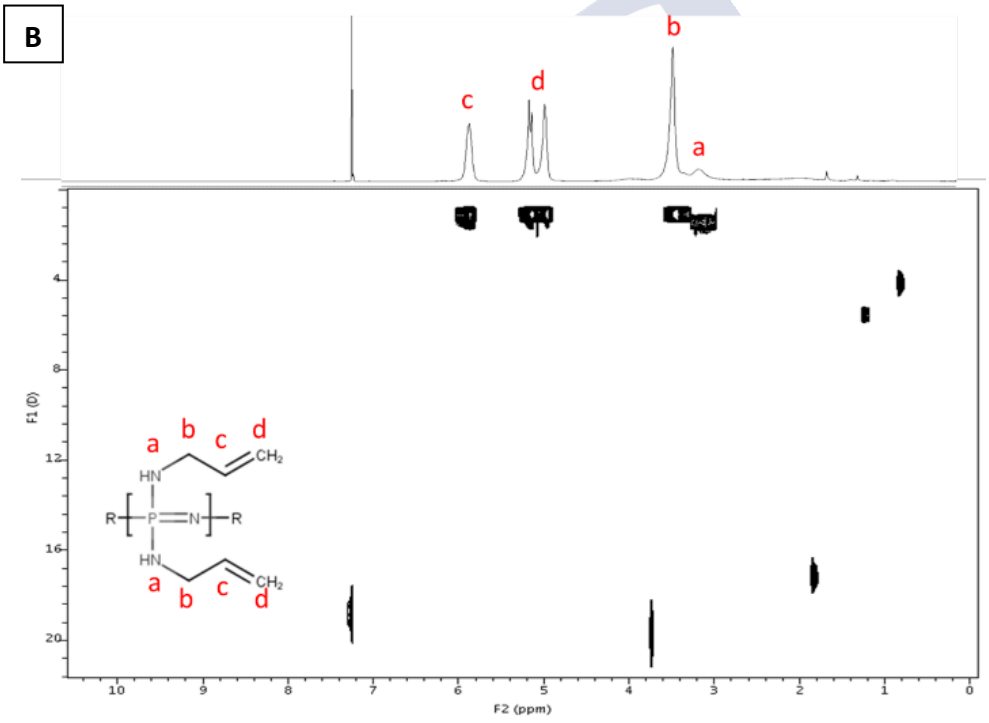
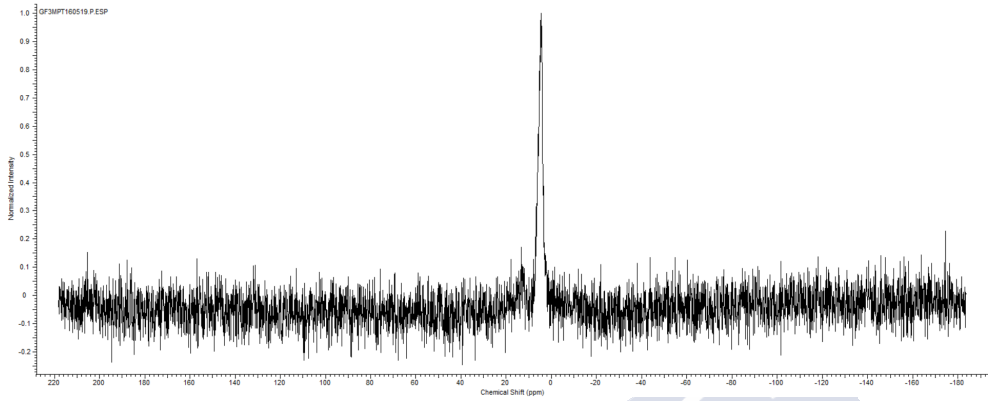
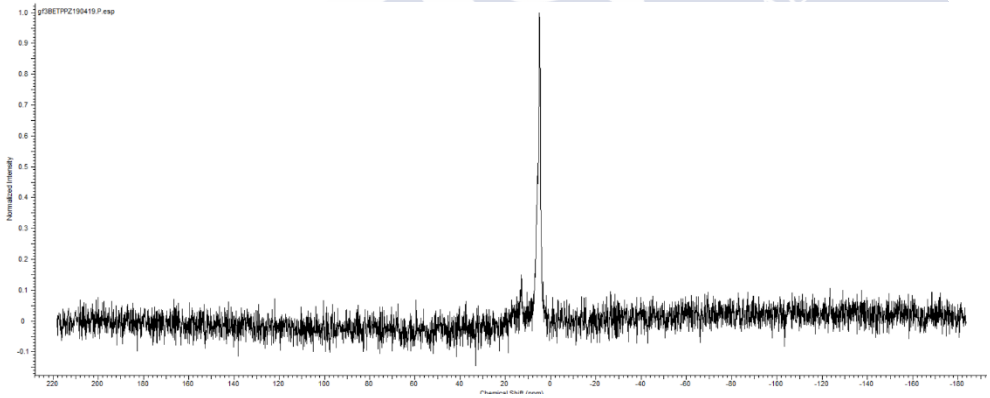


Figure S3.2. ^{31}P-NMR spectra of different substituted polyphosphazenes.

