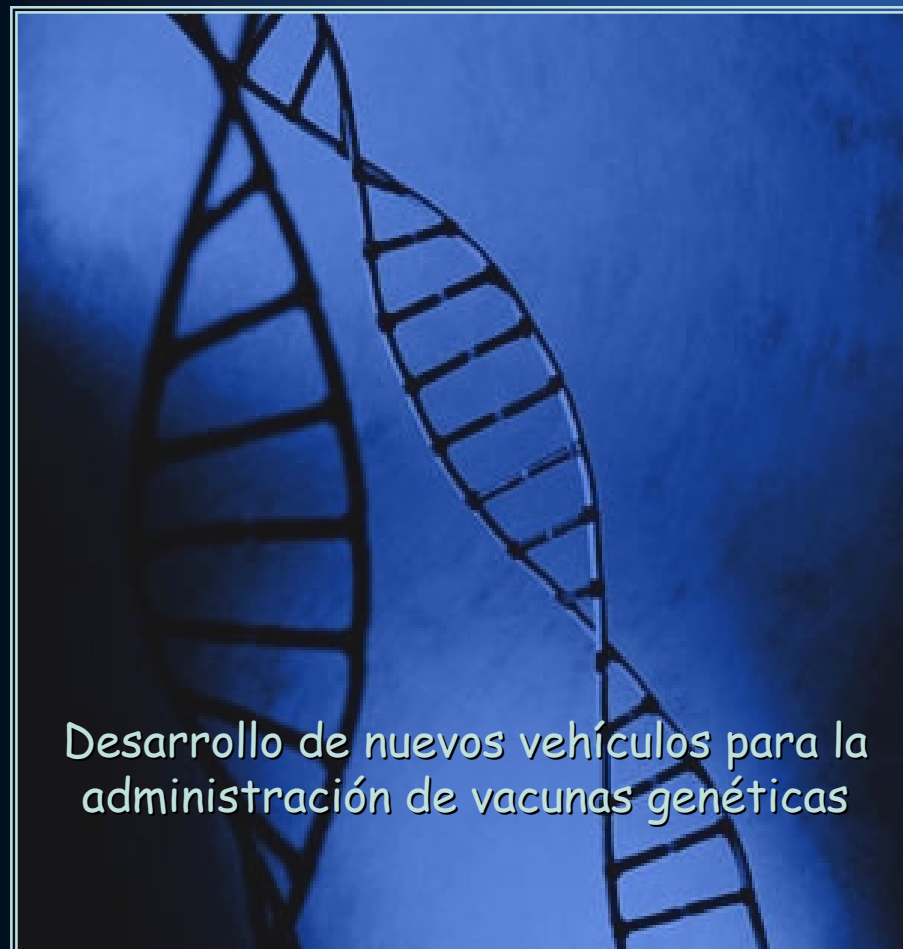


El objetivo principal de este trabajo ha sido el desarrollo de nuevos sistemas nanoparticulares para la vehiculización de ADN. Estas nanoestructuras, que se basan en una composición novedosa de mezclas de ácido (poliláctico-glicólico) con una serie de derivados de polioxietileno (poloxameros y poloxaminas), se prepararon mediante una nueva técnica de emulsificación-difusión. Como consecuencia de la composición original de estos nanosistemas y de las suaves condiciones utilizadas en su preparación, se ha logrado una mejora de la estabilidad del ADN nanoencapsulado así como un importante grado de control de su liberación. Estas nanopartículas mostraron la capacidad de atravesar membranas celulares y barreras biológicas como la mucosa nasal. Además, los estudios "in vivo" de expresión genética han puesto de manifiesto la eficiencia de estas nuevas nanoestructuras -tras su administración por vía nasal- de inducir respuestas inmunes sistémicas prolongadas y específicas a la proteína codificada. Finalmente, los resultados también indicaron que estos nuevos vehículos podrían también ser potencialmente aplicados como sistemas transportadores para liberación controlada de otras moléculas bioactivas de tipo proteico.

The main objective of the present work has been the development of new nanoparticulate carriers for gene delivery. These nanostructures are based on a novel blend composition of poly (D,L-lactic-co-glycolic acid) and a series of polyoxyethylene derivatives (poloxamers and poloxamines), and were prepared by a new emulsification-diffusion technique. This new and versatile matrix composition, together with the mild preparation conditions have led to an improvement of the stability of the nanoencapsulated DNA and also to adjustable controlled release characteristics of the encapsulated plasmid DNA. These nanoparticles exhibited the ability to cross the cell membrane and to overcome biological barriers such as the nasal mucosa. Moreover, the "in vivo" gene expression studies revealed the efficacy of these new blend nanostructures for inducing specific and prolonged systemic immune responses against the encoded protein upon nasal administration. Finally, results also indicate that the application of these novel carriers could potentially be extended to the delivery and controlled release of other type of bioactive molecules such as proteins.

Desarrollo de nuevos vehículos para la administración de vacunas genéticas

Universidad de Santiago de Compostela
Departamento de Farmacia y Tecnología
Farmacéutica



Desarrollo de nuevos vehículos para la
administración de vacunas genéticas

A Mis Padres.

Szüleimnek sok szeretettel.

Noémi Stefánia Csaba

Santiago de Compostela, 2005



**UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
FACULTAD DE FARMACIA**

Departamento de Farmacia y Tecnología Farmacéutica

**Desarrollo de nuevos vehículos para la administración de
vacunas genéticas**

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COMPOSTELA

CERTIFICAN:

Que la presente Memoria Experimental, titulada **“DESARROLLO DE
NUEVOS VEHÍCULOS PARA LA ADMINISTRACIÓN DE
VACUNAS GENÉTICAS”**, elaborada por la Licenciada en Farmacia **Dña
Noémi Stefánia Csaba**, ha sido realizado bajo su dirección en el
Departamento de Farmacia y Tecnología Farmacéutica y, hallándose
concluida, autorizan su presentación a fin de que pueda ser juzgada por el
tribunal correspondiente.

Y para que conste, expiden y firman la presente certificación en Santiago de
Compostela, el 3 de mayo de 2005.

Fdo. M. J. Alonso Fernández

Fdo. A. Sánchez Barreiro

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Introducción

Introducción

El objetivo de esta introducción es el de recoger una serie de conceptos básicos sobre el uso de material genético con fines terapéuticos, así como las características físico-químicas de ADN más relevantes desde un punto de vista farmacéutico y el interés del desarrollo de vacunas basadas en ácidos nucleicos. Asimismo, también se presenta una revisión general de los vectores sintéticos utilizados en la actualidad para la vehiculización del ADN. Teniendo en cuenta que esta tesis doctoral se centra en el desarrollo de nanopartículas poliméricas biodegradables, una revisión más detallada sobre estos sistemas se recoge en los capítulos siguientes de la presente memoria.

1. El ADN plasmídico como fármaco

La unidad básica del ADN es el nucleótido, formado por una molécula de desoxi-D-ribosa, un grupo fosfato y una de las cuatro bases heterocíclicas nitrogenadas de purina (adenina y guanina) o de pirimidina (citosina y timina).¹ Estos nucleótidos se unen formando una cadena de ADN mediante enlaces fosfodiéster. En general, y como describieron Watson y Crick en 1953, la molécula de ADN está constituida por dos cadenas de nucleótidos unidas entre sí formando una estructura de doble hélice. Las dos cadenas de nucleótidos que constituyen una molécula de ADN se mantienen unidas por puentes de hidrógeno entre las bases nitrogenadas de ambas

¹ J.D. Rawn, Bioquímica, 1989, Ed. McGraw-Hill

cadena que quedan enfrentadas. Este enlace está condicionado químicamente, de forma que la adenina sólo se unirá con la timina y la guanina con la citosina. La estructura de un determinado ADN está definida por la secuencia de las cuatro bases en la cadena de nucleótidos, residiendo precisamente en esta secuencia de bases la información genética del ADN.²

Los plásmidos son unidades circulares de ADN extracromosómico de una longitud entre 5000 – 10000 pares de bases que se replican de manera autónoma. Su composición puede ser diseñada para la inserción de la secuencia del gen de interés que codifica la proteína terapéutica o antigénica.³

Además del gen de interés, el ADN plasmídico también debe incluir otras secuencias esenciales para su fabricación y su posterior funcionamiento. Estas unidades son: el origen de replicación bacteriano y el gen de resistencia antibiótica, que permiten su producción en cultivos de bacterias. Además, el ADN plasmídico también debe contener las secuencias promotoras, potenciadoras y de poliadenilación, que son los tres mediadores de la transcripción correcta del gen de interés insertado. La correcta selección de estos elementos puede contribuir al éxito de la transfección.^{4,5}

El ADN plasmídico es una macromolécula hidrofílica, con alta carga negativa que proviene de la presencia de los grupos fosfato en los nucleótidos. Los plásmidos

² J. D. Watson y F. H. C. Crick. A structure for deoxyribose nucleic acid. *Nature* 171:737, 1953.

³ C. R. Middaugh y col. Analysis of plasmid DNA from a pharmaceutical perspective. *J.Pharm.Sci.* 87 (2):130-146, 1998.

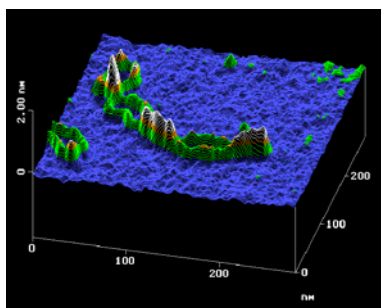
⁴ K. E. Shroff y col. Potential for plasmid DNAs as vaccines for the new millennium. *PSTT* 2 (5):205-212, 1999.

⁵ N. S. Yew. Controlling the kinetics of transgene expression by pasmid design. *Adv. Drug Deliv. Rev.* 57:769-780, 2005.

tienen un peso molecular elevado, superior a los 2.000.000 Daltons y un diámetro hidrodinámico normalmente superior a 100 nm.^{6,7}

La estructura terciaria de un plásmido intacto es la superenrollada. Cuando una de las cadenas de la molécula se corta, se pierde la forma superenrollada/superhelicoidal y el plásmido pasa a una forma más relajada, denominada circular abierta (Figura 1.). Cuando ambas cadenas se rompen en una misma región, la conformación cambia a lineal, una forma biológicamente menos activa.⁸

A.



B.

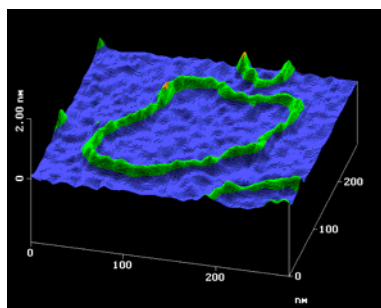


Figura 1. Microfotografías de AFM (microscopía de fuerza atómica) del ADN plasmídico en forma superhelicoidal (A) y circular abierta (B)⁹

La administración “in vivo” de estas estructuras de ADN plásmidico en solitario (conocidas como “plásmidos desnudos”), está limitada por la rápida degradación

⁶ F. D. Ledley. Pharmaceutical approach to somatic gene therapy. *Pharm.Res.* 13 (11):1595-1613, 1996.

⁷ M. Nishikawa y col. Theoretical considerations involving the pharmacokinetics of plasmid DNA. *Adv. Drug Deliv. Reviews* 57:675-688, 2005.

⁸ C. R. Middaugh y col. Analysis of plasmid DNA from a pharmaceutical perspective. *J.Pharm.Sci.* 87 (2):130-146, 1998.

⁹ <http://www.kip.uni-heidelberg.de/> (del Instituto Kirchoff de Física, Heidelberg)

enzimática que sufren por las nucleasas presentes en el suero y en el medio extracelular. Concretamente, según estudios realizados en roedores (mediante PCR y radiomarcaje), la vida media de un plásmido administrado por vía intravenosa no supera los 5 minutos.^{10,11} Por otro lado, el elevado tamaño y alta carga negativa del plásmido dificultan seriamente su penetración a través de barreras biológicas como los epitelios o las membranas celulares.¹²

Una vez internalizado, el plásmido ha de resistir los posibles procesos de degradación durante del tránsito intracelular (pH extremo de los endolisosomas, enzimas del citosol) y, finalmente, ha de acceder al núcleo (Figura 2.).¹³ Debido a la escasa permeabilidad de la membrana nuclear, este último paso representa uno de los mayores obstáculos en la terapia génica.

Estos factores, que en conjunto resultan en un número de células transfectadas insuficiente, limitan seriamente la efectividad de los plásmidos administrados parenteralmente, e imposibilitan, por el momento, su administración por vías mucosas. Consecuentemente, para paliar los problemas biofarmacéuticos asociados al ADN, es necesario usar sistemas de administración que puedan protegerlo y proporcionarle mejores características de transporte a través de barreras biológicas y de transfección.¹⁴

¹⁰ B. E. Houk y col. Pharmacokinetics of plasmid DNA in the rat. *Pharm.Res.* 18:67-74, 2001.

¹¹ Y. K. Oh y col. Prolonged organ retention and safety of plasmid DNA administered in polyethylenimine complexes. *Gene Therapy* 8:1587-1592, 2001.

¹² A. P. Rolland. From genes to gene medicines: recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 15 (2):143-198, 1998.

¹³ D. Lechardeur y col. Intracellular routing of plasmid DNA during non-viral gene transfer. *Adv. Drug Deliv. Rev.* 57:755-767, 2005.

¹⁴ F. D. Ledley. Pharmaceutical approach to somatic gene therapy. *Pharm.Res.* 13 (11):1595-1613, 1996.

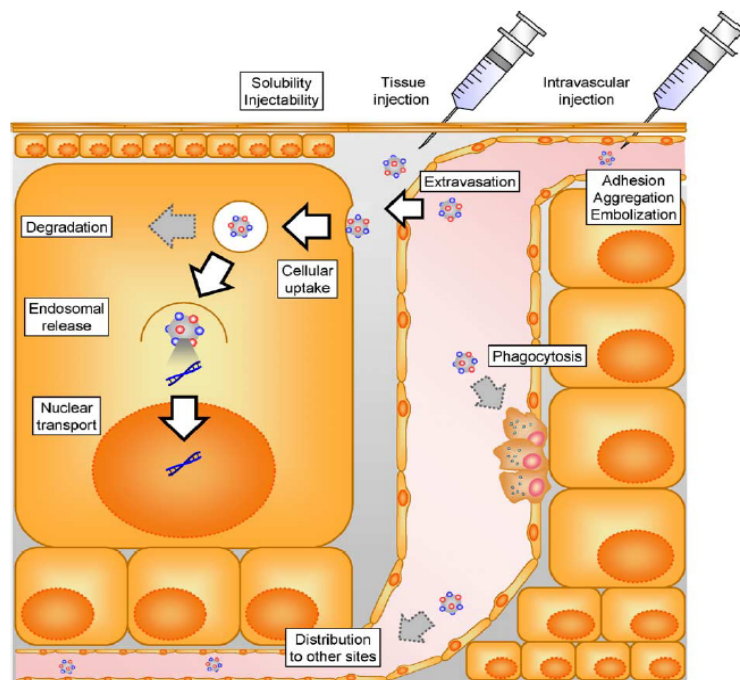


Figura 2. Problemas farmacocinéticos asociados a la administración de ADN “in vivo”. Nishikawa et al.¹⁵

Desde un punto de vista biofarmacéutico, un vehículo ideal tendría que cumplir las siguientes etapas en el organismo:

- Transporte o acceso a un tejido particular en el cuerpo
- Disminuir su captura y respuestas inmunes no deseadas
- Unión a una estructura específica de las células diana
- Internalización al citoplasma (escape de los lisosomas)
- “Targeting” y transporte al núcleo (o mitocondria o cualquier otro

¹⁵ M. Nishikawa y col. Theoretical considerations involving the pharmacokinetics of plasmid DNA. *Adv. Drug Deliv. Rev.* 57:675-688, 2005.

- organelo potencialmente interesante para terapia génica)
- Liberación controlada del material genético transportado para su transcripción óptima¹⁶

2. Vacunas ADN

La terapia génica se define como la introducción de material genético en las células del paciente para aumentar, alterar o inhibir la expresión de un gen o para reemplazar un gen defectuoso. Aunque mayoritariamente las investigaciones se han centrado en el tratamiento del cáncer y en la corrección de las enfermedades provocadas por el defecto en un gen singular (fibrosis quística, hipercolesterolemia familiar, etc.), la terapia génica podría ser útil también para el tratamiento de enfermedades adquiridas como las cardiovasculares, neurológicas o enfermedades infecciosas como el SIDA (Figura 3.).^{17,18} Por otro lado, un área en el que la administración de material genético está cobrando un gran interés es el de la vacunación genética.^{19,20}

La importancia de este concepto, basado en el uso de plásmidos codificantes de antígenos, radica en que dichas vacunas genéticas poseen una serie de ventajas sobre las vacunas tradicionales que se describen a continuación.

¹⁶ R. J. Mersny. Tissue- and cell specific targeting for the delivery of genetic information. en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC press, 2005, 5-27.

¹⁷ R.G. Crystal. Transfer of genes to humans: early lessons and obstacles to success. *Science* 270:404-410, 1995.

¹⁸ I. M. Verma y N. Somia. Gene therapy - promises, problems and prospects. *Nature* 389:239-242, 1997.

¹⁹ H. Tighe y col. Genetic vaccination: plasmid DNA is more than just a blueprint. *Immunology Today* 19 (2):89-97, 1998.

²⁰ D. B. Lowrie. DNA vaccination exploits normal biology. *Nature Med.* 4 (2):147-148, 1998.

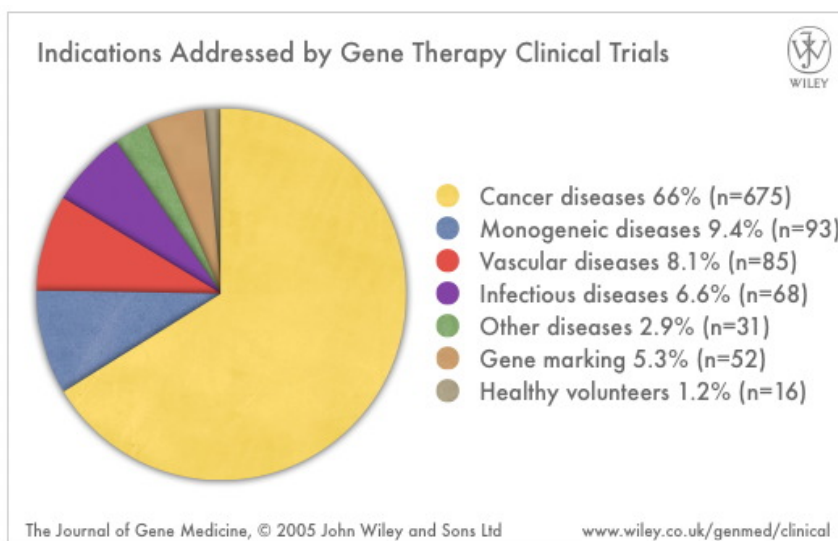


Figura 3. Indicaciones y número de los ensayos clínicos sobre terapia génica (número total = 1020).²¹ Datos actualizados en enero de 2005

Las vacunas convencionales se pueden clasificar en vacunas basadas en bacterias o virus atenuados/inactivados y vacunas derivadas de patógenos muertos, subunitarias y recombinantes.²² El primer grupo, que se basa en el empleo de patógenos vivos, provoca una respuesta fuerte y combinada de inmunidad celular y humoral pero, a su vez, conlleva un riesgo de virulencia.

Este efecto adverso grave se observó por ejemplo en el caso de la vacuna de poliovirus²³, y las vacunas contra sarampión²⁴ y pertussis²⁵.

²¹ <http://www.wiley.co.uk/genmed/clinical> (The Journal of Gene Medicine)

²² T. Storni y col. Immunity in response to particulate antigen-delivery systems. *Adv. Drug Deliv. Rev.* 57:333-355, 2005.

²³ E. A. Cheraskova y col. Long-term circulation of vaccine-derived poliovirus that causes paralytic disease. *J. Virol.* 76:6791-6799, 2002.

Debido a estos problemas, la administración de vacunas basadas en patógenos vivos no es aplicable a las personas inmunodeprimidas, mujeres embarazadas y niños de corta edad.

El segundo grupo de vacunas anteriormente mencionado no presenta riesgos de virulencia o mutación, pero da lugar a respuestas inmunes débiles y, por tanto, se requieren dosis altas y repetidas para la obtención de respuestas inmune. Esta administración repetida conlleva un riesgo considerable de incumplimiento por parte del paciente y hace más costosos y complejos los programas de vacunación.²⁶ Además, estas vacunas inducen únicamente una respuesta humoral, siendo incapaces de provocar respuestas inmunes de tipo celular (mediada por los linfocitos citotóxicos). Esto resulta una desventaja importante en el caso de patógenos intracelulares, ya que en este caso el mecanismo de defensa fundamental del organismo es la activación de los linfocitos CTL (citotóxicos).^{27,28}

La futura generación de vacunas basadas en ácidos nucleicos representa una nueva estrategia, que combina las ventajas de los dos grupos de vacunas anteriormente descritos. Ciertamente, estas vacunas no contienen patógenos, sino ADN codificante del antígeno de interés, lo cual evita problemas de seguridad. De hecho,

²⁴ S. Piyasirisilp y T. Hemachudha. Neurological adverse events associated with vaccination. *Curr.Opin.Neurol.* 15:333-338, 2002.

²⁵ S. Donnelly y col. Whole-cell but not acellular pertussis induce convulsive activity in mice: evidence of a role for toxin induced interleukin-theta in a new murine model for analysis of neuronal side effects of vaccination. *Infect.Immun.* 69:4217-4223, 2001.

²⁶ M Friede y M. T. Aguado. Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. *Adv. Drug Deliv. Rev.* 57 :325-331, 2005.

²⁷ S. Raychaudhuri y K. L. Rock. Fully mobilizing host defense: building better vaccines. *Nature Biotech.* 16:1025-1031, 1998.

²⁸ T Storni y col. Immunity in response to particulate antigen-delivery systems. *Adv. Drug Deliv. Rev.* 57:333-355, 2005.

hasta la fecha no existe ninguna evidencia de que la administración de ácidos nucleicos genere efectos tóxicos. En este sentido, conviene recordar que en las vacunas tradicionales el ADN de los patógenos es habitualmente administrado como contaminante natural sin que ello haya producido efectos adversos.²⁹

Asimismo, las vacunas genéticas generan una respuesta inmune fuerte y eficaz, con un perfil similar a la obtenida tras la administración de patógenos atenuados, incluyendo respuestas celulares.³⁰ La presencia de la respuesta celular en este caso podría explicarse por la producción “de novo” de antígeno en el interior de las células del sistema inmune, tras la transfección y consiguiente expresión de la proteína codificada. Por tanto, su procesamiento por las células presentadoras de antígenos (APC) sería similar a la que tiene lugar para los patógenos intracelulares como por ejemplo los virus.³¹ Adicionalmente, puede darse un proceso denominado “cross priming”, en el que las células comunes también traspasan los antígenos producidos a las células presentadoras, potenciando así las respuestas celulares obtenidas.³² Aparte de su seguridad y su capacidad de generar defensas muy adecuadas y fuertes, las vacunas genéticas también muestran otras características ventajosas muy relevantes.^{33,34}

²⁹ C. W. Beard y P. W. Mason. Out of the farm with DNA vaccines. *Nature Biotech.* 16:1325-1328, 1998.

³⁰ J. R. Haynes y col. Accell particle-mediated DNA immunization elicits humoral, cytotoxic and protective immune responses. *AIDS research and human retroviruses* 10 (2):S43-S45, 1994.

³¹ J. L. Whitton y col. DNA immunization: mechanistic studies. *Vaccine* 17:1612-1619, 1999.

³² D. M. Feltquate. DNA vaccines: vector design, delivery and antigen presentation. *J.Cell.Biochem.* 30:304-311, 1998.

³³ M. Giese. DNA-antiviral vaccines: New developments and approaches - A review. *Virus Genes* 17 (3):219-232, 1998.

Estas ventajas adicionales son:

- la fabricación relativamente barata de los plásmidos (una vez identificada una secuencia que codifica la molécula antigénica) comparado con el elevado coste de obtener patógenos atenuados o antígenos específicos
- la existencia de métodos adecuados para su eficiente purificación, lo que permite eliminar los agentes contaminantes, típicamente presentes en las vacunas convencionales
- la elevada estabilidad de los ácidos nucleicos, que hace pensar en la posibilidad de evitar la cadena fría para el almacenamiento y transporte de las vacunas de ADN, otro factor que contribuiría a reducir los gastos de producción y aplicación de las mismas.

Todas estas ventajas reflejan el gran potencial clínico del ADN plasmídico y el interés de su empleo para evitar las limitaciones que plantea el uso de proteínas (reducida biodisponibilidad, problemas farmacocinéticos, elevado coste de fabricación, etc.).^{35, 36}

3. Vectores sintéticos como sistemas de liberación de plásmidos: nuevas tendencias y últimos avances

Los sistemas de liberación de ADN se clasifican tradicionalmente en vectores virales y vectores sintéticos. Debido a que los virus tienen la capacidad natural de

³⁴ M. Friede y M. T. Aguado. Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. *Ad. Drug Deliv. Rev.* 57 :325-331, 2005.

³⁵ H. L. Robinson. Nucleic acid vaccines: an overview. *Vaccine* 15 (8):785-787, 1997.

³⁶ A. P. Rolland. From genes to gene medicines: recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 15 (2):143-198, 1998.

transfectar células, son considerados como los vectores más efectivos para el transporte y expresión del material genético.³⁷ Sin embargo, por el momento, su uso está limitado debido al posible riesgo de mutaciones o recombinaciones. Otros inconvenientes de los vectores virales son su toxicidad, la incapacidad de transportar cantidades elevadas de ADN y su elevado coste de producción.³⁸

A pesar de estas limitaciones, actualmente la mayoría de los ensayos clínicos en terapia génica se centran en el empleo de vectores virales como sistemas transportadores del material genético (Figura 4.).

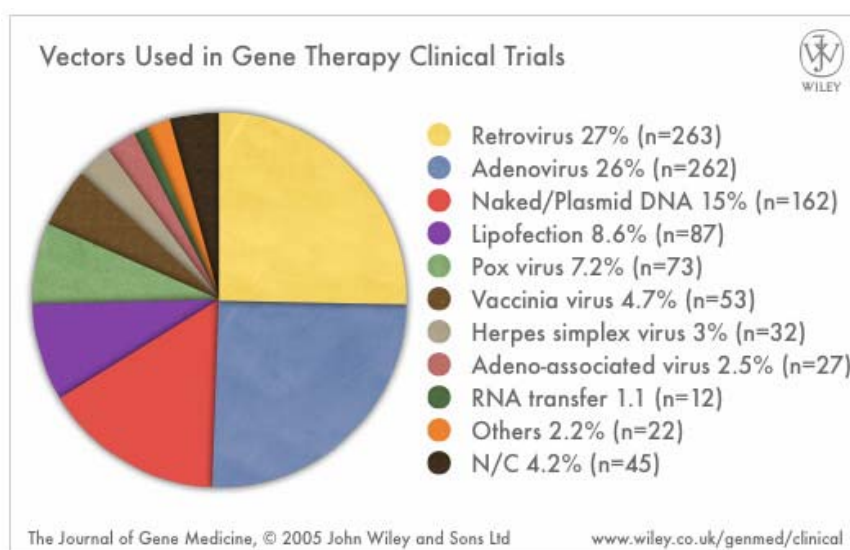


Figura 4. Vectores usados en los ensayos clínicos de terapia génica. Datos actualizados en enero de 2005.³⁹

³⁷ A. Bout. Gene Therapy. In: *Pharmaceutical Biotechnology*, Ed. D. J. A. Crommerlin y R. Sindelar, Harwood Academic Publishers, 1997, 167-183.

³⁸ A. P. Rolland. From genes to gene medicines: recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 15 (2):143-198, 1998.

³⁹ <http://www.wiley.co.uk/genmed/clinical> (The Journal of Gene Medicine)

No obstante, casos preocupantes como la muerte del voluntario J. Gelsinger y el desarrollo de leucemia en niños tras tratamientos virales contra enfermedades genéticas han puesto recientemente de manifiesto los serios problemas asociados a la aplicación de vectores virales en humanos.^{40,41}

Debido a estas razones, el interés de los vectores sintéticos es cada vez mayor, ya que estos sistemas no tienen el riesgo de reproducción o recombinación y además, su fabricación y modificación es relativamente fácil.⁴² Otra ventaja importante de los vectores sintéticos reside en su capacidad para vehiculizar moléculas de ADN independientemente de su tamaño. Como consecuencia, en las últimas décadas se han propuesto un gran número de sistemas basados en métodos físicos y/o químicos como alternativas al uso de los vectores virales en la terapia génica. Entre estos sistemas destacan los vectores poliméricos, que se pueden clasificar en: polímeros catiónicos, polímeros neutros no condensantes y polímeros biodegradables.

3.1. Polímeros catiónicos

La utilización de polímeros catiónicos se basa en la capacidad de estos polímeros de condensar el ADN plasmídico mediante fuerzas electrostáticas, dando lugar a unas estructuras más pequeñas (normalmente de forma globular o toroidal) y, a la vez, enmascarar la carga negativa de la molécula.⁴³ La reducción de tamaño y carga

⁴⁰ D. Ferber. Gene therapy: safer and virus-free? *Science* 294 (5547):1638-1642, 2001.

⁴¹ D. B. Kohn y col. Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat.Rev.Cancer* 3 (7):477-488, 2003.

⁴² D. Luo y W. M. Saltzman. Synthetic DNA delivery systems. *Nature Biotech.* 18:33-37, 1999.

⁴³ M. C. Tang y F. C. Szoka. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Therapy* 4:823-832, 1997.

tras la complejación podría favorecer la interacción del plásmido con las células, y por tanto, podría mejorar la eficiencia de transfección. Sin embargo, la carga positiva de los complejos obtenidos puede causar la activación del sistema de complemento con la consiguiente opsonización y eliminación de los sistemas antes de llegar a sus células diana.^{44,45}

3.1.1. Polilisina y derivados

El hecho de que los péptidos que condensan el ADN en la naturaleza (como las histonas o los péptidos virales) contengan un alto porcentaje de aminoácidos básicos ha sugerido la utilización de moléculas peptídicas como vectores para terapia génica. Entre ellos, el más utilizado ha sido la polilisina (PLL) (Figura 5).

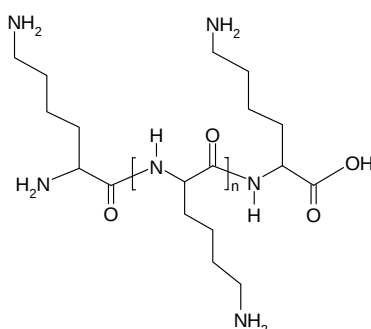


Figura 5. Estructura química de la polilisina

⁴⁴ S. C. de Smedt y J. Demesteer. Cationic polymer based gene delivery systems. *Pharm.Res.* 17 (2):113-126, 2000.

⁴⁵ D. Finsinger y col. Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. *Gene Therapy* 7:1183-1192, 2000.

Debido a su elevada citotoxicidad y su carácter no biodegradable, en los últimos años las investigaciones sobre estos polímeros han ido dirigidas al desarrollo de polímeros basados en la depolimerización y la pegylación de la poly(L-lisina),⁴⁶ o en su conjugación con polímeros biodegradables como polisacáridos o poliésteres.⁴⁷

Otro objetivo de las modificaciones químicas de la polilisina ha sido mejorar la eficiencia de transfección del complejo PLL-ADN. Así por ejemplo, se ha sintetizado una amplia variedad de derivados de la PLL que poseen ligandos para favorecer su transporte a las células diana, aumentando la endocitosis del complejo mediante su unión a receptores específicos.

Los más eficientes en esta categoría son los conjugados:

- PLL-transferrina, PLL-folato y PLL-anticuerpo, destinados a facilitar el acceso a células cancerígenas^{48,49}
- PLL-asialoorosomucoid y PLL-lactosa/galactosa, destinados a conseguir la vehiculización hacia las células hepáticas⁵⁰

⁴⁶ M. Mannisto y col. Structure activity relationships of poly(L-lysines): effects of pegylation and molecular shape on the physicochemical and biological properties. *J.Control.Rel.* 83:169-182, 2002.

⁴⁷ J. H. Jeong y T. G. Park. Poly(L-lysine)-g-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. *J.Control.Rel.* 82 (1):159-166, 2002.

⁴⁸ C. M. Ward y col. Modification of PLL-DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo. *J.Gene.Med.* 4:1-12, 2002.

⁴⁹ W. Suh y col. Anti-JL1 antibody-conjugated poly(L-lysine) for targeted gene delivery to leukaemia T cells. *J.Control.Rel.* 72:171-178, 2001.

⁵⁰ M. Nishikawa y col. Targeted delivery of plasmid DNA to hepatocytes in vivo: optimisation of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. *J.Pharmacol.Exp.Ther.* 287:408-415, 1998.

También existen sistemas combinados como los conjugados PLL-PEG-lactosa, PLL-PEG-folato, PLL-PEG-AWGB (péptido para la unión al endotelio arterial) o el sistema terplex que consiste en estearil-PLL y una lipoproteína de baja densidad.⁵¹

Otra alternativa propuesta para aumentar la eficiencia del sistema se ha basado en la introducción de secuencias peptídicas específicas que permitan modificar su transporte intracelular. En esta línea destacan, por ejemplo, los péptidos fusogénicos, la histidina/polihistidina y las secuencias de localización nuclear.^{52,53}

3.1.2. Polietilenimina

La polietilenimina (PEI) está considerada como uno de los vectores más eficientes en terapia génica “in vitro” y también “in vivo” (Figura 6.). Debido al elevado número de átomos de nitrógeno protonables, la cadena polimérica posee una alta densidad de cargas positivas, lo que permite la formación de complejos de tamaños muy reducidos.⁵⁴

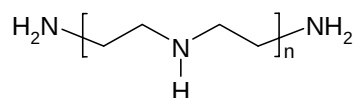


Figura 6. Estructura química de la polietilenimina

⁵¹ M. Lee y S. W. Kim. Poly(L-lysine) and copolymers for gene delivery. en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, 79-95.

⁵² E. Wagner y col. Polylysine-based transfection systems utilizing receptor-mediated delivery. *Adv. Drug Deliv. Rev.* 30:97-114, 1998.

