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Obtención y caracterización de nanopartículas poliméricas de ácido poli(láctico-co-glicólico)/ácido poli(gama-glutámico) conjugadas con ácido fólico para el transporte y liberación de doxorrubicina

T E S I S

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Abreviaturas

AF: ácido fólico

ClTx: clorotoxina

cRGDfK: ciclo(Arg-Gly-Asp-D-Phe-Lys)

DOX: doxorrubicina

DPPE: 1,2-dipalmitol-sn-glicero-3-fosfoetanolamina

EDA: etilendiamina

EDC: 1-etil-3-(3-dimetilaminopropil) carbodiimida

EE: eficiencia de encapsulación. ERO: especies reactivas de oxígeno

FR: receptor de folato

HSPC: Fosfatidilcolina hidrogenada de soya

NHSS: N-hidroxisulfosuccimida

NPs: nanopartículas

PEG: polietilenglicol

PGA: ácido poliglicólico

PLA: ácido poliláctico

PLGA: ácido poli(láctico-co-glicólico)

TPGS: D-α-tocoferol polietilenglicol succinato

γ-PGA: ácido poli(gama-glutámico)

1. RESUMEN

Las nanopartículas poliméricas presentan una alternativa a la terapia convencional contra el cáncer, pues permiten aumentar la selectividad del tratamiento, minimizando los efectos secundarios no deseados en tejidos sanos.

El PLGA y el γ-PGA, son polímeros utilizados en la obtención de nanopartículas para el transporte de fármacos. Los conjugados de ácido fólico (AF) pueden mejorar la entrega de fármacos a células tumorales por su interacción con receptores de folatos sobreexpresados en estos tejidos. Por ello, el objetivo de este estudio fue obtener y caracterizar un sistema de nanopartículas a base de PLGA y γ-PGA, funcionalizándolas con AF para el transporte y liberación de doxorrubicina (DOX), un agente antineoplásico.

En este trabajo, se obtuvieron nanopartículas de PLGA mediante el método de emulsificación-evaporación del disolvente. La modificación superficial con γ-PGA presentó una señal a los 208 nm en UV-vis, mientras que la conjugación con AF mostró dos bandas a 280 nm y 348 nm. Cambios en las señales de FT-IR entre 1250 cm^{-1} y 1780 cm^{-1} indicaron las correctas modificaciones en las nanopartículas. Las imágenes de TEM/SEM mostraron estructuras cuasi-esféricas, con la presencia de cúmulos de material polimérico. Los tamaños de partícula (DLS) permanecieron menores a 600 nm y se observó cambio del potencial zeta de - 10.28 ± 1.37 mV a 14.2 ± 2.69 mV, después de las modificaciones realizadas.

La eficiencia de encapsulado de doxorrubicina (DOX) fue de 47.97 ± 1.8 %, con una eficiencia de carga de 0.33 ± 0.012 %. El perfil de liberación de DOX-PLGA/γ-PGA-AF mostró responder a condiciones ácidas de pH (5.3), con un porcentaje de liberación de 55.42 ± 0.6 %. Finalmente, las nanopartículas DOX-PLGA/γ-PGA-AF fueron evaluadas en células HeLa, mostrando una disminución de la viabilidad celular de aproximadamente 60% a las 72 horas de exposición. La captación de las nanopartículas de PLGA/γ-PGA-AF mostró ser por endocitosis mediada por AF.

Con base a los resultados obtenidos, se concluyó que las NPs de PLGA/γ-PGA-AF son un sistema potencial de transporte y entrega direccionada de fármacos, como la DOX, a través del reconocimiento molecular de receptores de folato sobreexpresados en algunos tipos de cáncer, haciéndolo un candidato adecuado para futuras aplicaciones terapéuticas.

2. ABSTRACT

Polymeric nanoparticles have an alternative to conventional cancer therapy, since they allow to increase the selectivity of the treatment, minimizing undesirable side effects on healthy tissues.

PLGA and γ-PGA are polymers used to obtain nanoparticles in drug delivery. Folic acid (FA) conjugates have shown improved drug delivery to tumour cells by interaction with over-expressed folate receptors. Thus, the aim of this study was to obtain and characterize a PLGA/ γ-PGA-based nanoparticle system, functionalized with FA to delivery and release of doxorubicin (DOX), as a model antineoplastic agent.