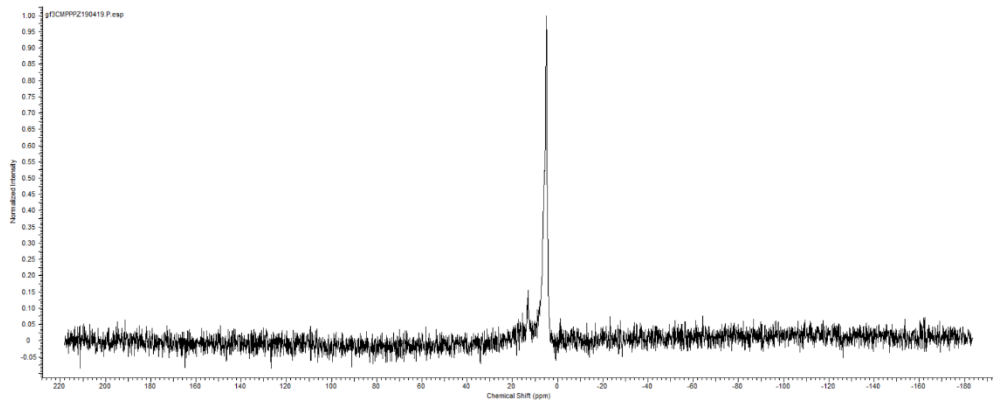
AliPPZ



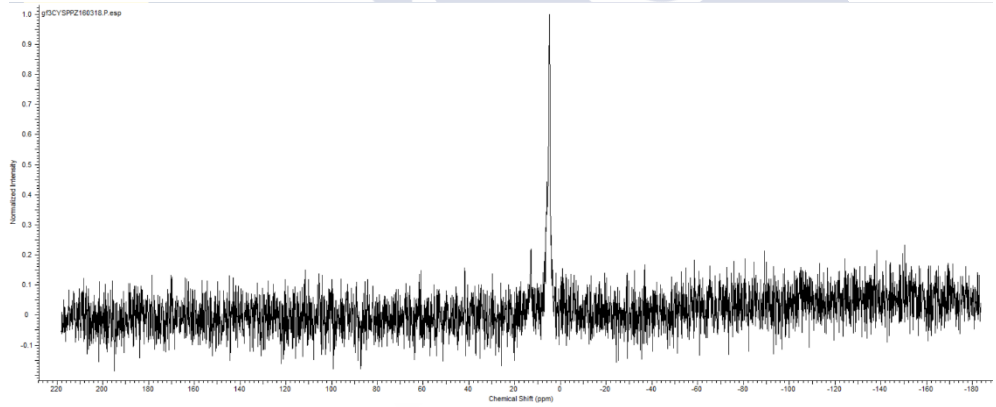
2AminePPZ



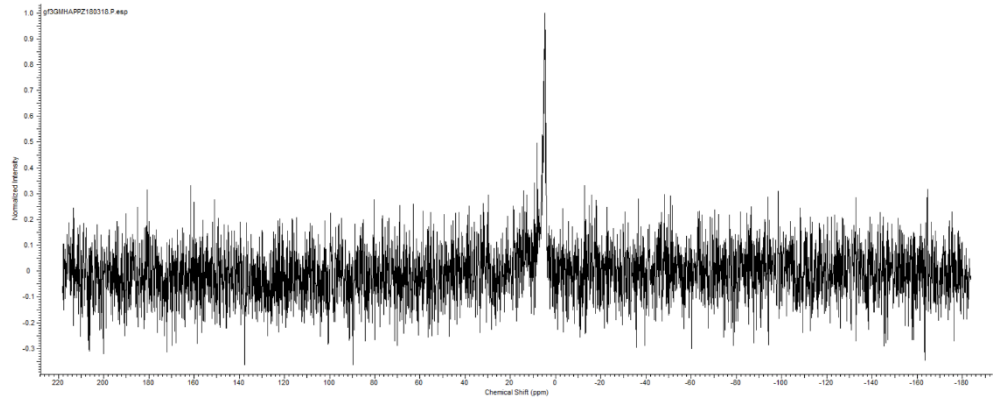
AminePPZ



HydroxiPPZ



6MHA-PPZ



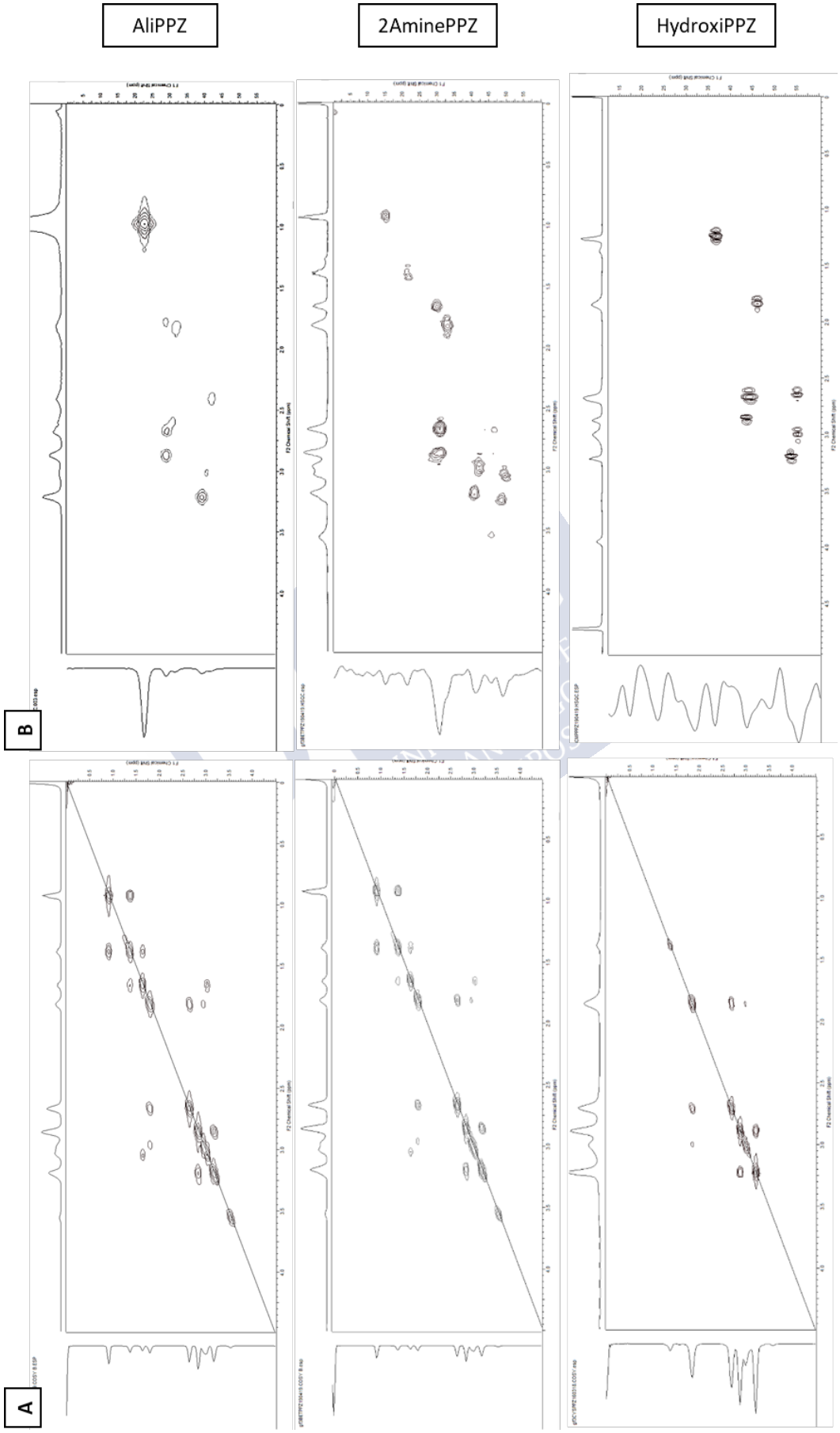


Figure S3.3. Comparison of bidimensional-NMR spectra of the different polymers. (a) ^{1}H-^{1}H COSY spectra and (b) ^{1}H-^{13}C HSQC spectra.

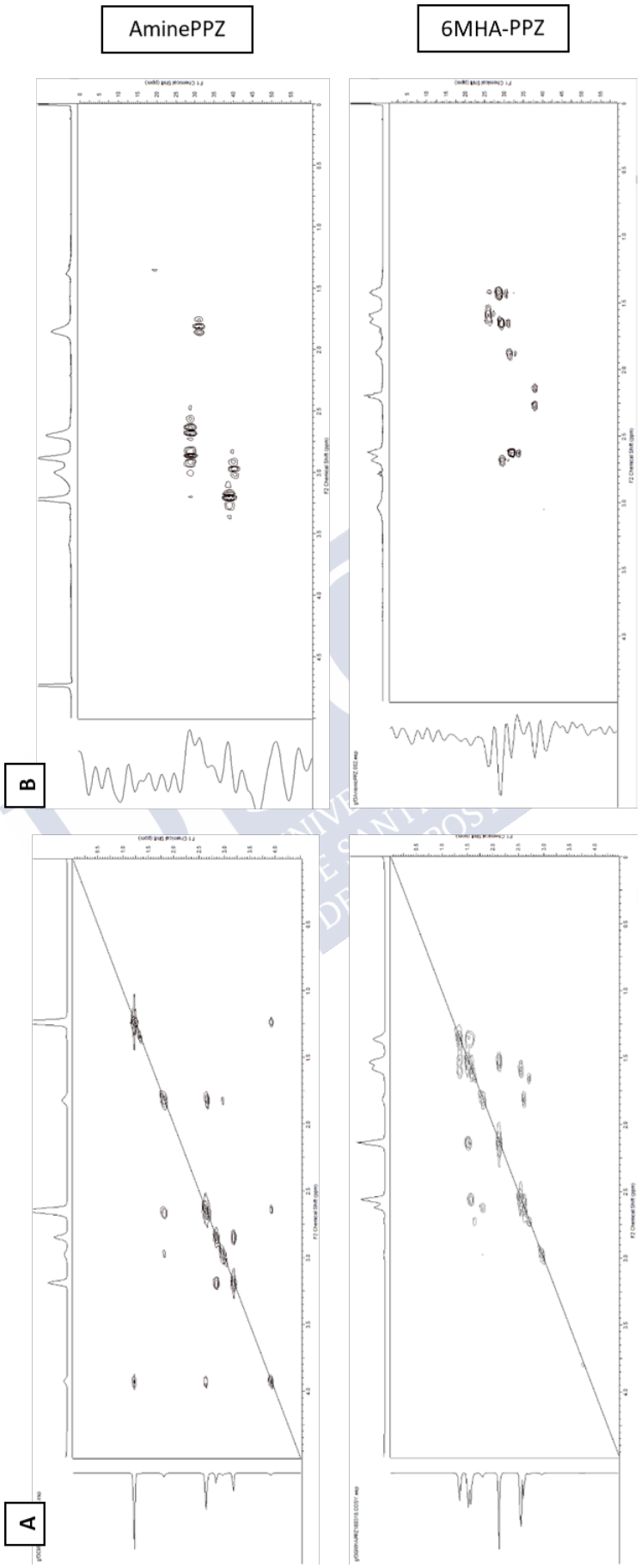
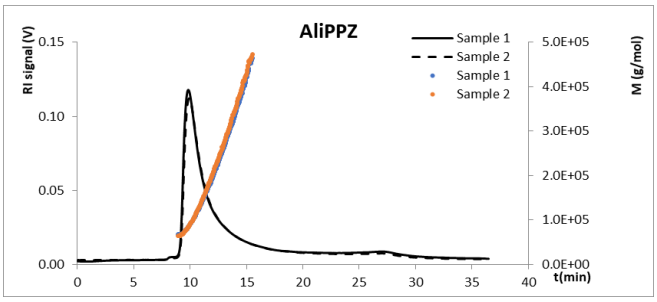
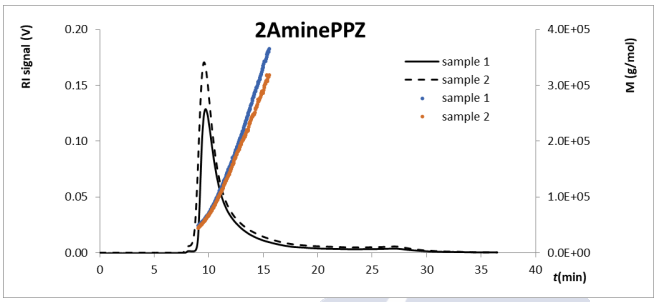
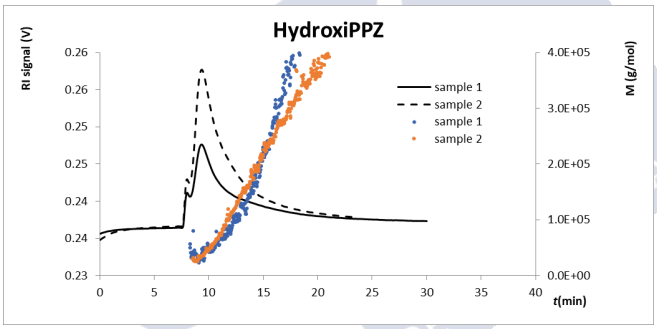
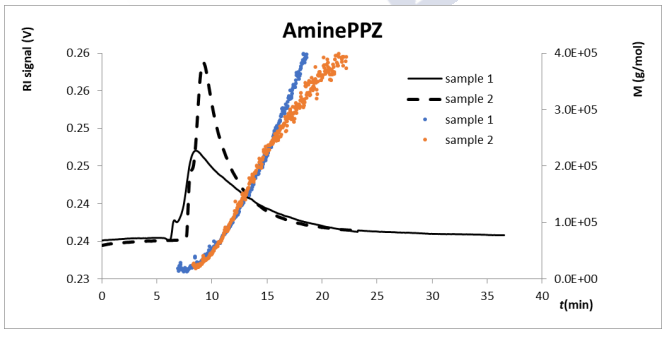

AminePPZ
6MHA-PPZ
B
A

Figure S3.4. Fractograms of Asymetric Flow Field-Flow Fraction (AF4) (n=2).









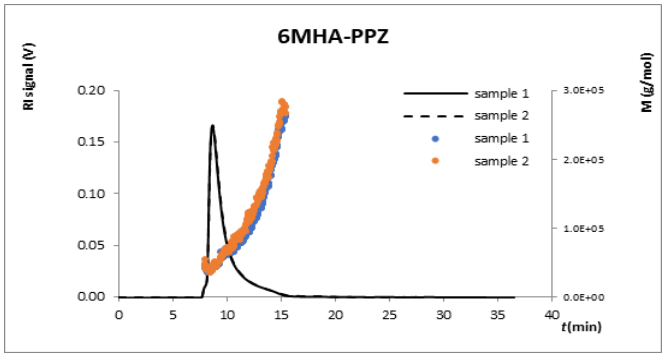


Figure S3.5. Relation between the intensity and the size of the nanoparticles measured by Nanoparticle tracking analysis.

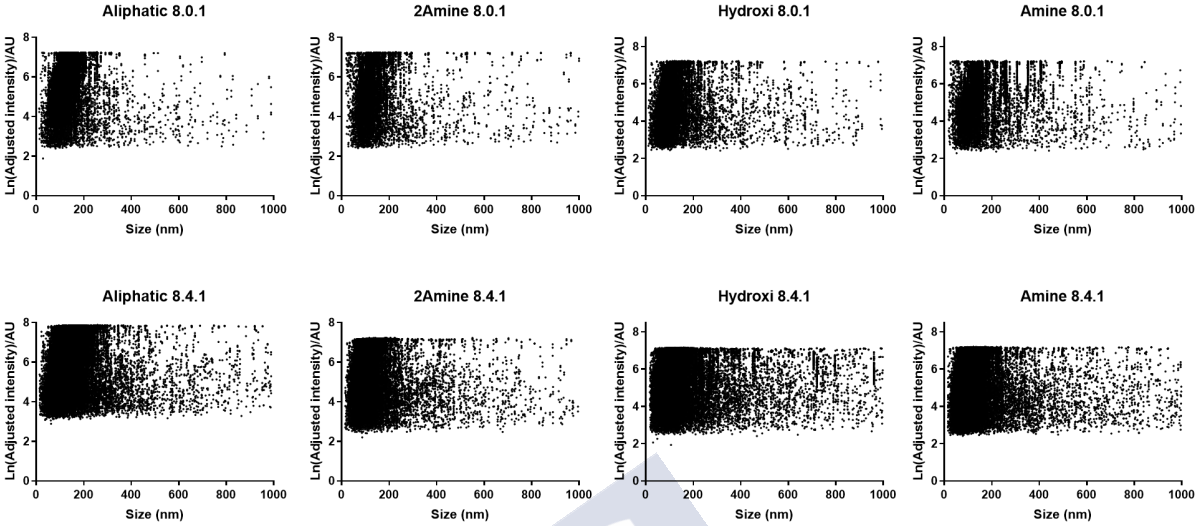


Figure S3.6. Logarithmic representations of nanoparticle concentration expressed as μg pDNA/cm^2, to determine LC50.