⁵³ C. K. Chan y col. Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines. *Gene Therapy* 7:1690-1697, 2000.

⁵⁴ M. C. Garrett. Gene delivery systems using cationic polymers. *Crit. Rev. Ther. Drug Carrier Syst.* 16 (2):147-207, 1999.

La presencia de estos grupos protonables en la estructura polimérica también tiene importancia en el proceso de internalización celular. Su capacidad de amortiguar los cambios de pH confiere protección al plásmido y posibilita su escape de los endolisosomas tras su internalización.⁵⁵

El potencial de PEI ha sido evaluado en varias líneas celulares (incluidas células de origen humano) y también “in vivo”. Los resultados obtenidos han puesto de manifiesto su elevada capacidad de transfección. No obstante, en la mayoría de los casos también se observaron efectos tóxicos importantes asociados a su capacidad permeabilizante de la membrana celular.⁵⁶ Para solventar este problema, recientemente se han sintetizado PEIs de bajo peso molecular a las que se atribuyen menor capacidad de disrupción de membranas y, por tanto, una menor toxicidad.⁵⁷ Además del peso molecular, la eficiencia/toxicidad del sistema también depende de la estructura polimérica (linear o ramificada) y la relación de cargas entre el polímero catiónico y el ADN.⁵⁸

Al igual que en el caso de la polilisina, la PEI también ha sido modificada químicamente con el fin de aumentar su eficiencia de transfección. Así por ejemplo, los polímeros pegilados y los conjugados con ligandos como galactosa,

⁵⁵ W. T. Godbey y col. Poly(ethylenimine) and its role in gene delivery. *J.Control.Rel.* 60 (149):160, 1999.

⁵⁶ W. T. Godbey y col. Poly(ethyleneimine)-mediated gene delivery affects endothelial cell function and viability. *Biomaterials* 22 (471):480, 2001.

⁵⁷ K. Kunath y col. Low-molecular weight polyethyleneimine as non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo biodistribution with high molecular weight polyethyleneimine. *J.Control.Rel.* 89:113-125, 2003.

⁵⁸ L. Wightman y col. Different behavior of branched and linear polyethileneimine for gene delivery in vitro and in vivo. *J.Gene.Med.* 3 (4):362-372, 2001.

transferrina o folato han dado lugar a eficacias de transfección superiores a las obtenidas con la polietilenimina original.^{59, 60} Asimismo, en la actualidad se están estudiando diversos sistemas basados en conjugados de β -ciclodextrinas con polietileniminas (lineares y ramificadas) para la vehiculización del ADN.⁶¹

3.1.3 Dendrímeros

Los dendrímeros propuestos como vehículos de ADN son polímeros catiónicos con una estructura simétrica tridimensional altamente ramificada. Estos polímeros normalmente tienen forma globular (Starburst) y poseen un elevado número de grupos amina protonables, que permiten la interacción del sistema con el ADN (Figura 7.).

Esta arquitectura macromolecular se consigue mediante reacciones repetidas de polimerización, dando lugar a la formación de nuevas generaciones de dendrímeros. Cada reacción aumenta en $\sim 1\text{nm}$ el diámetro de la estructura esférica, además de duplicar la masa molecular y el número de los grupos amina superficiales.⁶²

⁵⁹ M. Ogris. Gene delivery using polyethyleneimine and copolymers. en: *Polymeric gene delivery: principles and applications*, ed M. M. Amiji, CRC Press, 2005, 97-106.

⁶⁰ E. Wagner. Strategies to improve DNA polyplexes for in vivo gene transfer: Will artificial viruses be the answer? *Pharm.Res.* 21 (1):8-14, 2004.

⁶¹ S. H. Pun y M. E. Davis. Cyclodextrin-containing polymers for gene delivery. In: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, 187-210.

⁶² D. A. Tomalia y J. M. J. Frechet. Discovery of dendrimers: a brief historical perspective. *J.Polymer Sci.* 40:2719-2728, 2002.

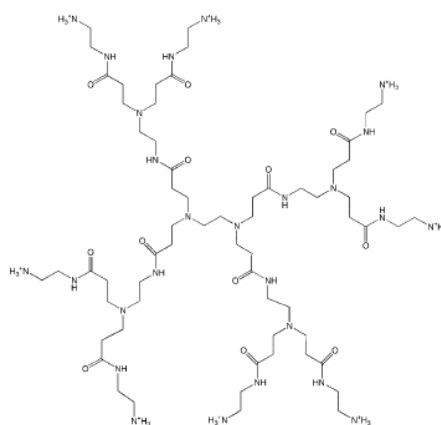


Figura 7. Estructura química de dendrímeros de poliamidoamina

En general, la toxicidad y la eficiencia de transfección de los polímeros catiónicos están relacionadas entre sí, de manera que los sistemas más eficientes conllevan efectos tóxicos más importantes. Sin embargo, en el caso de los dendrímeros, a pesar de su eficiencia, el problema de toxicidad es significativamente inferior al de otros polímeros catiónicos. De hecho, estudios llevados a cabo en cultivos celulares han puesto de manifiesto el siguiente orden de citotoxicidad: PEI>PLL>dendrímero.^{63,64}

Los dendrímeros más utilizados son los elaborados a partir de poliamidoamina (PAMAM starburst dendrimer). Una variedad de estos dendrímeros PAMAM está

⁶³ E. Tomlinson y A. P. Rolland. Controllable gene therapy, Pharmaceuticals of non-viral gene delivery systems. *J.Control.Rel.* 39:357-372, 1996.

⁶⁴ D. Fisher y col. In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and haemolysis. *Biomaterials* 24 (1121-1131), 2003.

comercializada con el nombre Superfect[®] y está considerada como uno de los agentes más eficientes en la transfección in vitro.⁶⁵

Otras modificaciones prometedoras para mejorar las características de esta clase de polímeros incluyen las que pueden producirse en la superficie con PEG o con L-arginina,⁶⁶ la conjugación con secuencias endosomolíticas (el péptido GALA),⁶⁷ así como la conjugación con ligandos como el EGF (factor de crecimiento epitelial) para el targeting a sus receptores específicos⁶⁸ y la conjugación con alfa-ciclodextrinas.⁶⁹

Recientemente se han investigado nuevos dendrímeros sintetizados a partir de polipropilenimina o dendrímeros bifuncionales obtenidos con la combinación de péptidos catiónicos y lípidos.^{70,71} Actualmente se está estudiando el potencial de estos nuevos sistemas que también podrían ser alternativas de interés para la vehiculización de ADN.

⁶⁵ <http://www1.qiagen.com/literature/handbooks/PDF/Transfection>

⁶⁶ J. S. Choi y col. Poly(ethylene glycol)-conjugated cationic dendrimers. In: *Polymeric gene delivery: principles and applications*, edited by M. M. Amiji, CRC Press, 2005, p. 159-173.

⁶⁷ J. Haensler and F. Szoka. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconj.Chem.* 4:372-379, 1993.

⁶⁸ R. F. Barth y col. Molecular targeting of epidermal growth factor receptor for neuron capture therapy of gliomas. *Cancer Res.* 13:1181-1185, 2002.

⁶⁹ S. H. Pun y M. E. Davis. Cyclodextrin-containing polymers for gene delivery. In: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, p. 187-210.

⁷⁰ L. A. Kubasiak y D. A. Tomalia. Cationic dendrimers as gene transfection vectors: dendri-poly(amidoamines) and dendri-poly(propylenimines). en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, 133-157.

⁷¹ H. K. Bayele y col. Versatile peptide dendrimers for nucleic acid delivery. *J.Pharm.Sci.* 94 (2):446-457, 2005.

3.1.4. Quitosano

El quitosano es un polisacárido de origen natural (compuesto por unidades de D-glucosamina y N-acetil-D-glucosamina unidas mediante enlaces glicosídicos β (1,4)) que se obtiene de la desacetilación de la quitina (Figura 8.). A diferencia de los sistemas catiónicos anteriormente descritos, el uso de quitosano no se restringe a las aplicaciones en terapia génica. Debido a su biodegradabilidad, mucoadhesividad y sus características de biocompatibilidad favorables, este polímero ha sido utilizado ampliamente para la vehiculización de un gran número de moléculas biológicamente activas.⁷²

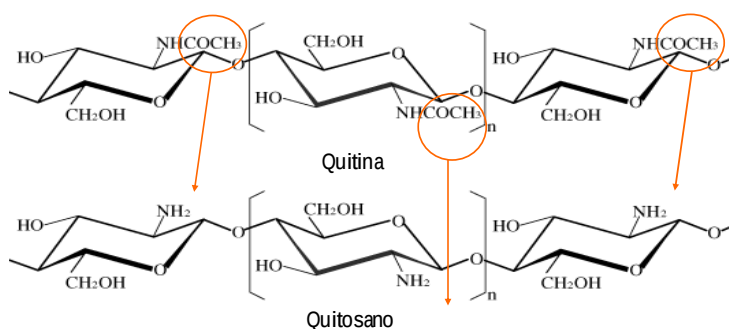


Figura 8. Desacetilación de la quitina a quitosano

Además, en el caso del quitosano las posibilidades de asociación de ADN no se limita complejación electrostática, sino que también existe la posibilidad de elaboración de micro- y nanopartículas poliméricas en las que se puede encapsular el material genético.⁷³

⁷² K. A. Janes y col. Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug Deliv. Rev.* 47:83-97, 2001.

⁷³ W. Paul y C. P. Sharma. Chitosan, a drug carrier for the 21st century: a review. *S.T.P. Pharma Sciences* 10 (1):5-22, 2000.

En este contexto, se ha investigado ampliamente el potencial de quitosano con especial interés en los efectos del grado de desacetilación y el peso molecular. Como tendencia general, se puede concluir que:

- un grado de acetilación baja facilita la interacción del quitosano con el ADN, dando lugar a la formación de complejos nanométricos⁷⁴
- el bajo peso molecular (combinado con una pureza muy alta) lleva asociada una menor toxicidad⁷⁵ y favorece además, la disociación intracelular del complejo quitosano-ADN, mejorando así la eficiencia de transfección del sistema⁷⁶

Además del análisis de la influencia de las características intrínsecas del quitosano, otros estudios han investigado los efectos de ciertas modificaciones químicas en la eficiencia de complejación y transporte del ADN. Estas modificaciones y sus efectos en las características físico-químicas de los vehículos y en su eficacia de transfección, se encuentran recogidos en la Tabla 1.

⁷⁴ T. Kiang y col. The effect of degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials* 25:5293-5301, 2004.

⁷⁵ M. Koping-Hoggard y col. Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethyleneimine in vitro and after lung administration in vivo. *Gene Therapy* 8:1108-1121, 2001.

⁷⁶ S. C. W. Richardson y col. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. *Int.J.Pharm.* 178:231-243, 1999.

Derivados de quitosano	Objetivo	Sistema	Transfección	Ref.
QS-deoxicólico	↑ solubilización ↑ estabilidad	autoagregados	↓	[1,2]
Cuaternizado	↑ solubilización ↑ carga positiva	complejos	↑	[3]
Dodecilado	↑ estabilidad ↑ protección	complejos	sin determinar	[4]
Pegilado	↑ estabilidad ↓ toxicidad	complejos, nanopartículas	↑	[5,6]
Lactosilado	targeting	complejos	↓	[7]
Galactosilado	targeting	complejos coacervados	↑ en células hepáticas	[8,9]
Galactosilado y pegilado	targeting ↑ estabilidad	complejos coacervados	↑ en células hepáticas	[10]
Transferrina	targeting	complejos	↑	[11]
Proteína KNOB	targeting	complejos	↑	[12]
Péptido GM227.3	↑ endosomolisis	complejos	↑	[13]

Tabla 1. Derivados de quitosano propuestos como vehículos de ADN

Referencias de la Tabla 1.

1. W. G. Liu y K. D. Yao. Chitosan and its derivatives - a promising non-viral vector for gene transfection. *J.Control.Rel.* 83:1-11, 2002.
2. K. Y. Lee y col. Preparation of chitosan self-aggregates as a gene delivery system. *J.Control.Rel.* 51:213-220, 1998.
3. M. Thanou y col. Quaternized chitosan oligomers as gene delivery vectors in vivo. *J.Control.Rel.* 87:294-295, 2003.
4. W. G. Liu y col. Formation of a DNA/N-dodecylated chitosan complex and salt induced gene delivery. *J.Appl.Polym.Sci.* 82 (14):3391-3395, 2001.
5. H. Q. Mao y col. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J.Control.Rel.* 70:399-421, 2001.
6. N. Csaba y col. Ionically crosslinked chitosan nanoparticles as gene delivery systems: effect of pegylation on *in vitro* and *in vivo* gene transfer, sometida para su evaluación, 2005
7. P. Erbacher y col. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. *Pharm.Res.* 15 (9):1332-1339, 1998.
8. I. K. Park y col. Galactosylated chitosan (GC)-graft-poly(vinyl pyrrolidone) (PVP) as hepatocyte-targeting DNA carrier: Preparation and physicochemical characterization of GC-graft-PVP/DNA complex. *J.Control.Rel.* 86:349-359, 2003.
9. I. K. Park y col. Galactosylated chitosan-graft dextran as hepatocyte targeting DNA carrier. *J.Control.Rel.* 69 (1):97-108, 2000.
10. I. K. Park y col. Visualization of transfection of hepatocytes by galactosylated chitosan-graft-poly(ethylene glycol)/DNA complexes by confocal laser scanning microscopy. *Int.J.Pharm.* 257:103-110, 2003.
11. E. Wagner y col. Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Adv.Drug Deliv.Rev.* 14 (1):113-135, 1994.
12. T. Azzam y A. J. Domb. Cationic polysaccharides for gene delivery. en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, p. 279-299.
13. S. C. de Smedt y J. Demesteer. Cationic polymer based gene delivery systems. *Pharm.Res.* 17 (2):113-126, 2000.

Finalmente, una propuesta reciente ha sido la encapsulación del ADN en estructuras nanoparticulares obtenidas mediante gelificación iónica, empleando un agente reticulante como el tripolifosfato. Estas nanoestructuras, que ya han sido utilizadas con éxito para la encapsulación de proteínas y antígenos, ofrecen considerables ventajas en comparación con los complejos anteriormente descritos, como son:

- alta reproducibilidad, distribución de tamaño más homogénea
- protección eficiente de la macromolécula en el interior de la matriz polimérica
- liberación controlada
- posibilidad de coencapsular (sin modificaciones químicas del sistema) el ADN con otras moléculas que podrían facilitar su transporte en el organismo o su internalización celular/nuclear.^{77,78}

3.2. Polímeros no condensantes

Este grupo incluye una serie de polímeros que no contienen grupos cargados y cuya interacción con el ADN ocurre principalmente mediante enlaces débiles como puentes de hidrógeno y enlaces Van der Waals, manteniendo una estructura flexible no condensada.⁷⁹

⁷⁷ P. Calvo y col. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharm.Res.* 14 (10):1431-1436, 1997.

⁷⁸ K. A. Janes y col. Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug Deliv. Rev.* 47:83-97, 2001.

⁷⁹ A. Prokop y col. Maximizing the in vivo efficiency of gene transfer by means of nonviral polymeric gene delivery vehicles. *J.Pharm.Sci.* 91 (1):67-76, 2002.

En este caso los complejos resultantes tienen carga neutra o carga negativa moderada.⁸⁰ Este tipo de polímeros han resultado particularmente eficaces tras su administración intramuscular, probablemente debido a la combinación de múltiples factores, como facilitar una mayor penetración y distribución del ADN asociado en el tejido muscular y a su efecto protector frente a las endonucleasas.^{81,82} No obstante, este hecho no limita la aplicación exclusiva de estos sistemas a efectos locales musculares, ya que las células del tejido muscular tienen una elevada capacidad de sintetizar proteínas, que pueden ser útiles para el tratamiento de varias deficiencias de proteínas o para vacunación genética.⁸³

Los representantes más importantes de este grupo son los polivinilos y los polímeros tribloque tipo poloxámero. Estos polímeros son biocompatibles, con amplio historial de aplicación farmacéutica como excipientes solubilizantes, lubricantes, estabilizantes y como materiales de recubrimiento.⁸⁴

3.2.1. Polivinilos

Estos homopolímeros estructuralmente relacionados, como la polivinilpirrolidona (PVP) y el polivinilalcohol (PVA), también son conocidos en el campo de terapia génica con el nombre PINC, por tratarse de polímeros interactivos no

⁸⁰ J. R. Mumper y col. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. *Pharm.Res.* 13:701-709, 1996.

⁸¹ A. P. Rolland. From genes to gene medicines: recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 15 (2):143-198, 1998.

⁸² P. Lemieux y col. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Therapy* 7:986-991, 2000.

⁸³ Q. L. Lu y col. Non-viral gene delivery in skeletal muscle: a protein factory. *Gene Therapy* 10 (2):131-142, 2003.

⁸⁴ *Handbook of pharmaceutical excipients*, ed. Rowe, R.C y col., Pharmaceutical Press, 2003

condensantes.⁸⁵ Son moléculas altamente hidrosolubles, capaces de formar complejos con el ADN mediante enlaces secundarios, típicamente en el rango de pH 4-6 encontrándose esta interacción condicionada por la fuerza iónica del medio acuoso.

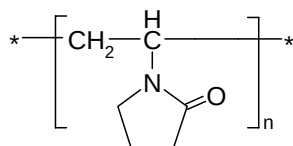


Figura 9. Estructura química de la polivinilpirrolidona

En particular, se ha descrito que la presencia de PVP (el polímero PINC más estudiado) facilita la interacción del ADN con las membranas biológicas y, por otro lado, una vez internalizado el complejo, impide su procesamiento por endolisosomas. Este efecto es probablemente debido a la desestabilización de las membranas del endosoma antes de su conversión a lisosomas degradativos.⁸⁶

3.2.2 Poloxameros

Los poloxameros (Pluronic[®]) son copolímeros neutros tribloque, constituidos por cadenas flexibles de polioxietileno (POE) y polioxipropileno (POP). Similarmente a los polivinilos, son muy solubles en agua pero, en este caso, debido a su carácter anfifílico, a partir de cierta concentración los poloxameros tienden a formar

⁸⁵ J. R. Mumper y col. Protective interactive noncondensing (PINC) polymers for enhanced plasmid distribution and expression in rat skeletal muscle. *J.Control.Rel.* 52:191-203, 1998.

⁸⁶ K. Ciftci and R. J. Levy. Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts. *Int.J.Pharm.* 218:81-92, 2001.

micelas. Las propiedades (solubilidad, HLB) de los poloxameros varían según la relación de monómeros OE:OP y el peso molecular de sus cadenas.⁸⁷

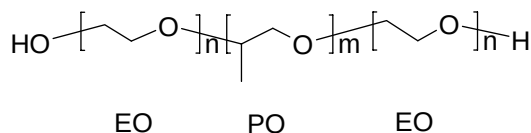


Figura 9. Estructura química de los poloxameros

En estos últimos años se ha descubierto que estos polímeros muestran propiedades interesantes, como por ejemplo facilitar la penetración de fármacos a través de la barrera hematoencefálica, o modificar el funcionamiento del complejo glicoproteína P (sistema de transporte activo en la membrana celular).^{88,89}

En el contexto de la terapia génica, los poloxameros se pueden aplicar en forma de:

- complejos y nanogeles, combinando poloxameros de distintas características, como en el sistema SP1017, una mezcla de Pluronic[®] L61 (hidrofóbico, Mw bajo) con el Pluronic[®] F127 (hidrofilico, Mw alto) que resultó muy eficaz como vehículo de transfección en cultivos celulares y también tras su administración por vía intramuscular⁹⁰

⁸⁷ A. V. Kabanov y col. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. *J.Control.Rel.* 82:189-212, 2002.

⁸⁸ S. M. Moghimi y A. C. Hunter. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *Tibtech* 18:412-420, 2003.

⁸⁹ V. A. Kabanov y V. Alakhov. Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. *Crit. Rev. Ther.Drug Carrier Syst.* 19 (1):1-73, 2002.

⁹⁰ P. Lemieux A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Therapy* 7:986-991, 2000.

- componente estructural en vehículos basados en polímeros catiónicos como el Pluronic 123-polietilenimina o el Pluronic 127-polilisina. En estos casos la eficiencia de transfección característica de los polímeros catiónicos se vio ligeramente disminuida. No obstante, esta disminución podría verse compensada por el efecto positivo que ejercen los poloxameros en la estabilidad de estos sistemas policationicos⁹¹

3.3. Polímeros biodegradables

Los vehículos elaborados a partir de materiales poliméricas biodegradables tienen la capacidad de encapsular moléculas terapéuticas y liberarlas de manera controlada. Los materiales más utilizados para la preparación de estos sistemas son los poliésteres ácido poliláctico (PLA) y el ácido poliláctico-glicólico (PLGA).

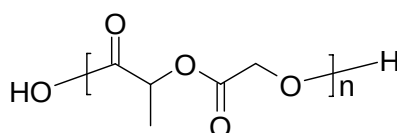


Figura 10. Estructura química del ácido poliláctico-glicólico

Estos polímeros, conocidos por su biocompatibilidad y seguridad, se degradan por hidrólisis no enzimática dando lugar a productos naturales que se eliminan en el organismo sin causar efectos adversos.⁹² Varios estudios han mostrado la capacidad de estos polímeros para encapsular ADN plasmídico en micro-, y recientemente,

⁹¹ A. V. Kabanov y col. Pluronic block copolymers for nonviral gene delivery. en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, 313-328.

⁹² D. E. Perrin y J. P. English. Polyglycolide and polylactide. en: *Handbook of biodegradable polymers*, ed. A. J. Domb, Harwood Academic Publishers, 1997, 3-27.

también en nanopartículas, lográndose una liberación controlada de ADN a partir de las mismas.^{93,94,95 96,97}

En estos últimos años se han desarrollado una serie de estrategias con el fin de optimizar las características originales de estos vehículos biodegradables. Estas modificaciones y sus efectos en las características de las micro- y nanopartículas se encuentran recogidos en la Tabla 2.

Aunque muchas de estas modificaciones resultan prometedoras, la mayor limitación de estas partículas biodegradables sigue siendo su deficiente estabilidad “in vivo”. Tras su administración, estas partículas son rápidamente eliminadas de la circulación por el sistema fagocítico mononuclear, imposibilitando el transporte del material encapsulado a las células diana. En este sentido, el uso de diversas combinaciones de poliésteres con polioxietileno y sus derivados podría ofrecer una alternativa interesante, ya que la presencia de las cadenas hidrofílicas de polioxietileno confiere mayor estabilidad (tanto física como biológica) al sistema al producir un efecto de repulsión/protección estérica.^{98,99}

⁹³ T. Hao y col. Biological potency of microsphere encapsulated plasmid DNA. *J.Control.Rel.* 69:249-259, 2000.

⁹⁴ E. Walter y col. Gene delivery systems to phagocytic antigen presenting cells. *S.T.P.Pharmaceutical Sciences* 11 (1):45-56, 2001.

⁹⁵ D. Luo y col. Controlled DNA delivery systems. *Pharm.Res.* 16 (8):1300-1308, 1999.

⁹⁶ V. Labhasetwar y col. Gene transfection using biodegradable nanospheres: results in tissue culture and a rat osteotomy model. *Colloid.Surf.B: Biointerf.* 16:281-290, 1999.

⁹⁷ S. Prabha y col. Biodegradable nanoparticles as a gene expression vector. en: *Polymeric gene delivery: principles and applications*, ed. M. M. Amiji, CRC Press, 2005, 357-366.

⁹⁸ R. Gref y col. Biodegradable long circulating polymeric nanospheres. *Science* 263:1600-1603, 1994.

⁹⁹ M. J. Alonso. Nanomedicines for overcoming biological barriers. *Biomedicine&Pharmacotherapy* 58:168-172, 2004.

Modificación	Sistema	Preparación	Efecto	Ref.
ADN-polilisina *	Micropartículas	Evaporación del solvente	↑ encapsulación	[1,2]
ADN-lípido catiónico *	Micropartículas	Difusión del solvente	↑ encapsulación ↑ estabilidad	[3]
ADN-PEI-PEG	Micropartículas	Evaporación del solvente	↑ transfección ↑ estabilidad	[4]
ADN-fosfato cálcico *	Micropartículas	Evaporación del solvente	↑ estabilidad	[5]
Criopreparación	Micropartículas	Evaporación del solvente	↑ estabilidad	[6]
Coencapsulación de sales básicas	Micropartículas	Atomización	↑ estabilidad	[7]
Coencapsulación de Lipid A	Micropartículas	Evaporación del solvente	↑ internalización	[8]
Recubrimiento con lípidos catiónicos **	Micropartículas	Evaporación del solvente	↑ transfección	[9,10]
Recubrimiento con GM-CSF (citokina)	Micropartículas	Atomización	targeting	[11]
Recubrimiento con quitosano **	Nanopartículas	Evaporación del solvente	↑ internalización	[12]
Recubrimiento con PEI **	Nanopartículas	Evaporación del solvente	↑ asociación ↑ internalización	[13]
Coencapsulación con calcio	Nanopartículas	Evaporación del solvente	↑ encapsulación ↑ transfección	[14]

* complejación/precipitación del ADN antes del proceso de encapsulación

** ADN adsorbido en la superficie de los vehículos preformados

Tabla 2. Ejemplos de las propuestas de modificación de micro- y nanopartículas de PLGA destinadas a la vehiculización de ADN

Referencias de la Tabla 2.

1. Y. Capan y col. Influence of formulation parameters on the characteristics of poly(D,L-lactide-co-glycolide) microspheres containing poly(L-lysine) complexed plasmid DNA. *J.Control.Rel.* 60:279-286, 1999.
2. Y. Capan y col. Preparation and characterization of poly(D,L-lactide-co glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA. *Pharm.Res.* 16 (4):509-513, 1999.
3. S. Hirosue y col. Plasmid DNA encapsulation and release from solvent diffusion nanospheres. *J.Control.Rel.* 70 (1) (2):231-242, 2001.
4. K. A. Howard y col. Formulation of a microparticle carrier for oral polyplex-based DNA vaccines. *Biochim et Biophys Acta* 1674 (2):149-157, 2004.
5. Y. P Li y col. Stability and release characteristics of poly(D,L-lactide-co-glycolide) encapsulated CaPi-DNA coprecipitation. *Int.J.Pharm.* 269 (1):61-70, 2004.
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7. E. Walter y col. Microencapsulation of DNA using poly(D,L-lactide-co-glycolide): stability issues and release characteristics. *J.Control.Rel.* 61:361-374, 1999.
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9. K. S. Denis-Mize y col. Plasmid DNA absorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. *Gene Therapy* 7:2105-2112, 2000.
10. M. Singh y col. Cationic microparticles: a potent delivery system for DNA vaccines. *PNAS* 97 (2):811-816, 2000.
11. B. Mandel, M. Kempf, H. P. Merkle, and E. Walter. Immobilisation of GM-CSF onto particulate vaccine carrier systems. *Int.J.Pharm.* 269 (259):265, 2004.
12. R. Kumar y col. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. *Biomaterials* 25:1771-1777, 2004.
13. M. Bivas-Benita y col. PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium. *Eur.J.Pharm.Biopharm.* 58 (1):1-6, 2004.
14. H. Cohen y col. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Therapy* 7:1896-1905, 2000.

Además, como ha sido descrito en la sección anterior, los derivados tribloque y multibloque de polioxietileno-polioxipropileno podrían ofrecer ventajas adicionales como mejorar la interacción del sistema particular con el entorno biológico y/o aumentar su eficiencia de trasfección.^{100,101}

Una introducción más profunda a las características fisicoquímicas y biofarmacéuticas de estos vehículos, su diseño y morfología, así como su capacidad de encapsular y liberar ADN en forma activa y de manera controlada, y su utilidad “in vivo” como transportadores de material genético, se recoge en los capítulos siguientes de la presente memoria:

- Revisión 1. **“Nanoparticles made of poly(lactic acid) and poly(ethylene oxide) as carriers of plasmid DNA”**, en *Polymeric gene delivery: principles and applications*, ed. M.M. Amiji, capítulo 23, 367-385, CRC Press, 2005

y en

- Revisión 2. **“Poly(lactic acid) and poly(ethylene oxide) nanoparticles as carriers for gene delivery”** en *DNA Delivery/Gene Transfer Vector Lab Manual*, III. Non-viral techniques and vectors, ed. T. Friedmann y J. Rossi, capítulo 7, CSH Lab. Press, en prensa

¹⁰⁰ A. Prokop y col. Maximizing the in vivo efficiency of gene transfer by means of nonviral polymeric gene delivery vehicles. *J.Pharm.Sci.* 91 (1):67-76, 2002.

¹⁰¹ A. V. Kabanov y col. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. *J.Control.Rel.* 82:189-212, 2002.

Revisión 1

Nanoparticles made of poly(lactic acid) and poly(ethylene oxide) as carriers of plasmid DNA

N. Csaba, C. Pérez, A. Sánchez y M.J. Alonso

en: Polymeric Gene Delivery, Principles and Applications, CRC Press 2005, Capítulo 23, 367-385, ed. M.M. Amiji

CHAPTER 23

Nanoparticles Made of Poly(lactic acid) and Poly(ethylene oxide) as Carriers of Plasmid DNA

Noémi Csaba, Celso Pérez, Alejandro Sánchez, and Maria José Alonso

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23.1 INTRODUCTION

The advantageous properties of biodegradable colloidal systems have already been clearly evidenced during the past few decades. These carriers can be successfully used for the delivery of

a wide variety of drugs, including macromolecules that are poorly soluble or that are unstable in the environmental conditions of the living body.¹⁻³ The association of the drug molecules can be carried out by encapsulation inside the particle matrix or by adsorption onto the particle surface. There exist already numerous techniques for the formation of nanoparticles, the selection of which normally depends on the characteristics of the drug molecule, and also those of the polymer.^{4,5} A particularly important feature of these vehicles is that, due to their nanometric size, they are able to overcome biological membranes and even well-organized mucosae, thereby transporting the associated active compound.^{6,7} This specific ability of nanoparticles to provide intracellular penetration for the drug molecules has a great importance in gene delivery. Nevertheless, besides particle size, factors such as the surface characteristics and composition also have strong influence on the stability and on the *in vivo* fate of both the drug and the carrier.^{8,9}

Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are probably the most commonly used biodegradable polymers used for the formation of particulate drug delivery systems. These polymers are well known for their biodegradability, biocompatibility, and ability to control drug release.¹⁰ However, upon degradation, these polymers are converted into highly reactive oligomers, which generate a very acidic microenvironment inside the particles with dramatic consequences on the stability of the incorporated molecules.^{11,12} This is especially important in the case of macromolecules, such as proteins and genes, which can lose their biological activity due to these undesired interactions with the surrounding nanoparticle matrix. The incorporation of additional excipients to the polymer matrix, such as poly(ethylene oxide) and derivatives, has been proposed as an efficient approach for overcoming this limitation.¹³

Interactions between PLA- and PLGA-based nanoparticles and the *in vivo* environment can also compromise their efficiency as drug delivery systems. It has been described that, when administered intravenously, these particles are easily recognized by the mononuclear phagocyte system (MPS) and rapidly cleared from the blood stream.¹⁴ This effect can also be attributed to the hydrophobic character of the particle forming polymers that favour the adsorption of blood components, such as opsonins; a process that represents the first step of their elimination from the circulation. On the other hand, it has also been shown that polyester-based nanoparticles can suffer significant aggregation or flocculation in biological media that are rich in enzymes, such as gastrointestinal fluids.¹⁵ The development of surface-modified nanoparticles appears to be an appropriate and efficient solution for these problems.¹⁶

23.2 POLY(ETHYLENE OXIDE) AND DERIVATIVES: PHYSICOCHEMICAL PROPERTIES AND BIOLOGICAL BEHAVIOR

The terms poly(ethylene oxide) (PEO) and poly(ethylene glycol) (PEG) refer to the same compound. However, the term PEO is normally preferred when referring to high-molecular-weight polymers and also, irrespective of the molecular weight, when referring to block copolymers such as poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO). The usual names of these latter copolymers, as indicated in the USPNI, are poloxamer and poloxamine.

PEO and its derivatives (poloxamers and poloxamines) are neutral and hydrophilic molecules with flexible polymer chains. Additionally, in the case of poloxamine derivatives, apart from the PEO and PPO units, the polymer also comprises an ethylenediamine molecule as a linking element. The chemical structure of these polymers is presented in Figure 23.1. The differences between these molecules rely on their chain lengths, and their PEO and PPO content. These variations in the chemical structure have a consequence on their solubility and surface active properties. Poloxamers and poloxamines can be obtained from BASF Corporation under the commercial names Pluronic® and Tetronic®, respectively. These commercial names are usually followed by a number of 2 or 3 digits in which additional information is given about the composition and the approximate molecular weight of the molecules. In the case of poloxamers, this additional information also

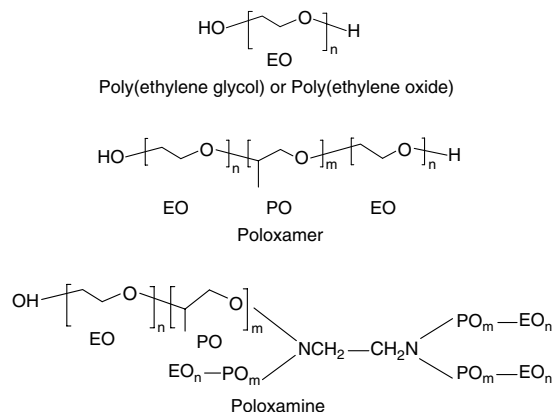


Figure 23.1 Chemical structure of PEG and its derivatives.

includes a letter that indicates the physical form of the compound. According to this, the designation “L,” “P,” and “F” stand for liquid, paste, and solid (flake) forms.

PEO and its derivatives (poloxamers and poloxamines) have been extensively used as biomaterials. More recently, due to their good biocompatibility and low toxicity, these biomaterials have attracted great attention for the design of advanced drug delivery systems. However, these polymers are not biodegradable and, consequently, their potential application in drug delivery will largely depend on their molecular weight.

Thus, while very high molecular weight polyethylene oxide (up to 7000 kDa) is acceptable for the design of oral drug delivery systems,^{17,18} only those with a relative low molecular weight (normally less than 30 kDa and preferably 5–10 kDa) have been proposed for parenteral administration.^{19–21} This is obviously due to the difficulties for the glomerular filtration and, hence, elimination of high molecular weight compounds from the blood circulation.