In this research, PLGA nanoparticles were obtained by emulsification-solvent evaporation technique. The superficial modification with γ-PGA displayed a band at 208 nm, whilst FA conjugation showed two bands at 280 nm and 248 nm, by UV-Vis spectrocopy. Changes in signals in the range of 1250 cm^{-1} y 1780 cm^{-1} by FT-IR spectroscopy demonstrated the successful modifications made to nanoparticles. TEM/SEM micrographs exhibited quasi-spherical nanoparticles, with cumulus of polymeric material. After modifications, particle size (DLS) remained lower than 600 nm, and it was observed a change in zeta potential from - 10.28 ± 1.37 mV a 14.2 ± 2.69 mV.

DOX encapsulation efficiency was of 47.97 ± 1.8 %, with a loading efficiency of 0.33 ± 0.012 %. Release profile of DOX-PLGA/γ-PGA-FA nanoparticles responded to acidic conditions (pH 5.3), showing a release percentage of 55.42 ± 0.6 %. Finally, DOX-PLGA/γ-PGA-FA were evaluated on HeLa cells, displaying a viability decrease of 60 %, approximately, after 72 h of exposure. Cellular uptake was attributed to FA receptor-mediated endocytosis.

Based on these results, it was concluded that PLGA/γ-PGA-FA nanoparticles are a potential carrier for transport and delivery of drugs, such as DOX, through molecular recognition of folate receptors over-expressed in some types of cancer, making it a suitable candidate for further therapeutic applications.

M A R C O
T É O R I C O

3. ANTECEDENTES

3.1. Nanotecnología

La nanotecnología se basa en la manipulación de átomos y moléculas individuales para producir materiales con aplicaciones muy por debajo del nivel submicroscópico.

El término nanopartícula describe una sub-clasificación de sólidos ultrafinos con dimensiones entre 1 y 100 nm que poseen propiedades novedosas que los distinguen de los materiales macroscópicos. Las nanopartículas basadas en polímeros presentan un caso especial en su clasificación como nanopartícula pues, por limitaciones en metodologías para su obtención, pueden ser incluidas en el límite entre la nanotecnología y la química macromolecular. De acuerdo a lo anterior, los materiales poliméricos particulados con diámetros menores a 1,000 nm son considerados nanopartículas (Adams y Barbante, 2013; Arnaldi, 2014; Safari y Zarnegar, 2014).

3.2. Nanotecnología aplicada en la medicina

La nanomedicina se ha definido como la aplicación y desarrollo de la nanotecnología para diagnosticar, tratar y prevenir enfermedades a nivel celular y molecular (Mei *et al.*, 2013).

Una de las aplicaciones más importantes es el diagnóstico y tratamiento de cáncer. Según la Organización Mundial de la Salud (OMS), el cáncer está definido como un proceso de crecimiento y diseminación incontrolado de células, con elevadas tasas de replicación y metabolismo desregulado (OMS, 2014).

Esta enfermedad es una de las principales causas de morbilidad y mortalidad en todo el mundo. De acuerdo con cifras de la OMS (2014), en 2012 hubo unos 14 millones de nuevos casos y se le atribuyeron 8,2 millones de las muertes ocurridas en ese año. Además, se prevé que los casos anuales de cáncer aumentarán de 14 millones en 2012 a 22 millones en las próximas dos décadas. Los principales tipos de cáncer reportados respecto al número de defunciones a las que dieron origen, son los siguientes:

- Pulmonar (1,59 millones)
- Hepático (745 000)
- Gástrico (723 000)
- Colorrectal (694 000)
- Mamario (521 000)
- Cáncer de esófago (400 000)

Por lo tanto, el empleo de la nanotecnología para la administración de agentes terapéuticos cobra un interés relevante en la terapia contra el cáncer; siendo el objetivo una administración dirigida para el transporte y liberación de fármacos a sitios deseables, minimizando efectos secundarios no deseados en tejidos sanos (Safari y Zarnegar, 2014).

3.3. Sistemas de liberación y transporte de fármacos por nanopartículas poliméricas

Las estrategias más populares y potenciales utilizadas en la administración de fármacos incluyen micelas, dendrímeros, liposomas y nanopartículas poliméricas. Las nanopartículas (NPs) poliméricas, debido a su tamaño, pueden penetrar en los sitios tumorales y depositar su carga, de aquí el interés por su aplicación en la elaboración de sistemas de administración de fármacos anticancerosos (Ye y Squillante, 2013).