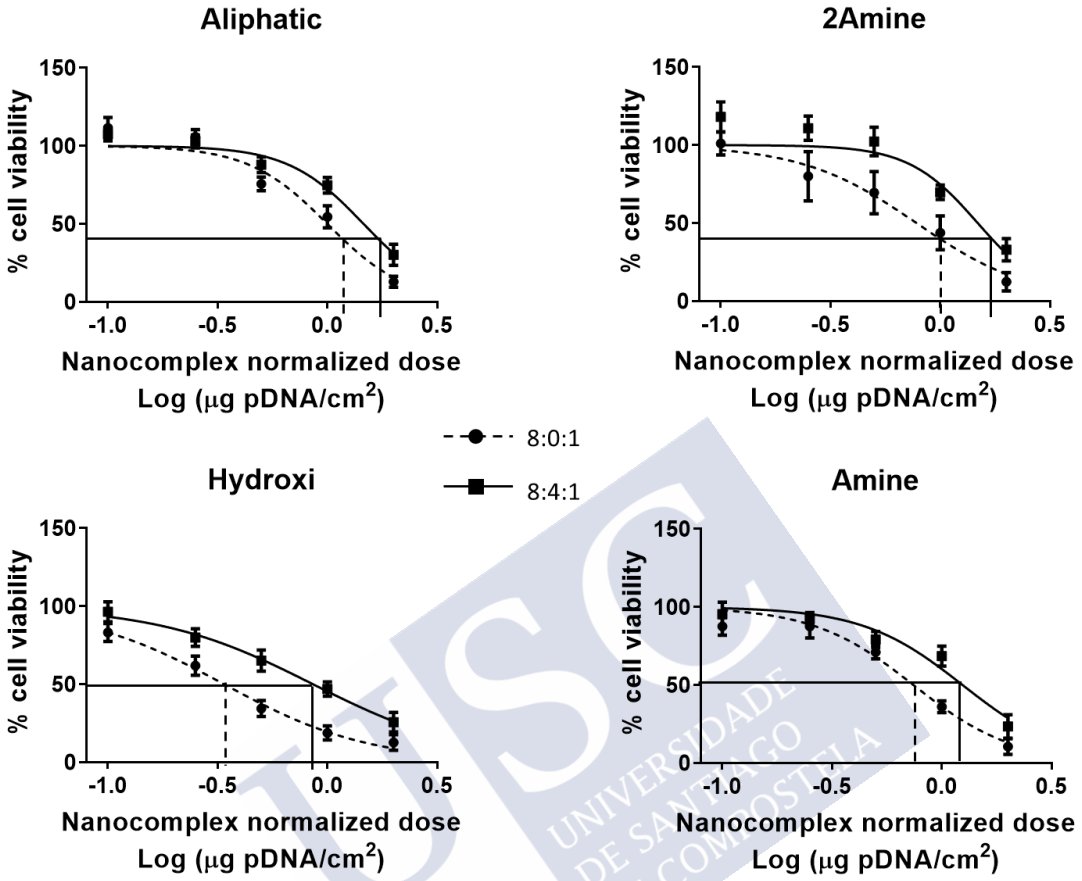


Figure S3.7. 3D toxicity assay, evolution of the neurosphere area after the treatment with the different prototypes.

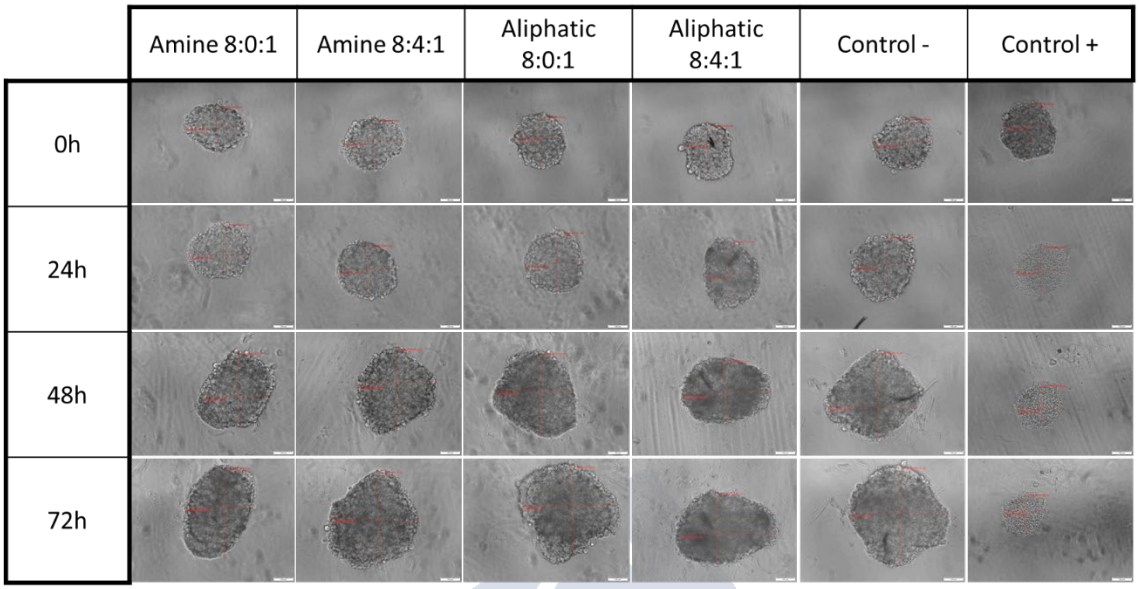
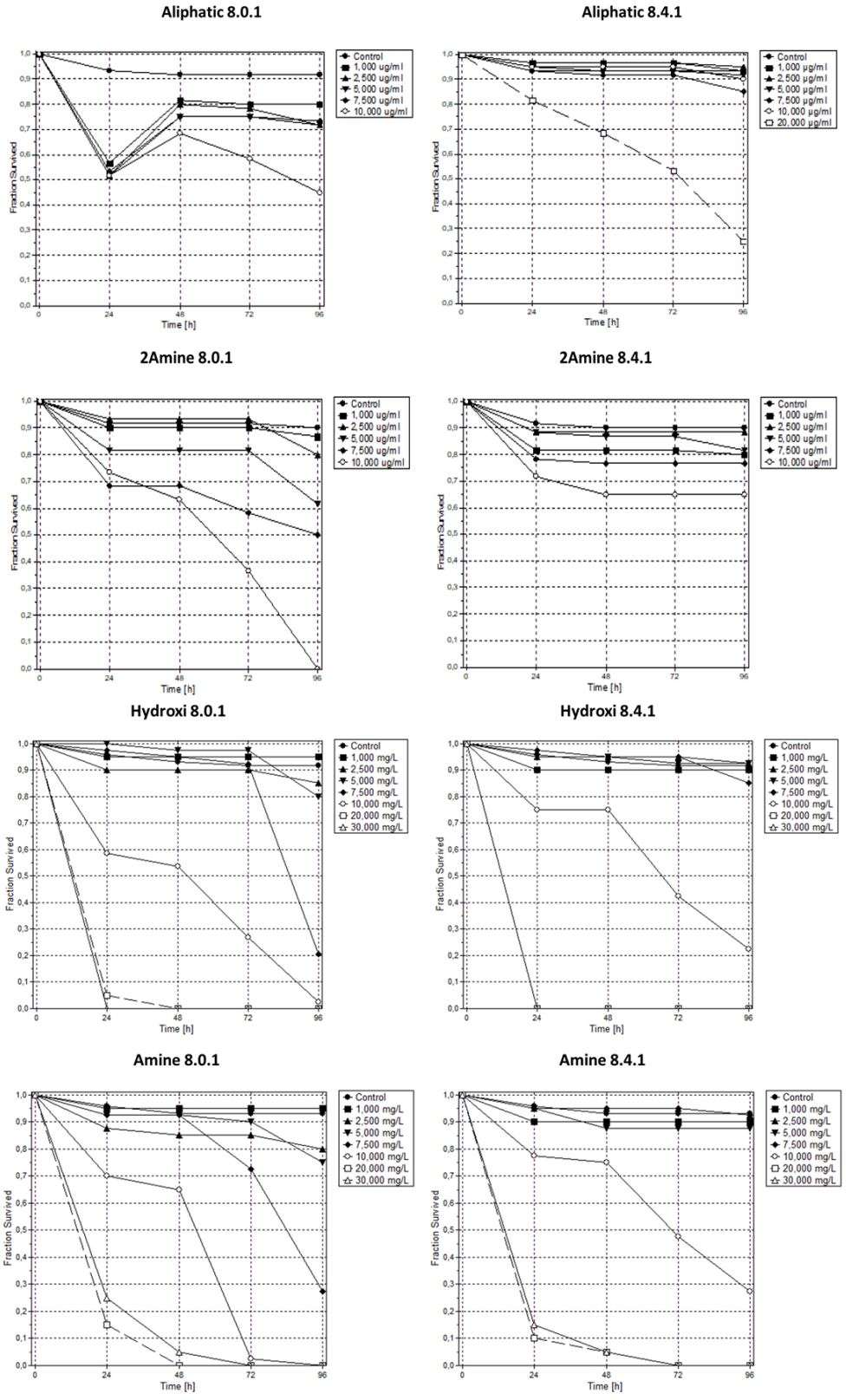


Figure S3.8. Survival of the introduced Danio rerio as observed under presence of the nanocomplexes at different times. The concentrations are expressed in µg of cationic polyphosphazene/mL. Control of survival: osmosis water.



Chapter 4

In vitro and *in vivo* efficacy of polyphosphazene based nanoparticles in a glioblastoma model

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Abstract

One of the therapeutic strategies to tackle cancer stem cells (CSCs) is to induce their differentiation towards a less malignant genotype sensitive to conventional chemotherapy. Bone morphogenic proteins (BMPs) are involved in cell differentiation and have shown to be effective in treating numerous tumors. In this work, a plasmid encoding BMP-4 has been included in an optimized polymeric gene delivery system. The nanoparticles obtained with the therapeutic plasmid have adequate physicochemical properties for their use in gene therapy and were shown to significantly reduce *in vitro* tumor cells clonogenicity. This effect was even more marked after the association of the nanoparticles with a conventional chemotherapeutic agent (Temozolomide). *In vivo* efficacy was tested in a xenograft glioblastoma model, where a significant reduction in tumor size and an increase in survival was observed after the coadministration of the therapeutic nanoparticles and temozolomide. The expression of CSC markers in dissected tumors was also studied. Tumors treated with Temozolamide (Tz) showed an induction of chemotherapy resistance genes. We observed that tumors treated with the BMP4 nanoparticles and Tz presented a normalization in the expression of markers of chemotherapy resistance. These results suggest the potential of these polyphosphazene nanoparticle formulation as a potential therapy for the suppression of glioblastoma.

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4.1. Introduction

Cancer stem cells (CSCs) are undifferentiated tumor cells that have the ability to generate a new tumor and are resistant to conventional chemotherapeutic agents. Some therapies against CSCs are based on agents that induce their differentiation to tumor cells, which makes them sensitive to conventional chemotherapy [1].