In the particular case of poloxamers, their potential has been continuously increasing due to their versatility and their intrinsic biological behavior, which varies depending on their composition. For example, some specific poloxamers have the ability to evolve reversibly from a sol to a gel state upon physiological conditions, usually upon changes in temperature.^{22,23} Due to this interesting behavior these polymers are known as thermosensitive and “*in situ* gelling systems.” They have already been used for a wide variety of purposes, their application for ocular drug delivery being of special interest.^{24,25}

Another interesting property of poloxamers relies on their ability to increase the permeability of very well-organized epithelia. This specific property and its connotations in drug delivery have been very well illustrated by their successful application as penetration enhancers through the blood-brain barrier.^{26–28}

The potential that poloxamers offer as carriers in gene therapy is especially promising. More specifically, there is recent evidence of the ability of these polymers to complex and protect plasmid DNA (pDNA), thereby enhancing gene expression. Furthermore, the efficacy of a combination of two poloxamers, Pluronic® L61 and Pluronic® F 127, as a gene carrier was also confirmed *in vivo*.^{29,30}

Besides the intrinsic potential of PEO and its derivatives, which will be extensively reviewed in a different chapter of this book, these polymers are probably the most popular materials for the surface modification of drug delivery vehicles (liposomes, micelles, and nanoparticles). This surface modification can be achieved by adsorbing them onto preformed nanoparticles or by covalent linking to the core forming polymer (i.e., PLA, PLGA) prior to particle formation. More recently, the interest of introducing them into the nanoparticle matrix has also been taken into consideration.

23.3 POLY(ETHYLENE OXIDE) AND DERIVATIVES: NEW APPROACHES FOR IMPROVING DRUG DELIVERY FROM NANOPARTICULATE CARRIERS

Due to the interesting biological properties of PEO and derivatives, there has been a growing interest in developing technological approaches for engineering nanoparticles consisting of a combination of polyesters and PEO. These approaches have been based upon two main principles: (1) physical entrapment or adsorption of PEO and derivatives into or onto PLA and PLGA nanomatrices, and (2) chemical modification of the matrix-forming polymer (PLA and PLGA) by covalent linking with PEO. An illustration of the different structures that can be formed is shown in Figure 23.2. In the first case, the modification will be in the core or on the coating, depending on whether PEO is introduced in the nanoparticles preparation process (Figure 23.2A) or simply adsorbed onto preformed particles (Figure 23.2B). In the second case, the nanostructural organization of the PEG fragments of the copolymer will largely depend on the nanoparticles preparation technique. Accordingly, PEG chains will be oriented toward the surface of the particles (Figure 23.2C) and they can also form small reservoirs in the core of the particles (Figure 23.2D).

As indicated in the introductory section, there are two main purposes for the incorporation of PEO and derivatives to the classical PLA and PLGA nanoparticles. One purpose has been to avoid the interaction of these hydrophobic particles with proteins and enzymes by providing them with a protective hydrophilic stealth. The original idea was to prevent the rapid opsonization of the particles, thus prolonging their circulation time following intravenous administration. At present, it is known that this hydrophilic coating is also beneficial for preventing aggregation of the particles, following mucosal administration (oral, nasal, ocular) and their subsequent transport across mucosal barriers. Moreover, the PEG coating is now considered a very good strategy for introducing ligands on the particle surface and, hence, designing active-targeting delivery vehicles.^{31,32} On the other hand, the purpose of the incorporation of PEO and derivatives into the nanoparticle matrix has been the improvement of the stability of nanoencapsulated molecules. As indicated above, the

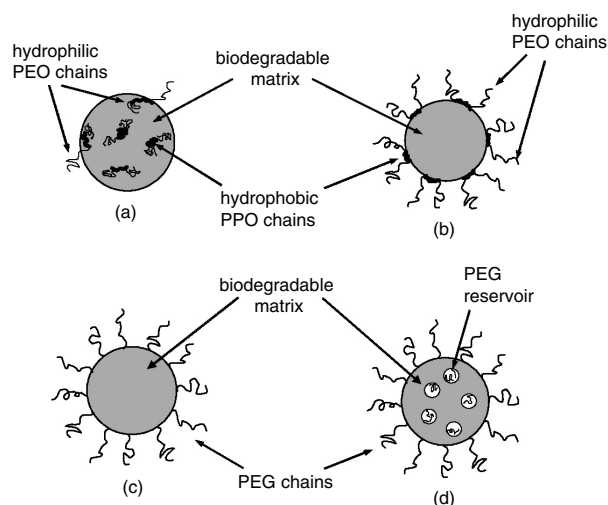


Figure 23.2 Schematic view of the various structures of nanoparticles made of polyesters and PEO and derivatives (poloxamer/poloxamine). (A) Poloxamer/poloxamine-containing nanoparticle. (B) Poloxamer/poloxamine-coated nanoparticle. (C), (D) Nanoparticles made of PLA-PEG copolymers.

dilution of PLA and PLGA with these polymers, forming an intimate blend, will reduce the acidification and, hence, provide a more friendly environment for proteins, oligonucleotides and plasmid DNA. Additionally, it has been hypothesized that the presence of poloxamer and poloxamine, together with plasmid DNA in the nanomatrix, might help improving the transfection efficiency. The next section describes the technological approaches for introducing polyoxyethylene and its derivatives within and/or onto the nanostructures.

23.4 DESIGN OF NANOPARTICLES MADE OF POLYESTERS, PEO, AND DERIVATIVES

23.4.1 Poloxamer/Poloxamine-Coated PLGA Nanoparticles

The easiest approach for surface modification of PLA and PLGA nanoparticles consisted of a simple physical adsorption of poloxamers and poloxamines. It has been reported that this straightforward surface modification permits the stabilization of polymeric nanoparticles in biological fluids and prolongs their half-life in the blood stream.^{21,33} The adsorption of these polymers onto hydrophobic nanoparticles was possible since the hydrophobic part can easily anchor to the nanoparticles surface whereas the hydrophilic part protrudes toward the aqueous surrounding medium (Figure 23.2B). The protective effect of these polymers can be explained by the steric hindrance that the hydrophilic coating material displays over the hydrophobic matrix polymer. Additionally, from the results of studies performed with radiolabeled particles, it was concluded that these coatings are able to modify the biodistribution of the vehicle. For example, it was observed that some poloxamers and poloxamines enhance the lymphatic drainage and the uptake of particles by the macrophages of the regional lymph nodes.^{34,35} This effect could also be advantageous in the development and administration of vaccines and drugs whose target is the lymphatic or the immune system.

Despite the number of articles reporting the biological significance of these coatings, some authors have argued that due to the nonspecific attachment, these polymers can be easily displaced by serum proteins.³⁶ This unwanted displacement reduces the stability of the vehicles in the circulation and exposes the particles to aggregation. As indicated above, an alternative approach to provide the nanoparticles with a PEG coating would be the synthesis of a copolymer with a PEG fragment (PLA-PEG, PLGA-PEG) and the use of an adequate manufacturing technique that favours the orientation of the PEG fragment toward the particles surface. This will be discussed in the corresponding section.

23.4.2 Poloxamer/Poloxamine-Containing PLGA Nanoparticles

The incorporation of poloxamers into PLA or PLGA micro- and nanoparticles has been a useful tool for the modification of the release profile and for the improvement of the stability of the encapsulated molecules.^{13,37-39} The internal organization of poloxamers within the particle structure varies depending on the particles preparation technique. An illustration of a possible structure is presented in Figure 23.2A. For example, we have produced PLGA nanoparticles containing poloxamer reservoirs using the double emulsion-solvent extraction/evaporation technique.⁴⁰ In this case, poloxamer 188 was dissolved in the inner water phase of the emulsion, thus forcing its partial entrapment upon precipitation of PLGA. The idea behind this approach was to stabilize the macromolecule that was coencapsulated with the poloxamer. However, this technique is limited to the incorporation of a small amount of a poloxamer with a high HLB.

More recently, we have developed a more versatile technique that allowed us to obtain PLGA-poloxamer/poloxamine blends in the form of nanoparticles. The technique is based upon the principle of the solvent diffusion or solvent displacement. In this case, both PLGA and poloxamer/poloxamine are codissolved in the same organic solvent and, then, coprecipitated upon

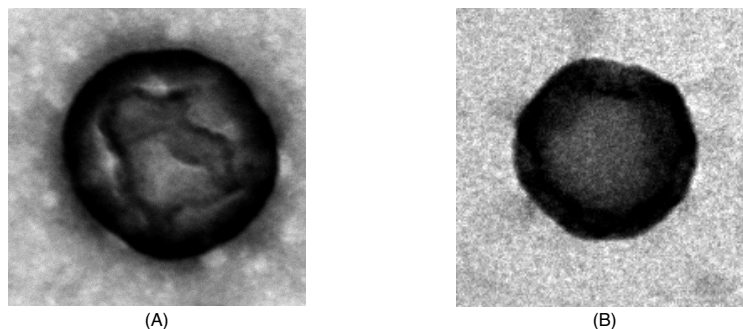


Figure 23.3 Nanoparticles prepared from PLGA:poloxamer (Pluronic® F68, HLB = 29) (A) and PLGA:poloxamine (Tetronic® 904, HLB = 14.5) blends (B).

addition to a miscible organic solvent. This technique allowed the effective incorporation of a series of poloxamers and poloxamines with different HLB values into PLGA nanoparticles, as confirmed by $^1\text{H-NMR}$ analysis.⁴¹ The formation of these mixed systems is based on the phenomenon that polyesters and poloxamers/poloxamines are readily miscible via hydrogen bonding. Similar to other matrices based on an intimate polymer mixture,¹³ the incorporation efficiency of the poloxamer/poloxamine into the particle matrix structure is determined by its thermodynamic compatibility with the PLGA component and also by the solubility of the poloxamer/poloxamine in the nanoparticles external formation medium. Thus, the composition of the nanomatrix can be adjusted by selecting different polyether types and varying the preparation conditions. The physical appearance of some of these nanoparticles is shown in Figure 23.3.

The recognized advantages of the biodegradable PLGA nanoparticles and the previously mentioned transfection-enhancing effect of poloxamer-based gene delivery systems suggest that these new nanostructures formed by an intimate blend of these two components could be very interesting devices for DNA encapsulation. This will be a specific subject of discussion in the corresponding section.

23.4.3 Design of Nanoparticles from Copolymers of PLA and PEG

Although PEG has also been physically entrapped into PLGA matrices for improving the stability of the encapsulated peptides,⁴² the main purpose of using this polymer in the field of colloidal drug carriers has been to modify their surface properties. The only successful approach for forming surface-modified PLA–PLGA nanoparticles with a stable PEG protective coating has been the covalent attachment of PEG to the hydrophobic PLA–PLGA polymers. These copolymers consisting of PLA or PLGA and PEG can be synthesized by a ring opening polymerization reaction of lactide monomers on the hydroxyl-end of a monomethoxy-poly(ethylene glycol) component in the presence of stannous octoate as catalyst.¹⁹ Depending on the weight ratio of the reaction mixture, a series of PLA–PEG copolymers with different solubilities and hydrophilicities can be obtained. Moreover, besides varying the molecular weights, it is also possible to synthesize multiblock copolymers, for example, consisting of PLGA–PEG–PLGA or PLA–(PEG)₃ type block structures.^{20,43}

These amphiphilic copolymers have been used to produce surface-modified particles in two different ways: they can be adsorbed onto preformed PLA–PLGA nanoparticles and used as coating materials or, alternatively, they can be processed in order to make nanoparticles with the appropriate surface-core configuration. With respect to the first approach, the adsorption of

PLA-PEG onto the particles is favored, as compared to that of PEG alone, due to the amphiphilic properties of the copolymer. However, in this situation, it is necessary to use low molecular weight PLA-PEG since it must be dissolved in the coating incubation medium. The few reports on the use of this approach indicated that this kind of coating permits a prolongation of the blood circulation time of nanoparticles.^{44,45}

The alternative approach, based on the processing of the copolymer to produce PEG-coated PLA nanoparticles, has been the subject of an important number of articles.^{19,20,46-48} The results published so far have indicated that not only the exact composition of the polymer, but also the nanoparticles manufacturing technique have a major effect on the polymer organization within the nanostructures and, hence in their surface composition. Most commonly, nanoparticles have been prepared by emulsion-solvent evaporation or by solvent diffusion techniques.^{49,50} Because of the different solubility of the PLA and the PEG block, a phase separated structure is formed between the organic solvent and the aqueous phase during particle preparation, leading to the projection of the hydrophilic PEG chains toward the aqueous medium, thus overlaying the solidifying nanoparticle core.⁴⁶ The formation of this so-called core-shell structure has been confirmed by various surface analysis techniques¹⁹ and by ¹H-NMR.^{20,51}

Classically, the emulsion-solvent extraction/evaporation techniques have required high energy sources (sonication, homogenization) and also the use of surfactants, i.e., cholic acid or polyvinyl alcohol, in order to form submicrometric emulsions. However, very recently, we presented the formation of these PLA-PEG nanoparticles without surfactants and using a mild agitation technique. The key for the formation of these particles relied on the appropriate selection of formulation variables such as polymer molecular weight and concentration.⁵²

For an appropriate steric hindrance effect, it is necessary to have a homogeneous distribution of the hydrophilic chains on the nanoparticle surface. We have investigated the influence of some formulation parameters (copolymer molecular weight and preparation technique) on the density of the PEG coating, as determined by ¹H-NMR.^{52,53} The results showed that the PEG coating density (amount of PEG per weight of particles) can be modulated by adjusting the formulation conditions. This is particularly important if we take into account that the density of the PEG brush has a remarkable influence on plasma protein adsorption and particle aggregation. Indeed, it has been shown that a high PEG surface density is desirable for preventing protein adsorption⁵³ and also phagocytic uptake.^{47,55,56} In addition, we have observed that the PEG coating has a key role in preventing particle aggregation following oral and nasal administration and facilitating particle permeation across mucosal surfaces.^{57,58} More specifically, in a recent work performed in our laboratory we found that nanoparticles prepared by solvent evaporation (single or double emulsion) crossed the nasal mucosa more easily than those prepared by nanoprecipitation, whose PEG surface density was less important.⁵³

Depending on the preparation technique, some of the PEG chains can be situated inside the polymeric matrix. For example, using the double emulsion-solvent evaporation technique, some PEG molecules have the chance of being oriented toward the internal aqueous phase of the double emulsion, thus forming, upon solidification, little reservoirs in which the active molecule is entrapped. The presence of these PEG reservoirs increases water uptake, thereby influencing the degradation of the core forming polymer and also the release characteristics of the vehicles.⁵⁹ The PEG component present in the interior of the particle can also act upon the stability of the entrapped molecules by blocking unfavorable interactions of the drug with the surrounding environment or with the degradation products of the core polymer. Additionally, the fact that the reactive carboxylic end group of the polyester remains blocked by the attachment of the PEG molecule can also help in preserving the structural integrity of the drug. These protective effects could be confirmed in the case of tetanus toxoid, which maintained its biological activity when encapsulated inside PLGA-PEG-PLGA microspheres⁴³ and PLA-PEG nanospheres.⁶⁰

23.5 ENCAPSULATION AND DELIVERY OF OLIGONUCLEOTIDES AND PLASMID DNA FROM NANOPARTICLES

Surprisingly, despite the important amount of work in the design of nanostructures consisting of polyesters and PEO and derivatives, there are only a few examples in the literature aimed at exploring the advantages of these novel carriers for gene/oligonucleotide delivery. The main purpose of this section is to describe in detail the advances made so far on the nanoencapsulation and controlled release of these complex macromolecular compounds.

23.5.1 Encapsulation of Oligonucleotides and Plasmid DNA into PLA-PEG Nanoparticles

An interesting approach for the association of oligonucleotides to PLA-PEG nanoparticles was explored by Emile et al.⁶¹ Two different antisense oligonucleotide sequences containing the same bases in reverse orientation, INV-val 13 and AS-val 13, were used for these studies.^{62,63} In order to reduce their negative charge, these oligonucleotides were complexed with lysine-containing cationic oligopeptides, prior to their encapsulation. After this charge neutralizing process, the complex was solubilized together with the PLA-PEG copolymer in acetone and then coprecipitated in an aqueous medium. Nanoparticles were prepared of PLA-PEG copolymers with different PEG chain lengths (mol wt 2000 and 5000 Da) and constant PLA content (mol wt 30000 Da). The results showed that the charge neutralization of the oligonucleotide by the positive charges of the oligopeptide is an effective method for improving the encapsulation efficiency (from 5% to about 55%). On the contrary, the type of oligonucleotide and oligopeptide did not have strong influence on the encapsulation. Despite the efficacy of this formulation approach, no information regarding the stability of the oligonucleotide and its release from the nanoparticles has been reported so far. Nevertheless, the previous research on the microencapsulation of oligonucleotides within PLGA micro- and nanospheres (to be covered in Chapters 22 and 28 of this book), suggests that there are formulation options that remain to be explored in order to optimize encapsulation and release of oligonucleotides from nanoparticles.

We have recently investigated the effect of the preparation technique and the presence of protective excipients on the pDNA (pCMV-Luc) encapsulation and delivery from PLA-PEG nanoparticles.⁶⁴ For this purpose, we selected an optimized water-in-oil-in-water (w/o/w) solvent evaporation⁴⁰ and a newly adapted water-in-oil (w/o) solvent diffusion technique. In both cases, the emulsification energy (sonication) was minimized in order to avoid damage of the pDNA molecules. In addition, we evaluated the potential benefit of the co-encapsulation of protective interacting non-condensing polymers, such as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP). These so-called PINC polymers were reported to be able to protect DNA by complex formation by hydrogen bonds and through this improved stability, they can also enhance the efficiency of transfection.^{65,66} Therefore, in the above-mentioned study we incubated the plasmid DNA with PVA and PVP, allowing their complexation in an aqueous medium prior to their encapsulation within PLA-PEG nanoparticles. The scheme for the formation of the pDNA-loaded nanoparticles is presented in Figure 23.4.

Under these conditions, all resulting formulations were of spherical shape (Figure 23.5), in nanometric size range (less than 300 nm), and had surprisingly high DNA loadings.

The physico-chemical characteristics of the formulations were shown to be dependent on the nanoparticle preparation method, however, they were not affected by the co-encapsulation of PVA and PVP. The w/o/w solvent evaporation technique yielded nanoparticles that were larger (270–300 nm) and less negative (~20 mV) than those prepared by the solvent diffusion technique (125–130 nm, between -22 and -33 mV). These changes were attributed to the different mechanisms of nanoparticle formation and, in particular, to the environment that the different external phases (ethanol or water) provide for particle precipitation.

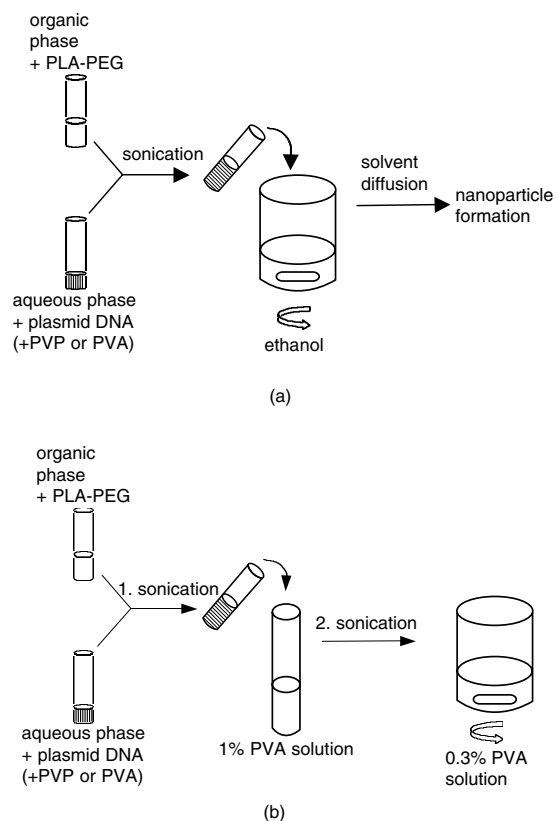


Figure 23.4 (A) Encapsulation of plasmid DNA into PLA-PEG nanoparticles by the emulsification-solvent diffusion technique. (B) Encapsulation of plasmid DNA into PLA-PEG nanoparticles by the water/oil/water double emulsion technique.

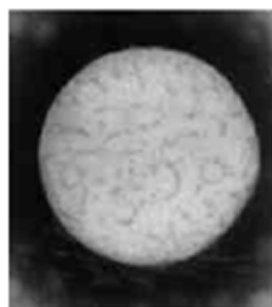


Figure 23.5 TEM micrograph of a plasmid-loaded nanoparticle prepared by the solvent evaporation technique. (From Perez, C., Sanchez, A., Putnam, D., Ting, D., Langer, R., and Alonso, M.J., Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA, *J. Control. Release* 75, 211–224, 2001. With permission.)

Interestingly, similar tendencies could be observed for the encapsulation efficiencies that were influenced by the particle preparation technique but not by the presence of PVA or PVP. In all cases, the encapsulation efficiencies of the pCMV-Luc were extremely high, around 60–80% for the solvent evaporation and 80–90% for the solvent diffusion method. The higher efficiency provided by the latter technique can be explained by the very quick nanoprecipitation of the polymer upon the diffusion of the polymer dissolving solvent into the polymer precipitating solvent. An additional fact that facilitates high entrapment is the complete insolubility of DNA in the ethanolic external phase of this formulation. However, it was also observed that the addition of the plasmid led to a significant increase in the negative zeta potential of the particles prepared by the solvent diffusion technique. This result suggests that a considerable amount of the plasmid may be entrapped near or associated to the surface of the nanoparticles.

One of the most interesting observations in the above-mentioned work was the positive effect that the PEG in the polymer chain has in the encapsulation efficiency. Indeed, the encapsulation efficiency of pCMV-Luc into PLA nanospheres without PEG was as low as 25%, thus suggesting that PEG favors the interaction and, consequently, the entrapment of the plasmid into the nanoparticles. This interaction could be confirmed by incubating blank PLA-PEG nanospheres with free pDNA. The results showed that 29% of the plasmid was bounded to the surface of the nanoparticles, thus evidencing the interaction between the PEG chains and DNA.

The positive role of PEG in the encapsulation of plasmids was later corroborated for PLA-PEG microspheres⁶⁷ where the encapsulation efficiencies of DNA were significantly improved as compared to microspheres prepared from PLGA alone.^{68,69} These authors indicated that the hydrophilic PEG chains are able to increase the compatibility between the DNA and the polymer molecules.⁷⁰ Other investigations of Liu et al. also revealed the impact of the DNA structure and molecular weight on its encapsulation efficiency in PLA-PEG particles. Their results suggested that small and compact DNA molecules can be more easily incorporated into the particle matrix than large molecules.⁷¹

23.5.2 Encapsulation of Plasmid DNA into PLGA Nanoparticles Containing Poloxamers and Poloxamines

Very recently, we have investigated the potential of PLGA-poloxamer and PLGA-poloxamine blend nanostructures for DNA delivery.^{41,72} Previous results obtained for proteins encapsulated into poloxamer-containing blend matrices suggested that this system might be beneficial for the stability of the DNA molecule. With this objective in mind, we applied a very mild technique that permits the incorporation of poloxamers (Pluronic® F68 and L121) and poloxamines (Tetronic® 908 and 904) within the nanoparticle's structure. This emulsification-solvent diffusion technique differs from the previously applied version⁶⁴ in the sense that it does not require any sonication steps (Figure 23.6). The complete elimination of high-energy sources was expected to greatly contribute to the preservation of the initial configuration of the encapsulated plasmid.

The above-described technique yielded plasmid-loaded blend particles in the size range of 150–300 nm, with a spherical shape and a negative surface charge. In general, pDNA encapsulation did not significantly alter particle size. The only exception that could be observed was in the case of the nanoparticles composed of a PLGA-poloxamine 908 blend where nanoparticle size increased significantly (from 170 to 270 nm) upon pDNA incorporation. This phenomenon could probably be explained by a different kind of interaction with DNA that might not have been produced with the other derivatives used. The efficiencies of plasmid encapsulation into all blend formulations were found to be higher (35%) than those of pure PLA vehicles (25%).

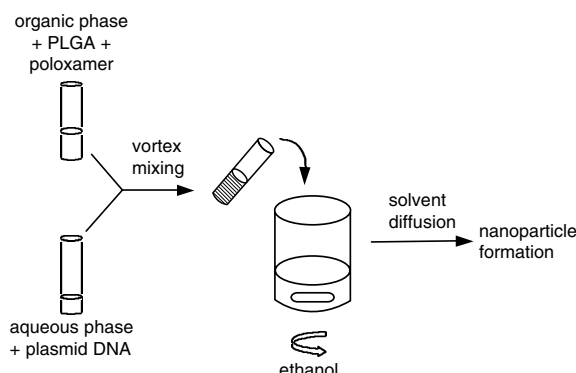


Figure 23.6 Encapsulation of plasmid DNA into PLGA-poloxamer blend nanoparticles by a modified emulsification-solvent diffusion technique.

23.5.3 Controlled Release of Plasmid DNA from Nanoparticles of PLA-PLGA and PEO and Derivatives

The release of macromolecules, i.e., proteins, from PLA-PEG matrices has been studied and documented.^{50,59,60} It has been generally accepted that macromolecules are released by a combined mechanism that includes polymer degradation followed by erosion, and also diffusion through the aqueous channels, the dimensions of which increase during polymer degradation and protein release.⁵⁹ As compared to the classical PLA-PLGA matrices, those containing PEG are known to have a greater water uptake which might potentially contribute to the release of the encapsulated macromolecule. Nevertheless, despite the work dedicated toward this aim, a clear conclusion of the effect of PEG on protein release has not been established yet. This is due to the additional benefit on the stability of encapsulated macromolecules that has been attributed to PEG, as will be commented on in the following section. Similarly, the co-incorporation of poloxamer within PLGA matrices has been found to affect protein release and stability of macromolecules such as tetanus toxoid and alpha interferon.^{13,38}

To our knowledge, the only report on the release of plasmid DNA from PLA-PEG matrices was the one presented by our group for PLA-PEG nanoparticles.⁶⁴ The results of this work showed that the nanoparticle's preparation technique had a great impact on the pDNA release, whereas the co-encapsulation of the protective excipients, such as PVP or PVA, did not alter the plasmid release pattern. More specifically, nanoparticles prepared by the solvent diffusion technique led to a very quick plasmid release, whereas those prepared by the double emulsion-solvent evaporation technique provided a slow and continuous release.

In other words, for the nanoparticles prepared by the solvent diffusion technique, the polymer matrix did not appear to have a role in the release, a situation that could in part be attributed to the presence of the previously mentioned surface-associated DNA molecules. A complementary explication was additionally found in the nonaqueous nature of the external phase of this preparation method. The plasmid encapsulated by this anhydrous process can create a considerable osmotic pressure inside the particles that can induce a very quick release by breaking up the particles. The development of this osmotic pressure in the interior of a particle has already been observed for proteins encapsulated similarly in nonaqueous conditions.⁷³ On the other hand, the nanoparticles

prepared by the double emulsion technique displayed a typical biphasic pattern that provides plasmid release in its active form for at least a time period of 28 days. These formulations have an initial fast release that corresponds to the dissociation of the surface-bound DNA, followed by a second, continuous phase that is governed by the degradation of the PLA matrix and by the erosion of the particles.^{74,75} On the other hand, in agreement with what was reported for PLGA nanoparticles,⁷⁶ we observed that changes in the theoretical plasmid loadings (0.5–1–2%) did not alter significantly the release pattern of PLA–PEG nanoparticles. These results together with the lack of effect of the PINC polymers co-encapsulated with the plasmid suggest that the degradation of the polymer matrix is the most important factor in determining the release characteristics of the nanoparticles prepared by the double emulsion technique.

As indicated in the previous section, we have recently attempted the substitution of a pure polyester matrix by a blend structure comprising a poloxamer-type compound and evaluated its effect on the controlled release of pDNA. A major advantage of these blend systems is that they usually reduce significantly the initial typical burst release of the encapsulated molecules. More specifically, it has been argued that the presence of the poloxamer in the particle can prevent rapid release of the drug by filling the pores of the polymeric core. In addition, poloxamer affects the water uptake of the vehicles and, consequently, the polymer degradation and drug release.^{37,77} In agreement with these mechanisms, we have recently observed that the incorporation of poloxamers and poloxamines into PLGA nanoparticles led to an important reduction of the pDNA burst release (normally less than 10% of the encapsulated molecule), as compared to that typically observed for PLGA and PLA–PEG nanoparticles (more than 20%). This reduction of the burst effect was particularly remarkable for the nanostructures that comprise poloxamers and poloxamines with relatively low HLB values (i.e., Pluronic® L121 HLB = 1 and Tetronic® 904 HLB = 14.5). Moreover, the HLB of the poloxamers and poloxamines incorporated into the nanostructures was observed to affect the pDNA release rate, the rate being slower for those having a lower HLB. Interestingly, for these blend systems, a closely linear pDNA release profile could be observed during a time interval of 2 weeks. Consequently, these results indicated that, as expected, the incorporation of poloxamers and poloxamines into the nanoparticle's structure opens new prospects on the controlled release of pDNA from nanocarriers.

Before making an extrapolation of the *in vitro* release behavior of pDNA from nanoparticles to the *in vivo* situation one must be very cautious. Indeed, a firm conclusion from the important amount of information accumulated in the protein delivery field is that *in vitro* conditions do not reflect the *in vivo* environment to which biodegradable colloidal systems are exposed. Furthermore, it has been clearly shown that *in vitro* release conditions often have a deleterious effect on the stability of the macromolecule being released from the particles.³⁸

23.5.4 Stability of Plasmid DNA Released from Nanoparticles of PLA–PLGA and PEO and Derivatives

One of the major challenges in plasmid encapsulation into nanoparticles is the protection of the structural integrity and conformation of the DNA molecules. Indeed, pDNA, as a macromolecule, is very sensitive against the shear and high-energy sources that are normally required for the nanoparticle formation.^{78,79} Being conscious of this important limitation, a few years ago Ando et al. proposed the microencapsulation of pDNA within PLGA microspheres using very low temperatures during the emulsification process.⁸⁰ The authors indicated that using this cryopreparation approach the exposure of the frozen pDNA to the sonication/homogenization energy was minimized. However, using this technique the authors were able to produce PLGA microparticles rather than nanoparticles. More recently, we investigated the effect of the nanoencapsulation conditions on the stability of pDNA incorporated into PLA–PEG nanoparticles. More specifically, we attempted to minimize the plasmid exposure to shear and cavitation-induced stress by developing a new solvent diffusion technique and using protective polymers (PVA, PVP). The electrophoretic analysis of the

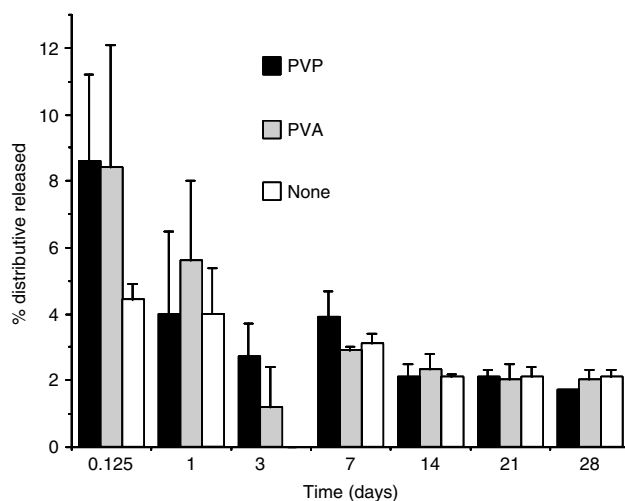


Figure 23.7 *In vitro* release of plasmid DNA from PLA-PEG nanoparticles prepared by double emulsion technique, encapsulated in a free form or associated with PVP or PVA. (From Perez, C., Sanchez, A., Putnam, D., Ting, D., Langer, R., and Alonso, M.J., Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA, *J. Control. Release* 75, 211–224, 2001. With permission.)

plasmid released from the particles after a 1-day incubation period revealed a conversion of the supercoiled form to the relaxed isoforms. This conversion is not too critical if we take into account that open-circular form still retains an important transfection capacity. However, these results indicate as well that further improvements in particle preparation are necessary in order to maintain intact the original plasmid conformation. An important discovery in this regard is the recently reported approach for producing PLA-PEG nanoparticles without high energy sources and without surfactants.⁵² Therefore, it could be assumed that using this single and straightforward procedure the deleterious effect of sonication could be avoided. These new developments hold promise for an optimized DNA nanoencapsulation approach.

Unfortunately, in addition to the shearing and cavitation forces used during encapsulation, the microenvironment generated in the course of the polymer degradation was found to be very critical for the stability of the encapsulated plasmids.^{69,81} As indicated in the introductory section, the pH inside PLA-PLGA matrices increases significantly in the course of the polymer degradation due to the accumulation of oligomers.⁸² In this regard, it is important to keep in mind that, due to their smaller size and, hence, more important specific surface area, nanoparticles are expected to release more easily the polymer degradation products than microparticles. On the other hand, this very harmful environment, to which proteins are also very sensitive, could be improved thanks to the incorporation of poly(oxyethylene) derivatives. For example, we have verified the positive effect of this strategy for very delicate proteins such as tetanus toxoid and interferon alpha.^{13,38} More recently, we have also investigated the stability of pDNA encapsulated in PLGA-poloxamer blend nanostructures and concluded that the incorporation of poloxamer has a very positive effect in the preservation of the pDNA conformation.⁸³

Overall, the results published until now indicate that a combination of a mild nanoencapsulation technique with the appropriate stabilizing excipients is required for the adequate formulation of pDNA in nanoparticles. Within this frame, the use of poloxamers and poloxamines as stabilizing excipients appears to be a promising approach.