En los sistemas de NPs, el fármaco puede ser atrapado o encapsulado, adsorbido físicamente o ligado químicamente a la superficie de la nanopartícula o en la matriz de ésta. Los diferentes materiales utilizados en la preparación de NPs poliméricas presentan una cinética de liberación del fármaco diversa: la desorción del fármaco, la difusión a través de la pared de polímero de la NP, o la erosión de la matriz de la NP. Además, los fármacos pueden ser liberados todos a la vez en un mecanismo de ráfaga o de forma sostenida. Muchos de los fármacos contra el cáncer con alta citotoxicidad o muy baja biodisponibilidad pueden ser administrados por liberación sostenida a través de nanopartículas poliméricas (Chronopoulou *et al.*, 2013; Lai *et al.*, 2014).

Es importante recordar que no existe una "bala mágica" cuando se habla de la terapia contra el cáncer por lo que, para conseguir un incremento en la respuesta terapéutica, es necesario la aplicación, en combinación sinérgica, de las propiedades de los múltiples agentes terapéuticos. Además, debido a que los tumores poseen poblaciones mixtas de células donde algunas de ellas presentan metabolismos resistentes a quimioterapia, se requiere un sistema de suministro eficaz que mantenga concentraciones terapéuticas óptimas del fármaco en el tumor, con fluctuación mínima (Zhu *et al.*, 2014).

Las nanopartículas de polímeros naturales y sintéticos presentan características deseables en la administración de fármacos, tales como alta estabilidad, alta capacidad portadora, liberación controlable, reducción de la captación celular no específica, la combinación de

diversos mecanismos de daño derivados de las propiedades inherentes de la partícula y de las moléculas de vectorización y la encapsulación de diferentes fármacos para el tratamiento múltiple. Además, es posible modificar la superficie de una NP con moléculas que permitan la orientación hacia un tejido u órgano específico (mecanismo mediado por ligantes y receptores), y/o permitan incrementar la circulación sistémica al aumentar el tiempo de residencia por disminución de la depuración (Chronopoulou *et al.*, 2013; Lai *et al.*, 2014; Zhu *et al.*, 2014).

Para la obtención de NP con aplicaciones médicas, los materiales empleados deben ser biocompatibles y biodegradables. Los poliésteres a base de ácido poliláctico, ácido poliglicólico, y sus copolímeros, como el ácido poli(láctico-co-glicólico), son algunos de los biomateriales más empleados en la preparación de NP para el transporte y liberación de fármacos, además de estar aprobados por la FDA (Food and Drug Administration, USA) y la Agencia Europea de Medicamentos (Gajendiran *et al.*, 2013; Lai *et al.*, 2014; Safari y Zarnegar, 2014).

3.3.1. Sistemas de nanopartículas a base de PLGA

El ácido poli(láctico-co-glicólico) es un copolímero de naturaleza hidrofóbica, y aunque no es soluble en agua puede ser degradado por ella. La ventaja de la alta biodegradabilidad del PLGA radica en que el resultado de su hidrólisis son los ácidos láctico y glicólico, metabolitos naturales presentes en el cuerpo humano (ver figura 1).

El PLGA es ampliamente requerido en el campo de la medicina debido a su facilidad para formar nanopartículas, su bioabsorbilidad, su seguridad clínica, sus propiedades de liberación controlada eficientes, la capacidad de transportar pequeñas moléculas, péptidos, proteínas y fármacos, y sus características de degradación favorables (Gyulai *et al.*, 2013; Heo, Cho y Lim, 2014; Shen *et al.*, 2013; Ye y Squillante, 2013).

En la terapia del cáncer, la liberación del fármaco puede modularse mediante la manipulación de parámetros relacionados con la degradación del PLGA, tales como el pH. Debido a que en los tejidos tumorales existe un pH ácido (aproximadamente pH 5,3), la erosión de PLGA ocasiona que el fármaco sea liberado en el microambiente del tumor (Lai *et al.*, 2014; Shen *et al.*, 2013).