BMPs are endogenous ligands of the TGF-β superfamily that transduce their biological activities by SMAD phosphorylation. Phosphorylated SMAD migrates to the nucleus, where it regulates the expression of genes involved in the differentiation of CSCs [2–4]. There are 14 proteins that belong to the family of BMPs, but BMP-2/-4 and -7 are the most widely investigated for CSC treatment.

BMP-2 showed contradictory effects in tumor therapy: in most cases it is able to reduce tumor growth, cell migration and expression of CSC markers by inducing CSCs differentiation. However, in some tumors it has also shown to activate oncogenes, initiate metastasis and worsen cancer progression [5–7]. BMP-4 promotes differentiation and apoptosis, reduces self-renewal capacity, and resistance to chemotherapy [8,9]. This protein has shown promising effects in tumors such as glioblastoma [7,10–12], breast cancer [13] or lung squamous cell carcinoma [14]. Finally, BMP-7 has also been shown to increase the expression of cell cycle inhibitors by inducing senescence, and reducing metastasis and cancer stemness markers [7,15].

The major problem for the use of BMPs as therapeutic agents is their low half-life *in vivo*. One strategy to solve this limitation is to encapsulate the proteins in a controlled release device. For example, a formulation consisting of PLGA-microspheres was able to release BMP-7 for over two months without loss of protein bioactivity. The intratumoral implantation of these microspheres resulted in a reduction of tumor size *in vivo* and in the modulation of tumor cell expression patterns towards a less malignant phenotype [16,17]. Another strategy to generate local sustained levels of BMPs relies on the delivery of nucleic acids encoding BMPs. Such approach was followed by Mangraviti et al., who developed poly (beta-amino ester) nanoparticles for the *in vitro* delivery of a plasmid encoding BMP-4 to mesenchymal

stem cells, and then implanted those cells as an antitumoral therapy. This cell therapy exerted a therapeutic effect on brain tumor CSCs upon intranasal administration [18].

We have previously reported a formulation of nanoparticles for gene delivery based on a combination of two polyphosphazenes, one cationic, substituted with cysteamine and 2-methyl-1-propanethiol (Aliphatic-PPZ), and another anionic, substituted with 6-mercaptohexanoic acid (6MHA-PPZ). This formulation showed excellent efficacy, with transfection levels 3-fold higher than those achieved with Lipofectamine 2000 (Chapter 3). Our aims were to test the capacity of these nanoparticles to deliver a BMP-4 plasmid upon intratumoral injection, and the capacity of this gene therapy to generate an antitumoral effect on glioblastoma models, both in absence and in the presence of temozolomide, the first-line chemotherapeutic for this tumor.

4.2. Materials and Methods

4.2.1 Materials

Polymers were synthetized in our lab as previously described (Chapter 3). Temozolomide and Xylazine were bought from Sigma-Aldrich (USA). Ethanol, Crystal violet and acetic acid (glacial) were purchased from Merk (Germany). Ketamine for the anesthesia was provided by Pfizer (USA). BMP4 plasmid was bought from Sino Biological Inc. (China). Bone Morphogenic Protein 4 (BMP-4) was purchased from Peprotech (UK). Cell medium EMEM, Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (P / S) were provided by Gibco (ThermoFisher, USA). All the products were used as received.

4.2.2 Amplification of BMP4 plasmid

To perform the nanoparticle efficacy tests we selected a plasmid encoding BMP4 (Sino Biological,China). The plasmid was amplified in our lab.

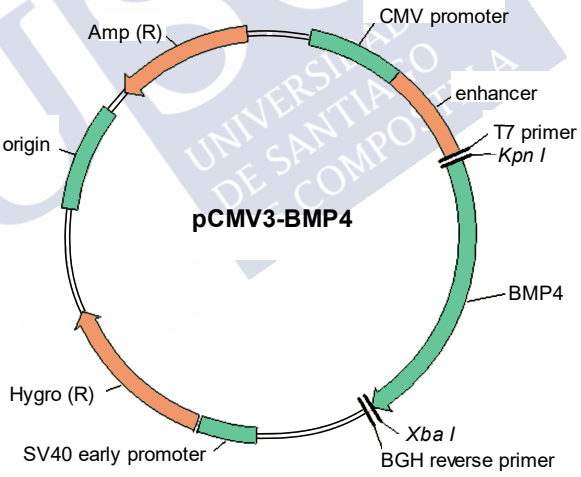


Figure 4.1. Plasmid vector encoding Bone Morphogenic Protein 4 (BMP-4).

Competent E. coli DH5α bacteria were transformed with BMP4 plasmid. After that, bacteria were resuspended in Luria-Bertani (LB) medium and incubated for 2 h at 37 °C and orbital agitation. In order to select the transformed bacteria, they were subsequently seeded at different concentrations in Petri dishes with antibiotic supplemented LB medium (50 µg ampicillin / mL) and incubated at 37 °C for 24 hours. The next day, a colony was isolated and

grown in liquid supplemented LB in the orbital incubator. The culture was amplified until reaching enough number of bacteria to perform plasmid extraction.

Plasmid extraction was performed with an Invitrogen™ PureLink™ HiPure Plasmid Gigaprep Kit (Thermo Fisher, USA) following the manufacturer's instructions. Briefly, bacteria were centrifuged at 5000 rcf for 15 min, the pellet was resuspended in a RNase solution to remove the RNA and mixed with a lysis buffer to release the intracellular content. In order to remove the cellular debris, a precipitation buffer was added to the solution and filtered. Then, the pDNA was purified using the by columns provided in the kit. Finally, the plasmid was precipitated, washed, and quantified by UV absorption (Nanodrop, ThermoFisher, USA).

4.2.3 Nanoparticle preparation

Nanoparticles were prepared by ion complexation using the Aliphatic-PPZ as cationic polymer associated with the Anionic-PPZ, as detailed in the chapter 3. Nanoparticles were prepared for a final concentration of 83.3 µg of pDNA / ml. In this case, a pDNA encoding Bone Morphogenic Protein 4 (BMP4) was used as nucleic acid.

4.2.4 Nanoparticles characterization

4.2.4.1 Nanoparticle size

Nanoparticles size was determined by Dynamic Light Scattering (DLS) using a Nanosizer ZS instrument (Malvern, UK) after the dilution 1 : 10 in 1 mM KCl. Each analysis was performed in triplicate at 25 °C, with a backscatter angle of 173 °.

4.2.4.2 Binding efficiency of nanoparticles

Test was performed to verify that the nanoparticles can associate the pDNA and dissociate it under suitable conditions. A concentration of nanoparticles corresponding to 0.33 µg of plasmid was mixed with a nucleic acid visualization reagent (SYBR® Gold nucleic acid stain) and loading agent (30% glycerol and 0.25% bromophenol blue), this mixture was loaded on an agarose gel (1% w/v in Tris-EDTA 1x buffer) and allowed to run for 30 min at 100V in the electrophoresis cell (Wide Mini-Sub Cell GT Systems, BioRad, USA). For dissociation test, the same amount of nanoparticles was incubated with heparin (20:1 w/w heparin: pDNA) for 1 h at 37 °C, and the resulting sample processed as described before.

4.2.5 *In vitro* evaluation

4.2.5.1 Clonogenicity assay

Two lines of human glioblastoma cells (U87MG and U251) have been used in this experiment. Both cell lines were cultivated in EMEM medium supplemented with 10% (v / v) heat inactivated Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin/Streptomycin (Gibco, USA) and were maintained in culture to a confluence of 75% at 37 °C, 95% relative humidity and 5% CO_2 conditions.

For the clonogenicity assay, 10^5 cells/well were seeded in a 12-multiwell plate. After 24 h the treatment was added, the cells were treated with seven different treatments: Medium, BMP4 protein, Blank-NPs, pBMP4-NPs, Temozolamine (Tz), BMP4-protein + Tz combination and pBMP-NPs + Tz combination. The concentration was 23.2 µg of nanoparticles/mL, 30 ng BMP4-protein/mL and 2.4 µg temozolamide/mL. Two days later, 500 cells/well were re-seeded on a 6-multiwell plate and allowed to grow 12 days more.

The cells were stained with a preparation of 50% ethanol, 5% acetic acid and 0.5% crystal violet, washed with water twice and the number of colonies was counted per well.

4.2.5.2 Evaluation of the combination effect by the coefficient of drug interaction (CDI)

The effect of the co-administration of the temozolomide and the BMP4 protein/ pBMP4-NPs was evaluated by coefficient of drug interaction (CDI). It was calculated by the following formula: CDI = AB / (A × B). AB is the number of colonies formed (fraction of the control group) from the combined effects of both treatments. A or B is the number of colonies formed (fraction of the control group) of each treatment separately. If CDI <1 indicates that the combination of treatments has a synergistic effect, if CDI = 1 it is additive and if CDI> 1 it is antagonistic.

4.2.6 *In vivo* antitumor efficacy

4.2.6.1 *In vivo* glioblastoma xenograft mouse model and treatment administration

This experiment was carried out during the predoctoral stay at the Université Catolique de Louvain, under the supervision of Prof. Veronique Préat. The experiment was approved by the

ethical animal care committee of the Université Catholique de Louvain (2019/UCL/MD004) and was performed according the Belgian National Guidelines in accordance with European Directive. Animals had free access to water and food all the time.

Briefly, eight-week-old NMRI female nude mice (Janvier, France) were anesthetized intraperitoneally with 150 µL of a solution of 10 mg / ml ketamine (Pfizer, USA) and 1 mg / ml xylazine (Sigma, USA), and two million of fresh U87MG cells were administered subcutaneously in the flank. The tumor was allowed to grow for ten days before the administration of the treatment.

When the tumor size was around 35 mm^3, mice were anesthetized, randomized in six groups and the treatment was administered intratumorally, four doses in four consecutive days. Experimental groups were: (1) Group Control (n=7); (2) Group Temozolomide (Tz) (n=6); (3) Group Blank nanoparticles (n=6); (4) Group BMP4 + Tz (n=6); (5) Group pBMP4 nanoparticles (n=7) and (6) Group pBMP4 nanoparticles + Tz (n=7). As control we use saline solution (Figure 4.2). The doses administered were 5 µg / g of temozolomide intraperitoneally, 1.54 µg / g of BMP4-pDNA nanoparticles and 2 ng / g of BMP4 protein.

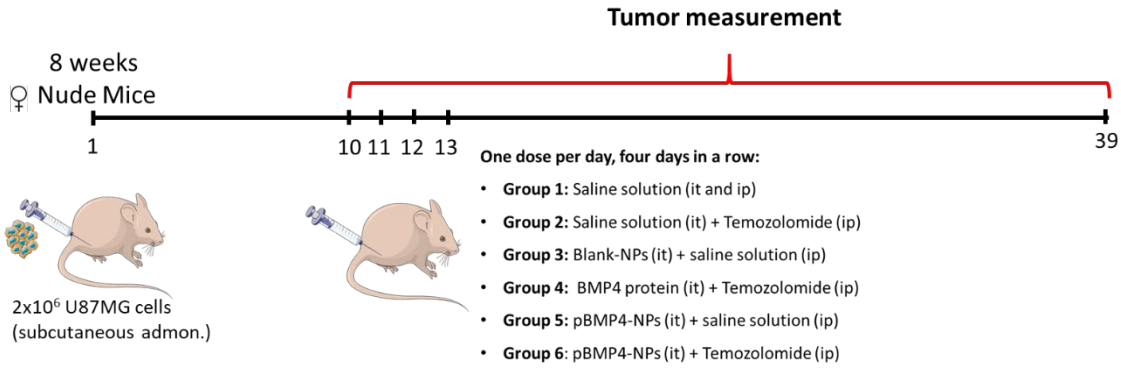


Figure 4.2 Scheme of the *in vivo* assay in glioblastoma xenograft model in 5-week-old female nude mice, the tumor was generate after the administration of $2x10^6$ cells U87MG/mouse. The treatment was administrated after 10 days, when the tumors were an average volume of 35 mm^3. Mice were divided in six groups and treated with four doses administered four days in a row. The evolution of the tumors and animal weight was studied until mice sacrificed. NPs: nanoparticles; BMP4: Bone morphogenic protein 4; Tz: temozolomide; it: intratumoral; ip: intraperitoneally.