23.6 *IN VIVO* ADMINISTRATION OF PLASMID-CONTAINING PLA-PEG NANOPARTICLES

Although nanoparticles composed of a PLA-PEG copolymer were originally conceived with the idea of increasing blood circulation times of drugs administered intravenously, the beneficial effects provided by the PEG molecules have proven to be promising for other drug delivery applications. For example, a few years ago we reported the positive effect of a PEG coating around PLA nanoparticles on the nasal/intestinal transport of nanoencapsulated proteins.⁵⁸ Moreover, following nasal administration of a model protein (tetanus vaccine), we observed a high and long-lasting immune response.⁸⁴ In our attempt to find an explanation for this special behavior we observed that, while PLA nanoparticles aggregated massively upon contact with proteins and enzymes present in mucosal fluids, those coated with PEG remained quite stable. More recently, we were able to visualize the nasal transport of PLA-PEG fluorescent nanoparticles using confocal fluorescent microscopy, and concluded that these PEG-coated particles can cross mucosal surfaces and that the intensity of their transport is affected by the PEG coating.⁵³ Whether or not this is a direct consequence of their improved stability in the nasal mucosa requires further investigation. Nevertheless, this work clearly supports the potential application of PEG-coated nanoparticles as transmucosal carriers for macromolecules.

Fortunately, the special behavior of PEG-coated particles as transmucosal carriers could be corroborated further for plasmid DNA delivery. In this study PLA-PEG nanoparticles containing the plasmid-encoding beta galactosidase (pCMV β -Gal) were administered intranasally to mice⁵¹ and the immune response elicited by β -Gal determined by enzyme-linked immunosorbent assays (ELISA). As shown in Figure 23.8, the IgG levels elicited by the encapsulated plasmid were significantly higher (80 ng/ml) than those corresponding to the naked plasmid (20 ng/ml). Moreover, the levels were similar to those elicited by the naked plasmid following intramuscular injection. Consequently, these results clearly show that PLA-PEG nanoparticles act as transmucosal carriers for pDNA. In addition, the fact that the encapsulated plasmid DNA could induce systemic immune response after the intranasal administration indicates that its incorporation into PLA-PEG nanoparticles does preserve the biological function of the plasmid.

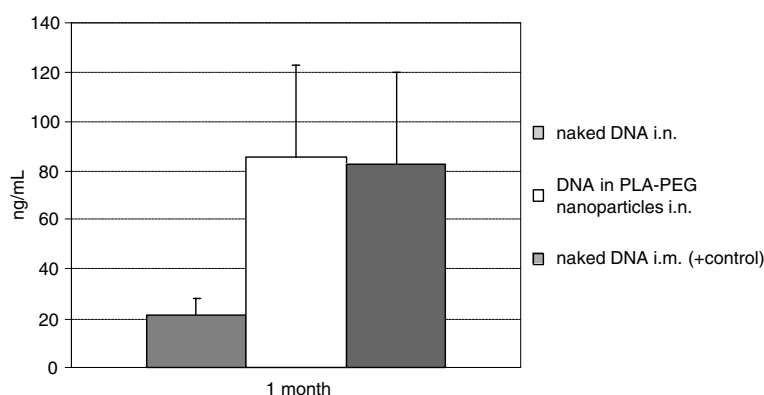


Figure 23.8 IgG antibody levels in mice after intranasal (i.n.) and intramuscular (i.m.) administration of DNA, free and encapsulated in PLA-PEG. (From Vila, A., Sanchez, A., Perez, C., and Alonso, M.J., PLA-PEG nanospheres: New carriers for transmucosal delivery of proteins and plasmid DNA, *Polym. Adv. Technol.* 13, 851–858, 2002. With permission.)

As a main conclusion, these studies reinforced that PLA-PEG nanoparticles hold great potential as carriers through mucosal surfaces. Furthermore, from these studies it has become clear that the use of these vehicles can be extended from protein drugs to pDNA, with the subsequent consequences for the development of new genetic vaccines.

23.7 CONCLUDING REMARKS

Over the last few years, important advances in the design of nanoparticles consisting of biodegradable polyesters (PLA–PLGA) and PEO and derivatives have led to the development of nanostructures with a variety of well-defined core and coat compositions. Even though the number of reports on the utility of these systems for gene delivery is still very limited, a clear conclusion from the results published so far is that these optimized compositions can be beneficially adapted for the adequate encapsulation and controlled release of plasmid DNA. Furthermore, preliminary *in vivo* experiments have evidenced the potential of these new systems as transmucosal DNA carriers. It is expected that additional future information on the intracellular fate of these new carriers will help with their further optimization and, hopefully, their exploitation as gene delivery systems.

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Revisión 2

Poly(lactic acid) and poly(ethylene oxide) nanoparticles as carriers for gene delivery

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Non-viral techniques and vectors, CHS Lab Press 2005,
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Poly(lactic acid) and poly(ethylene oxide) nanoparticles as carriers for gene delivery

Rationale

Among the strategies explored so far to achieve efficient gene transfer, the design of biodegradable nanoparticles based on a combination of PLA and PEO derivatives (in form of copolymers or blends) represents a promising alternative for in vivo applications. The development of these systems was intended to address three key issues: (i) the stabilization of the DNA molecules in the nanoparticulate matrix, (ii) the controlled release of the encapsulated genetic material for extended periods of time and (iii) the facilitation of the effective transport of the genetic material across mucosal surfaces.

Introduction

New biodegradable nanoparticle formulations based on the combination of poly(L-lactic acid) (PLA) and poly(D,L lactic-co-glycolic acid) (PLGA) with polyoxyethylene (PEO) derivatives have already shown their potential as carriers of bioactive macromolecules (proteins and antigens) across mucosal surfaces (Tobío et al. 1998, Sánchez et. al 2003, Vila et al. 2004a). Taken into consideration these promising results, we have recently evaluated the potential of these carriers for transmucosal gene delivery.

These nanostructures are composed of either PLA-PEG copolymers or physically entangled PLGA: poloxamer or PLGA: poloxamine blends. These nanoparticles have a size in the size range of 100-400 nm and a negative surface charge (between -45 mV and - 25 mV). These parameters can be modulated by adjusting the composition (copolymer or blend structure, type of PEO derivative) of the nanoparticles (Pérez et al. 2001, Csaba et. al. 2004). The DNA encapsulation

efficiency of these nanocarriers is high and dependent on their composition, varying between 30 and 90%. Moreover, these systems exhibit the ability to control the release of the encapsulated plasmid DNA for variable periods of time, from one week to several weeks. More specifically, nanocarriers based on blends of PLGA and PEO derivatives released the encapsulated plasmid for 1-2 weeks, whereas those made of PLA-PEG copolymers gave a more extended release (Pérez et. al, 2001, Csaba et. al 2005). This different controlled release behavior indicates the possibility of appropriate carrier selection, depending on application requirements

Materials List

Poly (L-lactic acid)-poly (ethylene glycol) copolymer, poly (D,L-lactic-co-glycolic acid), poloxamers with different HLB values and chain lengths: Pluronic[®] F68, Pluronic[®] L121, poloxamines with different HLB values and chain lengths: Tetronic[®] 904, Tetronic[®] 908, pEGFP-C1 vector and pCMV-BGal vector.

Protocols and Procedures:

Nanoparticle preparation:

PLGA:polyoxyethylene blend nanoparticles:

These nanoparticles were prepared by the emulsification-solvent diffusion technique. The polymer blend components (50 mg PLGA and 50 mg PEO derivative) were dissolved in 2 ml of methylene chloride and then, emulsified with a small aqueous phase (200µl) containing 500 µg plasmid DNA. This emulsification was carried out by vortex mixing in order to avoid shearing stress of DNA. This emulsion was poured onto a polar solvent (25 ml ethanol), thus leading to immediate polymer precipitation in form of nanoparticles. Finally, in order to facilitate the recovery of the nanoparticles, the desired volume of water was added and the organic solvents evaporated under vacuum (Csaba et al. 2004).

PLA-PEG nanoparticles:

These nanoparticles can be prepared by either the emulsion-solvent diffusion technique or by the double emulsion technique. In this latter case, the emulsion of the plasmid aqueous phase in the external organic phase is emulsified into an external aqueous phase, leading to the formation of a double emulsion (Pérez et al. 2001)

Analytical techniques:

The size and zeta potential of the nanoparticles were determined by photon correlation spectroscopy and laser-Doppler anemometry, respectively. Particle morphology was analyzed by transmission electron microscopy. DNA association efficiency and release profiles were determined by fluorimetric assays using PicoGreen[®] reagent. Structural integrity of plasmid DNA was analysed by agarose gel electrophoresis. In vitro and in vivo gene expression was studied by fluorescent microscopy and ELISA assays, respectively. In vitro and in vivo nanoparticle uptake was analysed by confocal laser scanning microscopy using fluorescently labeled carriers.

Advantages and limits

The major limitation of the above described system is their low transfection efficiency as compared to the cell transfection reagents commercialized for “in vitro” applications. An additional limitation could be related to the necessity of using organic solvent for the nanoencapsulation of the genetic material into the polymer nanomatrice.

The main advantages of nanoparticles consisting of PLA and PEO and derivatives are related to their biodegradability and low toxicity. Consequently, these nanocarriers should be seen as potential candidates for in vivo gene delivery. An additional advantage of these nanosystems is related to their ability to preserve the

stability and to control the release of the encapsulated DNA for extended periods of time. Finally, an evidenced advantage of these nanocarriers relies on their capacity to overcome not only cellular barriers but also mucosal barriers. For example, using confocal microscopy we were able to visualize the transport of fluorescent nanoparticles (made of PLA-PEG and also made of PLGA and poloxamer blends) across the nasal mucosa and conclude that the presence of PEO and derivatives had a critical role in their stability and further transport across the nasal mucosa (Figure 1.) (Tobío et al. 2000, Vila et al. 2004b, Csaba et al. 2005b).

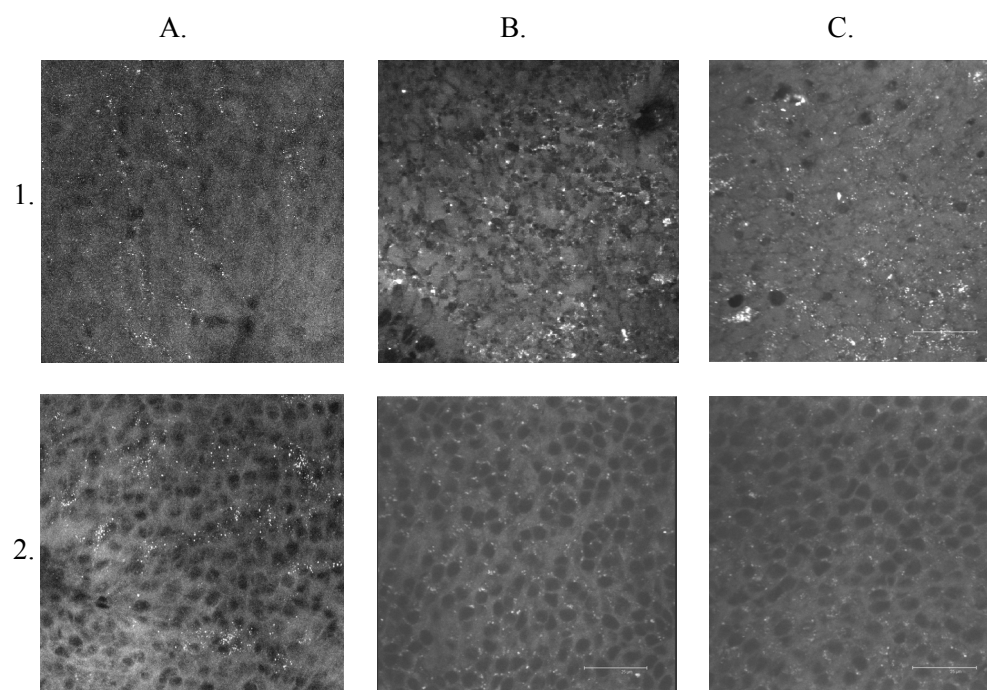


Figure 1. Confocal laser scanning micrographs of (1) surface and (2) accumulated internal cross sections of nasal mucosa excised following administration of rhodamine loaded nanoparticles prepared of (A) PLA-PEG copolymer, (B) PLGA:poloxamer blend and (C) PLGA:poloxamine blend

Furthermore, using pCMV-BGal encoding beta-galactosidase as a model plasmid, we could verify that both types of nanocarriers, based on block copolymer (PLA-PEG) or physical blends (PLGA-poloxamer) were able to transport the associated plasmid DNA across the nasal mucosa. Indeed, as shown in Figure 2, a single dose of these DNA-loaded PLA-PEG nanoparticles administered intranasally to mice led to a significant systemic immune response to the encoded protein (Vila et. al. 2002), this response being significantly higher than that corresponding to the naked DNA.

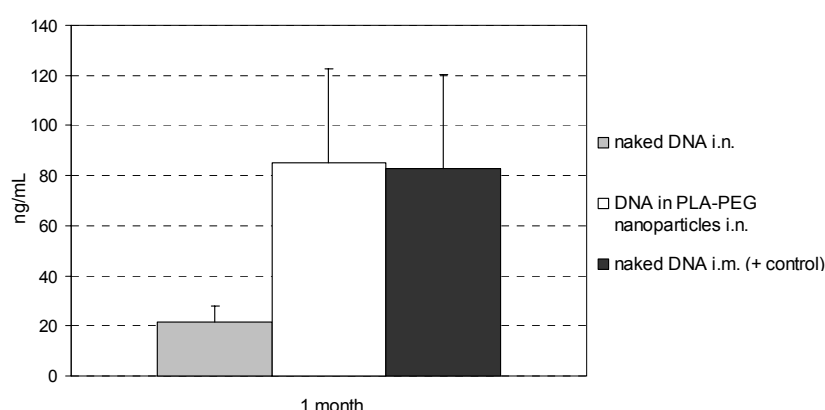


Figure 2. IgG antibody levels after (i.n.) and intramuscular (i.m.) administration of DNA, free and encapsulated in PLA-PEG nanoparticles (adopted from Vila et al. 2002 with permission).

A very similar pattern was recently observed for the blend nanocarriers consisting of PLGA and poloxamer. Consequently, overall, these results highlight the positive behavior of these nanoparticles as transmucosal gene delivery carriers and their potential application for mucosal delivery of DNA vaccines.

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Antecedentes, hipótesis y objetivos

Antecedentes

1. Las nanopartículas basadas en los polímeros biodegradables ácido poliláctico y poliláctico-glicólico presentan una alternativa prometedora para la vehiculización de ADN¹. No obstante, los procedimientos actualmente empleados para su encapsulación en estos sistemas y la degradación del propio polímero pueden afectar a la estabilidad de esta macromolécula².
2. Polímeros anfifílicos como los poloxámeros han mostrado la capacidad de complejar y proteger el ADN, y de mejorar su eficacia de transfección³.
3. Por otro lado, en el caso de proteínas encapsuladas en partículas micrométricas elaboradas a partir de ácido poliláctico y glicólico, se ha demostrado que la modificación de la matriz polimérica mediante la formación de mezclas físicas con poloxámeros permite minimizar la degradación de las macromoléculas bioactivas encapsuladas y mejorar su perfil liberación⁴.

¹ V. Labhasetwar y col. Gene transfection using biodegradable nanospheres: results in tissue culture and a rat osteotomy model. *Colloid.Surf.B: Biointerf.* 16:281-290, 1999.

² T. Hao y col. Biological potency of microsphere encapsulated plasmid DNA. *J.Control.Rel.* 69:249-259, 2000.

³ P. Lemieux y col. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Therapy* 7:986-991, 2000.

⁴ M. Tobio y col. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm.Res.* 16 (5):682-688, 1999.

Hipótesis

1. La estabilidad del ADN encapsulado en nanopartículas biodegradables podría mejorarse mediante
 - el empleo de una nueva técnica de encapsulación que evite el uso de fuentes con aporte de energía considerable.
 - el desarrollo de matrices poliméricas compuestas por una mezcla íntima del ácido poliláctico-glicólico con distintos derivados de polioxietileno como poloxámeros o poloxaminas
2. Esta modificación en la composición de los vehículos también serviría para ajustar los perfiles de liberación de los vehículos, minimizando la fase “burst”, característica típica de las micro- y nanopartículas poliméricas de PLGA.
3. Asimismo, la interacción entre el ADN y los polioxietilenos también podría mejorar la eficacia de transfección del sistema.
4. La administración por una vía mucosa, como la vía nasal, de material genético codificador de una proteína antigénica incluido en las nanopartículas anteriormente señalados debería dar lugar al desarrollo de una respuesta inmune frente a dicho antígeno.

Objetivos

Teniendo en cuenta los antecedentes expuestos, el objetivo global del presente trabajo se ha dirigido al diseño de sistemas nanoparticulares innovadores y a la evaluación del potencial que ofrecen estas partículas como sistemas de liberación controlada de ADN destinados a la inmunización (vacunas genéticas) por una vía

no invasiva como la nasal. Desde el punto de vista práctico, este objetivo global puede dividirse en los siguientes objetivos específicos:

1. Desarrollo y caracterización de nanopartículas compuestas por PLGA y diversos derivados de polioxietileno

Resultados recogidos en Artículo I:

“Design and characterisation of new nanoparticulate polymer blends for drug delivery” *Journal of Biomaterials Science, Polymer Editions* 15, 9, 1137-1151, 2004

2. Asociación de ADN a nanopartículas elaboradas a base de mezclas PLGA:poloxamer y PLGA:poloxamina y evaluación de las propiedades de liberación controlada in vitro

Resultados recogidos en Artículo II.:

“PLGA-poloxamer and PLGA-poloxamine blend nanoparticles: new carriers for gene delivery” *Biomacromolecules*, 2005, 6, 271-278

3. Potencial de nanopartículas elaboradas a base de mezclas PLGA:poloxamer y PLGA:poloxamina: evaluación de su capacidad transportadora de ADN en cultivos celulares y de su utilidad para inducir respuestas inmunes tras su administración por vía nasal.

Resultados recogidos en Artículo III:

“PLGA:poloxamer and PLGA:poloxamine blend nanostructures for transmucosal delivery of DNA: in vitro and in vivo evaluation of uptake and transfection” sometida para su evaluación

Artículo 1

Design and characterisation of new nanoparticulate polymer blends for drug delivery

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Design and characterisation of new nanoparticulate polymer blends for drug delivery

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Abstract—The aim of the present work was the design of novel nanoparticle compositions based on poly(lactic acid/glycolic acid) (PLGA): poloxamer and PLGA: poloxamine blend matrices. For this purpose, we have applied a modified solvent diffusion technique that allows the preparation of the nanoparticles without the use of high energy sources. Nanoparticles have been prepared with different PLGA: poloxamer and PLGA: poloxamine ratios using PEO-derivatives with different molecular weights (M_w) and hydrophilia–lipophilia balance (HLB) values. Our results show that the physico-chemical characteristics of the nanoparticles, such as size and zeta potential, are influenced by the type of PEO-derivative associated to the PLGA matrix. The ^1H -NMR analysis of the different nanoparticle compositions showed that the extent of incorporation of the PEO-derivative depends strongly on its HLB and also on the nanoparticles preparation conditions. The capacity of these nanoparticles as drug delivery devices was evaluated using bovine insulin as a model drug. The insulin-encapsulation efficiency was shown to be dependent on the composition of the nanoparticles, those containing hydrophilic PEO-derivatives being the most effective in entrapping the drug molecules. The formation of the blend system displayed positive effects on the release characteristics of the nanoparticles. Nanoparticles exhibited a reduced initial burst and a nearly linear, constant release rate over a time period of two weeks.

Key words: PLGA; polyoxyethylene derivatives; nanoparticle; polymer blend; protein delivery.

INTRODUCTION

Biodegradable particulate carrier systems have gained considerable interest in the recent years because of their potential use for the encapsulation, protection and controlled release of biologically active agents. The most commonly used materials for the preparation of these vehicles are poly(lactic acid) (PLA) and its copolymers with glycolic acid (PLGA), polyesters that are well known for their safety and

biocompatibility. Micro- and nanoparticulate carriers made of PLA or PLGA have already been used for the delivery of many therapeutic molecules and there is a large number of studies directed to the formulation and release characteristics of these systems [1–4].

A particularly important feature of PLA and PLGA particles is that their degradation characteristics give the ability to control the release time and rate of the encapsulated molecules. This degradation is governed by hydrolytic processes and leads to the formation of acidic oligo- and monomers that cause a significant pH decrease inside the particles [5, 6]. In the course of time, this acidic microclimate and the presence of an increased number of carboxylic end groups can affect the stability and biological activity of the encapsulated molecules [7]. This acidification-induced inactivation or degradation presents an obstacle in the use of these delivery systems that still has to be overcome.

It has already been proposed by a number of authors that the co-encapsulation of protective excipients into PLGA particles could possibly prevent the unwanted interactions between the drug and the polymer as well as neutralize the acidity generated in the course of polymer degradation [7–10]. Among the alternatives, the use of block copolymers containing polyoxyethylene and polyoxypropylene units (i.e. poloxamers and poloxamines) (Fig. 1) could be a possible choice for pro-

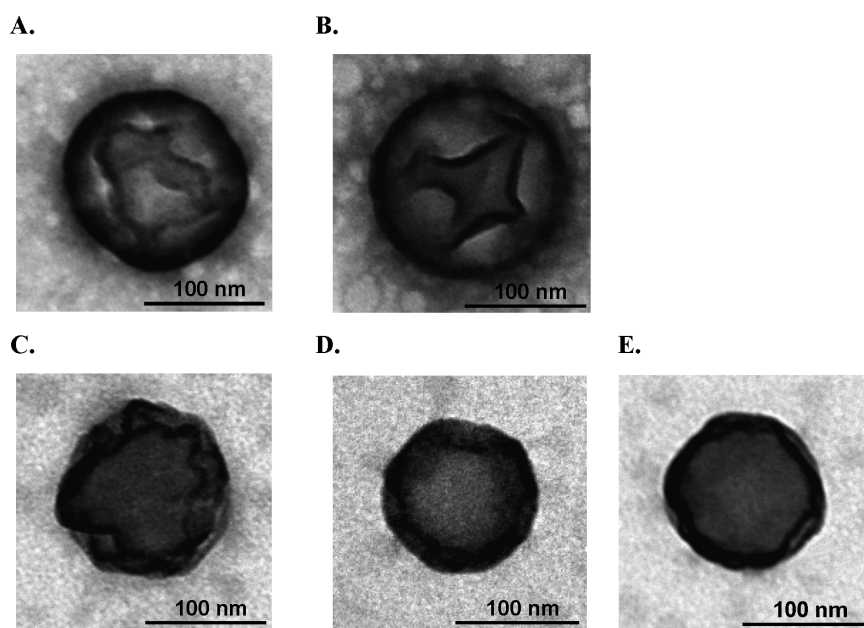


Figure 1. TEM micrographs of different blend nanoparticles with polymer ratios 50 : 50. (A) PLGA : Pluronic® F68; (B) PLGA : Pluronic® L121; (C) PLGA : Tetronic® 908; (D) PLGA : Tetronic® 904; (E) PLGA : Tetronic® 901.

viding the encapsulated macromolecules with a more stable environment. These polyoxyethylene-polyoxypropylene block copolymers have already been widely used for particle engineering as surface active agents and coating materials in the preparation of surface modified, long circulating vehicles [4, 11–13]. Moreover, poloxamer-type copolymers have extensively been investigated for other pharmaceutical applications, mainly in form of gels and micelles. The results of these investigations also include substantial information on the biocompatibility and safe use of these polyoxyethylene derivatives *in vivo* [14–17].

Previous studies carried out in our laboratory have shown that the incorporation of poloxamer-type polyoxyethylene-polyoxypropylene block copolymers into poly (lactic acid/glycolic acid) microparticles and nanoparticles is a useful approach for improving the stability of proteins encapsulated into them. In these previously reported nano- and microparticles the poloxamer was co-encapsulated in small amounts with proteins, such as BSA and interferon alpha [18, 19]. As an alternative approach, we have also developed a composite microparticulate system that is based on a blend structure of PLGA and poloxamer [20]. This blend system exhibited improved encapsulation efficiency while providing a controlled, pulsatile release profile for the protein in its active form. The formation of similar polyester–poloxamer blends in the form of films and microparticles has also been described as an approach for improving the release behaviour of these systems [21, 22]. Surprisingly, despite the recognized value of these blend matrices, this strategy has not been applied yet to the design of nanostructures. Based on this previous information, the main goal of the present work was the design of novel nanoparticle compositions based on blend matrices of PLGA : poloxamer and PLGA : poloxamine. For the preparation of the particles, we have optimised a new, modified solvent diffusion technique. The resulting blend nanoparticles have been characterised with respect to their physico-chemical characteristics, morphology, composition and also to their suitability for encapsulation and controlled release of model compounds such as bovine insulin.

MATERIALS AND METHODS

Materials

The polymer poly (D,L-lactic acid/glycolic acid) 50 : 50 (PLGA) was purchased from Boehringer-Ingelheim (Ingelheim, Germany) under the commercial name of Resomer® RG 503. The poloxamer Pluronic® F-68 with HLB = 29 and poloxamer Pluronic® L121 with HLB = 1 were obtained from Sigma Aldrich (Madrid, Spain) and BASF (Ludwigshafen, Germany). The poloxamines Tetronic® 901, 904 and 908 (HLB = 2.5, 14.5 and 30.5) were kindly donated by Bascom Belgium (Brussels, Belgium). Bovine insulin was purchased from Sigma Aldrich. All other solvents and chemicals used were of the highest grade commercially available.

Preparation of blank PLGA blend nanospheres

Nanoparticles with different PLGA : poloxamer and PLGA : poloxamine ratios were prepared by a modified emulsion–solvent diffusion technique. First, 50 mg of PLGA and the different quantities of Pluronic® or Tetronic® polymer (0, 25, 50 and 75 mg, respectively) were dissolved in 2 ml of methylene chloride and this organic solution was mixed for 30 s with a small aqueous phase by vortex (2400 min⁻¹, Heidolph, Germany). Then, the obtained emulsion was poured under moderate magnetic stirring onto a larger polar phase (25 ml ethanol), leading to immediate polymer precipitation in the form of nanoparticles. The formulations were diluted with 25 ml MilliQ water and the stirring was maintained for 10 min more. After solvent evaporation under vacuum at 30°C (Rotavapor, Büchi R-114, Flawil, Switzerland) nanoparticles were collected and concentrated in an aqueous medium. For further analysis, nanoparticles were isolated by centrifugation (1 h, 8000×g, 15°C, Avanti 30, Beckman, Barcelona, Spain) and optionally freeze dried (primary drying step for 48 h at –34°C, secondary drying step until the temperature gradually rose to 25°C, Labconco, Kansas City, MO, USA).

Physicochemical characterisation of PLGA blend nanoparticles

Nanoparticle size, polydispersity and zeta potential were determined by photon correlation spectroscopy and laser-Doppler anemometry, respectively (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK). Mean values were obtained from 4 different batches, each measured 3 times. In the case of the determination of zeta potential, measurements were performed in 1 mM KCl.

The morphology of the nanoparticles was examined by transmission electron microscopy (CM 12 Philips, Eindhoven, The Netherlands) using samples stained with a 2% phosphotungstic acid solution.

The composition of the nanoparticle matrix was analysed by ¹H-NMR (Bruker AMX-300, Bruker, Barcelona, Spain) studies using samples of isolated and freeze-dried formulations dissolved in deuterated chloroform. Quantitative analysis was performed by the integration of the characteristic peaks in the spectra of the dissolved particles, using the software MestRe-C version 2.3. The amount of the poloxamer or poloxamine associated to the nanoparticles, calculated from the ¹H-NMR spectra was represented as a percentage with respect to the total mol quantity of PLGA and PEO derivative in the nanoparticles.

The thermal behaviour of the blend matrices was examined by differential thermal calorimetry (DSC) between –30°C and 80°C at a rate of 5°C/min (DSC-50, Shimadzu, Tokyo, Japan). Sample analysis was performed after nanoparticle isolation and freeze-drying.

Encapsulation of bovine insulin, determination of the encapsulation efficiency

Bovine insulin was introduced into the internal aqueous phase (200 µl) of the formulations before the emulsification step and encapsulated with 1% theoretical

loading. In order to provide conditions for the complete dissolution of insulin, in this case the internal aqueous phase of the formulation also comprised 0.01 M HCl. Theoretical loadings were calculated with respect to the total weight of the PLGA in the formulation. Physico-chemical characterisation of the insulin loaded formulations was performed as described previously for the blank nanoparticles.

Encapsulation efficiency was calculated from the amount of insulin that remained in the supernatant samples recollected by the centrifugation (1 h, 8000×g, 15°C, Avanti 30, Beckman) of the nanoparticle suspension. The amount of this insulin was determined by the standard micro BCA protein assay (Pierce, Rockford, IL, USA). For these evaluations we used calibration curves prepared from the supernatant of the corresponding blank formulations.

In vitro release study

Nanoparticles were prepared with 1% theoretical loading of insulin, according to the solvent diffusion technique described above. After the solvent evaporation step, nanoparticles were recollected and concentrated in phosphate-buffered saline (PBS, pH 7.4). The final concentration of this suspension was adjusted to 2 mg particles/ml release medium. This nanoparticle suspension was divided in volumes of 4 ml into individual tubes, each corresponding to a time interval of the release experiment ($t = 3$ h and 1, 3, 7, 14 days). These release samples were incubated at 37°C with horizontal shaking (120 min⁻¹, Promax 1020, Heidolph, Schwabach, Germany). Samples were recollected at the predetermined time intervals by centrifugation for 1 h at 8000×g and 15°C (Avanti 30, Beckman). The amount of insulin released at each time point was determined from the isolated supernatants by the micro BCA protein assay as described above. For the calculation of the cumulative release profiles we used calibration curves prepared from the supernatant of the corresponding blank formulation.

RESULTS AND DISCUSSION

In the present work, we have prepared biodegradable nanoparticles using a solvent diffusion technique that was conveniently adapted for the encapsulation of hydrosoluble macromolecules while minimizing the use of high energy sources. Indeed, the incorporation of an aqueous phase (in which the macromolecule can be dissolved) into the polymer (PLGA) solution was achieved by means of vortex mixing, and the formation of nanostructures was a consequence of the controlled diffusion of the polymer dissolving phase (methylene chloride) into the external polar phase (ethanol).

Besides its mildness and simplicity, the technique developed in the present work allowed us to incorporate several types of PEO-PPO-PEO block copolymers with different characteristics (Table 1) into the nanoparticles structure, forming an intimate blend with PLGA. Based on previous studies performed by our group,

Table 1.

Composition, molecular weight, HLB value and cloud point of the poloxamers and poloxamines used in this study

Commercial name	Composition	$M_w (\times 10^{-3})$	HLB	Cloud point ($^{\circ}\text{C}$)
Pluronic [®] F68	2 $\times$ 75 EO + 30 PO	8.5	29	>100
Pluronic [®] L121	2 $\times$ 5 EO + 68 PO	4.4	1	14
Tetronic [®] 901	4 $\times$ 3 EO + 4 $\times$ 17 PO	4.7	2.5	20
Tetronic [®] 904	4 $\times$ 15 EO + 4 $\times$ 17 PO	6.7	14.5	74
Tetronic [®] 908	4 $\times$ 119 EO + 4 $\times$ 17 PO	25	30	>100

we hypothesized that these modifications would display positive effects on the stability and controlled release of protein drugs encapsulated into them [13]. Hence, the influence of the incorporation of PEO derivatives on the physico-chemical characteristics of the nanoparticles, as well as on their encapsulation capacity and controlled release properties was investigated.

Physico-chemical characteristics of the nanoparticles

By adjusting the preparation conditions, the above described method leads to the formation of particles of different composition in the desired nanometric size range, between 150 and 350 nm (Tables 2–6). The size distribution of the nanoparticles was, in general, homogeneous, having polydispersity values generally lower than 0.20. An exception was represented by the PLGA : Tetronic[®] 901 blend series, which displayed the largest particle size and broader size distribution (polydispersity index between 0.162 and 0.308). This could be explained by the fact that the cloud point of this particular poloxamine is close to the ambient temperature (20 $^{\circ}\text{C}$) and, consequently, this could affect particle formation.

The yield of the preparation process, based on the PLGA component, was always higher than 75%. Morphological analysis by transmission electron microscopy (TEM) showed that irrespective to their composition, nanoparticles had spherical shape (Fig. 1). The TEM images also show some irregularities in the nanoparticles structure, which could be related to the presence of two differentiated polymers (PLGA and a PEO derivative) in the nanostructures.

Tables 2–6 show the influence of the quantity and type of the polyether component on the particle size and zeta potential of the nanoparticles. As compared to the pure PLGA nanoparticles, an initial decrease in particle size due to the incorporation of the PEO derivative could be observed in all series. This effect was previously described for PLGA microspheres containing surfactants such as polyoxyethylenes or polysorbates [23] and was attributed to the surface active properties of the polyethers present in the formulations. After this initial decrease, the size of the particles containing more hydrophilic block copolymers was either maintained (Pluronic[®] F68, Tetronic[®] 904) or further decreased (Tetronic[®] 908). On the contrary, in the case of the most hydrophobic block copolymers (Pluronic[®] L121,

Table 2.

Mean particle size, polydispersity index, zeta potential and relative percentage of Pluronic® F68 (average $\pm$ SD, $n = 4$) in the nanoparticles prepared with different theoretical polymer ratios

PLGA : Pluronic® F68	Size (nm)	P.I.	ζ -potential (mV)	Pluronic® F68	
				Mol%	Weight%
50 : 0	191.5 $\pm$ 7.1	0.046	−60.1 $\pm$ 7.4	—	—
50 : 25	162.8 $\pm$ 4.4	0.079	−50.2 $\pm$ 0.8	7.9 $\pm$ 0.7	2.0 $\pm$ 0.2
50 : 50	163.2 $\pm$ 5.1	0.135	−43.1 $\pm$ 6.4	10.8 $\pm$ 0.5	2.8 $\pm$ 0.1
50 : 75	159.8 $\pm$ 6.5	0.163	−38.5 $\pm$ 0.6	10.6 $\pm$ 0.6	2.7 $\pm$ 0.2

Table 3.