Adicionalmente, con la intención de mejorar el sistema de administración de fármacos se han desarrollado diversas estrategias en la obtención de NP de PLGA, como la modificación de su superficie. En este sentido, se han creado sistemas de NP a base de PLGA ligadas a fármacos como paclitaxel, en los que se mejora la formulación con el empleo de agentes como la caseína (Narayanan *et al.*, 2014) o el TPGS (Wang *et al*., 2013), descrito como un inhibidor de las proteínas de la bomba de eflujo de multirresistencia, que se sobreexpresa en las células cancerosas (Zhu *et al.*, 2014; Tao *et al.*, 2013). El quitosano también ha sido elegido como un agente para modificar la superficie de las NP de PLGA para la entrega de fármacos como la dexametasona (Chronopoulou *et al.*, 2013), la doxorrubicina y el verapamilo (Shen *et al.*, 2013).

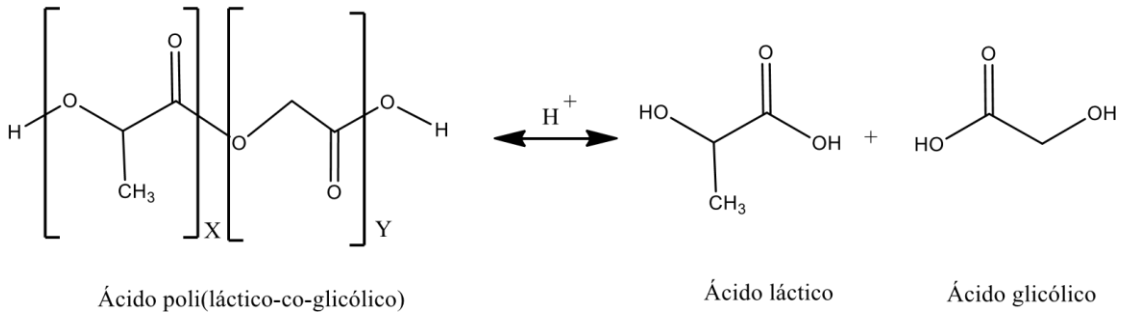

Figura 1. Estructura del PLGA y la formación de sus productos de degradación.

3.3.2. Sistemas de nanopartículas a base de γ-PGA

El ácido poli(γ-glutámico), es una homopoliamida aniónica compuesta de unidades de ácido D- y L-glutámico (ver figura 2), producido naturalmente por fermentación bacteriana, soluble en agua, no tóxico, pH-sensible, biocompatible y biodegradable (Guan *et al.*, 2014; Hellmers *et al.*, 2013; Xu *et al.*, 2014).

A la fecha, han sido desarrollados varios sistemas de NP compuestos de γ-PGA, entre ellos el sistema conjugado de γ-PGA y L-ésteretílico-fenilalanina para la entrega de antígenos y adyuvantes de vacunas que pueden generar respuestas inmunes y/o ser utilizadas en la inmunoterapia contra el cáncer (Shima *et al.*, 2013; Toita *et al.*, 2013).

Se han preparado también sistemas de NP de γ-PGA y quitosano para la liberación controlada de proteínas alcalinas (lisozimas) con aplicaciones antimicrobianas (Liu *et al.*, 2013), la

administración oral de insulina (Sonaje *et al.*, 2010) y como portadores de fármacos como la doxorrubicina (Hellmers *et al.*, 2013).

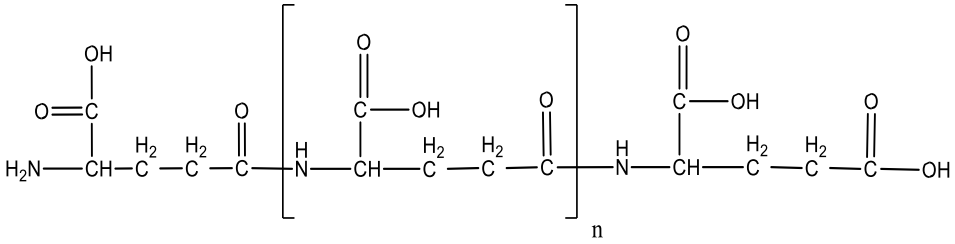

Figura 2. Estructura del ácido poli(γ-glutámico) (γ-PGA).

Así mismo, el γ-PGA ha sido empleado como acarreador de fármacos tóxicos con el fin de reducir efectos adversos. En 2006, Ye *et al.* lo utilizaron junto con cisplatino; Tomiya *et al.* (2013) lo unieron a primaquina; y más recientemente el γ-PGA se utilizó como funcionalizador de nanopartículas de oro para el desarrollo de una sonda colorimétrica basada en su propiedad pH-sensible (Guan *et al.*, 2014).