4.2.6.2 Tumor growth and survival rate

After the administration of the different treatments, tumor size and body weight were measured every 2 days. Tumor size was measured by callipers and volume was calculated according the following formula V= L x W x H, wherein L is the length, W is the width and H is the height of the tumor. Relative tumor volume was calculated as V/Vo (Vo is the tumor volume before the first administration). Mice were considered to be dead and sacrificed either when the tumor volume was higher than 1500 mm^3, on the appearance of necrosis or ulcers, with > 20 % weight loss or in presence of distress signs.

4.2.6.3 Tumor RNA extraction and Real time-PCR

For RNA extraction, tumors were homogenized in 1 ml of TRI-Reagent (ThermoFisher) by GentleMACS Dissociator (Miltenyi Biotec, Germany). The homogenized tumor was centrifuged to remove the fatty layer and tissue debris. The homogenate was mixed with 200 µl chloroform / mL TRI-Reagent by repeated turning and left to stand 15 min at room temperature. Then, samples were centrifuged and the aqueous phase was transferred to an eppendorf containing 500 µl of isopropanol / mL of TRI-Reagent, mixed by repeated turning, and cooled to -20 °C for 15 min. The sample was centrifuged and the supernatant was decanted, the pellet was washed twice with ethanol and allowed to dry at room temperature and resuspended in DNAse/RNAse water. The concentration was determined by NanoDrop

2000 (ThermoFisher). RNA samples were reverse transcribed using the kit RevertAid First Strand cDNA Synthesis Kit (ThermoFisher). Real time-PCR (RT-PCR) was made using the kit NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, Portugal). Both PCR was carried out using the Mastercycler Nexus (Eppendorf, Germany). GAPDH was used as housekeeping gene to normalize gene expression. Primer sequences for the genes of interest are shown in Table 4.1.

Table 4.1. Lyophilized primers obtained from ThermoFisher were resuspended in DNAse / RNAse water to a stock solution of 100 µM. The thermal cycler conditions were as follows: 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 10 seconds, and 68 °C for 20 seconds.

	Forward	**Reverse**
GAPDH	5´-GCCAAGGTCATCCATGACAACT-3´	5´-AGGGCCATCCACAGTCTTCTG-3´
Sox2	5´-ACACCAATCCCATCCACACT-3´	5´-GCAAACTTCCTGCAAAGCTC-3´
Nestin	5´-CCTCCTGGAGGCTGAGAACTC-3´	5´-AAGGCTGGCACAGGTGTCTC-3´
Oct4	5´-CCCGCCGTATGAGTTCTGTGG-3´	5´-CCGGGTTTTGCTCCAGCTTCTC-3´
Nanog	5´-CCGCGCCCTGCCTAGAAAAGAC-3´	5´-AGCCTCCCAATCCCAAACAATACG-3´
BMP4	5´-AATGTGACACGGTGGGAAACT-3´	5´-CCCGCTGTGAGTGATGCTTA-3´
MDR1	5´-AACAACGCATTGCCATAGCTCGTG-3´	5´-AGTCTGCATTCTGGATGGTGGACA-3´
MRP1	5´-CATCGTTCTGTTTGCTGCCCTGTT-3´	5´-AGTACGTGGTGACCTGCAATGAGT-3´
ABCG2	5´-GCCACGTGATTCTTCCACAA-3´	5´-TTCTGCCCAGGACTCAATGC-3´

4.2.7 Statistical analysis

Data were represented as mean ± standard deviation (SD). Statistical differences were calculated using the Student's t-test, one-way and two-way ANOVA in combination with Tukey's multiple comparisons test. The significance was set to $p < 0.05$. All the experiments were repeated three times, if not stated otherwise.

4.3 Results and Discussion

4.3.1 Nanoparticle characterization

The nanoparticles (NPs) used for plasmid DNA delivery was formed by the condensation of 6-mercaptohexanoic acid substituted poly(phosphazene) (PPZ), (2-methyl-1-propanethiol) (cysteamine) substituted PPZ, and plasmid DNA (pDNA), in a ratio of amines/carboxyls and DNA phosphates of 8:4:1. This nanoparticle formulation was selected due to its excellent capacity to mediate cells transfection, reaching levels higher than those achieved with Lipofectamine (Chapter 3). The change of the plasmid from one encoding GFP/Luciferase (Chapter 3) for another encoding BMP4 (pBMP4) did not significantly affect the physicochemical characteristics of the nanoparticles. The nanoparticles presented a size below 150 nm and a polydispersity index below 0.2 (Figure 4.3.a). As previously observed, the nanoparticles were able to achieve tight plasmid association and to release the polynucleotide when in the presence of heparin (Figure 4.3.b).

A

	Size (nm)	PDI
Control_NPs	143±4	<0.2
pBMP4_NPs	125±3	<0.2

B

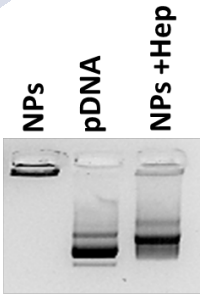


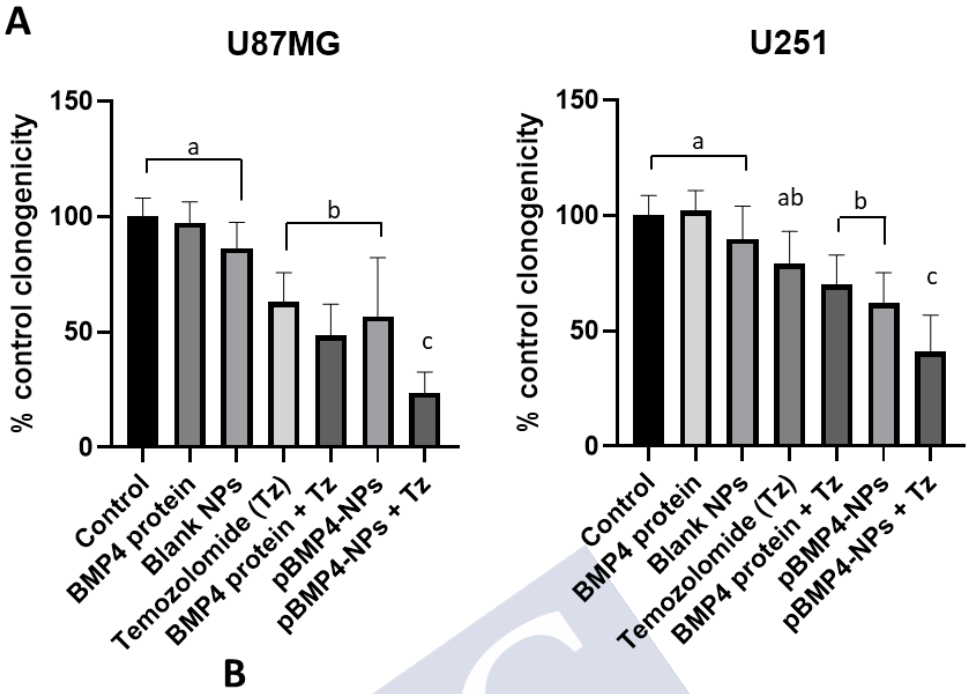
Figure 4.3 pBMP4-Nanoparticle characterization. A. Nanoparticle size and polydispersity of nanoparticles prepared with therapeutic plasmid. B. Gel retardation assay to test the association of the nucleic acid and the dissociation in presence of a competitor. NPs: nanoparticles, Hep: heparin.

4.3.2 Clonogenicity assay

The association of drugs that reduce the aggressiveness of tumors and antitumoral can generate synergism against cancer stem cells (CSCs). As previously described by Zhao, the association of two anti-tumoral agents (Temozolomide and Paclitaxel) at low concentrations reduces the number of colonies in a very marked way, and this effect could not be achieved

by increasing the concentration of the drug monotherapies [19]. Herein we investigated the combined effects of pBMP4-nanoparticles and temozolomide (Tz), the first line chemotherapeutic agent used for glioblastoma. This assay was performed to see if this association reduces the clonogenicity of tumor cells compared to the separate treatments. In these experiments, the temozolomide dose selected corresponded to its IC50 (Concentration to inhibit half of the cell population) [19].

Clonogenicity trend was similar for both cell lines (Figure 4.4.a), but the effect is more marked in U87MG, which is surprising since this line has a lower percentage of cancer stem cells than U251 [20]. The group that combines pBMP4-nanoparticles and temozolomide showed a significant decrease in the number of colonies compared to the treatments given separately. BMP-4 alone did not reduce the clonogenic capacity of the cells, although according to several reports this protein can reduce their stem cell markers [21]. The effect was even higher in the groups treated with the pBMP4-nanoparticles compared to the administration of the protein directly; this could be because either the dose was insufficient or the protein was unstable in the culture medium. In order to determine the effect of the association of pBMP4-nanoparticles and temozolomide, their coefficient of drug interaction (CDI) was calculated. Both the association of BMP4 protein and Tz, and the association of the pBMP4-nanoparticles and Tz resulted in CDI <1, indicating a synergistic effect. This synergistic association effect was observed both in U87 and U251. Moreover, this synergistic effect was higher for the Tz/pBMP4-nanoparticles association (Figure 4.4.b).



B

CDI	U87MG	U251
BMP4 protein + Tz	0.66	0.84
pBMP4-NPs +Tz	0.79	0.87

Figure 4.4 Clonogenicity assay in two glioblastoma cell lines (U251 and U87). A. Clonogenic capacity of the cells after the incubation during 48h with the different treatments, data are expressed in number of colonies formation (untreated cells were assume as 100%). B. Calculation of CDI value to evaluate the combination effect. The treatments in statistically homogeneous groups are marked with the same letters ($p < 0.05$). Tz: temozolomide; NPs: nanoparticles; BMP4: Bone morphogenic protein-4.