Mean particle size, polydispersity index, zeta potential and relative percentage of Pluronic® L121 (average $\pm$ SD, $n = 4$) in the nanoparticles prepared with different theoretical polymer ratios

PLGA : Pluronic® L121	Size (nm)	P.I.	ζ -potential (mV)	Pluronic® L121	
				Mol%	Weight%
50 : 0	191.5 $\pm$ 7.1	0.046	−60.1 $\pm$ 7.4	—	—
50 : 25	164.5 $\pm$ 6.3	0.156	−27.3 $\pm$ 7.1	45.4 $\pm$ 3.5	9.5 $\pm$ 1.2
50 : 50	185.5 $\pm$ 6.0	0.195	−30.0 $\pm$ 8.0	58.3 $\pm$ 1.5	15.0 $\pm$ 0.8
50 : 75	257.3 $\pm$ 0.0	0.179	−24.5 $\pm$ 5.5	63.0 $\pm$ 1.5	17.6 $\pm$ 0.9

Table 4.

Mean particle size, polydispersity index, zeta potential and relative percentage of Tetronic® 908 (average $\pm$ SD, $n = 4$) in the nanoparticles prepared with different theoretical polymer ratios

PLGA : Tetronic® 908	Size (nm)	P.I.	ζ -potential (mV)	Tetronic® 908	
				Mol%	Weight%
50 : 0	191.5 $\pm$ 7.1	0.046	−60.1 $\pm$ 7.4	—	—
50 : 25	189.2 $\pm$ 4.6	0.202	−30.9 $\pm$ 3.9	3.9 $\pm$ 0.5	2.8 $\pm$ 0.3
50 : 50	174.0 $\pm$ 5.4	0.271	−26.9 $\pm$ 1.2	5.5 $\pm$ 0.1	4.0 $\pm$ 0.1
50 : 75	171.2 $\pm$ 3.2	0.235	−24.1 $\pm$ 1.0	6.4 $\pm$ 0.5	4.6 $\pm$ 0.4

Table 5.

Mean particle size, polydispersity index, zeta potential and relative percentage of Tetronic® 904 (average $\pm$ SD, $n = 4$) in the nanoparticles prepared with different theoretical polymer ratios

PLGA : Tetronic® 904	Size (nm)	P.I.	ζ -potential (mV)	Tetronic® 904	
				Mol%	Weight%
50 : 0	191.5 $\pm$ 7.1	0.046	−60.1 $\pm$ 7.4	—	—
50 : 25	160.2 $\pm$ 5.6	0.188	−40.0 $\pm$ 4.6	14.7 $\pm$ 1.5	3.2 $\pm$ 0.4
50 : 50	168.7 $\pm$ 9.4	0.179	−38.4 $\pm$ 3.3	18.8 $\pm$ 2.0	4.2 $\pm$ 0.5
50 : 75	168.8 $\pm$ 2.5	0.160	−39.6 $\pm$ 2.0	19.5 $\pm$ 0.6	4.4 $\pm$ 0.1

Table 6.

Mean particle size, polydispersity index, zeta potential and relative percentage of Tetronic® 901 (average $\pm$ SD, $n = 4$) in the nanoparticles prepared with different theoretical polymer ratios

PLGA : Tetronic® 901	Size (nm)	P.I.	ζ -potential (mV)	Tetronic® 901	
				Mol%	Weight%
50 : 0	191.5 $\pm$ 7.1	0.046	-60.1 $\pm$ 7.4	—	—
50 : 25	205.3 $\pm$ 54.5	0.162	-25.4 $\pm$ 5.0	23.3 $\pm$ 0.2	3.9 $\pm$ 0.0
50 : 50	277.4 $\pm$ 102.9	0.308	-28.9 $\pm$ 5.4	26.3 $\pm$ 6.1	4.6 $\pm$ 1.3
50 : 75	333.7 $\pm$ 82.1	0.275	-38.2 $\pm$ 8.3	28.8 $\pm$ 1.1	5.1 $\pm$ 0.3

Tetronic® 901), the nanoparticle size gradually increased as more polyether was incorporated into the formulations.

With respect to the surface characteristics of the formulations, all nanoparticles had negative surface charge. However, it can also be seen that all PLGA : poloxamer and PLGA : poloxamine nanospheres have less negative zeta potentials than the PLGA ones. This suggests that a part of the polyoxyethylene component can be situated on-, or close to the surface of the nanoparticles. As has been described for other nanoparticles with PEG, poloxamer or poloxamine coatings, these polyoxyethylene derivatives can displace the shear plane of the diffuse layer to a larger distance from the particle surface and, thus, reduce the zeta potential [24–26].

Analysis of the particle composition

The composition of the nanoparticle series was determined by ^1H -NMR studies, using samples of isolated and freeze-dried formulations. The spectrum of the control particles prepared from pure PLGA contains the three characteristic peaks corresponding to the $-\text{CH}-$ (5.2 ppm) and $-\text{CH}_3$ (1.6 ppm) groups of the lactic acid and to the $-\text{CH}_2-$ (4.8 ppm) group of the glycolic acid component of the polymer. The presence of new peaks was noted between 1.1 and 1.2 ppm, and between 3.3 and 3.7 ppm in the spectra of the blend nanoparticles (Fig. 2). These peaks correspond to the $-\text{CH}-$ and $-\text{CH}_2-$ groups of the PEO-PPO blocks and indicate the successful incorporation of poloxamer or poloxamine into the nanoparticles. Moreover, the intensity of these peaks increased with respect to those of PLGA when the amount of the PEO-PPO block copolymer incorporated into the nanoparticle matrix was increased. This suggests that the composition of the nanoparticles can be modified by adjusting the initial polymer ratios.

The quantitative analysis of the spectra of the formulations containing poloxamers showed that Pluronic® F-68 can be incorporated up to 11 mol% with respect to the total quantity of PLGA and poloxamer in the particle. This percentage was achieved at the theoretical PLGA : poloxamer ratio of 50 : 50 and remained unmodified for the 50 : 75 ratio. A very different situation was observed in the case of Pluronic®

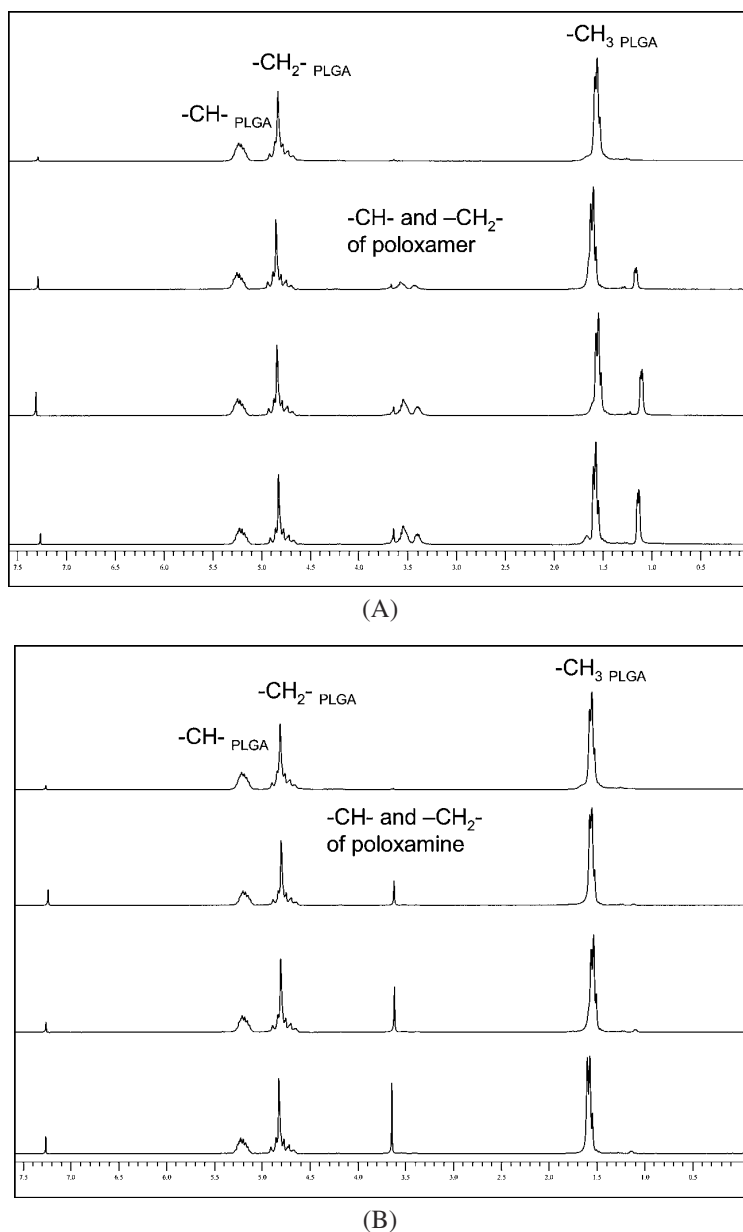


Figure 2. Typical ¹H-NMR spectra of PLGA:poloxamer and PLGA:poloxamine blend formulations. (A) PLGA:Pluronic® L121; (B) PLGA:Tetronic® 908.

L121, for which the association was much higher and was not limited at the ratios investigated, reaching the value of 63 mol% (Tables 2 and 3).

These differences in composition for the two types of nanoparticles could be explained by the different thermodynamic compatibility of these polyoxyethylene derivatives with the PLGA component. As described previously, the organization of these blend structures relies basically on chain entanglement and the formation of hydrogen bonds between the carboxylic end group of the polyester and the hydroxyl terminal of the polyether [27]. Characteristics such as the PEO:PPO ratio, the chain lengths and the hydrophobicity of the poloxamer/poloxamine is expected to determine its miscibility with PLGA and, thus, it will also influence the structure and composition of the particle matrix. In addition, the different nanoparticle sizes, as indicated above, could be considered as a consequence of the formation of different blend structures.

These results are in accordance with those of a previous quantitative FT-IR study performed by Yeh *et al.* [21], in which they analysed the composition of blend PLGA microparticles containing different types of poloxamers. In this previous work it was shown that the hydrophilic poloxamer Pluronic® F127 was less effectively incorporated into the particles than the more hydrophobic Pluronic® L121. This tendency underlines the importance of the HLB as a parameter for the prediction of the degree of miscibility of the two polymers to be blended.

Apart from the influence of the thermodynamic compatibility, the differences in the incorporation amounts of the polyethers into the nanoparticle structures may also be attributed to the preparation conditions. In our case, the different solubility of the polyoxyethylene derivatives in the external medium of the particle formation should logically affect their incorporation within the matrix. The low solubility of the poloxamer Pluronic® L121 prevents its partition towards the external ethanol/water phase and facilitates its incorporation at higher amounts. However, the association of the poloxamer Pluronic® F68 into the nanoparticle matrix is probably limited by its hydrophilic character [27].

Tendencies similar to those observed for the poloxamer-containing nanomatrices could be identified following the analysis of the poloxamine-containing nanostructures (Tables 4–6). The hydrophobic character of the poloxamines Tetronic® 904 and 901 allows their efficient association to the polyester matrix while in the formulations comprising of the very hydrophilic Tetronic® 908 this efficiency was limited by the tendency of this poloxamine to diffuse from the organic phase to the aqueous continuous phase of the nanoparticles formation medium.

The comparison of the incorporated amounts of the different PEO derivatives (Fig. 3) shows that this parameter is clearly influenced by their HLB. Thus, the minimum value was observed for Tetronic® 908 (HLB = 30) and the maximum incorporation was achieved for the lipophilic Pluronic® L121 (HLB = 1). The tendency of the poloxamines to associate to the polyester matrix could also be affected by the molecular weight. In fact, as seen in Fig. 3, their association went from less than 10% for Tetronic® 908 (M_w 25×10^3) to nearly 30% for Tetronic®

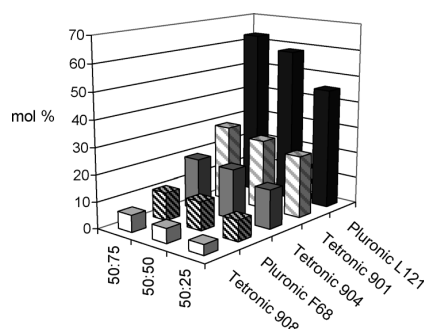


Figure 3. Incorporated amounts determined by $^1\text{H-NMR}$ for the different poloxamers and poloxamines into the nanoparticle series with different initial polymer ratios.

901 (M_w 4700). However, since this parameter is not independent of the HLB, it is not possible to draw a conclusion about its possible role in the incorporation of the PEO derivatives into the PLGA matrix. On the other hand, the comparison of the behaviour of Tetronic® 901 and Pluronic® L121, both of them hydrophobic and with a similar M_w , led us to suggest that not only the hydrophobicity but also their different chemical composition may affect their entanglement within the PLGA network.

Finally, further evidence of the incorporation of the PEO derivatives in the nanomatrices was found in the DSC thermograms (Fig. 4). The processing of the co-dissolved PLGA : PEO derivatives to form nanoparticles led to a shift in the glass transition temperature (T_g) from 51.34°C, corresponding to the PLGA nanoparticles, to 48.61°C/50.27°C observed for the blend PLGA : poloxamer/poloxamine nanoparticles, respectively. Additionally, a significant change in the shape of the endotherms could be noted. Instead of the marked T_g characteristics of PLGA, the nanoparticles containing poloxamer or poloxamine displayed a broad endotherm. The reduction in the glass transition temperature could be attributed to the PLGA : poloxamer/poloxamine miscibility [27]. In fact, the more hydrophobic Pluronic® L121 can be more readily intermixed with the PLGA polymer chains than Tetronic® 908, thus leading to a higher modification in the thermal event. This would also agree with the more important association of Pluronic® L121 to the nanomatrix as compared to Tetronic® 908.

Insulin encapsulation into the nanoparticles

In order to investigate the potential of our blend nanoparticles for the delivery of macromolecules, we encapsulated bovine insulin as a model compound. For these studies we selected the formulations with theoretical polymer ratio 50 : 50 from the following compositions : PLGA : Pluronic® F68 and L121, PLGA : Tetronic® 908 and 904. The results clearly show the influence of the different PEO derivatives on the success of the encapsulation of insulin (Table 7). In the case of the

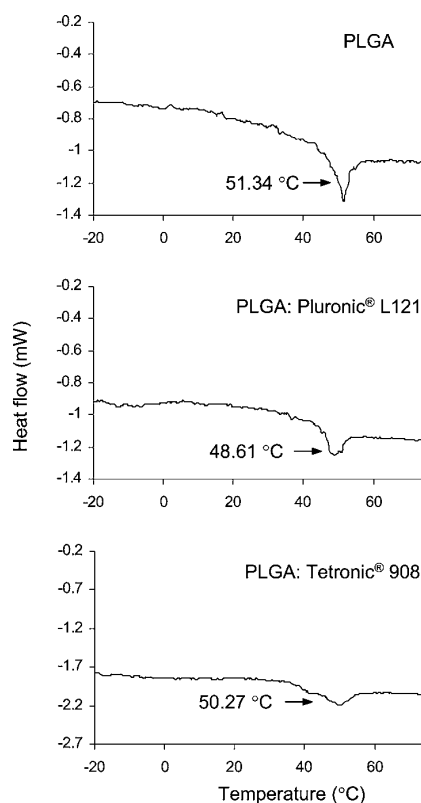


Figure 4. DSC flow curves of PLGA, PLGA : Pluronic® L121 and PLGA : Tetronic® 908 nanoparticle formulations with polymer ratio 50 : 50 (T_g values of the different formulations marked by arrows).

Table 7.

Mean particle size, zeta potential and encapsulation efficiency of insulin-loaded nanoparticles with polymer ratio 50 : 50 ($n = 3$)

Polymer type	Size (nm)	ζ -potential (mV)	Encapsulation efficiency (%)
Pluronic® F68	310.3 ± 1.9	-29.9 ± 5.5	39.4 ± 12.2
Pluronic® L121	— ^a	—	—
Tetronic® 908	269.2 ± 0.4	-14.4 ± 6.0	44.2 ± 0.9
Tetronic® 904	183.6 ± 3.0	-30.8 ± 6.5	19.3 ± 0.4

^a No determination possible due to precipitation.

more hydrophobic blend system comprising the poloxamer Pluronic® L121, the insulin-loaded nanoparticles precipitated rapidly during the solvent evaporation step after particle formation. The characteristics of the insulin-loaded particles

improved when the more hydrophilic PEO derivatives were present in the blend. Simultaneously, a remarkable increase could be observed in the encapsulation efficiencies. More specifically, the poloxamer Pluronic® F68 and the poloxamine Tetronic® 908 (both having HLB values around 30) led to the most effective insulin association reaching encapsulation efficiencies of around 40%. These values were also accompanied by a significant increase in particle size (between 100 and 150 nm) and by a reduction of about 10 mV in the zeta potential. This could be considered as a further confirmation of the successful incorporation of the peptide. Additionally, the decrease of the negative zeta potential also suggests that insulin is partially located on the surface of the nanoparticles. On the other hand, it was interesting to note that PLGA nanoparticles containing insulin could not be formed due to the uncontrolled precipitation of the polymer caused by the presence of insulin. Consequently, the presence of PEO derivatives in the formulation process was critical for the association of insulin.

Previous studies directed to the definition of the drug incorporation characteristics of polymeric blend microparticles also led to the conclusion that the presence of the polyether component has beneficial effects on the encapsulation of proteins such as ovalbumine and tetanus toxin [20, 21, 28]. This positive effect has been attributed to an interaction between the protein and the poloxamer [21]. In the present study this effect could not be observed given the unfeasibility to form PLGA nanoparticles containing insulin without using PEO derivatives. Consequently, it appears that in the experimental conditions defined in the present work, the presence of insulin interferes with the particle formation giving an uncontrolled precipitate. However, this interference could be overcome by facilitating the accommodation of insulin due to the presence of the PEO derivatives.

In vitro release of insulin from the nanospheres

Polymeric micro- and nanoparticles typically exhibit a biphasic release profile consisting of an initial rapid release, followed by a sustained release phase [29, 30]. This initial, so-called burst effect is attributed to the release of the surface-associated molecules which are easily detached upon dilution of the particles. This initial burst is more pronounced in the case of nanometric vehicles where the large surface area facilitates the loss of the associated drug molecules. Following this initial burst, the release is controlled by the polymer degradation and the diffusion of the polymer degradation products. A different behaviour has been reported for polymeric blend films and microparticles, for which an improvement of the controlled release properties was observed [21, 27, 28]. More specifically, these previous studies showed a reduced burst, followed by a continuous release of the associated protein. This different release pattern, as compared to the typical one observed for simple polyester matrices, was attributed to the matrix hydration process, followed by a retarded release of the PEO derivative due to its entanglement with the polyester chains.

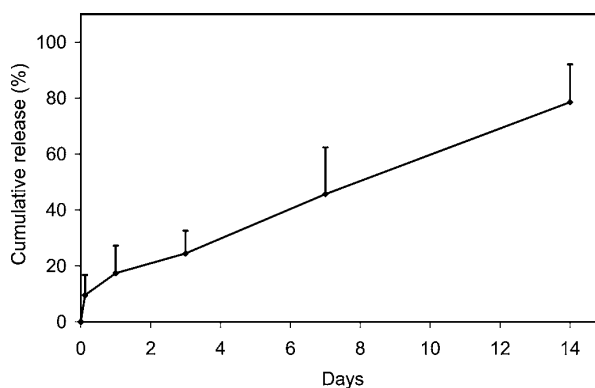


Figure 5. Cumulative release profile of insulin from PLGA:Tetronic® 908 50:50 nanoparticles ($n = 3$).

Taking the previous information into account, our approach was to evaluate the possibility of controlling the release of the model peptide insulin from the newly developed nanomatrices. With this purpose in mind, we selected the formulation PLGA:Tetronic® 908 for investigation of its release behaviour, based on its favourable physico-chemical properties and insulin encapsulation efficiency. Figure 5 illustrates the *in vitro* release profile obtained for this formulation, representing the percent of release with respect to the total amount of insulin encapsulated. It can be seen that these nanoparticles exhibit a controlled release of insulin with a negligible burst effect and a very constant, closely linear release rate over a time period of two weeks.

This release behaviour, which agrees with those previously reported for PEO-containing microspheres [21, 28] suggests that the release of the encapsulated macromolecule is governed by the erosion of the nanomatrice, this process being not simply controlled by the polyester degradation but also by the swelling and release of the associated PEO. Therefore, these results led us to confirm that the modification of the particle matrix by a PEO derivative is a useful approach for the adjustment of the release profile of polyester-based nanoparticles.

CONCLUSIONS

We have developed a new nanoparticulate drug carrier consisting of a blend nanomatrix of PLGA and a PEO derivative using an optimised solvent diffusion method. The amount of PEO derivative incorporated into the nanomatrix can be modulated by selecting the derivative with the adequate HLB. These new nanoparticles could associate insulin and release it in a controlled manner, avoiding the burst effect typically observed for protein containing PLGA micro- and nanospheres.

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Artículo 2

PLGA:Poloxamer and PLGA:Poloxamine blend nanoparticles: new carriers for gene delivery

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PLGA:Poloxamer and PLGA:Poloxamine Blend Nanoparticles: New Carriers for Gene Delivery

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The main objective of the present work was the development of new nanoparticulate carrier systems for the delivery of plasmid DNA. These new carriers consist of a blend matrix formed by a poly(lactic-co-glycolic acid) (PLGA) copolymer and polyoxyethylene derivatives. More specifically, we have prepared nanostructures with different PLGA:poloxamer and PLGA:poloxamine compositions by an optimized emulsification–solvent diffusion technique and studied the potential of these carriers for the encapsulation and controlled release of plasmid DNA. Depending on the particle composition, the encapsulation efficiency of the model plasmid pEGFP–C1 varied between 30% and 45%. All formulations provided continuous and controlled release of the plasmid with minimal burst effect. In addition, the release rate and duration was dependent on the composition of the particle matrix. Moreover, gel electrophoresis and cell culture (MCF-7 cell line) assays allowed us to confirm that the biologically active form of the plasmid was preserved during the particle preparation process and also during its release. Cell culture experiments also indicated that the new nanoparticles do not exhibit toxic effects on these cells at concentrations up to 5 mg/mL. Altogether, these results indicate that these composite nanostructures present a promising approach for the delivery of plasmid DNA.

1. Introduction

At present, it is widely accepted that the success of gene therapy and genetic vaccination depends strongly on the design of a carrier that could efficiently protect and deliver DNA to the target cells. One of the strategies toward this aim has been its encapsulation into polymeric particles.¹ In particular, the systems based on poly(L-lactic acid) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA) hold promise because of their history of successful and safe use for the delivery and controlled release of macromolecules.²

So far, it has been shown that PLGA particles have good potential for the encapsulation of plasmid DNA, and indeed, their use for genetic vaccination seems to be plausible since satisfactory immune responses could be obtained after the administration of DNA-loaded PLGA microparticles.^{3,4} Recently, special interest has been paid to nanoparticulate carriers that, due to their small size, are able to overcome biological barriers, thereby improving the transport of the encapsulated molecules. Additionally, it was also suggested that PLGA-based nanoparticles may have the ability to escape rapidly from the endosomal compartments after their uptake into the cells.⁵ These characteristics facilitate the intracellular distribution of the vehicles and make them very appropriate as carriers for DNA, especially in those cases

where controlled, long-term delivery to the target tissue is preferred. This intracellular distribution of PLGA nanoparticles has also been confirmed by fluorescent imaging techniques where both the nanospheres and the released plasmid DNA could be detected in the cytoplasm of the cells.⁶ In accordance with these findings, PLGA nanoparticles have been shown to be more efficient than microparticles in transfecting cells, where this efficiency is influenced by the molecular weight of the polymer, their size, surface composition, and charge.^{7,8} Nevertheless, despite these interesting features, there are some elements that may be further improved with regard to the application of PLGA nanoparticles in gene delivery. First, their preparation usually requires the application of shearing forces and that can compromise the structural integrity of the DNA.^{9,10} Second, the degradation of the polymeric matrix can lead to the development of an acidic microclimate inside the particles, and it can also affect the stability of the plasmid molecule.^{11,12} Several solutions have been proposed in order to provide protection against these unwanted processes, such as the cryopreparation technique,¹³ the co-encapsulation of basic salts¹⁴ or the complexation of the DNA molecule with poly(L-lysine)¹⁵ prior to its encapsulation.

As another approach for gene delivery, amphiphilic polymers with the ability of interacting with DNA by van der Waals or hydrogen bonds can also be useful for the protection of DNA from degradation and for the facilitation of gene expression. For example, recent studies have shown

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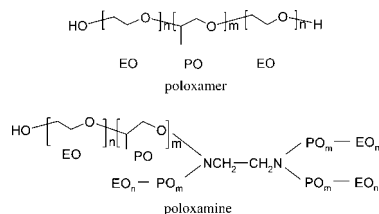


Figure 1. Chemical structure of poloxamers and poloxamines (EO = ethylene oxide unit, PO = propylene oxide unit).

that polyoxyethylene–polyoxypropylene–polyoxyethylene type polymers (poloxamers, Figure 1) can be successfully applied for the enhancement of the transfection efficiency in muscle.¹⁶ In this case, the mechanism of this positive effect was suggested to be related to the formation of complexes between the polymer and plasmid DNA, without condensation. Similar results were found in another *in vivo* study where a large amount of polymers were tested for their capacity of enhancing gene expression. It was observed that neutral or slightly positively charged polymers were more successful in increasing transfection efficiency of DNA than negatively charged polymers. One of the most effective polymers was a group of polyoxyethylene derivatives (poloxamines, Figure 1). These findings indicate again the utility of interaction and hydrogen bonding between amphiphilic polymers and DNA as a possible approach for gene delivery and enhanced transfection.¹⁷

Considering the advantages of the two above-described systems, nanoparticles and polyoxyethylene (PEO) derivatives, in the present work, we have designed new carriers in which the biodegradable polyester PLGA is combined with polyoxyethylene derivatives, poloxamers, and poloxamines in the form of nanoparticles. In addition, previous studies carried out in our laboratory have shown that the combination of PLA or PLGA with poloxamers can help preserve the biological activity of proteins to be delivered.^{18,19} Therefore, we hypothesized that our new nanoparticle composition could also have positive effects on the stability of the plasmid DNA during its encapsulation and sustained release.

2. Materials and Methods

2.1. Materials. The polymer PLGA 50:50 Resomer® RG 503 was purchased from Boehringer Ingelheim (Ingelheim, Germany). The poloxamer Pluronic F68 with hydrophilic-lipophilic balance (HLB) = 29 and poloxamer Pluronic L121 with HLB = 1 were obtained from Sigma Aldrich (Madrid, Spain) and BASF (Ludwigshafen, Germany). The poloxamines Tetronic 904 and 908 (HLB = 14.5 and 30.5) were kindly donated by BASCOM Belgium (Brussels, Belgium). Plasmid DNA encoding green fluorescent protein with CMV promoter (pEGFP-C1) was purchased from Elim Biopharmaceuticals (San Francisco, CA). Trizma base, agarose, xylene cyanole, bromophenol blue, and ethidium bromide (purity 95%) were all obtained from Sigma Aldrich (Madrid, Spain). One kb DNA ladder was obtained from Life Technologies (Barcelona, Spain).

All other solvents and chemicals used were of the highest grade commercially available.

2.2. Preparation of PLGA:Poloxamer and PLGA:Poloxamine Blend Nanospheres Containing Plasmid DNA. Nanoparticles with PLGA:poloxamer or PLGA:poloxamine ratios of 50:50 were prepared by a modified solvent diffusion technique. First, 50 mg of PLGA and 50 mg of poloxamer or poloxamine were dissolved in 2 mL of methylene chloride. This organic solution was then mixed by vortex agitation (2400 min⁻¹, Heidolph, Germany) for 30 s with an aqueous phase (200 µL) containing 200 µg of plasmid DNA. Then, the obtained emulsion was poured onto a polar phase (25 mL ethanol) under moderate magnetic stirring, leading to immediate polymer precipitation in the form of nanoparticles. The formulations were diluted with 25 mL of milli-Q water and the stirring was maintained for 10 min more. After solvent evaporation under vacuum at 30 °C (Rotavapor, Büchi R-114, Switzerland), nanoparticles were collected and concentrated in milli-Q water or in Tris-EDTA buffer 7.5. For further analysis, nanoparticles were isolated by centrifugation (1 h, 8000 RCF, 15 °C, Avanti 30 Beckman, Barcelona, Spain) and optionally freeze-dried (primary drying step for 48 h at -34 °C, secondary drying step until the temperature gradually rose to 25 °C, Labconco Corp., Kansas City, MI).

2.3. Physicochemical Characterization of PLGA:Poloxamer/Poloxamine Nanoparticles. The size, polydispersity, and zeta potential of the nanoparticles were determined by photon correlation spectroscopy and laser-Doppler anemometry, respectively (Zetasizer 3000 HS, Malvern Instruments, England). In the case of the determination of the zeta potential, measurements were performed in 1 mM KCl. Mean values were obtained from three different batches, each of them measured three times.

The morphology of the nanoparticles was examined by transmission electron microscopy (CM 12 Philips, Eindhoven, The Netherlands) using samples stained with a 2% phosphotungstic acid solution.

2.4. Determination of Plasmid DNA Encapsulation Efficiency. The theoretical DNA loading was 0.4% with respect to the total amount of PLGA in the nanoparticles. For the determination of the real plasmid loading, we attempted to extract the plasmid upon dissolution of the particles in chloroform. However, this procedure did not permit an effective recovery of the total amount of plasmid encapsulated. Consequently, the efficiency was calculated from the amount of nonencapsulated plasmid, which was recovered in the supernatant samples collected upon the centrifugation of the nanoparticles. The amount of this plasmid was determined by spectrofluorimetry (LS 50B luminescence spectrometer, Perkin-Elmer) using Pico Green reagent (Molecular Probes, OR).

2.5. In Vitro Release of Plasmid from PLGA-Blend Nanoparticles. The final concentration of the formulations was adjusted to 2 mg of particles/mL of release medium. Nanoparticle suspensions were incubated in Tris-EDTA buffer pH = 7.5 at 37 °C under horizontal shaking (100–110 cycles min⁻¹). At time intervals of 3 h and 1, 3, 7, and 14 days, the supernatant of the nanoparticle suspension was

collected by centrifugation at 8000 RCF, 15 °C for 1 h. The amount of plasmid released at each time interval was determined from the supernatant samples (diluted with Tris-EDTA buffer pH= 7.5 when it was necessary) by fluorimetric assay with PicoGreen reagent (Molecular Probes, OR).

2.6. Extraction of Plasmid DNA from the Nanospheres. Nanoparticles were freeze-dried, weighed and resuspended in 1 mL of Tris-EDTA buffer pH = 7.5. To solubilize the nanospheres, 500 μ L of chloroform was added to the particle suspension. This mixture was maintained in circular rotation at room temperature for 1 h for the extraction of the DNA from the organic PLGA–chloroform phase into the aqueous phase. The samples were centrifuged at 12000 RCF for 8 min (Avanti 30 Beckman, Barcelona, Spain) and 4–500 μ L of the supernatant was removed for the further analysis of the extracted plasmid.

2.7. Structural Integrity of the Encapsulated Plasmid DNA. The stability of plasmid DNA released from the different nanoparticle formulations (section 2.5) was analyzed by agarose gel electrophoresis (1% agarose containing ethidium bromide, 50 V, 120 min, Sub-Cell GT 96/192, Bio-Rad Laboratories Ltd., England). When it was required, samples were concentrated prior to the analysis by ultrafiltration at 4000 RCF using Nanosep 100K Omega centrifugal devices (Pall Corporation, MI). In all cases, 1 kb DNA ladder and untreated pEGFP–C1 plasmid were used as control.

2.8. Cell Culture Experiments. MCF-7 human epithelial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 ui/ml and 100 μ g/mL, respectively), and were maintained at 37 °C with a 5% CO₂ humidified atmosphere.

2.9. Biological Activity of the Encapsulated Plasmid. MCF-7 cells were plated at a density of 250 000 cells per well into 6 multiwell plates and were allowed to grow for 24 h before transfection. Plasmid DNA, either untreated, extracted as indicated in section 2.6, or released from the different nanoparticle compositions according to section 2.5, was complexed with FuGENE 6 (Roche, Indianapolis, IN) transfection reagent according to the manufacturer's instructions. At 24 h post-transfection, positive cells were detected by fluorescence microscopy (Estativo Eclipse TE 2000S, Nikon, Spain). Relative transfectivity of the different extraction and release samples was calculated referring to the number of GFP-positive cells transfected by the control, untreated plasmid.

2.10. Cytotoxicity Assay. MCF-7 cells were plated at a density of 10⁵ cells per well into 96 multiwell plates and were allowed to grow for 24 h before the experiment. The different concentrations (from 1 mg/mL to 5 mg/mL) of the nanoparticle formulations PLGA:Pluronic F68, PLGA:Pluronic L121, PLGA:Tetronic 908, and PLGA:Tetronic 904 resuspended in 100 μ L of DMEM were added to the cells and incubated for 24 h. After incubation, the cell culture medium containing nanoparticles was then replaced by fresh medium and cell viability was measured by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) colorimetric assay (Promega Corp., WI) at 490 nm, after a recuperation period of 24 h.

Table 1. Mean Particle Size, Polydispersity Index, ζ Potential of the Different Blank PLGA:Pluronic and PLGA:Tetronic Blend Nanoparticles (mean $\pm$ S. D., $n = 3$)

Poloxamer/Poloxamine type	size (nm)	P. I.	ζ potential (mV)
without	191 $\pm$ 7	0.046	–60.1 $\pm$ 7.4
Pluronic F68	163 $\pm$ 5	0.135	–43.1 $\pm$ 6.4
Pluronic L121	185 $\pm$ 6	0.195	–30.0 $\pm$ 8.0
Tetronic 908	174 $\pm$ 5	0.271	–26.9 $\pm$ 1.2
Tetronic 904	168 $\pm$ 9	0.179	–38.4 $\pm$ 3.3

2.11. Statistical Analysis. Statistical analysis of the experimental data was performed by applying one-way ANOVA tests ($\alpha < 0.01$) using the software package SPSS 12.0.

3. Results and Discussion

New nanoparticle compositions have been prepared by a blending process for which we have applied an emulsification–solvent diffusion technique. The suitability of this method for the incorporation of several types of polyoxyethylene derivatives into the polyester particle matrix has already been confirmed by different analytical techniques such as ¹H NMR and differential scanning calorimetry.²⁰ The preparation technique has been modified and optimized in order to avoid the use of high energy sources that could cause structural damages in the DNA molecule.⁹ This modified technique does not require the use of high energy sources; thus, it provides very mild conditions for the formation of plasmid-loaded blend particles and prevents shear-induced degradation during particle preparation. In addition, we have investigated the physicochemical characteristics and morphology of the resulting blend nanoparticles. Finally, with the expectation that the new composition would be beneficial for the delivery of macromolecules, we have also studied their capacity to encapsulate plasmid DNA and to release it in a sustained manner while preserving its biologically active forms.