3.3.3. Sistema conjugado de nanopartículas de PLGA/γ-PGA.

A pesar de las diferentes aplicaciones que tienen por separado los sistemas de nanopartículas de PLGA y de γ-PGA, el sistema conjugado PLGA/γ-PGA ha sido poco estudiado. Kuo y Yu (2011) describen la obtención de un sistema PLGA/γ-PGA como acarreador de saquinavir a través de las células endoteliales de la microvasculatura cerebral.

El PLGA es un copolímero que carece de cadenas reactivas dificultando su interacción con sistemas biológicamente activos (Yang *et al.*, 2010) y al añadir un polímero con características hidrofílicas como el γ-PGA se contribuye a mejorar su hidrofilicidad.

3.3.4. Sistemas de transporte de Doxorrubicina

La doxorrubicina es un antibiótico antraciclina utilizado como un agente quimioterapéutico antineoplásico para el tratamiento de cáncer. Su mecanismo de acción consiste en inhibir la topoisomerasa II, interfiriendo con el complejo topoisomerasa II-ADN, produciendo rupturas de la doble hebra, o la intercalación directa con el ADN (ver figura 3); por lo que se inhiben el proceso de replicación y la transcripción de ARNm y finalmente lleva a la célula a

apoptosis. También se cree que la DOX produce ERO creando un efecto citotóxico en las células (Agudelo *et al.*, 2014; Lanz-Landázuri *et al.*, 2014).

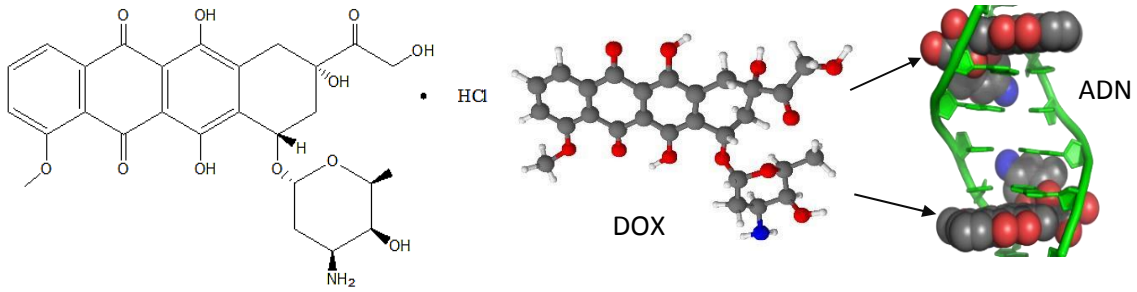

Figura 3. Estructura de la doxorrubicina (DOX) y su interacción con el ADN como mecanismo de acción. (Frederick *et al.*, 1990)

Varios sistemas de nanopartículas se han utilizado en la terapia contra el cáncer para la entrega y liberación de doxorrubicina (ver tabla 1).

3.4. Mecanismos de captación de nanopartículas

Las nanopartículas pueden penetrar fácilmente las membranas celulares debido a su tamaño nanométrico, teniendo una farmacocinética dependiente de las vías de administración y composición química.

3.4.1. Captación pasiva y activa

La captación pasiva está dada por un proceso de difusión caracterizado por la ley de Fick, donde existe un gradiente de concentración que origina un flujo idealmente irreversible de NP, con el fin de devolver el sistema a su estado de equilibrio. Se sabe que las nanopartículas se acumulan pasivamente en el tejido tumoral como consecuencia del efecto de aumento en la permeabilidad y retención (EPR por sus siglas en inglés "Enhanced Permeation Retention"), favorecido para aquellas de un tamaño entre 10 a 500 nm; este efecto se traduce en diferencias de permeabilidad y retención de moléculas en la vasculatura de un tumor en comparación con la del tejido no maligno, lo cual es causado por varias anomalías presentes en el tejido tumoral como su alta densidad vascular, el aumento de la permeabilidad de sus vasos, la defectuosa arquitectura de su vasculatura y el drenaje linfático defectuoso (Krais *et al.*, 2014; Burger *et al.*, 2014; Hellmers *et al.*, 2013).