4.3.3 *In vivo* efficacy

4.3.3.1 Tumor growth

Following the promising *in vitro* data, we tested the efficacy of pBMP4-NPs in a U87MG xenograft mice model. After administration of the different treatments, tumor size was followed for one month. Given that, there is always some heterogenicity at the beginning in the tumor size, the mice were distributed keeping the groups comparable. Besides, the results were also corrected respect to the initial tumor volume (Figure S4.1.b). In any case, thanks to this procedure for animal allocation to the different groups, there is no important changes in

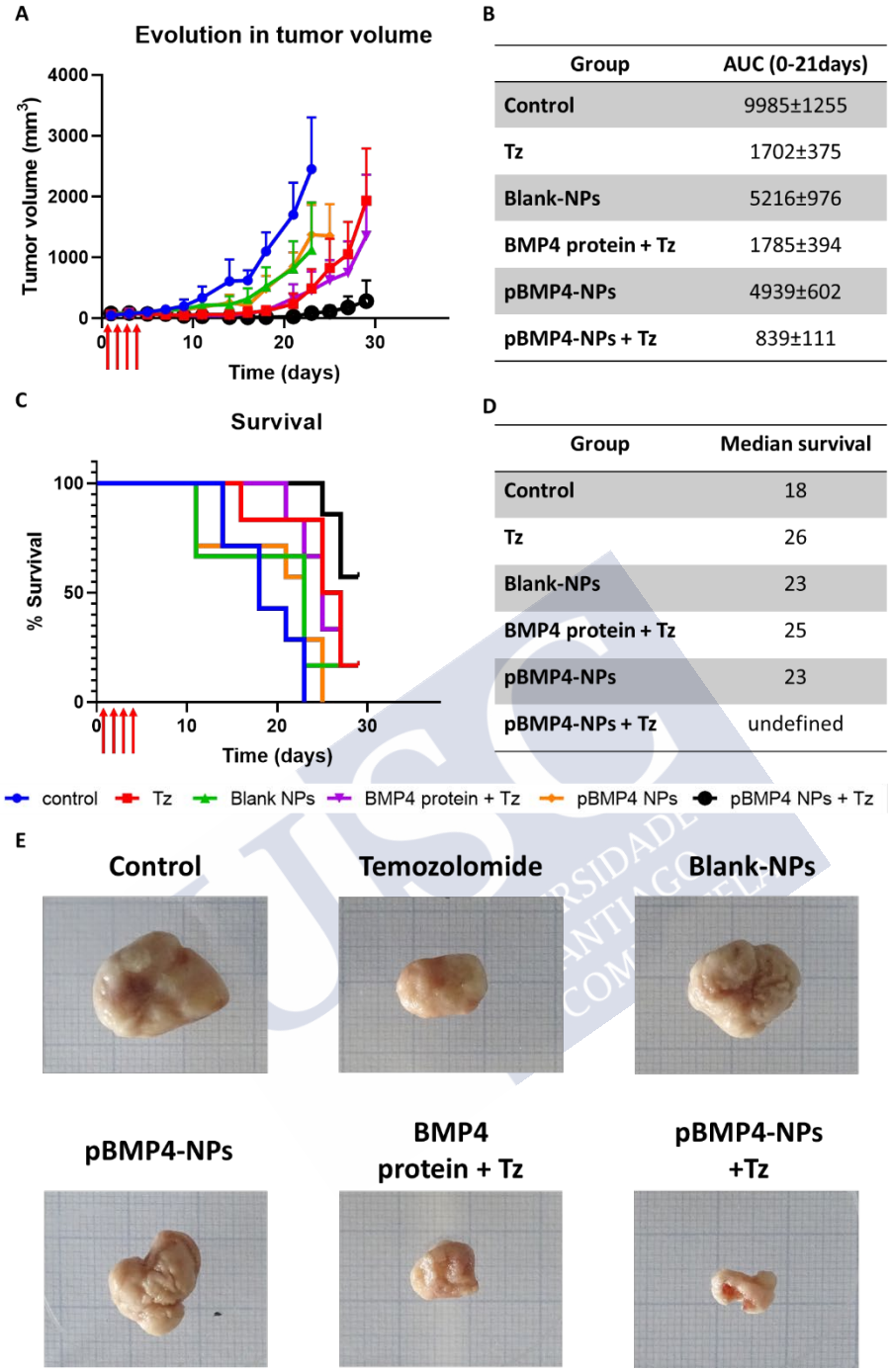
relative trends between the groups whether analyzing corrected or non-corrected tumor volume (see Figure S4.1.b vs. Figure 4.5.a).

No variations in mice weight were observed, indicating there was no toxicity during the experiment in any of the different treatments (Figure S4.1.a). However, differences were observed in tumor size, Blank NPs and pBMP4-NPs showed a very slightly tumor size reduction. A higher tumor reduction was observed when the animals were treated with temozolomide (Tz) or with pBMP4-NPs. The maximus reduction was observed with the association of Tz and pBMP4-NPs (Figure 4.5.a). This enhanced efficacy of the association of a cytostatic and a gene therapy for BMP4 was in agreement with previous reported *in vitro* and *in vivo* results [22].

The fact that pBMP4-NPs did not reduce tumor size significantly compared to the Control NPs group was unexpected; more considering that we had observed a biological effect *in vitro*. However, it is known that BMP4 action mechanism is through directed differentiation of CSCs of the tumor, and U87MG cells do not have a clear CSC phenotype, particularly when not cultured under conditions for enrichment in this subpopulation. Around 50 % of primary tumors from glioblastoma, on the other hand, are known to be sensitive to this BMP signalling [7]. In any case, we believe that this case is good illustration of the necessity of combining CSCs-specific and conventional cytostatic drug to treat glioblastoma as we have previously discussed [1].

Lastly, an increase in the survival of the animals of the group given the association of Tz and pBMP4-NPs was observed in comparison to all other groups. The groups given Blank NPs and pBM4-NPs alone did not show difference with respect to the control, nor did BMP4 protein + Tz show any difference with respect to Tz alone (Figure 4.5.c). The issue about BMP sensitivity for U87MG cells need to be considered here also. Besides, when analyzing the *in vivo* performance of BMP4 used as protein, we have to consider also its extremely short half-life under physiological conditions. Median survival was also calculated to observe the differences more clearly. This parameter is not shown for the pBMP4-NPs + Tz group because most animals in this treatment groups had survived by the end of the assay (Figure 4.5.d).

After sacrificing the animals, the tumors were extracted for imaging and morphological analysis (Figure 4.5.e), and for further processing for PCR. When considering differences in tumor volume it must be taken into account that the animals from the Control group were sacrificed at day 23 or before due to tumor burden. Some of the mice from the Blank NPs and the pBMP4-NPs were also euthanized before day 25 due to necrosis at the injection site.



Group	AUC (0-21days)
Control	9985±1255
Tz	1702±375
Blank-NPs	5216±976
BMP4 protein + Tz	1785±394
pBMP4-NPs	4939±602
pBMP4-NPs + Tz	839±111

Group	Median survival
Control	18
Tz	26
Blank-NPs	23
BMP4 protein + Tz	25
pBMP4-NPs	23
pBMP4-NPs + Tz	undefined

Figure 4.5. Efficacy *in vivo* of the formulation alone and in combination with the chemotherapeutic agent, temozolomide. A. Evolution of tumor volume, significant differences have been found between the groups, Control = pBMP4-NPs = Blank-NPs > Tz = BMP4 protein + Tz > pBMP4-NPs + Tz. B. Evolution of tumor volume expressed in AUC of each treatment after 21 days. C. Percentage of survival of the mice. D. Median survival of the mice after the administration of the different treatments. e. Images of the tumors after the euthanasia. It should be considering that the animals with tumor necrosis or a size greater than 1500 mm^3, were sacrificed in order to reduce the suffering of the animal. The red arrows illustrate the four administrations on consecutive days. Tz: Temozolomide, NPs: Nanoparticles; BMP4: Bone morphogenic protein-4.

4.3.3.2 Real time PCR

Tumors extracted at the end of the experiment were analyzed by quantitative Real time-PCR to study changes in the gene expression patterns resulting from our therapy. The expression of the transgene used, BMP4, was also analyzed to check efficient transfection by end of the experiment. Indeed, BMP4 expression were > 1000-fold higher in the groups with pBMP4-NPs as compared to the control. None of the other groups showed intrinsic BMP4 signalling (Figure 4.6).

Nestin [23], Nanog [24], Oct4 [25] and Sox2 [26] are genes implicated in the CSCs properties and are overexpressed in glioblastoma [27]. We have shown in previous studies with primary glioblastoma models that BMP signalling can reduce the expression of these markers. However, in this work there was not a very clear tendency in the data besides some small changes reaching statistical significance (Figure 4.6). Again, this might be related to a lack of sensitivity of the cell line (U87MG) to BMP4, because the CSCs population is less than 1% under normal conditions [28–30].

We also analyzed the expression of MDR, a drug efflux pump involved in chemotherapy resistance [31]. We observed that after the administration of Tz this gene was induced by 300% expression as compared to control cells. The association of Tz with BMP4 protein and pBMP4-NPs normalized MDR expression to basal levels, probably explaining the synergistic effect of the association of these therapies.

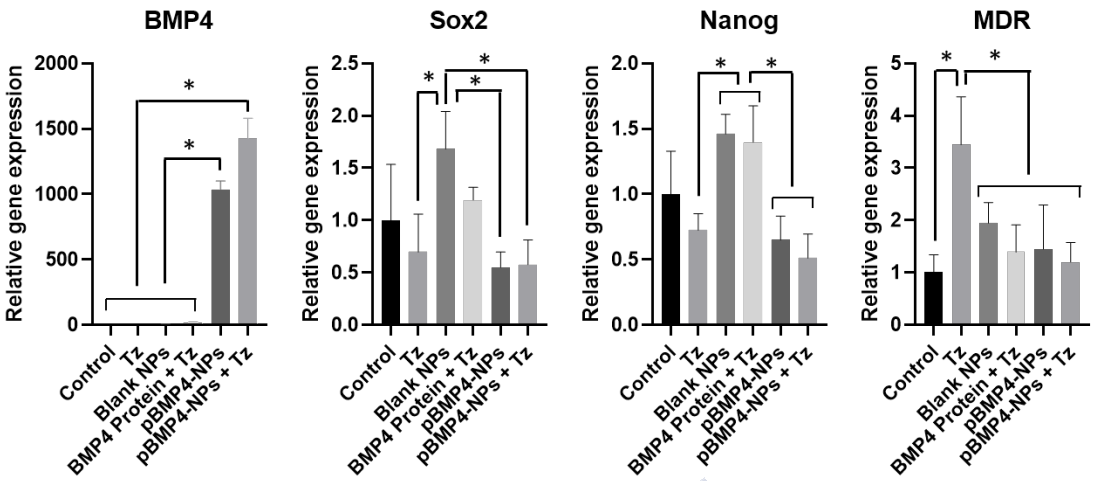


Figure 4.6. RNA expression of CSCs markers and efflux pumps after the administration of the different treatments in a glioblastoma xenograft model, measured by real time-PCR. Other genes were also studied, but no differences were observed between the different treatments (Figure S4.2). *Statistical analysis at $p < 0.05$.