These vehicles, as designed, could be administered parenterally or transmucosally. Following parenteral administration, these systems are expected to control the release of the encapsulated drug. On the other hand, regarding their potential for transmucosal administration, previous studies carried out in our laboratory have shown that the combination of PLGA with PEO in nanoparticulate carriers has positive effects in terms of overcoming biological barriers and improving the transport of the encapsulated molecules.^{21,22} Within the context of DNA delivery, nanoparticulate compositions based on PEO derivatives could improve transport across mucosal surfaces (i.e., nasal, intestinal), were the MALT (mucosal-associated lymphoid tissue) a target tissue for genetic vaccination.

3.1. Characterization of the Plasmid DNA-Loaded Nanoparticles. The solvent-diffusion technique described above yields both blank and plasmid-loaded blend particles with sizes less than 300 nm (Tables 1 and 2). In all cases, nanoparticles have negative surface charge, and as it can be observed on the transmission electron microscopy micrographs, they are spherical (Figure 2).

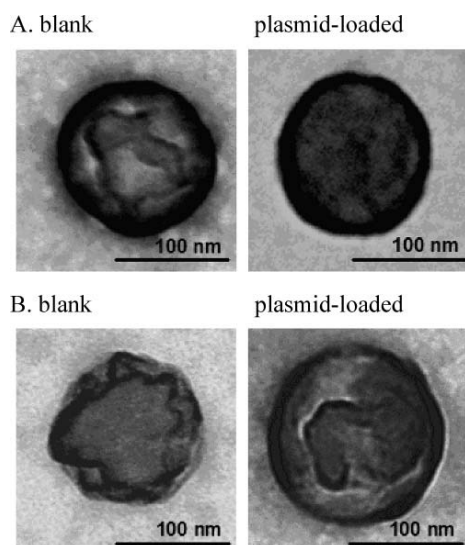


Figure 2. TEM micrographs of blank and plasmid-loaded (A) PLGA: poloxamer (Pluronic F68) and (B) PLGA:poloxamine (Tetronic 908) blend nanoparticles.

Table 2. Mean Particle Size, Polydispersity Index, ζ Potential, and Encapsulation Efficiencies of the Different Plasmid-Loaded PLGA:Pluronic and PLGA:Tetronic Formulations (mean $\pm$ S. D., $n = 3$)

Poloxamer/ Poloxamine type	size (nm)	P. I.	ζ potential (mV)	E. E. (%)
without	234 $\pm$ 13	0.187	-72.7 $\pm$ 3.7	32.9 $\pm$ 13.2
Pluronic F68	183 $\pm$ 6	0.114	-50.8 $\pm$ 3.6	35.2 $\pm$ 8.9
Pluronic L121	217 $\pm$ 5	0.154	-23.5 $\pm$ 1.4	31.3 $\pm$ 3.8
Tetronic 908	269 $\pm$ 11	0.437	-35.0 $\pm$ 0.9	32.0 $\pm$ 3.7
Tetronic 904	161 $\pm$ 7	0.154	-54.1 $\pm$ 2.0	44.1 $\pm$ 4.3

Generally, the encapsulation of plasmid DNA caused slight changes in the size and polydispersity of the blend nanoparticles (Tables 1 and 2). More important variations could be observed in the case of the nanoparticles composed of PLGA and of the poloxamine Tetronic 908, where the size of the nanostructures rose from 170 to 270 nm upon the incorporation of the plasmid molecules and the polydispersity was also significantly increased. This effect could be explained by the fact that, as compared to the other PEO-derivatives used, this particular poloxamine has considerably higher molecular weight with long ethylene oxide chains (see Table 3).

This specific characteristic may lead to a different kind of interaction between the PEO derivative and DNA and, hence, to a different architectural organization of the plasmid and the polymer blend.

With respect to the surface charge of the particles, no significant changes could be observed between blank and plasmid-loaded PLGA:poloxamer formulations (with Pluronic F68 or L121). However, a remarkable modification

Table 3. Composition, Molecular Weight, and Hydrophilia–Lipophilia Balance (HLB) Value of the Poloxamers and Poloxamines Used in This Study

commercial name	composition	Mw (Da)	HLB
Pluronic F68	2 $\times$ 75 EO + 30 PO	8500	29
Pluronic L121	2 $\times$ 5 EO + 68 PO	4400	1
Tetronic 904	4 $\times$ 15 EO + 4 $\times$ 17 PO	6700	14.5
Tetronic 908	4 $\times$ 119 EO + 4 $\times$ 17 PO	25000	30

was appreciated in the case of the PLGA:poloxamine compositions (with Tetronic 908 or 904) and the control formulation (only PLGA) which displayed higher negative surface charges upon DNA loading. Similar effects of drug loading have already been observed for other nanoparticulate carriers, where these changes were attributed to the surface-association of the drug, and to different orientation of the composing polymers due to the presence of the drug molecules.²³

3.2. Encapsulation of Plasmid DNA. For the characterization of the DNA loading capacity, we have chosen a model plasmid encoding green fluorescent protein (pEGFP–C1). This macromolecule was encapsulated into the different nanoparticle compositions with a fixed theoretical loading (0.4%). Table 2 shows the encapsulation efficiencies obtained under these conditions. It can be seen that all formulations have a good capacity of incorporating plasmid DNA, with the encapsulation efficiencies being generally higher than 30%.

These results are similar to those reported previously for conventional PLGA micro- and nanoparticles where the encapsulation efficiency of plasmid DNA usually varies between 20 and 50%.^{1,24} Among our blend formulations, the nanoparticles made of a PLGA:Tetronic 904 blend ended up being the most suitable for the encapsulation of the pEGFP–C1 plasmid, reaching in this case an efficiency of 44%. In the other cases, little influence of the polyoxyethylene type could be appreciated on the encapsulation capacities.

It has been suggested that the presence of certain surfactants such as Tween 80 or poly(vinyl alcohol) could improve the compatibility of the hydrophilic DNA molecule with the hydrophobic PLGA polymer. This effect may be understood by their interaction with DNA that could make it more hydrophobic, and this could facilitate the incorporation of the plasmid into the particle matrix.^{25,23} The affinity of these compounds to plasmid DNA is based on hydrogen bonding and other weak interactions¹⁶ and is mainly determined by the characteristics of the applied polymer (hydrophilia: lipophilia, chain lengths and composition). We suggest that in the particular case of the Tetronic 904, its intermediate HLB value (14.5) and its molecular composition are probably the most suitable to achieve this effect. Additionally, the presence of the amine groups in poloxamines (and the ability of displaying a low positive charge) is also thought to facilitate their interaction with the DNA molecule.¹⁷ In conclusion, the apparently higher encapsulation efficiency of the Tetronic 904 containing blend system may be attributed to a combination of the appropriate hydrophobicity and electrostatic interactions. Nevertheless, further studies on the mechanism of poloxamer/poloxamine–DNA interac-

tions would be necessary for better understanding and for efficient polymer selection.

3.3. In Vitro Release of the Encapsulated Plasmid. In general, macromolecules are released from biodegradable matrixes by a mechanism involving polymer degradation, followed by matrix erosion, and diffusion of the macromolecules through the aqueous channels generated during the erosion process. Typically, the release of an encapsulated macromolecule from PLGA micro- and nanospheres follows a tri-phasic release pattern where the initial phase corresponds to the detachment of the surface-localized drug molecules. The second phase, characterized by the absence or very limited release, is prolonged until significant PLGA degradation and matrix erosion take place. Once this critical point is reached, the encapsulated drug releases suddenly, very often in a pulsatile manner.^{26,27} This classically accepted release pattern is very often modified, especially in the case of large proteins and plasmid DNA due to unwanted interactions between the protein and the polymer degradation products and also due to the acidic microenvironment generated in the course of the polymer degradation.^{14,28} As a consequence, these first generation PLGA microspheres were found to be unsatisfactory as protein/DNA delivery vehicles since they exhibited an initial fast release followed by the absence of active protein/DNA release.

To overcome the above indicated limitations of PLGA microspheres, we proposed a number of years ago the substitution of a pure polymeric matrix by a composite blend structure consisting of PLGA and poloxamer 188.^{29,18} This modification in the matrix composition turned out to be very successful in terms of preventing the stability and controlling the release of the encapsulated protein, tetanus toxoid. On the basis of these positive results, our recent goal within this context was to develop an innovative technology that enables the encapsulation of proteins within nanoparticulate PLGA: poloxamer blends. Interestingly, these nanoparticles exhibited an improved and continuous protein release.²⁰

In the present work, taking into account the positive results obtained for proteins, we expected that a blend nanomatrix would represent a promising alternative for the efficient encapsulation and controlled release of plasmid DNA. The in vitro release experiments showed that the presence of the poloxamine or poloxamer component in the particle matrix led to an important modification of the plasmid DNA release profile. As it can be observed in Figure 3, parts A and B, all blend nanoparticle compositions provided a continuous (closely linear) release of the plasmid DNA molecules. In addition, it can be noted that the type of PEO derivative incorporated within the nanostructures affects the release rate of plasmid DNA. More specifically, the release rate appears to be dependent on the HLB of the PEO derivative. The nanoparticles composed of a blend of PLGA with the hydrophilic poloxamer Pluronic F68 or with the hydrophilic poloxamine Tetronic 908 release plasmid DNA within a period of one week, whereas the formulations composed of a blend of PLGA with a more hydrophobic component (Pluronic L121 or Tetronic 904) can prolong the release up to two weeks while maintaining the controlled manner.

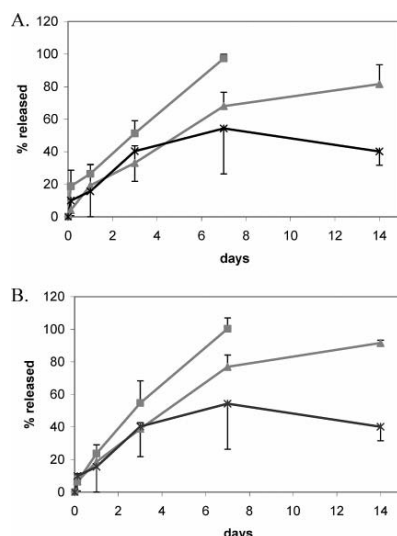


Figure 3. (A) Release profiles of plasmid DNA from PLGA:Pluronic F68 (■) and PLGA:Pluronic L121 (▲) blend nanoparticles and from the control PLGA (x) nanoparticles (mean $\pm$ S. D., $n = 3$). (B) Release profiles of plasmid DNA from PLGA:Tetronic 908 (■) and PLGA:Tetronic 904 (▲) blend nanoparticles and from the control PLGA (x) nanoparticles (mean $\pm$ S. D., $n = 3$).

These findings are in agreement with those found for PLGA:poloxamer microparticles where the improvement of the release profile was explained by the different matrix hydration processes because of the presence of the poloxamer.^{30,31} Therefore, we could assume that in the case of the nanoparticulate carriers described here the polyoxyethylene derivatives could play a similar role. Essentially, poloxamers and poloxamines could fill up the pores of the particle matrix and, depending on their HLB values, modify the rate of the release of the encapsulated plasmid.

Additionally, as a control experiment, and in order to assess the role of the PEO derivative in the controlled release mechanisms of these nanostructures, we have also studied the behavior of pure PLGA nanoparticles. The results presented in Figure 3, parts A and B, show that these PLGA nanoparticles have a limited capacity to control the release of the encapsulated plasmid. Indeed, although the early plasmid release was similar to that of the new compositions, after a few days the process reached a plateau when only a 40% of the encapsulated plasmid was released. Moreover, the plasmid DNA release from these nanomatrixes was highly variable (very large standard deviations) as compared to that observed for the new blend nanosystems. This behavior could be attributed to a slower erosion of the PLGA matrix as compared to that of the blend matrixes and to a lower stability of the plasmid DNA in the absence of the poloxamer/poloxamine component. Indeed, as described above, there might be unfavorable interactions between DNA and a polyester particle matrix that could explain the lower plasmid recovery.

3.4. Structural Integrity of the Encapsulated Plasmid DNA. One of the major challenges of plasmid DNA encapsulation into polymeric vehicles is the preservation of its structural integrity and, thus, of its biological activity. It has been generally accepted that there are two important steps in which the stability of the plasmid DNA encapsulated into PLGA particles can be compromised. One is related to the nanoencapsulation technique, whereas the second step is related to the polymer degradation process. In the present case, the cavitation-induced degradation during encapsulation is not expected to happen, due to the changes introduced in the preparation technique that exclude the exposure of the DNA to shearing stresses. With regard to the degradation process, as indicated above, it is known that the development of an acidic microclimate in the interior of the particles causes the inactivation of the encapsulated plasmid.^{11,32} Nevertheless, this phenomenon is expected to be attenuated by the presence of poloxamers or poloxamines in the nanostructures that they may act as blocking agents against unfavorable interactions between the PLGA degradation products and the encapsulated macromolecules.^{18,19}

The quantification of the supernatants of the release samples was performed using the PicoGreen reagent which is a specific fluorescent cyanine dye for double stranded DNA. Since close to 100% of the encapsulated plasmid could be recovered and detected by these fluorimetric assays, we can assume that no DNA fragmentation was induced either during the encapsulation processes or during the release of the plasmid. However, apart from the conservation of the double stranded structure, it is also necessary to maintain the biologically active form of the plasmid molecule. Therefore, we examined the release samples by agarose gel electrophoresis and we have compared them with the intact plasmid.

It can be seen in Figure 4 that the untreated pEGFP-C1 plasmid is present in the two forms that are considered to possess high biological activity.^{11,24} More specifically, the plasmid is predominantly in the supercoiled form (over 60%) and, in a smaller percent, in the opened circular form (less than 40%). Interestingly, the plasmid released from the nanoparticles was observed to maintain its initial structure. Indeed, the plasmid detected was present in both the supercoiled and circular forms, although a slow conversion from the supercoiled to the circular form was noted at late release times. On the other hand, very little or no conversion to the linear form could be detected in the release samples. This observation is very important because the linear form is the biologically less active one and more susceptible to further degradation and fragmentation than the circular forms.^{11,14} Overall, these results show the role of the PEO derivatives in preventing the structural changes of the encapsulated plasmid typically observed for the classical PLGA particles.¹

3.5. Biological Activity of the Encapsulated Plasmid DNA. As indicated above, we have shown that plasmid DNA encapsulated in different blend nanoparticles is released mainly in supercoiled and opened circular forms in the time range of one or two weeks, depending on the blend composition. To confirm that DNA preserves its biological

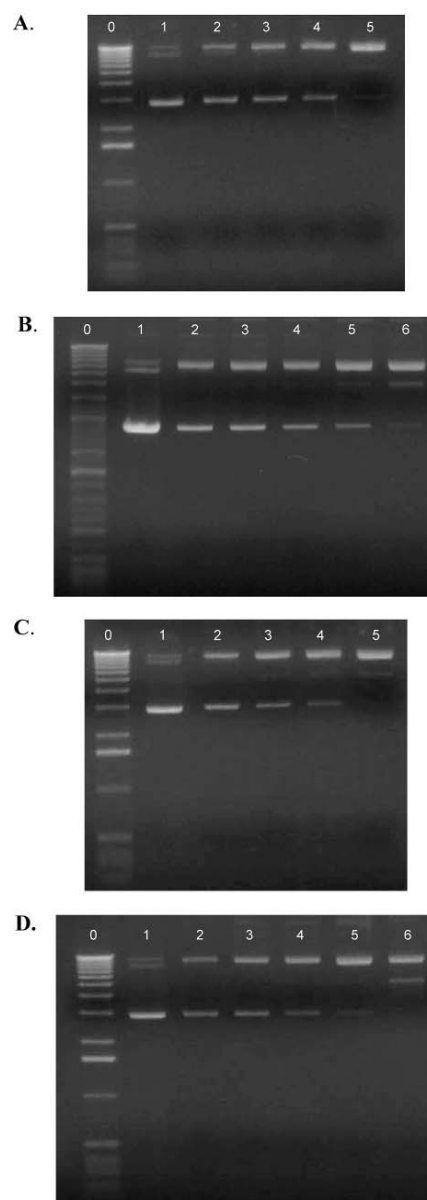


Figure 4. Agarose gel electrophoresis of plasmid DNA released from blend nanoparticles composed of (A) PLGA:Pluronic F68; (B) PLGA:Pluronic L121; (C) PLGA:Tetronic 908; (D) PLGA:Tetronic 904. Lane 0, 1 kb DNA ladder; lane 1, pEGFP-C1 control; lane 2, 3 h release; lane 3, 1 day release; lane 4, 3 day release; lane 5, 1 week release; lane 6, 2 weeks release.

activity throughout its encapsulation and release, we have also assessed its transfectivity in a cell culture model after

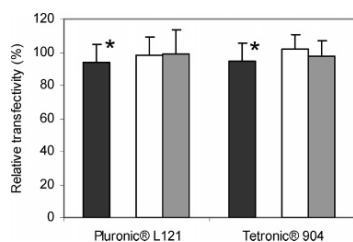


Figure 5. Transfectivity of plasmid DNA extracted (black bars) and released after 1 week (white bars) and 2 weeks (gray bars) incubation from nanoparticles composed of PLGA:Pluronic L121 and PLGA:Tetronic 904 (mean $\pm$ S. E., $n = 6$). Transfectivity levels are expressed as a percentage with respect to the level obtained for the positive control (untreated plasmid). * statistically different from the control ($\alpha < 0.01$).

its extraction or release from the formulations. For these experiments we have selected the nanoparticle compositions PLGA:Pluronic L121 and PLGA:Tetronic 904 because these formulations provided more extended release time for the plasmid (two weeks). Since the incubation of “naked” plasmid samples with cells results in very low, undetectable transfection levels, we have used the FuGene 6 reagent for the complexation of the pEGFP-C1 plasmid in the transfection assays. It can be seen in Figure 5 that plasmid DNA encapsulated and extracted from the blend formulations reaches about the 93% of the transfection level of the control sample (unencapsulated pEGFP-C1).

As expected, this shows that the modified solvent diffusion technique used in this study provides optimal conditions for the incorporation of DNA into the nanoparticles. Nevertheless, in some previous reports, satisfactory results could be obtained for plasmid extraction samples even in the case when the encapsulation process caused some DNA conversion to opened circular forms.³³

On the other hand, in Figure 5, it can also be seen that the relative transfectivity of the plasmid DNA released after 1 and 2 weeks remained practically unchanged (over 97% of the control), and this result is in agreement with those of the electrophoresis assays. As compared to pure PLGA vehicles, where the structural integrity of plasmid DNA released after incubation periods longer than 1–2 days is usually found to be compromised,^{34,35} it can be seen that the new blend nanostructures provide improved protection for the encapsulated plasmid in the whole course of the polymer degradation process and release.

Statistical analysis of the experimental data showed a slight, but significant, difference between the transfectivity of the extraction and release plasmid samples. This difference could be explained by the exposition of the plasmid to organic solvents during the time course of the extraction procedure that could alter its biological activity.

3.6. Cytotoxicity of PLGA:PEO Blend Nanoparticles in Cell Culture. A very important aspect of the investigation of new polymeric drug delivery systems is the analysis of their tolerability and toxicity upon the interaction with their target (and nontarget) cells. The nanostructures presented in this report are composed of two types of polymers which

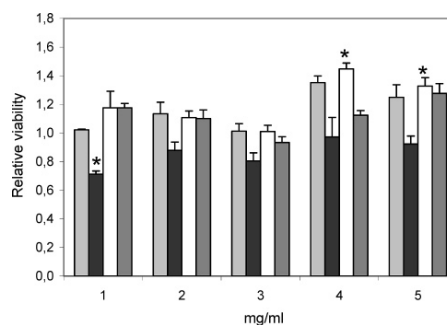


Figure 6. Cytotoxicity levels of nanoparticles composed of PLGA: Pluronic F68 (light gray bars), PLGA:Pluronic L121 (black bars), PLGA:Tetronic 908 (white bars), and PLGA:Tetronic 904 (dark gray bars) (mean $\pm$ S. E., $n = 4$). Viability levels are represented as a percentage with respect to the level obtained for the positive control (untreated cells). * statistically different from the control ($\alpha < 0.01$).

have already been used separately in various medical applications. In the case of the biodegradable PLGA, there are numerous references available on its biocompatibility and low toxicity.^{36,37} On the other hand, in the case of the polyoxyethylene component, the toxicity has also been reported to be acceptably low.¹⁶

Figure 6 shows the toxicity of the nanoparticles in the MCF-7 breast cancer cell line. The results indicate that nanoparticles did not present toxic effects on the cells since viability was always above 75–80% even at the highest nanoparticle concentrations (5 mg/mL). Interestingly, for some nanoparticle compositions, we observed an increase in the relative viability (differences are statistically significant in the case of the formulation comprising Tetronic 908 at 4 mg/mL and 5 mg/mL), with this effect probably being due to the presence of the polyoxyethylene component in the nanoparticles. Indeed, poloxamers are known to influence mitochondrial respiration.^{38,39} Taking into account that the technique used for the determination of cell viability is based on the measurement of the total respiratory activity,⁴⁰ it is possible that at high nanoparticle concentrations the presence of poloxamer- or poloxamine-type molecules could influence the cellular response.

On the other hand, the increased metabolic activity could also be explained by the long incubation period (24 h) in which the direct contact of the nanoparticles with the cells could provoke this stimulating, but not essentially harmful, effect. In addition, considering the fact that the effects caused by direct contact in such a high particle/cell concentration would be scarcely probable in the living body, it can be expected that these formulations would present a high safety margin for their in vivo administration.

4. Conclusions

The present report shows that it is possible to encapsulate plasmid DNA within novel PLGA:poloxamer and PLGA: poloxamine blend nanostructures while preserving its structural integrity and transfection capacity. Moreover, the

presence of the polyoxyethylene derivative has a positive effect on the release characteristics of the nanoparticles. The good tolerability of the formulations in cell cultures has also been confirmed. Accordingly, the incorporation of poloxamers and poloxamines into the particles structure offers new prospects for the delivery of plasmid DNA by nanocarriers.

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Artículo 3

**PLGA:poloxamer and PLGA:poloxamine blend
nanostructures for transmucosal delivery of DNA: in vitro
and in vivo evaluation of uptake and transfection efficiency**

N. Csaba, A. Sánchez y M.J. Alonso

Sometida para su evaluación

Abstract

Nanoparticles based on new compositions consisting of blends of poly (lactic-co-glycolic acid) (PLGA) and different polyoxyethylene derivatives have recently been developed by our group. These nanoparticles, obtained by a modified solvent diffusion technique, have shown good capacity to associate and release plasmid DNA in its biologically active form. In the present work we have further investigated the potential of these nanostructures as nasal gene delivery carriers. First, we studied the cellular uptake (HEK 293 cell line) of FITC-labelled plasmid DNA nanoencapsulated in PLGA-Pluronic F68[®] and PLGA-Tetronic T904[®] nanoparticles, by confocal microscopy. Second, we investigated the uptake of the rhodamine-loaded nanoparticles by the nasal mucosa following intranasal administration to mice. Third, we monitored the immune response generated by the nanoparticles containing the beta-galactosidase encoding gene, following nasal administration to mice, using the ELISA technique. The results of the in vitro cell culture studies showed that the new formulations exhibited the ability to enter the cells and transporting the associated DNA molecule. Moreover, the results of the in vivo administration indicated that both types of nanoparticles were able to overcome the nasal barrier. Finally, the results of the immune response showed that DNA-loaded PLGA-Pluronic F68[®] nanoparticles elicited a fast and high response, which was significantly more pronounced than that corresponding to the naked plasmid DNA for up to 6 weeks. Overall, these results suggest that these new nanoparticles have a potential as carriers for the delivery of DNA across the nasal mucosa.

Keywords: PLGA, polyoxyethylene derivatives, nanoparticle, gene delivery, nasal administration

1. Introduction

In vivo administration of plasmid DNA is a promising strategy in the clinical field which uses the body as a bioreactor for the production of molecules with therapeutic or prophylactic properties (1). In spite of the apparent simplicity of this gene therapy concept, the need of an elevated, prolonged and tissue-specific expression of plasmid DNA at specific target cell populations required in somatic gene therapy approaches (i.e., the treatment of genetic disorders) restricts the practical implementation of new gene-based medicines. In contrast to these difficulties encountered in gene therapy, the effective production of antigens from plasmid-based expression systems appears to be a more realistic approach whose success is expected to be evidenced in the very near future. Indeed, taking into account the mechanisms of inducing antibody responses, it is accepted that relatively short-term transfection by antigen-encoding plasmids at the right place could be sufficient for the activation the immune cascade (2). Moreover, it has also been reported that even very low levels of plasmid expression are able to elicit significant immune responses (3). In addition to these positive features, the development of genetic vaccines has received a great attention due to the important advantages which offer over conventional vaccines. These advantages include their ability to develop both humoral and cellular immune responses, their more cost-effective manufacture and higher stability, their improved storage and/or distribution possibilities and their improved safety issues (4, 5). Finally, these vaccines also offer the possibility of expressing multiple antigens or antigen/adjuvant combinations (6). Unfortunately, counterbalancing these clear advantages there is still a problem associated to the insufficient stability of these plasmid constructs “in vivo”, thus indicating the necessity to develop more efficient carriers for plasmid protection and delivery (7).

One of the promising alternatives for the delivery of these new class of vaccines consists on their encapsulation into biodegradable nanoparticles based on polyesters such as poly (L-lactic acid) (PLA) and poly (D,L lactic-co-glycolic acid) (PLGA). However, nowadays it is accepted that these widely investigated nanometric carriers could be further improved in terms of preserving the biological activity of the encapsulated DNA and also in terms of improving their interaction with the biological environment (8; 9). An efficient approach to deal with this latter problem has been based upon the coating of PLA nanoparticles with poly (ethylene glycol) (PEG). Indeed, it has been previously shown that PEG-coated PLA nanoparticles are able to cross mucosal surfaces and transport macromolecules, including classical vaccines (10; 11) and plasmid DNA (12). Nevertheless, so far there is no evidence of the potential of these surface modified nanoparticles for improving the DNA stability against degradation derived from the typical inner acidification of the nanomatrices.

With the attempt of resolving the problems inherent to PLGA nanomatrices, we have recently reported the development of novel, blended nanoparticle compositions of PLGA and polyoxyethylene derivatives (poloxamers and poloxamines). The design of these vehicles was based on multiple considerations such as the known protective effects observed for poloxamer containing micro- and nanoparticles encapsulating proteins, such as tetanus toxoid or interferon-alpha (13; 14) and also the recent reports indicating the potential of poloxamers/poloxamines for gene delivery (15; 16). So far, we have been able to show that these new PLGA:poloxamer and PLGA:poloxamine blend nanostructures can be prepared by an innovative technique that allows the encapsulation of plasmid DNA in its bioactive form. Moreover, due to the protective effects of the polyoxyethylene derivatives in the particle matrix, we could show that the biological activity of the plasmid is completely preserved during its controlled release (17).

Therefore, taking this previous information into account, the main objective of the present work was to investigate the ability of these blended nanoparticle compositions of PLGA and polyoxyethylene derivatives (poloxamers and poloxamines) to enter the cells, to overcome mucosal surfaces and, finally, to elicit an immune response. With this objective in mind we selected the beta-galactosidase reporter gene as a model DNA vaccine.

Materials and methods

2.1. Materials

The polymer poly (D, L-lactide-co-glycolide) 50:50 Resomer[®] RG 503 was purchased from Boehringer Ingelheim (Ingelheim, Germany). The poloxamer Pluronic[®] F68 with hydrophilia-lipophilia balance (HLB)=29 was obtained from Sigma Aldrich (Madrid, Spain). The poloxamine Tetronic[®] 904 was kindly donated by BASCOM Belgium (Brussels, Belgium). Plasmid DNA encoding green fluorescent protein with CMV promoter (pEGFP-C1) and plasmid DNA encoding beta-galactosidase with CMV promoter (pCMV-βGal) were purchased from Elim Biopharmaceuticals (San Francisco, CA, USA). Beta-galactosidase protein (βGal) and beta-galactosidase monoclonal antibody (Ab-1) were obtained from Calbiochem (Madrid, Spain). Rabbit anti-mouse IgG (H+L) horseradish peroxidase conjugate was from Southern Biotechnology (Birmingham, AL, USA) and ABTS substrate kit was from Zymed Laboratories (San Francisco, CA, USA). Ultra-high binding Immulon 4HBX 96-well microplates were obtained from Thermo Labsystems (Franklin, MA, USA). Male Swiss mice (6 weeks old, 20-22 g) from the Central Animals House of the University of Santiago de Compostela were used. The animals were kept in a 12 h light/dark cycle and they were allowed to access food and water ad libitum.

2.2. Preparation of PLGA:poloxamer and PLGA:poloxamine blend nanospheres

Nanoparticles with PLGA: poloxamer or PLGA: poloxamine ratios of 50:50 were prepared by a modified solvent diffusion technique developed by our group. First, 50 mg of PLGA and 50 mg of poloxamer or poloxamine were dissolved in 2 ml of methylene chloride. This organic solution was then mixed by vortex agitation (2400 min^{-1} , Heidolph, Germany) for 30 s with an aqueous phase (200 μl). Then, the obtained emulsion was poured onto a polar phase (25 ml ethanol) under moderate magnetic stirring, leading to immediate polymer precipitation in the form of nanoparticles. The formulations were diluted with 25 ml of milli-Q water and the stirring was maintained for 10 min more. After solvent evaporation under vacuum at 30°C (Rotavapor, Büchi R-114, Switzerland) nanoparticles were collected and concentrated in milli-Q water.

The size, polydispersity and zeta potential of the different nanoparticles were determined by photon correlation spectroscopy and laser-Doppler anemometry, respectively (Zetasizer 3000 HS, Malvern Instruments, England). In the case of the determination of zeta potential, measurements were performed in 1 mM KCl. Mean values were obtained from 3 different batches, each of them measured 3 times.

2.3. Plasmid DNA loaded blend nanoparticles

For its encapsulation, plasmid DNA was incorporated into the internal aqueous phase of the formulations prior to nanoparticle formation (section 2.2). The theoretical loading was 1 % with respect to the total amount of PLGA in the nanoparticles. Encapsulation efficiency was calculated from the amount of non-encapsulated plasmid, which was recovered in the supernatant samples collected upon the centrifugation of the nanoparticles. The amount of this plasmid was determined by spectrofluorimetry (LS 50B luminescence spectrometer, Perkin-Elmer) using Pico Green[®] reagent (Molecular Probes, OR, USA).

2.4. Rhodamine 6G loaded blend nanoparticles

The fluorescent marker rhodamine 6G was incorporated into the organic polymer solution (methylene chloride) of the formulations prior to nanoparticle formation.

The theoretical loading was 0.2% with respect to the total amount of PLGA in the particles. For the complete elimination of free rhodamine, nanoparticles were extensively washed by a 2-step procedure consisting on gel filtration (PD-10 desalting column with Sephadex G-25, Amersham Biosciences, Spain) and on particle isolation/resuspension by centrifugation (15 min, 8000 RCF, 15°C, Avanti 30 Beckman, Spain).

Encapsulation efficiency was calculated from the amount of the free rhodamine present in the aqueous phase of the formulation (obtained by centrifugation without particle washing). The amount of this rhodamine was determined by spectrophotometry (UV-Visible Spectrometer, UV-1603, Shimadzu, Spain).

In order to confirm that the fluorescent marker remains associated to the particles during the experiments to be carried out, nanoparticle suspensions (2 mg/ml) were incubated in HBSS pH=7.4 at 37°C. At different time intervals (1 and 4 hours) nanoparticles were centrifuged and the supernatants were analysed for rhodamine by fluorimetry (Luminescence Spectrometer LB-50, Perkin-Elmer, Spain) using the supernatants of the corresponding blank formulations for the preparation of the calibration curves.

2.5. FITC labelling of plasmid DNA

Fluorescent labelling of DNA was carried according to the diazonium-reaction described by Ishii et al (18). Briefly, fluorescein-5-isothiocyanate was reacted overnight with 2-(4-aminophenyl)-ethylamine in dimethyl-formamide under

magnetic stirring at 25 °C. The formation of FITC-aniline was monitored by thin layer chromatography (hexane: ethyl acetate 2:8 eluent) until the detection of a single mark at $R_f=0.26$. Then, FITC-aniline was reacted with sodium-nitrite in 0.5 M HCl at 0 °C under stirring. The reaction was stopped by the addition of 1M NaOH and subsequently, the obtained FITC-diazonium solution was mixed with pCMV β -Gal plasmid in 0.1 M borate buffer (pH=9). The reaction was carried out at 25 °C under stirring. The obtained FITC-plasmid was purified by ethanol precipitation and by gel exclusion chromatography using PD 10 desalting columns with Sephadex G-25 (Amersham Biosciences, Spain). Purified FITC-DNA was encapsulated into PLGA: Pluronic[®] F68 and PLGA: Tetronic[®] 904 formulations as described in section 2.3 using 0.4% theoretical loading. Nanoparticles were washed and isolated by centrifugation (15 min, 8000 RCF, 15°C, Avanti 30 Beckman, Spain) for the elimination of non-encapsulated FITC-DNA and finally, resuspended in 25 μ l of water.

2.6. In vitro cell transfection studies

In vitro transfection assays were performed in the epithelial human embryonic kidney cell line 293 (HEK 293). The HEK 293 cells were obtained from ATCC (Rockville, MD, USA) and were maintained according to the supplier's recommendations. The cells were seeded at 70% confluence in 24-well tissue culture plates (Costar, Cambridge, UK) 24 h before transfection.

Plasmid loaded PLGA: Pluronic[®] F68 and PLGA: Tetronic[®] 904 formulations were prepared as described in section 2.3., isolated by centrifugation (15 min, 8000 RCF, 15°C, Avanti 30 Beckman, Spain) for the elimination of non-encapsulated DNA and resuspended in 25 μ l water. Cells were washed and then formulations containing 1 or 2.5 μ g DNA together with 500 μ l OptiMEM were added to the

cells. After 4 h incubation, the formulations were removed and 1 ml of fresh culture medium was added. The medium was changed every second day for experiments that exceeded 2 days. At the time points indicated, cells were investigated under a fluorescence microscope (Nikon Eclipse TE2000-S, Nikon UK Ltd., UK) for GFP positive cells.

2.7. Evaluation of particle uptake in HEK 293 cell culture

HEK 293 cells were seeded 24 hours before the uptake experiment onto sterile, poly(L-lysine) pre-treated glass coverslips in 24-well tissue culture plates (Costar, Cambridge, UK) at 70% confluence. Cells culture medium was replaced by HBSS (pH=7.4) and then nanoparticle formulations were added at 300 µg/ml concentration to each well. After 1 hour incubation, the formulations were removed and the wells were rinsed three times with HBSS. Samples were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X and then cell nuclei were stained with propidium iodide according to manufacturers instructions, including ribonuclease A pre-treatment.

Cell fluorescence was analyzed by confocal laser scanning microscopy (TCS-SP2, Leica GmbH, Germany) that allowed the simultaneous visualization of the two different fluorescent markers. Excitation wavelengths were 488 nm for FITC (Ar laser) and 633 nm for propidium iodide (He/Ne laser),

2.8. Evaluation of particle uptake through nasal mucosa

A total dose of 2.5 mg of rhodamine loaded PLGA:Pluronic[®] F68 or PLGA:Tetronic[®] 904 nanoparticles was resuspended in distilled water and administered by nasal instillation to mice (n=2). Nanoparticles were administered repeatedly (4 x 10µl administration with 5 min time intervals) in order to allow a high dose of nanoparticles while avoiding their overflow into the gastrointestinal

tract. At 15 min post-administration the mice were sacrificed by light ether anaesthesia followed by cervical dislocation. The nasal mucosa was excised, washed and then fixed with 4% formaldehyde solution during 30 min. The whole mucosa was directly observed by vertical confocal laser scanning microscopy (TCS-SP2, Leica GmbH, Germany) at 488 nm excitation wavelength (Ar laser).

2.9. Immunisation protocol

The three groups of mice (n=9) were immunised by intranasal inoculation with three weekly doses of pCMV-βGal plasmid. For each dose, nanoparticles were resuspended in 30 µl distilled water of which 10 µl was dropped 3 times (with 15 min intervals) to the nasal cavity, using a micropipette. Group 1 received 25 µg of unencapsulated pDNA, group 2 and 3 received 25 µg of pDNA encapsulated in PLGA:Pluronic® F68 and PLGA:Tetronic® 904 nanoparticles, respectively. All animals were unanaesthetised during the administrations.

Blood samples were taken from the tail-vein of all animals at different time intervals post-administration. Blood samples were left at room temperature for 15 min, then centrifuged for serum isolation (3000 x g, 5 min, 4 °C) and stored at 20 °C until their further analysis.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Total anti-βGal specific serum IgG antibodies were determined using an ELISA protocol as follows. First, 50 µl of a 5 µg/µl standard βGal protein solution was added to each well of a 96-well microplate (Immulon 4HBX) and incubated for 3 hours at room temperature. Then the microplate was washed once with PBS/0.05% Tween 20 buffer (PBS/T). Then 150 µl of PBS/1% BSA was added to the wells and incubated for 2 hours at room temperature in order to block non-specific interactions of the primary and secondary antibodies with the wells bottom. Plates

were washed once with PBS/T and 100 μ l of serum samples (10 x diluted) were added serially to the first row of the well and 75 μ l PBS was added to the remaining wells. The first column was kept as a blank sample (only PBS), the second column was kept as a negative control (serum of non-immunised mice (NMS)) and the last column was used as a positive control (standard anti- β Gal antibody diluted to 2 μ g/ μ l in PBS: NMS 10:1). All samples were titrated 1:4 steps downplate and the microplate was incubated at 4 °C overnight. The next day wells were washed 4 times with PBS/T and then incubated for 1 hour with 100 μ l of rabbit anti-mouse IgG (H+L) HRP conjugate (Southern Biotechnology, AL, USA) diluted 1:2000 in PBS. Plates were washed 3 times with PBS/T and 100 μ l of freshly prepared ABTS solution (ABTS substrate kit, Zymed Lab., CA, USA) was added to the wells, incubated for 30 min at room temperature. Finally, O.D. were measured at $\lambda=405$ by a microplate reader (3550-UV, BioRad, CA, USA). Results were expressed as total serum IgG antibody concentrations.

2.11. Statistical analysis

Statistical analysis of the experimental data was performed by applying a Kruskal-Wallis test ($p < 0.05$) to the IgG levels elicited by the formulations at the different time points of the study.

3. Results and Discussion

Nanoparticles made of PLGA: polyethylene oxide blends have been proposed as promising vehicles for the delivery of sensitive macromolecules such as proteins or plasmid DNA. One of the most important features of these systems is the protective environment created during their preparation and release, which prevents structural alteration of the encapsulated molecules. Indeed, we have previously reported that

these blend compositions provide suitable, closely linear release profiles for plasmid DNA in its biologically active form (19). Based on these characteristics, in the present study we aimed to investigate the potential of the selected blend compositions for gene delivery in cell cultures and through mucosal surfaces *in vivo*.

3.1. Characterisation of plasmid loaded nanoparticles

The first attempt in this study was the optimisation of the vehicles designed in a previous work regarding their DNA loading. More specifically, we have increased the theoretical loading of plasmid DNA from the previously applied 0.4% to 1% and studied the effects of this modification on the properties of the nanoparticle formulations. Table 1 shows that nanoparticle size was not affected by this increased amount of DNA in the formulation. Similarly to the corresponding blank formulations, all DNA-loaded nanoparticles remained in the size range of 160-190 nm with very low polydispersity values, thus evidencing the formation of a very homogeneous population of nanoparticles (close to unimodal). This particle size is lower than those observed for other, DNA-loaded PLGA or PLA-PEG carriers, where the applied solvent diffusion or double emulsion techniques yield more heterogeneous particle populations, typically between 200 – 700 nm (20-22).

Formulations	DNA loading	Size (nm)	P.I.	ζ – potential (mV)	E. E. (%)
PLGA: Pluronic® F68	0	163 ± 5	0.135	-43.1 ± 6.4	-
	0.4%	183 ± 6	0.114	-50.8 ± 3.6	35.2 ± 8.9
	1%	184 ± 5	0.160	-43.4 ± 1.1	33.6 ± 4.3
PLGA: Tetronic® 904	0	168 ± 9	0.179	-38.4 ± 3.3	-
	0.4%	161 ± 7	0.154	-54.1 ± 2.0	44.1 ± 4.3
	1%	187 ± 7	0.174	-40.3 ± 2.0	48.7 ± 7.5

Table 1. Size, polydispersity index (P.I.) and DNA encapsulation efficiency (E.E. %) of PLGA: Pluronic® F68 and PLGA: Tetronic® 904 blend nanoparticles

Interestingly, as compared to the previously obtained results with 0.4% DNA loading, the encapsulation efficiencies using 1% DNA loading remained in the same range (between 35% - 48%), being higher for the poloxamine containing vehicles. In agreement with our objective, these results show an efficient increase of the final DNA loading for both nanostructures, reaching 3.4 µg plasmid for each mg of the PLGA: poloxamer blend nanoparticles and 4.8 µg plasmid/mg for the PLGA: poloxamine blend nanoparticles. These values are comparable to those observed for PLGA-based particles, but they still remain below the exceptionally high loadings obtained previously for PLA-PEG nanocarriers (21). Nevertheless, they could be considered sufficiently high for the in vivo administration of the desired quantity of DNA without the necessity of administering excessively high amounts of nanoparticles.

3.2. Characterisation of rhodamine-loaded nanoparticles

As indicated above, one of the purposes of the present work was to investigate the mechanism of interaction of the nanoparticles with the nasal mucosa. The idea behind was to elucidate whether or not these nanoparticles could work as transmucosal carriers for DNA vaccines. For the investigation of the transmucosal transport of the nanoparticles by confocal microscopy, it was necessary to associate a suitable fluorescent marker. Based on the satisfactory results obtained for PLA-PEG nanoparticles prepared by a similar solvent diffusion technique (23), we have chosen rhodamine 6G for particle labelling. Table 2 shows that rhodamine can be very efficiently associated to the nanoparticles, independently of the PEO type present in the blend composition. It can also be seen that rhodamine encapsulation does not influence the size or polydispersity of the formulations.

Formulations	Size (nm)	P.I.	E. E. (%)
PLGA: Pluronic [®] F68	183 ± 5	0.156	67.9 ± 5.3
PLGA: Tetronic [®] 904	162 ± 6	0.176	69.6 ± 5.7

Table 2. Size, polydispersity index (P.I.) and rhodamine encapsulation efficiency (E.E. %) of PLGA: Pluronic[®] F68 and PLGA: Tetronic[®] 904 blend nanoparticles

The in vitro release studies showed that rhodamine remains stably associated to the vehicles during the time period of four hours. Both blend compositions exhibited a minimal (less than 2%) release during the first hour of the experiment. After the first hour, no further rhodamine release could be observed from any of the formulations. Consequently, this small amount released was considered to have a negligible effect on the observation of the nanoparticles by confocal imaging.

3.3. *In vitro cell transfection efficiency*

In a previous study we have analysed the biological activity of plasmid DNA extracted and released (after 2 weeks) from different PLGA: polyoxyethylene blends (19). In these studies the extracted/released plasmid was complexed with a commercial transfection agent and added to cell cultures. The results of this experiment confirmed that DNA maintains completely its original transfection capacity upon its encapsulation/release from these blend matrices. In the present work our aim was to test the efficiency of the DNA-loaded blend nanoparticles by adding them directly to HEK 293 cell culture. For this purpose we chose the plasmid DNA encoding GFP. Unfortunately, the level of transfection observed by fluorescence microscopy was very low. More specifically, we observed an important number of cells, but exhibiting very low fluorescence intensity. Consequently, it was difficult to clearly identify the number of GFP positive cells. Interestingly, this low transfection intensity was maintained very similar in appearance throughout the duration of the experiment (10 days). Taking into account that the plasmid DNA released from the nanoparticles was completely active (17), for the explanation of these results we could formulate three different hypothesis: (i) the nanoparticles were not internalized by the cells, (ii) the plasmid DNA was released at a very slow rate (iii) the plasmid DNA was degraded inside the cells upon release. The first hypothesis could not be taken as a major cause for low transfection since the number of cells apparently transfected was high. The second hypothesis appears to be more realistic since it is known that these nanoparticles release the encapsulated plasmid in a controlled manner. The third hypothesis remains to be verified. However, given the positive effects reported for poloxamers and poloxamines as protective non-condensing carriers, and recently also for triblock PLGA-PEG-PLGA copolymer as cell culture additives for gene transfection (24). In this latter case, the enhancement of gene expression was

attributed to a facilitated particle endocytosis, provided by the presence of PLGA-PEG-PLGA.(25)

3.4. Evaluation of the internalization of plasmid DNA-loaded nanoparticles in HEK 293 cells

In order to further verify if the nanoparticles could transport the plasmid inside the cells we also examined their internalisation in cell cultures. For the localisation of the vehicles in the cells, we stained the cell nuclei with propidium iodide and we used FITC-labelled plasmid DNA. The FITC-conjugation was carried out using Ishii's method, that has already been used by several groups and was found to be a suitable tool for confocal imaging of plasmid DNA associated to nanometric gene carriers (18; 26).

Figure 1 shows the intracellular distribution of FITC-DNA in solution or encapsulated into PLGA:poloxamer and PLGA:poloxamine blend compositions after one hour incubation with HEK 293 cells. It can be seen that the naked plasmid is not able to enter the cell since the corresponding green signal can hardly be detected inside the cells. On the contrary, as indicated by the intense green fluorescence, the DNA-loaded vehicles penetrate very efficiently through the cell membrane. In the x-y sections (Figure 1A), the green spots corresponding to FITC labelled DNA appear homogeneously distributed in the cytoplasm of the cells and they can also be found in the nuclei. This nuclear localization can be better visualized in the x-z cross sections of the cell, in which the green fluorescence derived from the plasmid can be localized at the same depth as the red emission of the labelled nuclei (example shown on Figure 1B). These results are in agreement with those found by other authors for fluorescently labelled biodegradable

nanoparticles. The mechanism of this transcellular penetration is thought to be an energy dependent, endocytic pathway (27), (28).

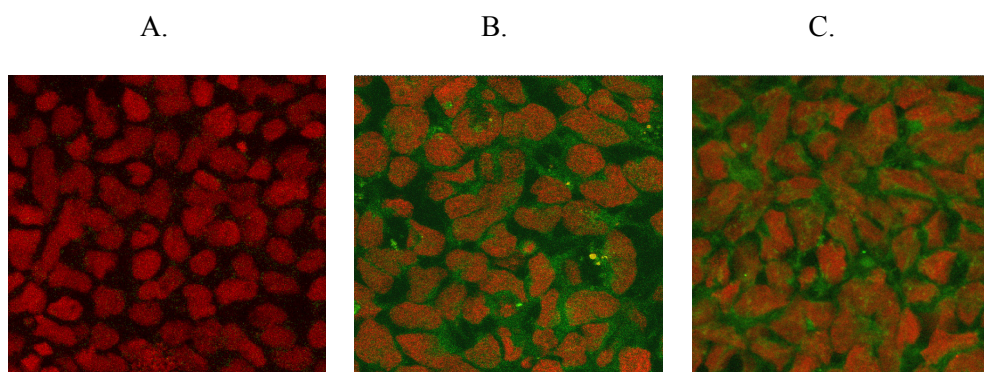


Figure 1A. Confocal laser scanning micrographs of HEK 293 cells incubated with FITC-DNA (green channel), x-y cross section. A. naked, B. encapsulated into PLGA:Pluronic® F68 nanoparticles and C. encapsulated PLGA:Tetronic® 904 nanoparticles. Cell nuclei are stained with propidium iodide (red channel)

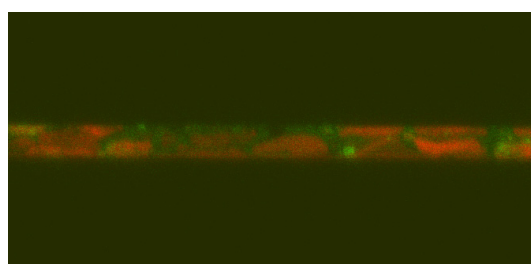


Figure 1B. Confocal laser scanning micrographs of HEK 293 cells incubated with FITC-DNA (green channel), encapsulated encapsulated into PLGA:Tetronic® 904 nanoparticles. Cell nuclei are stained with propidium iodide (red channel), x-z cross section

3.5. *Evaluation of transmucosal transport in vivo*

The transport of particles through mucosal surfaces has been extensively investigated in the last few years. A conclusion from these studies has been that the size of the particles is a critical factor that affects greatly their interaction and transport across mucosal surfaces and that, in general, particles smaller than 1 μm are more efficient in penetrating through mucosal barriers (29). In addition to the role of particle size, the surface chemical composition of the particles and, hence, their surface charge and hydrophilia are known to influence the interaction of the nanoparticles with the biological environment. For example, in this sense, we have recently found that the presence of PEG coating around the nanoparticles helps improving their stability in contact with mucosal fluids, and consequently, the ability of these nanoparticles to overcome the nasal mucosa (30).

Based on these previous finding, we hypothesised that given the structural similarity between PEG and PEO derivatives, the nanoparticles presented here could also be advantageous for their nasal uptake. Hence, we investigated the interaction of rhodamine-loaded PLGA: poloxamer and PLGA: poloxamine nanostructures with the nasal mucosa, following nasal administration to mice, using confocal microscopy. Figure 2 shows the images of three series obtained for three different groups of mice untreated and treated with PLGA: Pluronic[®] F68 or PLGA: Tetronic[®] 904 formulations, respectively. The series correspond to the mucosal surface (a) and to 5 μm (b) and 10 μm (c) separated cross sections that are fully representative of all animals receiving the same treatment (n=3). The first observation from these images is that, irrespectively of their composition, the nanoparticles remained stable upon contact with the nasal mucosa. The second important observation is that, as in the case of PLA-PEG nanoparticles (30), the PLGA: Pluronic[®] F68 and PLGA: Tetronic[®] 904 nanoparticles are able to enter the

nasal epithelium and are transported by a transcellular pathway, throughout the depth of the tissue ($\sim 25\text{-}30\text{ }\mu\text{m}$).

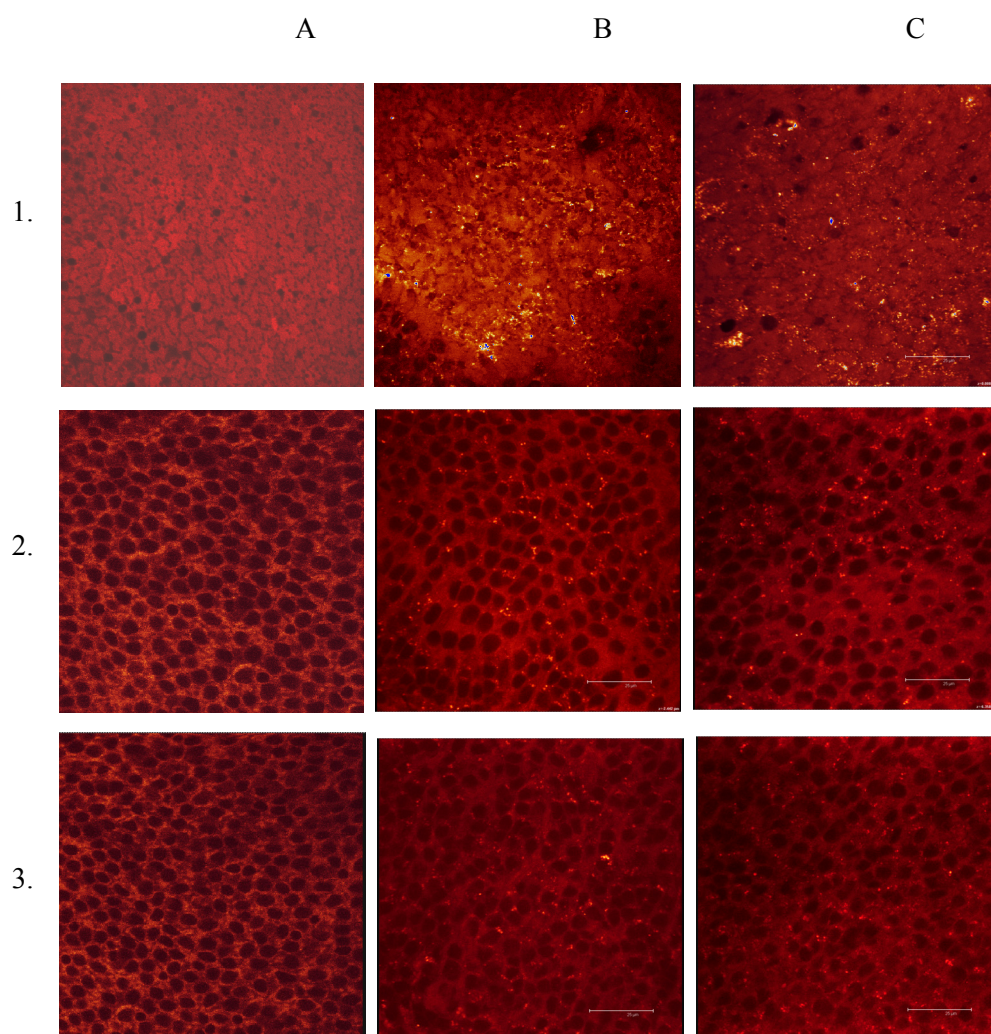


Figure 2. Confocal laser scanning micrographs of mouse nasal tissue: non treated (A), pre-treated with rhodamine loaded PLGA: Pluronic F68 nanoparticles (B) and pre-treated with rhodamine loaded PLGA: Tetronic 904 nanoparticles (C). Mucosal surface (1), cross section of $5\text{ }\mu\text{m}$ (2) cross section of $10\text{ }\mu\text{m}$ (3), scale bar corresponds to $25\text{ }\mu\text{m}$

These results are in agreement with the above mentioned statement that polyethylene oxide and its derivatives could play an important role in the in vivo behaviour of particulate carriers, providing improved stability and enhanced interaction with biological surfaces (31, 32). Likewise, they indicate the potential of the blend compositions described here as carriers for nasal drug delivery.

3.6. In vivo gene expression following intranasal administration

In order to further investigate the possible application of these PLGA: poloxamer and PLGA: poloxamine nanostructures as transmucosal gene delivery systems, we administered the nanoparticles intranasally and performed two kinds of analysis: first, we analyzed the qualitative β -galactosidase expression, at three days post-administration, using X-Gal staining and, second, we monitored the serum IgG antibody responses elicited, as a consequence of the gene expression.

Interestingly, the results of the qualitative β -galactosidase expression showed the typical blue coloration in the nasal tissue but not in the lung (data not shown). These results evidence the efficient interaction of the plasmid-DNA loaded nanoparticles with the nasal mucosa and, additionally, indicates that the particles administered intranasally did not reach the pulmonary region.

On the other hand, Figure 3 shows the systemic immune responses elicited by the naked plasmid and by the two different blend formulations at two weeks post administration. The results show that nanoencapsulated plasmid elicited β -galactosidase specific antibody levels that were significantly higher than those corresponding to the naked plasmid or to the controls (untreated mice). The maximum IgG values were reached at 4 weeks post-administration for all formulations, and then, decreased gradually, becoming negligible after 8 weeks. In the particular case of the poloxamer containing PLGA: Pluronic[®] F68

nanostructures, the IgG maximum was 3-fold higher than that elicited by the naked plasmid. Significantly improved immune could still be detected at the time point of 6 weeks, however, already in lesser extent (2-fold increase).

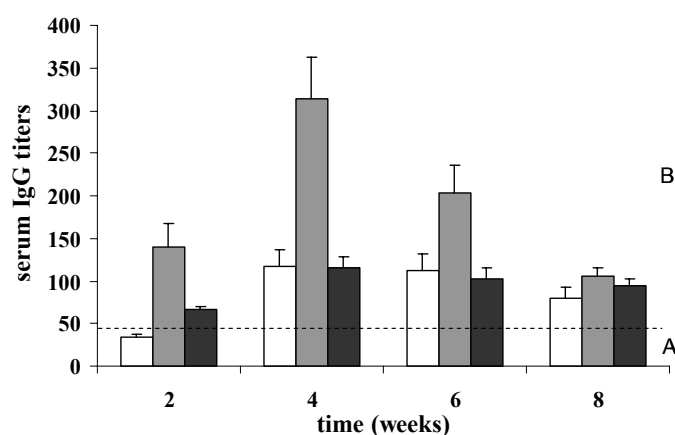


Figure 3. Serum IgG antibody levels following nasal administration of pCMV-βGal plasmid DNA, naked (□), encapsulated in PLGA: Pluronic® F68 (■) or in PLGA: Tetronic® 904 nanoparticles (■) region A: non-specific serum IgG, region B: specific anti βGal IgG

The results observed for the PLGA:Pluronic F68® nanoparticles are in good agreement, but slightly superior, to those corresponding to PLA-PEG nanoparticles containing the β-galactosidase encoding plasmid (21). More specifically, the highest levels obtained for PLA-PEG nanoparticles (containing 33 μg plasmid) were in the range of 100-120 ng/ml, while PLGA:Pluronic F68® nanoparticles (containing 25 μg plasmid) elicited IgG titers higher than 300 ng/ml at the same time point. Moreover, in this previous study it was shown that the IgG levels achieved for the nanoparticles were comparable to those elicited by the naked plasmid administered intramuscularly.

Surprisingly, the performance of the nanoparticles containing poloxamine (PLGA:Tetronic T904[®]) was similar to that of naked DNA. In fact, except for the 2-week time point, the IgG levels achieved for this formulation were not statistically different from those elicited by the naked plasmid. The different response observed for both types of nanoparticles (containing either poloxamer or poloxamine) can not be attributed to their different interaction with the nasal mucosa. Indeed, as indicated in section 3.5, both types of nanoparticles have the capacity to overcome the nasal mucosa. Therefore, we suggest that the difference between the efficacies of formulations at eliciting immune responses could possibly be related to their physico-chemical characteristics. Both formulations comprise nanoparticles in the size range of 150-200 nm, having PLGA as main component. However, the blended polyoxyethylene derivatives can confer different hydrophobicity to the particle matrix (33; 34). This different hydrophilia/lipophilia can remarkably modify the release characteristics of the particles, that is known to influence the immunogenicity of encapsulated antigens (35). Concretely, in our case the change from the poloxamer Pluronic[®] F68 (HLB=29) to the poloxamine Tetronic[®] 904 (HLB=14.5) has led to higher encapsulation efficiency and more sustained release of the encapsulated plasmid in vitro (19).

On the other hand, besides the differences in the release behaviour it might be possible that the two types of nanoparticles have different uptake by the nasal-associated lymphoid tissues (NALT) and, consequently, different delivery to the antigen presenting cells (i.e., M cells overlaying the follicle-associated epithelium of the NALT). In this sense, it is important to mention the observation by some authors about the correlation between the length of PEO chains of the block copolymers and their access to lymphatic structures (36). Finally, it should also be taken into account that poloxamer and poloxamine copolymers have an inherent adjuvant capacity and that this capacity is dependent of some physico-chemical

properties such as the HLB values (37). Therefore, from these hypotheses it could be concluded that more studies are necessary in order to fully elucidate what are the mechanism determinant of the success of these nanoparticles as carriers for DNA vaccines.

4. Conclusions

Nanoparticulate carriers based on different PLGA: polyoxyethylene blends exhibited good capacity of intracellular penetration both in cell cultures and in nasal mucosa. The transfection efficiency of the systems was shown to be dependent on the experimental conditions, showing better activities in vivo. The results of the genetic immunisation study using pCMV-BGal as a model plasmid showed that, as compared to the naked plasmid, the systemic IgG antibody response was significantly improved due to its nanoencapsulation into the blend nanoparticles. Altogether, these results support the potential application of controlled release, blend nanostructures as transmucosal gene delivery systems.

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Discusión general

Discusión

Las prometedoras perspectivas ofrecidas por la terapia génica y, en particular, por las vacunas basadas en ácidos nucleicos, junto a las características ventajosas de los vectores sintéticos como transportadores de genes, nos han llevado a investigar el potencial de nanoestructuras biodegradables como vehículos de ADN plasmídico.^{1,2} Concretamente, en la presente tesis hemos diseñado unos nanosistemas cuya novedosa composición permite evitar los frecuentes problemas de estabilidad de las macromoléculas encapsuladas en las micro- y nanopartículas de poliésteres, debidos al uso de tecnologías agresivas y al microclima ácido causado por la degradación de la matriz polimérica.^{3,4}

El diseño de estas nuevas composiciones nanoparticulares se inició tomando como referencia los resultados obtenidos previamente en nuestro laboratorio para proteínas encapsuladas en micropartículas elaboradas a base de mezclas PLGA y poloxámero.⁵ Estos resultados pusieron claramente de manifiesto que la presencia de derivados de polioxietileno (poloxameros o poloxaminas) en la matriz polimérica en forma de una mezcla íntima con el poliéster, permite preservar la estabilidad de la macromolécula bioactiva encapsulada.

Asimismo, en relación con la línea del presente trabajo, es importante destacar que investigaciones recientes han señalado el interés los de poloxameros y las

¹ T Storni y col. Immunity in response to particulate antigen-delivery systems. *Adv. Drug Deliv. Rev.* 57:333-355, 2005.

² C. S. Lengsfeld y col. Encapsulating DNA within biodegradable polymeric microparticles. *Curr. Pharm. Biotech.* 3:227-235, 2002.

³ S. Ando y col. PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. *J. Pharm. Sci.* 88 (1):126-130, 1999.

⁴ E. Walter y col. Microencapsulation of DNA using poly(D,L-lactide-co-glycolide): stability issues and release characteristics. *J. Control. Rel.* 61:361-374, 1999.

⁵ M. Tobio y col. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm. Res.* 16 (5):682-688, 1999.

poloxaminas como vectores de ADN,^{6,7} por lo que su incorporación en nanoestructuras de PLGA también podría aumentar la eficiencia de transfección de las mismas.

La técnica de nanoencapsulación utilizada en el presente trabajo para la elaboración de estos nanosistemas se basa en un proceso de emulsificación-difusión del disolvente, recientemente desarrollado en nuestro laboratorio.⁸ Esta técnica ha sido modificada convenientemente a fin de permitir el desarrollo de estos nuevos sistemas nanoparticulares constituidos por mezclas de ácido poliláctico-co-glicólico (PLGA) y derivados de polioxietileno (POE), evitando a la vez el uso de las altas energías (ultrasonidos o homogenización) que puedan dañar la macromolécula a encapsular.

1. Desarrollo y caracterización de nanoestructuras elaboradas a partir de mezclas de PLGA y derivados de polioxietileno

1.1. Preparación de nanopartículas PLGA:poloxamero y PLGA:poloxamina por el método de emulsificación-difusión

Como se ha descrito en la sección anterior, la técnica de preparación de las nanopartículas ha sido adaptada para la encapsulación de macromoléculas en condiciones especialmente suaves, utilizando simplemente agitación por medio de vortex en el proceso de emulsificación.

⁶ P. Lemieux y col. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Therapy* 7:986-991, 2000.

⁷ A. Prokop y col. Maximizing the in vivo efficiency of gene transfer by means of nonviral polymeric gene delivery vehicles. *J.Pharm.Sci.* 91 (1):67-76, 2002.

⁸ C. Perez y col. Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. *J.Control.Rel.* 75:211-224, 2001.

Esta técnica nos permitió obtener nanoestructuras de diversas composiciones de PLGA:poloxámero (Pluronic[®] F68 y Pluronic[®] L121) y PLGA:poloxamina (Tetronic[®] 901, 904 y 908) que presentan tamaños comprendidos 150-350 nm, dependiendo de la relación de los polímeros y del tipo de derivado POE utilizados en la preparación de las partículas (Tabla 1).

Nombre comercial	Composición	Mw (Da)	HLB
Pluronic [®] F68	2x75 EO + 30 PO	8500	29
Pluronic [®] L121	2x5 EO + 68 PO	4400	1
Tetronic [®] 901	4x3 EO + 4x17 PO	4700	2.5
Tetronic [®] 904	4x15 EO + 4x17 PO	6700	14.5
Tetronic [®] 908	4x119 EO + 4x17 PO	25000	30

Tabla 1. Características de los derivados de polioxietileno investigados
EO= unidades de oxietileno, PO=unidades de oxipropileno

El potencial zeta fue negativo en todos los casos, variando entre - 50 mV y -20 mV según la cantidad y las características del poloxámero o poloxamina incorporada (valor de HLB, peso molecular etc.). Al comparar estos valores con el potencial zeta de las nanopartículas elaboradas únicamente a base de PLGA (-60 mV), se puede observar que la presencia de los derivados de PEO, al igual que en el caso del recubrimiento de nanopartículas con POEs hidrofílicos, se traduce en una reducción de la carga eléctrica negativa, debido al desplazamiento del plano de deslizamiento de cargas en la superficie de partículas.^{9,10}

⁹ P. Quellec y col. Protein encapsulation within poly(ethylene glycol)-coated nanospheres I. Physicochemical characterization. *J.Biomed.Mater.Res* 42:45-54, 1998.

¹⁰ A. E. Hawley y col. Preparation of biodegradable, surface engineered PLGA nanospheres with enhanced lymphatic drainage and lymph node uptake. *Pharm.Res.* 14 (5):657-661, 1997.

1.2. Análisis de la morfología y composición de las nanoestructuras de PLGA:poloxámero y PLGA:poloxamina

El análisis morfológico de las nanoestructuras muestra que todas ellas tienen forma esférica, independientemente de su composición matricial. No obstante, en las imágenes tomadas por microscopía de transmisión electrónica se pueden observar unas irregularidades que podrían ser atribuidas a una estructura compuesta por distintos polímeros (Figura 1).

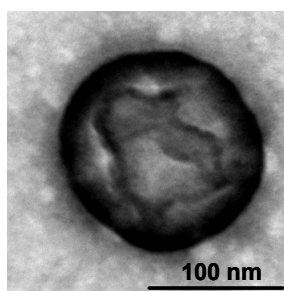


Figura 1. Microfotografía TEM de nanopartículas PLGA:Pluronic F68

El análisis de la composición matricial de las distintas nanoestructuras se realizó empleando las técnicas de resonancia magnética nuclear (RMN) y calorimetría diferencial de barrido (DSC). Los resultados de estos estudios confirmaron la incorporación eficiente de los derivados POE en las partículas en forma de mezclas íntimas con el PLGA. Asimismo, mediante la técnica de ^1H -RMN se pudo observar que el aumento de la cantidad inicial de los POE permite su incorporación en cantidades crecientes, posibilitando así la formación de vehículos de distintas relaciones de PLGA:poloxámero o PLGA:poloxamina. Los resultados obtenidos sugieren que la formación de estas mezclas íntimas poliméricas y la proporción en que ambos componentes se incorporan al nanosistema está determinada por la compatibilidad de los mismos; la cual, a su vez, guarda relación con el peso

molecular y la hidrofilia/lipofilia del derivado de POE.^{11,12} Los resultados de los estudios de ¹H-RMN cuantitativo muestran que, como tendencia general, los poloxameros y poloxaminas con carácter más hidrofóbico se mezclan de forma más eficiente con el también hidrofóbico PLGA y, por tanto, su incorporación en la matriz de las partículas es mayor (Figura 2). No obstante, el peso molecular de los POE también es un factor importante, ya que la formación de la mezcla íntima entre ambos se ve favorecida en el caso de poloxameros o poloxaminas con cadenas de polioxietileno y polioxipropileno más largas.¹³

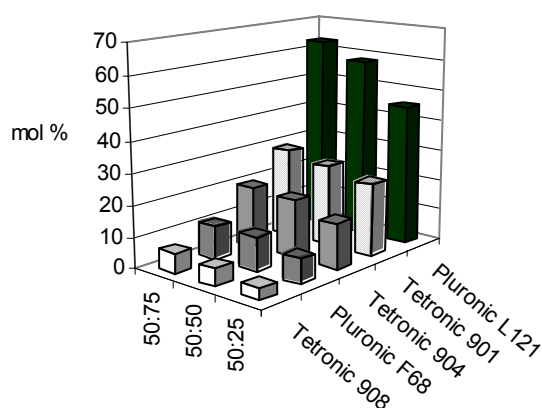


Figura 2. Eficiencia de incorporación en % de moles de distintos poloxameros y poloxaminas en nanopartículas de PLGA en función de la relación de polímeros (eje x) y del tipo de derivado de POE (eje z)

¹¹ T. G. Park y col. Controlled protein release from polyethyleneimine-coated poly(L-lactic acid)/Pluronic blend matrices. *Pharm.Res.* 9 (1):37-39, 1992.