4.4 Conclusions

A new gene nanocarrier based on the combination of two polyphosphazenes, one substituted with 6-mercaptohexanoic acid and another with cysteamine and 2-methyl-1-propanethiol was tested for its use as a therapy against glioblastoma. For that, the NPs were loaded with a plasmid encoding BMP4, a protein with specific activity against CSCs. *In vitro* studies showed that the NP formulation had a clear antitumoral effect, particularly when associated with the first line glioblastoma treatment Temozolomide (Tz), where this combination reached synergistic effects. The nanoparticles associated to Tz also showed remarkable antitumoral effects in glioblastoma xenograft models, significantly higher than those that could be achieved with the gene therapy or the cytostatic drug alone. The interest of this combination might be related to a capacity of BMP4 to supress the induction of efflux pumps caused by Tz. On the other hand, the activity of BMP4 was not mediated by a CSC differentiation mechanism, although this result is most likely related to a limitation of the model cell line. This study indicates great potential of the gene nanocarriers used, and it indicates high antitumoral activity for the BMP4 plasmid/ Tz association. The studies highlight the pertinence to pursue new studies in more advanced cellular models.

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Figure S4.1. Efficacy in vivo of the formulation alone and in combination with a chemotherapeutic agent. a. Evolution in the weight of the mice during the experiment. b. Evolution of tumor volume corrected for initial volume. The red arrows illustrate the four administrations on consecutive days. Tz: Temozolomide, NPs: Nanoparticles; BMP4: Bone morphogenic protein 4.

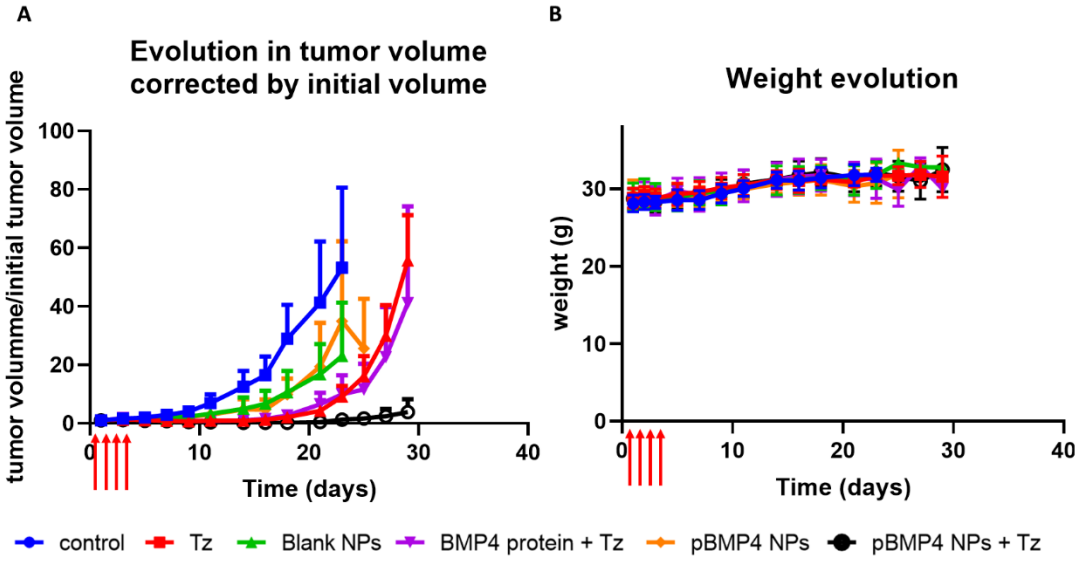
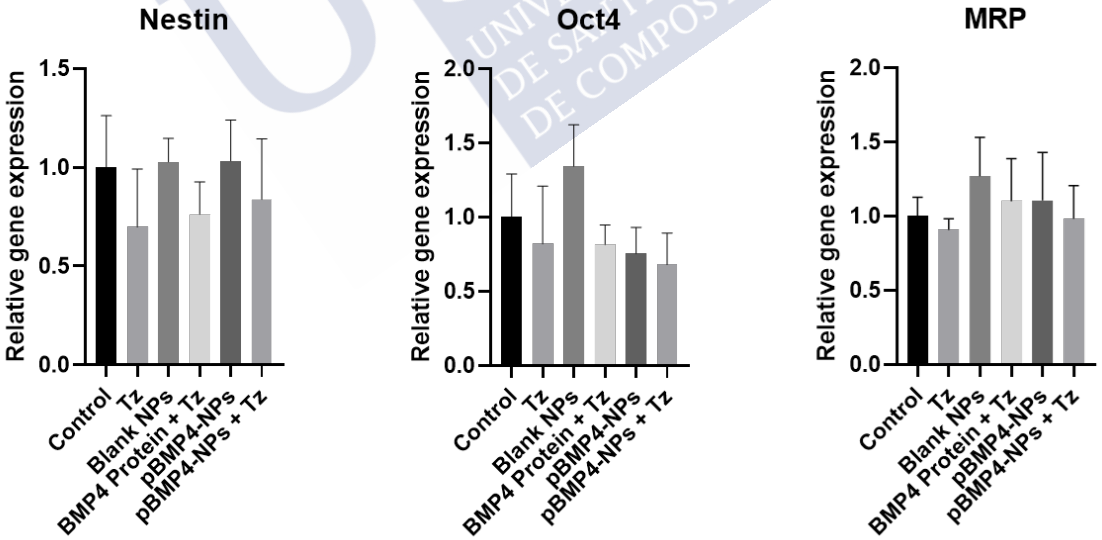


Figure S3.2 Expression of RNA corresponding to cancer stem cell markers and efflux pumps determined by Real time-PCR.



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Overall Discussion

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In most tumors, there is a cell subpopulation with stem cell characteristics, known as cancer stem cells (CSCs). These cells remain in a dedifferentiated state and have the ability of self-renewal, chemotherapy resistance, and higher migration capacity. Therefore, this small population is considered responsible for tumor recurrence and their suppression is a key issue in the fight against cancer. There are numerous strategies to modulate CSCs, acting specifically on this population or in the tumor niche that supports them [1,2].

Gene therapy has been postulated as an alternative to small drugs and proteins in the treatment of genetically based diseases such as cancer. Still, the main challenge of gene therapy has been the delivery of DNA to the target cells and finding ways to extend the duration of its expression [3]. Indeed, gene delivery requires overcoming several barriers such as rapid nucleic acid degradation in physiological media, and the penetration across several cellular membranes to reach the target site [4,5]. Initially, nucleic acids were included in viral vectors, because these vehicles allowed the protection and the release of genes at the site of action with high efficiency; unfortunately, they also presented safety and immunogenicity problems [6]. Nanotechnology had been proposed as a method to synthesize non-viral vectors that allow controlled release of nucleic acids and improve *in vitro* and *in vivo* gene expression, with a better safety profile. Furthermore, these nanocarriers can be chemically modified to direct the nucleic acid specifically to the target tissue and to increase its internalization [7].

Considering this background, the objective of this thesis has been the development of new polymeric nanoparticles for the efficient delivery of gene therapies. The polymers used in the design of gene nanocarriers have some common characteristics such as biodegradability, biocompatibility and the presence of cationic groups to complex the nucleic acids (generally guanidine, primary secondary, or tertiary amines) [8]. The main objective was the design of nanosystems with the appropriate physicochemical properties necessary to achieve an efficient transfection, through the rational synthesis of new cationic polymers and their subsequent association with an endosomolytic anionic polyphosphazene previously reported by our group [9]. The prototype that showed the best results was used for the generation of a new treatment for glioblastoma.

In the first chapter, we studied the influence of the cationic polymer structure and its association with an endosomolytic anionic polyphosphazene (6-Mercaptohexanoic acid substituted poly(phosphazene), 6MHA-PPZ), on nanocarrier toxicity and transfection. For that, we started with three commercial cationic polymers widely used in gene delivery experiments: PEI, chitosan, and protamine. Although the physicochemical properties of the nanoparticles were similar, remarkable differences were observed in toxicity and transfection. Among the three cationic polymers, PEI showed higher toxicity than the others, but also achieved much higher transfection, suggesting once again that the presence of secondary and tertiary amines in the polymer structure is a key factor to achieve increased transfection [10]. The association of 6MHA-PPZ to the nanoparticles did not produce important changes in physicochemical properties, neither reduced polymer toxicity in cell cultures. However, contrary to the results *in vitro*, the association of 6MHA-PPZ to the nanoparticles produced a slight reduction of toxicity as measured in zebrafish embryo tests.

The association of 6MHA-PPZ to the nanoparticles increased their transfection efficiency, at least for protamine and PEI. Therefore, the integration of the endosomolytic polymer 6MHA-PPZ seems to improve transfection efficiency in almost all tested nanoparticles and suggest that their effect might be translated to other nanoparticle systems**.** Overall, the PEI/6-MHA-PPZ NPs showed remarkable transfection efficacy, being 5-fold higher than that observed with PEI alone or with previous polyphosphazene prototypes from the group [9], and most importantly, up to 20-fold higher than the benchmark Lipofectamine 2000. The endosomolytic polymer 6MHA-PPZ seems to enhance transfection on a similar relative scale independent of the cationic polymer counterpart in the delivery system. Therefore, we suggest that optimal nanoparticle candidates could be generated by combining 6MHA-PPZ and other efficient polymers that present different amine types and/or other modifying groups.

After the results obtained in the previous chapter, new heteropolymers based on poly(phosphazenes) with primary amino groups were investigated. These materials were used in the design and optimization of gene delivery systems. The poly(phosphazenes) were synthesized following a click chemistry reaction previously developed in our laboratory, all having a terminal primary amine and different chemical side groups: hydroxyl, secondary

amine, or an aliphatic chain. As a reference, the homopolymer having all primary amines already reported by the laboratory [9] was also synthesized and tested.

The new cationic polyphosphazenes were used for the preparation of nanoparticles, by ionic condensation with a DNA plasmid (cationic nanoparticles), or by their condensation with both this plasmid and the 6MHA-PPZ. The physicochemical properties of the nanoparticles were suitable for being tested in gene therapy applications [11], since they had a size around 100-150 nm, positive surface charge and reversible association of the plasmid.

Regarding the toxicity of the nanoparticles, the addition of 6MHA-PPZ significantly reduced the toxicity of the particles in cell culture and in zebrafish embryo tests, probably because it reduces nanoparticle charge density [12]. The nanoparticle prototype containing the polyphosphazene with the aliphatic radical (Aliphatic-PPZ) showed the lowest toxicity.

Regarding nanoparticle transfection, the addition of 6MHA-PPZ improved once again gene expression for all prototypes due to its reported endosomolytic capacity [9]. The association of the Aliphatic-PPZ and the 6MHA-PPZ showed the highest transfection, which was 3-fold higher than the positive control benchmark (Lipofectamine 2000). This remarkable performance could be related to the hydrophobic chains of the Aliphatic-PPZ, which may interact with cell membranes facilitating nanoparticle internalization and endosomal escape [13–15]. Due to this high efficiency, this combination of polymers was chosen to be further tested *in vitro* and *in vivo* with a therapeutic nucleic acid sequence.

Bone morphogenic protein-4 is a protein that induces the differentiation of some stem cells and CSCs, reason why it can be used as treatment in cancer and also improves the sensitivity of the tumors towards conventional chemotherapy [16,17]. A plasmid encoding this protein was associated to the best performing nanoparticle prototype designed in the previous chapter (Aliphatic-PPZ/6MHA-PPZ nanoparticles).