¹² M. Yeh y col. Improving protein delivery from microparticles using blends of poly(DL lactide co-glycolide) and poly(ethylene oxide)-poly(propylene oxide) copolymers. *Pharm.Res.* 13 (11):1693-1698, 1996.

¹³ T. G. Park y col Poly(lactic acid)/Pluronic blends: characterization of phase separation behavior, degradation, and morphology and use as protein releasing matrices. *Macromolecules* 25:116-122, 1992.

Por otro lado, mediante la técnica de análisis térmico diferencial se pudo observar el desplazamiento en la temperatura de transición vítrea del PLGA debido a la presencia de los derivados de polioxietileno. Este desplazamiento se ha visto acompañado de un ensanchamiento de los picos, lo que en conjunto sugiere la formación de matrices constituidas por mezclas íntimas de PLGA:POE, corroborando los resultados obtenidos por ^1H -RMN.

1.3. Capacidad de nanopartículas PLGA:POE para la encapsulación y liberación controlada de moléculas bioactivas

En esta primera etapa de desarrollo y optimización, previa a la aplicación de estas nuevas nanoestructuras como transportadores de ADN, hemos utilizado la insulina como molécula bioactiva modelo para confirmar la capacidad de dichos sistemas para la encapsulación y liberación controlada de macromoléculas. En estos estudios, basándonos en los resultados de la caracterización físico-química, hemos fijado la relación teórica de PLGA:POE en 50:50 y hemos descartado la formulación PLGA:Tetronic[®] 901, cuya reproducibilidad fue inferior a la conseguida para las otras nanoestructuras.

Los resultados de estos estudios muestran que la encapsulación de la insulina se ve favorecida en presencia de los derivados POE más hidrofílicos como el Pluronic[®] F68 y el Tetronic[®] 908, logrando eficacias de encapsulación del orden de 40-45%, claramente superiores a las conseguidas en ausencia de estos derivados. Este resultado podría explicarse, de manera similar a lo descrito en la literatura en el caso de proteínas encapsuladas en micropartículas poliméricas, como consecuencia de una interacción favorable entre el polioxietileno y la proteína.^{14,15}

¹⁴ M. Tobio y col. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm.Res.* 16 (5):682-688, 1999.

¹⁵ M. Yeh y col. Improving the delivery capacity of microparticle systems using blends of poly(DL-lactide co glycolide) and poly(ethylene glycol). *J.Control.Rel.* 37:1-9, 1995.

Por el contrario, en el caso de las nanopartículas que contienen polioxietilenos más hidrofóbicos, no se pudieron concluir efectos positivos asociados a su presencia. Además en el caso del sistema que contiene el polioxietileno más hidrofóbico (Pluronic® L121) se observaron problemas de estabilidad a corto plazo que dieron lugar a la agregación de estas partículas.

Teniendo en cuenta estos resultados, para los estudios de liberación “in vitro” se seleccionó la formulación PLGA:Tetronic® 908. Como aparece reflejado en la Figura 3., el perfil de liberación de la insulina a partir estas nanoestructuras carece de la liberación inicial rápida “burst” (característica típica y no deseada de las micro- y nanopartículas de poliésteres) y se prolonga de manera lineal durante el tiempo de realización del estudio (2 semanas) (Figura 3.)

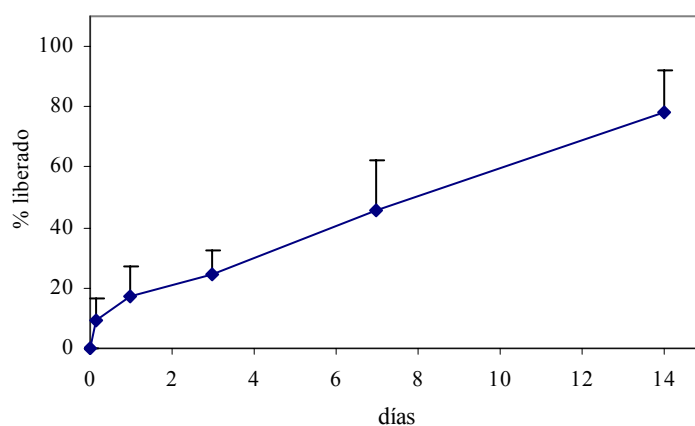


Figura 3. Perfil de liberación de insulina a partir de nanopartículas de PLGA:Tetronic® 908

Este comportamiento altamente positivo se puede atribuir a la óptima encapsulación del péptido propiciada por su interacción favorable con el derivado POE. Asimismo, es previsible que la presencia del derivado POE afecte al proceso de degradación y erosión de la matriz polimérica (gobernada por la hidrólisis del

PLGA), como ha sido anteriormente sugerido en el caso de películas y micropartículas constituidos por mezclas íntimas entre PLGA y poloxameros.^{16,17}

2. Asociación de ADN a nanopartículas elaboradas a base de mezclas PLGA:poloxámero y PLGA:poloxamina y evaluación de sus propiedades in vitro

Los resultados obtenidos en la primera etapa de la presente memoria experimental han puesto de manifiesto que mediante una técnica de emulsificación-difusión modificada es posible incorporar derivados de polioxietileno en matrices poliméricas de PLGA, lo cual permite la preparación de nanopartículas de composiciones novedosas que tienen el potencial de encapsular macromoléculas y liberarlas de manera controlada.

Esta segunda etapa ha ido dirigida a investigar las posibilidades que ofrecen dichas nanopartículas para la encapsulación de ADN, utilizando como plásmido modelo el que codifica la proteína verde fluorescente (pEGFP).

2.1. Asociación de ADN a las nanopartículas PLGA:poloxámero y PLGA:poloxamina

Todas las composiciones de PLGA:poloxámero (Pluronic® F68 y L121) y de PLGA:poloxamina (Tetronic® 904 y 908) mostraron una buena capacidad para encapsular el plásmido pEGFP. Más concretamente, las eficacias de encapsulación

¹⁶ A. Sanchez y col. Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. *Eur.J.Pharm. Sci.* 18:221-229, 2003.

¹⁷ M. Yeh y col. Improving protein delivery from microparticles using blends of poly(DL lactide co-glycolide) and poly(ethylene oxide)-poly(propylene oxide) copolymers. *Pharm.Res.* 13 (11):1693-1698, 1996.

observadas para una carga teórica del 0,4% (con respecto a la cantidad de PLGA en las partículas) están comprendidas entre 30 - 45% (Tabla 2). Estos valores son comparables a los obtenidos normalmente en el caso de micro- y nanopartículas elaboradas a partir de PLGA.^{18,19}

En un experimento posterior se procedió a explorar las posibilidades de aumentar la cantidad de ADN asociada a estas nanoestructuras. Para ello, se seleccionaron las formulaciones PLGA:poloxámero (Pluronic[®] F68) y PLGA:poloxamina (Tetronic[®] 904) y la carga teórica se aumentó de 0,4% a 1%. Curiosamente, ni la eficacia de encapsulación ni el tamaño de partícula sufrieron modificaciones como consecuencia del aumento de carga teórica de ADN, lo que se tradujo en un aumento significativo de carga real de ADN en las nanopartículas. Concretamente, la incorporación de ADN a las nanopartículas fue de 3.4 µg plásmido por mg de nanopartículas de PLGA:Pluronic[®] F68 y 4.8 µg por mg de la formulación PLGA:Tetronic[®] 904.

Tipo de poloxámero/poloxamina	Tamaño (nm)	P.I.	Potencial ζ (mV)	E. E. (%)
PLGA solo	234 ± 13	0.187	-72.7 ± 3.7	32.9 ± 13.2
Pluronic [®] F68	183 ± 6	0.114	-50.8 ± 3.6	35.2 ± 8.9
Pluronic [®] L121	217 ± 5	0.154	-23.5 ± 1.4	31.3 ± 3.8
Tetronic [®] 908	269 ± 11	0.437	-35.0 ± 0.9	32.0 ± 3.7
Tetronic [®] 904	161 ± 7	0.154	-54.1 ± 2.0	44.1 ± 4.3

Tabla 2. Tamaño, polidispersión (P.I.), potencial zeta y eficacia de encapsulación de ADN plasmídico (E.E.) (carga teórica 0,4%) en las diferentes nanopartículas

¹⁸ C. S. Lengsfeld y col. Encapsulating DNA within biodegradable polymeric microparticles. *Curr. Pharm. Biotech.* 3:227-235, 2002.

¹⁹ Y. Hsu y col. Comparison of process parameters for microencapsulation of plasmid DNA in poly(D,L-lactic-co-glycolic acid) microspheres. *J. Drug Targ.* 7 (4):313-323, 1999.

Una excepción a este comportamiento se encontró para la formulación de PLGA:Tetronic[®] 908, la cual experimentó un aumento de tamaño tras la asociación del ADN. Este aumento de tamaño se podría atribuir hipotéticamente al elevado peso molecular de esta poloxamina, cuyas largas cadenas podrían interaccionar con el plásmido de manera distinta que los otros derivados de polioxietileno.

Asimismo, es necesario mencionar que en algunos casos también se observó un incremento en la carga superficial negativa de las partículas, indicando posibles cambios en organización de los componentes en la nanoestructura por la presencia del ADN.²⁰

2.2. Liberación “in vitro” del ADN encapsulado

Al igual que se observó para la insulina, el ADN encapsulado se liberó de una forma continua y controlada a partir de todas las formulaciones ensayadas. Como se puede constatar en la Figura 4, los perfiles son casi lineales, y no muestran el efecto burst característico de otras formulaciones de nanopartículas convencionales. De nuevo, se observa que la naturaleza del componente POE es un factor determinante del comportamiento de las diferentes nanoestructuras. Asimismo, de estos resultados se desprende que las matrices más hidrofílicas (PLGA:Pluronic[®] F68 y PLGA:Tetronic[®] 908) liberan el ADN más rápido, durante una semana, mientras que las nanopartículas que contienen derivados de POE más hidrofóbicos (PLGA:Pluronic[®] L121 y PLGA:Tetronic[®] 904) dan lugar a una liberación más sostenida. Esta diferencia en la capacidad de control de la liberación se considera relacionada con la diferente hidratación de las matrices poliméricas

²⁰ C. Perez y col. Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. *J.Control.Rel.* 75:211-224, 2001.

basadas en mezclas PLGA:POE, y depende de la hidrofilia/lipofilia del derivado del polioxietileno incorporado.^{21,22}

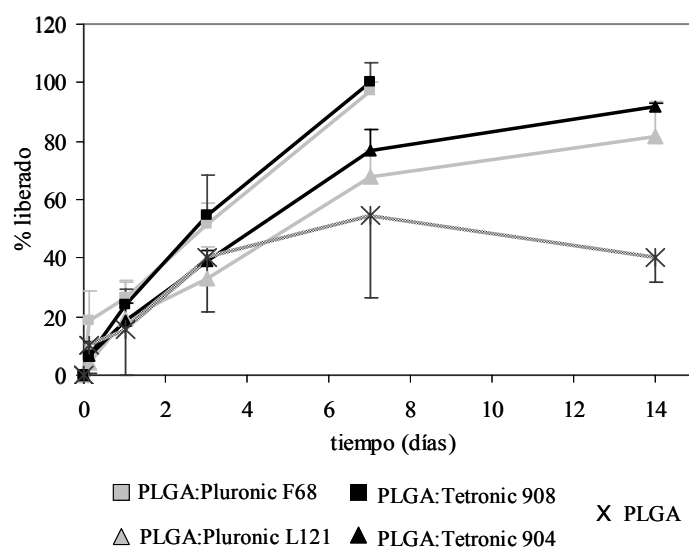


Figura 4. Perfiles de liberación de ADN a partir de las distintas nanopartículas

Por otro lado, en la Figura 4 se puede apreciar también que en el caso de las nanoestructuras PLGA:POE la liberación es más reproducible que en el caso de la formulación convencional elaborada a base de PLGA, recuperándose a partir de las primeras prácticamente el 100% del ADN encapsulado. Este último suceso es poco habitual en el caso de macromoléculas encapsuladas en micro- y nanopartículas de PLGA, en las que la liberación generalmente se ve afectada por interacciones no deseadas entre las moléculas encapsuladas y los productos de degradación

²¹ T. G. Park y col. Poly(lactic acid)/Pluronic blends: characterization of phase separation behavior, degradation, and morphology and use as protein releasing matrices. *Macromolecules* 25:116-122, 1992.

²² T. G. Park y col. Controlled protein release from polyethyleneimine-coated poly(L-lactic acid)/Pluronic blend matrices. *Pharm.Res.* 9 (1):37-39, 1992.

acumulados con el tiempo en el interior de las matrices poliméricas.^{23,24} Por tanto, en su conjunto, estos resultados permiten concluir el efecto positivo de las nuevas composiciones matriciales en la liberación del ADN encapsulado.

2.3. Estabilidad del ADN encapsulado

Teniendo cuenta que el objetivo principal de esta parte experimental ha sido el de diseñar nanopartículas capaces de encapsular y liberar el ADN en su forma biológicamente activa, un aspecto importante de esta etapa de trabajo fue la confirmación de la estabilidad del ADN encapsulado y liberado, aplicando diversos métodos analíticos, como son la cuantificación mediante el reactivo PicoGreen® (específico de ADN de doble cadena), el análisis conformacional en geles de agarosa y, finalmente, la determinación de la actividad biológica del ADN extraído y liberado de las partículas en cultivos celulares. Los resultados del análisis electroforético presentados en la Figura 5 muestran la ausencia de conversión a la forma menos activa biológicamente (lineal), y solamente se observan cambios de la forma superhelicoidal a circular abierta a tiempos largos de liberación.

Tal y como se describe en la literatura, este cambio no influye significativamente en la capacidad de transfección del ADN.²⁵ De hecho, los estudios en cultivos celulares indicaron que la actividad biológica del ADN se conserva perfectamente durante su encapsulación en las nanopartículas PLGA:POE y durante su posterior liberación.

²³ M. Tobio y col. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm.Res.* 16 (5):682-688, 1999.

²⁴ A. Sanchez y col. Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. *European Journal of Pharmaceutical Sciences* 18:221-229, 2003.

²⁵ C. R. Middaugh y col. Analysis of plasmid DNA from a pharmaceutical perspective. *J.Pharm.Sci.* 87 (2):130-146, 1998.

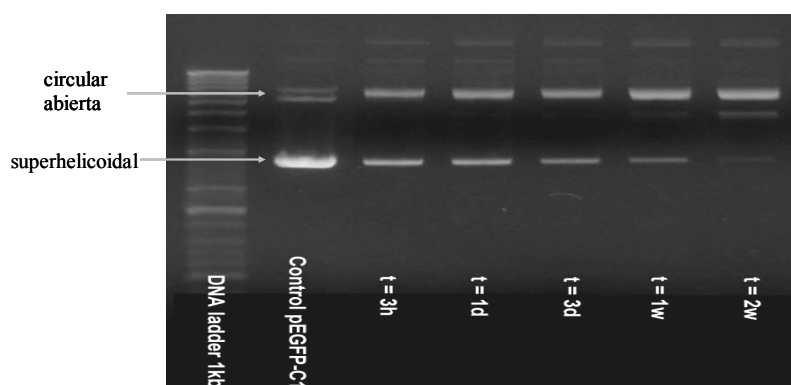


Figura 5. Análisis de la estabilidad estructural del plásmido pEGFP liberado a distintos tiempos a partir de nanopartículas elaboradas a base de una mezcla de PLGA:Pluronic® L121

En comparación con los resultados obtenidos para micro- y nanopartículas convencionales, estos estudios indican que la presencia de los poloxameros o poloxaminas en las nanoestructuras mejora significativamente la estabilidad del plásmido no solo durante su encapsulación, sino también en el transcurso de su liberación.^{26,27}

2.4. Toxicidad de las nanopartículas en cultivos celulares

Como es sabido, para la formación de las nanopartículas desarrolladas en este trabajo se han utilizado materiales conocidos por su adecuada biocompatibilidad y baja toxicidad.^{28,29}

²⁶ E. Walter y col. Hydrophilic poly(D,L-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. *J.Control.Rel.* 76:149-168, 2001.

²⁷ Y. S. Jong y col. Controlled release of plasmid DNA. *J.Control.Rel.* 47:123-134, 1997.

²⁸ D. E. Perrin y J. P. English. Polyglycolide and polylactide. en: *Handbook of biodegradable polymers*, ed. A. J. Domb y col, Harwood Acad. Publ., 1997, 3-27.

Por tanto, se podía pronosticar que las formulaciones de mezclas íntimas de PLGA con poloxameros y poloxaminas no deberían dar lugar a efectos tóxicos importantes. En efecto, como era de esperar, los ensayos de toxicidad mostraron que las nanopartículas no desencadenan signos de toxicidad en cultivos celulares (línea MCF-7) hasta concentraciones muy elevadas (5 mg/ml), y como consecuencia de una exposición prolongada (24h). Curiosamente, en algunos casos se observó un aumento en la viabilidad celular, probablemente debido a la combinación del leve estrés celular causado por las condiciones extremas de este experimento y la capacidad de los derivados de POE de interactuar con elementos de la respiración celular, parámetro medido por el método colorimétrico utilizado para determinar la viabilidad celular.^{30,31}

3. Estudio del potencial de nanopartículas de PLGA:poloxámero y PLGA:poloxamina como vectores sintéticos de ADN: evaluación de su interacción con células en cultivo y de su comportamiento in vivo tras su administración por vía nasal

3.1. Evaluación de la interacción de nanopartículas PLGA:poloxámero y PLGA:poloxamina en cultivos celulares

Para conseguir la visualización del proceso de la interacción de las nanopartículas con las células en cultivos, se ha empleado la técnica de microscopía confocal.

²⁹ S. M. Moghimi y A. C. Hunter. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *Tibtech* 18:412-420, 2003.

³⁰ G. P. Kirillova y col. The influence of Pluronics and their conjugates with proteins on the rate of oxygen consumption by liver mitochondria and thymus lymphocytes. *Biotechnol.Appl.Biochem.* 18:329-339, 1993.

³¹ C. J. Goodwin y col. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. *J.Immunol.Methods* 179 (1):95-103, 1995.

Para ello, ha sido necesaria la elaboración de nanopartículas marcadas con una etiqueta fluorescente. Más concretamente, se ha seleccionado la fluoresceína-isotiocianato (FITC, emisión en verde) para lograr el marcaje del ADN plasmídico previamente a su nanoencapsulación. Adicionalmente, para la localización precisa del ADN encapsulado en los cultivos celulares se consideró la utilización de un segundo marcador, el ioduro de propidio (emisión en rojo) como marcador del núcleo celular.³²

Esta combinación nos permitió observar que el ADN encapsulado penetra de forma eficiente al interior de las células. Las imágenes muestran una intensa señal fluorescente verde proveniente del FITC-ADN, que corresponde a un número importante de moléculas de ADN internalizadas en las células, con distribución intracelular homogénea (Figura 6). Por el contrario, en el caso de ADN en forma libre se puede observar que éste tiene una limitada capacidad de penetración en las células.

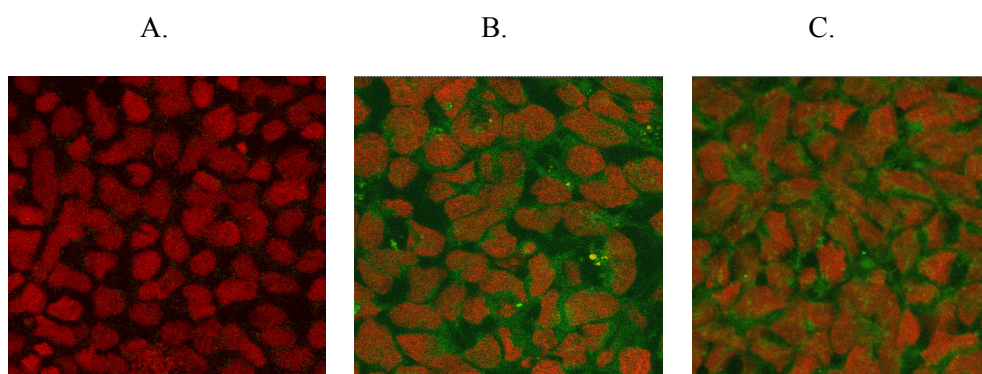


Figura 6. Imágenes de microscopía confocal de células HEK (núcleo marcado en rojo) tras su incubación con FITC-DNA (1) en forma libre (2) encapsulado en nanopartículas PLGA:poloxámero y (3) encapsulado en nanopartículas PLGA:poloxamina

³² T. Ishii y col. Facile preparation of a fluorescence-labeled plasmid. *Chem.Lett.*:386-387, 2000.

Estos resultados están en concordancia con las conclusiones de otros trabajos que indican que el uso de sistemas nanoparticulares aumenta la penetración celular de las moléculas encapsuladas mediante procesos endocíticos.^{33,34,35}

3.2. Evaluación del transporte de las nanopartículas tras su administración por vía nasal

En el planteamiento de los experimentos “in vivo”, se han tenido en cuenta los buenos resultados obtenidos en un estudio previo con un sistema nanoparticular elaborado a base de PLA-PEG, en el que se observó que la autofluorescencia del tejido era suficiente para la localización de las partículas marcadas con Rodamina 6G.³⁶ La suspensión de nanopartículas fluorescentes fue administrada a ratones por vía intranasal y al cabo de 15 minutos los ratones fueron sacrificados y la mucosa nasal extraída para su posterior observación por microscopía confocal. En la Figura 7 se presenta una serie de fotografías que corresponden a secciones transversales de la mucosa nasal. En dichas imágenes se puede identificar un número importante de puntos fluorescentes correspondientes a nanopartículas individuales. La falta de agregados en la superficie de la mucosa y la presencia de estas nanopartículas en el interior de las células, ponen en evidencia la capacidad de las mismas para atravesar superficies mucosas, y, por tanto, para transportar ADN encapsulado.

³³ J. Panyam y V. Labhasetwar. Dynamics of endocytosis and exocytosis of poly(D,L lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. *Pharm.Res.* 20 (2):212-220, 2003.

³⁴ J. Davda y V. Labhasetwar. Characterisation of nanoparticle uptake by endothelial cells. *Int.J.Pharm.* 233:51-59, 2002.

³⁵ I. Behrens y col. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. *Pharm.Res.* 19 (8):1185-1193, 2002.

³⁶ A. Vila y col. Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density. *J.Control.Rel.* 98 (2):231-244, 2004.

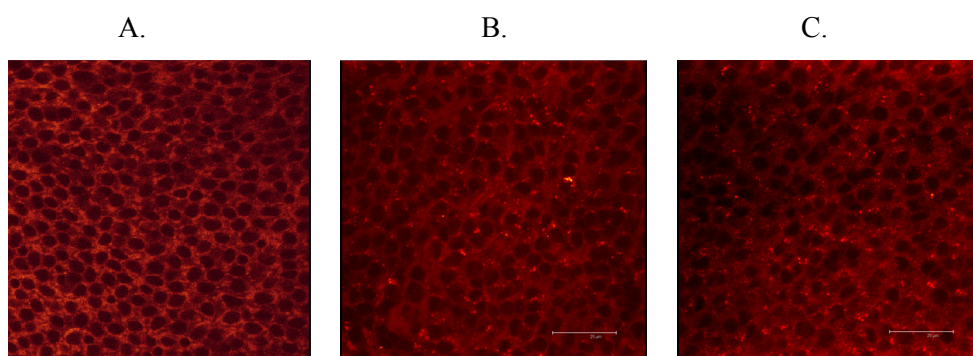


Figura 7. Imágenes de microscopía confocal de mucosa nasal (profundidad de 10 μm , corte único) (A) sin tratamiento (B) tras la administración de nanopartículas PLGA:poloxámero y (C) PLGA:poloxamina (escala= 25 μm)

En comparación con los resultados obtenidos para las micro y nanopartículas de PLGA, cuyo paso a través de la mucosa se vió muy limitado por su inestabilidad en este entorno biológico, se puede concluir que la incorporación de poloxámero y poloxamina mejora significativamente la capacidad de penetración de estas nanoestructuras.³⁷ Este efecto podría atribuirse a las propiedades estabilizantes del componente polioxietileno, probablemente debido a un mecanismo de protección estérica similar al conseguido con polietilenglicol en el caso de nanopartículas preparadas con el copolímero PLA-PEG (anteriormente descrito en detalle en la Revisión 1 y en la Revisión 2 de esta memoria).³⁸

³⁷ A. Vila y col. Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density. *J.Control.Rel.* 98 (2):231-244, 2004.

³⁸ M. Tobio y col. The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA nanoparticles following oral administration. *Colloid.Surf.B: Biointerf.* 18:315-323, 2000.

3.3. Evaluación de la capacidad de transfección de las nanopartículas PLGA:poloxámero y PLGA:poloxamina “in vitro”

Teniendo en cuenta los resultados satisfactorios obtenidos en la etapa 2 de la presente memoria experimental, que mostraron la eficiente asociación de ADN a las nanopartículas y la preservación de su actividad biológica tras su extracción/liberación de las mismas, en una etapa final de este trabajo consideramos importante el investigar el potencial de dos sistemas seleccionados (PLGA con el poloxámero Pluronic® F68 y PLGA con la poloxamina Tetronic® 908) de transfectar células “in vitro”. De la observación de estos resultados podría concluirse que el número de células transfectadas fue elevado, pero la intensidad de la transfección fue muy leve. De hecho, aunque visible, la tonalidad fluorescente emitida por las células fue insuficiente para permitir la cuantificación de las células transfectadas. Esta débil eficiencia de transfección en cultivos celulares podría relacionarse con el proceso de liberación sostenida del plásmido de la matriz polimérica.

Estos resultados son concordantes con la ausencia de información bibliográfica referente a la evaluación “in vitro” de la capacidad transfectiva de sistemas de liberación controlada. En efecto, en general, los resultados publicados hacen referencia a sistemas que liberan el plásmido asociado en el interior de la célula por un mecanismo de disociación que ocurre en un tiempo relativamente corto.^{39,40}

No obstante, a pesar de las dificultades para cuantificar la transfección “in vitro”, conviene resaltar que esta liberación del ADN encapsulado de forma lenta y prolongada ofrece un interesante atractivo en el campo de la vacunación genética.

³⁹ S. Gebredikan y col. Formulation and in vitro transfection efficiency of poly(D,L-lactide-co-glycolide) microspheres containing plasmid DNA for gene delivery. *AAPS PharmSciTech* 1 (4):28, 2000.

⁴⁰ T. Hao y col. Biological potency of microsphere encapsulated plasmid DNA. *J.Control.Rel.* 69:249-259, 2000.

3.4. Respuesta inmune generada tras la administración nasal de ADN encapsulado en nanopartículas de PLGA:poloxámero y PLGA:poloxamina

Este experimento, dirigido a la investigación del potencial “in vivo” de estos sistemas como vehículos de vacunas genéticas, consistió en la administración nasal del plásmido pCMV- β Gal (codificante de la proteína beta-galactosidasa) en una dosis única, en forma de solución y encapsulado en nanopartículas elaboradas a base de mezclas PLGA:poloxámero y PLGA:poloxamina. La respuesta inmune generada en ratones como consecuencia de la expresión de la proteína β Gal se analizó mediante la técnica de ELISA. Los resultados de estos ensayos pusieron de manifiesto que el plásmido encapsulado en las nanopartículas da lugar una respuesta importante que se manifiesta al cabo de 2 semanas, siendo esta respuesta significativamente superior a la conseguida para el plásmido libre.

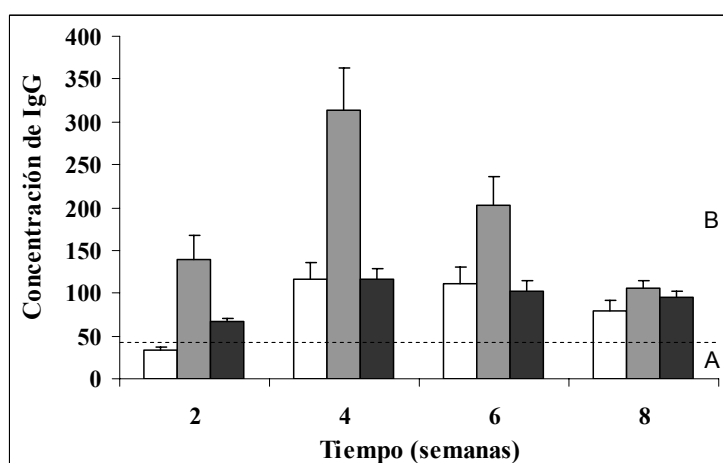


Figura 8. Niveles de IgG en suero (ng/ml) tras la administración de pCMV- β Gal en solución (□), encapsulado en nanopartículas PLGA:Pluronic® F68 (■) y en nanopartículas PLGA:Tetronic® 904 (■) Región A: nivel basal de IgG, Región B: IgG específica anti- β galactosidasa

Asimismo, en la Figura 8 puede observarse que los niveles de IgG fueron especialmente altos en el caso del sistema PLGA:poloxámero (Pluronic® F68), mientras la formulación PLGA:poloxamina (Tetronic® 904) generó respuestas similares a las del plásmido control. Finalmente, puede observarse que esta respuesta observada para la formulación de PLGA:poloxámero alcanza su máximo valor a las 4 semanas, si bien a las 6 semanas sigue siendo significativamente superior a la del control.

Las diferencias encontradas en la eficiencia de los dos tipos de nanopartículas no pueden atribuirse a su capacidad de transporte a través de la mucosa nasal, ya que su comportamiento fue muy similar. Por tanto, la explicación debe encontrarse en la naturaleza del componente POE y su interacción con el entorno biológico. En efecto, es posible que el carácter hidrofóbico del sistema PLGA:poloxamina (Tetronic® 904) de lugar a una degradación más lenta y, por tanto, a una liberación del ADN más sostenida en este entorno biológico. De hecho, la influencia del tipo de derivado de POE en la liberación pudo ser constatada “in vitro”, como se describe en la sección 2.2 de la presente memoria. Estas diferencias en el perfil de liberación pueden influir, como ya ha sido descrito por otros autores, en la inmunogenicidad de los antígenos encapsulados.⁴¹

Conviene resaltar, que la respuesta inmune observada en el caso de las partículas PLGA:poloxámero coincide con los resultados de otro sistema nanoparticular basado en el copolímero PLA-PEG, para el cual se lograron intensidades y perfiles de respuesta de inmune similares tras la administración nasal de pCMV-βGal, con

⁴¹ H. Tamber y col. Formulation aspects of biodegradable polymeric microspheres for antigen delivery. *Adv. Drug Deliv. Rev.* 57:357-376, 2005.

un nivel máximo de IgG después de un mes.⁴² Este comportamiento afín probablemente podría atribuirse a la similitud estructural del poloxámero y el polietilenglicol y, por otro lado, a la liberación del plásmido de manera rápida.

En resumen, los resultados de este estudio de inmunización y, en particular, aquellos obtenidos para el sistema PLGA:poloxámero, ponen en manifiesto el potencial de estas nanoestructuras basadas en mezclas de PLGA con derivados de POE como transportadores de vacunas genéticas a través de superficies mucosas. Asimismo, indican que el tipo de poloxámero/poloxamina incorporado en la estructura tiene una importancia crítica en la eficacia del sistema con respecto a su capacidad para generar respuestas inmunes.

⁴² A. Vila y col. PEG-PLA nanoparticles as carriers for nasal vaccine delivery. *J.Aerosol.Med.* 17 (2):174-185, 2004.

Conclusiones

Conclusiones

El trabajo experimental recogido en la presente memoria se ha dirigido al diseño de una serie de sistemas de nanoparticulares con composiciones novedosas destinadas a la encapsulación, liberación y vehiculización de ADN plasmídico en su forma biológicamente activa. Los resultados obtenidos en este trabajo permiten concluir lo siguiente:

1. Se ha desarrollado una nueva técnica de preparación de nanopartículas con ADN basada en la adaptación de una técnica de emulsificación-difusión. Esta técnica permite la encapsulación de ADN plasmídico en condiciones excepcionalmente suaves y da lugar a la consecución de nanoestructuras de tamaños comprendidos entre 150-350 nm, con carga superficial negativa y forma esférica.
2. El análisis morfológico y estructural de los nanosistemas desarrollados ha permitido comprobar la eficiente incorporación de polioxietilenos en la matriz polimérica de dichos vehículos. En otras palabras, estas nanoestructuras están compuestas por mezclas íntimas de PLGA y derivados de polioxietileno.
3. Estos nuevos sistemas muestran la capacidad de encapsular ADN plasmídico de manera eficiente, y de liberarlo de forma controlada. Asimismo, se ha observado que el ADN plasmídico extraído y liberado de las nanopartículas conserva completamente su integridad y su actividad biológica.
4. Las nanopartículas compuestas por PLGA:poloxamer y PLGA:poloxamina muestran una excelente capacidad de atravesar barreras biológicas tales como la membrana celular o las superficies mucosas.

5. El estudio de expresión genética “in vivo” mostró que la administración de ADN encapsulado en estas nanoestructuras PLGA:poloxamer y PLGA:poloxamina por vía nasal es capaz de originar respuestas inmunes sistémicas, específicas contra la proteína codificada.

6. Tanto las características físico-químicas como el comportamiento “in vitro” y el potencial de estos sistemas como vehículos de fármacos “in vivo” están fuertemente condicionados por la naturaleza del componente polioxietileno presente en la matriz polimérica, lo cual proporciona una gran versatilidad a esta nueva clase de nanoestructuras.

7. Además, más allá de cumplir los objetivos principales de este trabajo, los resultados obtenidos también han puesto en manifiesto el potencial de estas nanoestructuras como vehículos de otras moléculas diferentes del ADN plasmídico, tales como proteínas o fármacos lipofílicos de bajo peso molecular.

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