The nanoparticles were used to study the antitumoral effect of this gene therapy *in vitro* and *in vivo* in a glioblastoma model. Nanoparticles were administered alone or in combination with the first line chemotherapy drug for glioblastoma: Temozolamide (Tz) [18]. A decrease in cell clonogenicity was observed *in vitro* after the treatment with the nanoparticles containing the BMP4 plasmid (pBMP4-NPs), but this decrease was much more pronounced after the

association with Tz. As matter of fact, quantitative analysis showed that the two treatments generated a synergistic effect. Similar results were obtained in the *in vivo* tests in a glioblastoma xenograft model. However, in this case pBMP4-NPs alone were not able to reduce tumor size. On the other hand, the association of pBMP4-NPs with temozolomide produced again a clear synergistic effect. Not only tumor size was almost stable during all the experiment, but a neat increase in the survival of the animals was also observed.

At this point, we wanted to analyze the reasons behind the synergistic effect of pBMP4-NPs and Tz. For this, we studied the expression of genes involved in the CSC phenotype. The tumors were dissected and the expression of BMP-4, Nanog, Sox-2 and MDR was determined. BMP-4 expression in the tumor group treated with the BMP4-nanoparticles was more than 1000 times higher than the observed on the other groups, indicating an efficient delivery of the therapeutic gene that was maintained *in vivo*. The effect of pBMP4-NPs in the CSC phenotype (Sox-2 and Nanog) was not clear from these studies, probably due to an inherent limitation of the cell model used (U87MG), which has low proportion of this cell subpopulation. On the other hand, pBMP4-NPs were able to normalize the expression of efflux pump MDR, induced by the presence of Tz. This gene is related to chemotherapeutic resistance and is generally overexpressed in tumor cells after chemotherapy treatment [19]. The fact that pBMP4-NPs reduce MDR expression provides an explanation to the synergistic effect observed and indicates that these nanoparticles might work in association with other chemotherapeutic agents that are substrates to these pumps.

In summary, different prototypes of nanoparticles for gene therapy have been designed and tested *in vitro* and *in vivo*. Polymers that combine amino and hydrophobic groups, together with an endosomolytic anionic polymer were particularly promising and were able to provide a therapeutic effect in a glioblastoma model. These data indicate the interest of these types of nanocarriers as gene delivery systems and their use might be potentially extended in the future to other types of polynucleotides such as RNA.

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Conclusions

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The objective of this thesis has been to evaluate the potential of new nanoparticles for gene delivery based on polycations and the endosomolytic polymer 6MHA-PPZ, and to use these nanocarriers as a potential therapy against cancer stem cells (CSCs).

The experimental data of thesis led us to the following conclusions:

1. A variety of polyphosphazenes with primary amines and other functional groups can be synthesized by a click chemistry approach. These materials can form stable nanoparticles by condensation with a plasmid.
2. Nanoparticles can be formed from cationic commercial polymers and synthesized polyphosphazenes in combination with 6MHA-PPZ.
3. NPs based on the association of 6MHA-PPZ and commercial cationic polymers such as PEI and protamine show better transfection capacity *in vitro* than the same particles without 6MHA-PPZ. These results suggest that this endosomolytic polymer acts as a transfection enhancer for most nanoparticles.
4. The heterosubstituted poly(phosphazene) that combines amino-terminal and aliphatic groups in its structure (Aliphatic-PPZ), has shown less toxicity *in vitro* and *in vivo*.
5. The association of the 6MHA-PPZ reduces the toxicity of cationic polyphosphazene-based nanoparticles and improves their transfection *in vitro*.
6. Transfection/toxicity ratios are particularly remarkable for Aliphatic-PPZ/6MHA-PPZ and PEI/6MHA-PPZ nanoparticles, two formulations that largely beat the transfection of the positive control benchmark Lipofectamine 2000.
7. Aliphatic-PPZ/6MHA-PPZ nanoparticles loaded with a therapeutic plasmid encoding Bone Morphogenic Protein-4 (pBMP4-NPs) reduces cell clonogenicity *in vitro* and shows a synergistic effect with Temozolomide in two glioblastoma cell lines (U87MG and U251).
8. The coadministration of pBMP4-loaded nanoparticles and temozolomide reduces tumor volume and increases survival in a glioblastoma xenograft model, as compare to the negative controls, but also to temozolomide alone.

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List of abbreviations

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2AminePPZ: poly(phosphazene) substituted with cysteamine and 2-(butylamino)ethanethiol

6MHA: 6-mercaptohexanoic acid

6MHA-PPZ: 6-mercaptohexanoic acid substituted poly(phosphazene)

AAPPZ: allylamine substituted polyphosphazene

AF4: Asymmetric Flow Field-Flow Fractionation

ALDH: aldehyde dehydrogenase

AliphaticPPZ/AliPPZ: poly(phosphazene) substituted with cysteamine and 2-methyl-1-propanethiol

AminePPZ: cysteamine substituted poly(phosphazene)

APL: acute promyelocytic leukemia

ATRA: all trans retinoic acid

BET: 2-butylamino)ethanethiol

b-FGF: basic fibroblast growth factor

BMP: bone morphogenetic protein

BMP-4: bone morphogenetic protein type 4

CAF: carcinoma-associated fibroblasts

CDI: coefficient of drug interaction

Ch: cholesterol

CS: chitosan

CSC: cancer stem cells

Cys: cysteamine

Đ: polydispersity index

DLS: Dynamic Light Scattering

DMAE: 2-dimethylaminoethanol

DMAEA: 2-dimethylaminoethylamine

DMAES: 2-(dimethylamine)ethanethiol

DMEM: Dulbecco's Modified Eagle Medium

DMPA: 2,2-dimethoxy-2-phenylacetophenone

DOPC: 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine

DOTAP: dioleoyl-trimethylammonium propane

DOTMA: di-octadecenyl-trimethylammonium propane

ECM: extracellular matrix

EGFR: epidermal growth factor receptor (EGFR)

EPR: Enhanced Permeation and Retention

FBS: Fetal Bovine Serum

FESEM: Field Emission Scanning Electron Microscopy

FET: Fish Embryo Acute Toxicity

GM-CSF: granulocyte macrophage colony stimulating factor

H: height

Hep: heparin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF: hypoxia inducible factor

hpf: hours post-fertilisation

HPMA: N-(2-hydroxypropyl)methacrylamide

HydroxiPPZ: poly(phosphazene) substituted with cysteamine and 1-mercapto-2-propanol

IC50: concentration to inhibit half of the cell population

IFN: interferon

IL: interleukins

Ip: intraperitoneally

iRNA: interference RNA

kb: kilobase

kbp: kilobase pairs

Kcps: kilocounts per second

L: length

LB: Luria-Bertani

LC50: lethal concentration for the 50% of the cell population

LOEC: lowest observed effect concentration

LOX: lysyl oxidase

LPS: lipopolysaccharide (LPS)

MALS: Multi-Angle Light Scattering

MDR: multidrug resistance

miRNA: microRNA

Mn: number average molar mass

MP: mercapto-2-propanol

MPT: 2-methyl-1-propanethiol

mRNA: messenger RNA

MSCs: mesenchymal stem cells

mV: millivolts

Mw: molecular weight or weight average molar mass

N:C:P: number of amines of the polymer branches (N), number of terminal carboxylic groups (C) and the phosphates of the pDNA (P)

NLS: nuclear localization signal

nm: nanometers

NMR: nuclear magnetic resonance

NOEC: no observed effect concentration

NPC: nuclear pore complex

NPs: nanoparticles

nt: nucleotide

NTA: Nanotracking Analysis

OECD: Organization for Economic Cooperation and Development

PAGA: poly[α-(4-aminobutyl)-L-glycolic acid]

PAMAM: poly(amidoamine)

pBMP4: plasmid encoding Bone Morphogenic Protein 4

pBMP4-NPs: nanoparticles containing the plasmid encoding BMP-4

PC: phosphatidylcholine

PCL: poly(ε-caprolactone)

PDI: polydispersity index

pDNA: plasmid DNA

PE: phosphatidylethanolamine

PEDF: pigment epithelium-derived factor

PEG: poly(ethylene glycol)

pEGFP-Luc: plasmid encoding enhanced Green Fluorescent Protein and Luciferase

PEI: polyethyleneimine

PGA: poly-glutamic acid

PLA: poly-lactic acid

PLGA: poly(lactic-co-glycolic acid)

PLL: poly(L-lysine)

PMDS: poly(N-methyldietheneamine sebacate)

ppm: parts per million

PPZ: polyphosphazene

P/S: penicillin-streptomycin

PVP: polyvinyl pyrrolidone

RLU: Relative Luminescence Units

ROS: reactive oxygen species

Sc: subcutaneously

SD: standard deviation

siRNA: small interfering RNA

SSO: splice-switching oligonucleotides

STEM: scanning Transmission electron microscopy

TAM: tumor associated-macrophages

TEA: triethylamine

TFE: 2,2,2-trifluoroethanol

TGF- β: transforming growth factor-beta

THF: tetrahydrofuran

TICs: tumor initiating cells

TLR: Toll-like receptor

TN: tumor niche

TNF-α: tumor necrosis factor-alpha

TRP2: tyrosine related protein 2

Tz; temozolomide

VEGF: vascular endothelial growth factor

VHL: Von Hippel–Lindau

W: width

Ethical considerations and Permissions

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Animal studies

Fish Embryo Acute Toxicity Test (Chapter 2 and 3):

These experiments have been carried out in collaboration with the group of the Prof. Laura Elena Sánchez Piñón. All experiments and protocols have been approved by the animal care and use committee of the University of Santiago de Compostela and the standard protocols of Spain (CEEA-LU-003 and Directiva 2012-63-UE).

Antitumoral efficacy in a glioblastoma xenograft mice model (Chapter 4):

This experiment was carried out during the predoctoral stay at the Université Catolique de Louvain, under the supervision of Prof. Veronique Préat. The experiment was approved by the ethical animal care committee of the Université Catholique de Louvain (2019/UCL/MD004) and was performed according the Belgian National Guidelines in accordance with European Directive.

UCLouvain

Louvain Drug Research Institute (LDRI)
Advanced Drug Delivery and Biomaterials

Brussels, November 13th 2020,

To whom it may concern,

This letter certifies that Carla García Mazás made stayed in the lab I direct from September to November of 2019. During that time, she performed *in vitro* and in *vivo* studies with glioblastoma cells, these experiments were part of his PhD work. For the *in vivo* studies, we xenotrasplanted glioma cells in the flank of immunodeficient mice. When tumors became visible, animals were treated with intratumoral injections of polyphosphazene complexes, for effective delivery of a plasmid encoding BMP4 in the presence or in the absence of temozolomide (conventional chemotherapy for glioma patients). Tumor size was measured three times per week for a month. All these procedures were done under my supervision and all the protocols with animals were reviewed and approved by the Research Ethics and Animal Welfare Committee at our Institution Université Catholique de Louvain (2019/UCL/MD004), and was performed according the Belgian National Guidelines in accordance with European Directive.

Please do not hesitate to contact me if you need any other information related to this subject.

Best regards,

Véronique Préat

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Published content (Chapter 1)

International Journal of Pharmaceutics
Volume 523, Issue 2, 25 May 2017, Pages 490-505

Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

Carla Garcia-Mazas, Noemi Csaba, Marcos Garcia-Fuentes

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