El mecanismo de captación activa está dado por la interacción de ligandos (moléculas pequeñas, antígenos, péptidos, proteínas, etc.) con receptores presentes en la membrana plasmática de las células. En las células tumorales se ha demostrado que existe una sobreexpresión de determinados receptores, por lo que la incorporación de compuestos como el ácido fólico y/o γ-PGA a la superficie de las NP, contribuirá a mejorar la internalización de éstas en las células cuyos receptores específicos se encuentren sobreexpresados (Krais *et al.*, 2014; Liao *et al.*, 2012; Peng *et al.*, 2009).

Es importante señalar que el diseño de sistemas de NP debe estar basado en el principio de multimericidad, con el fin de producir efectos multivalentes, logrando fuertes interacciones ligando-receptor. Múltiples ligandos pueden enlazar múltiples sitios de reconocimiento, lo que maximiza los eventos de interacción de la superficie de la membrana celular y por lo tanto el aumento de la concentración de nanopartículas en un sitio de destino.

Tabla 1. Ejemplos de sistemas de NP utilizados para la entrega y liberación de doxorrubicina.

Nanopartícula	**Material**	**Descripción**	**% EE**	**Referencia**
Metálica	Au	Funcionalizadas con péptidos de penetración celular α-helicoidales.	----	Park, Tsutsumi y Mihara, 2014
		Recubiertas con dextrano.	----	Jang *et al.*, 2013
Ferromagnéticas	Fe_3O_4	Recubiertas con quitosano.	42.4	Sadighian *et al.*, 2014
		Recubiertas con quitosano, atrapadas en PLGA y funcionalizadas con cRGDfK	74.8	Shen *et al.*, 2013
		Autoensambladas con poliamidoaminas.	87.6	Chen *et al.*, 2014
Poliméricas	Quitosano	Funcionalizadas con ácido hialurónico.	44.2	Deng *et al.*, 2014
		Conjugadas con γ-PGA.	70	Hellmers *et al.*, 2013
	Poliuretanos	Sistema pH y termo-sensible.	~80	Wang *et al.*, 2013
	Gelatina	Conjugada con DPPE y ácido poliláctico.	92.7	Han *et al.*, 2013
	PLGA	Modificadas en su superficie con PEG.	47	Park *et al.*, 2009
Liposomas	Colesterol-HSPC	Modificación superficial con PEG y funcionalizados con ClTx.	99	Xiang *et al.*, 2011
		Conjugado con PEG y otros lípidos; funcionalizados con apoB100.	>85	Kopecka *et al.*, 2011

ClTx, clorotoxina; **cRGDfK**, ciclo(Arg-Gly-Asp-D-Phe-Lys); **DPPE**, 1,2-dipalmitol-sn-glicero-3-fosfoetanolamina; **EE**, eficiencia de encapsulación; **HSPC**, Fosfatidilcolina hidrogenada de soya; **PEG**, polietilenglicol.

3.4.2. El ácido fólico como ligando en la captación tumoral

El ácido fólico es un tipo de vitamina, esencial en las células eucariotas para la síntesis de ADN. Presenta una alta afinidad por su receptor (K_d < 1nM) y entra en las células malignas a través de un mecanismo de endocitosis mediada por receptores (ver figura 4). Entonces, el ácido fólico y/o sus conjugados se combinan con el receptor de folato situado en la superficie de las células cancerosas y se internalizan a compartimentos intracelulares para formar endosomas, donde el ambiente ácido (pH = 5,0 ~ 5,5) induce la liberación del receptor por el folato, condicionando que los receptores de folato regresen de nuevo a la superficie celular después de la disociación. Los conjugados de folato son posteriormente degradados por lisosoma o liberados en el citosol (Chen *et al.*, 2013; Huijuan *et al.*, 2013; Polyák *et al.*, 2013).

El receptor de folato tiene tres isoformas, FR-α, FR-β y FR-γ (ver tabla 2), y está presente sólo en algunos tejidos específicos del cuerpo humano. Sin embargo, su expresión está altamente inhibida en los tejidos normales y altamente expresada o sobreexpresada en varios tipos de cáncer (Huijuan *et al.*, 2013). Esta sobreexpresión del receptor de folato es una característica común de la transformación maligna, debido a las altas tasas de replicación; y hace a los FR una diana terapéutica muy atractiva para la elaboración de nuevos sistemas de administración de agentes anticancerosos.

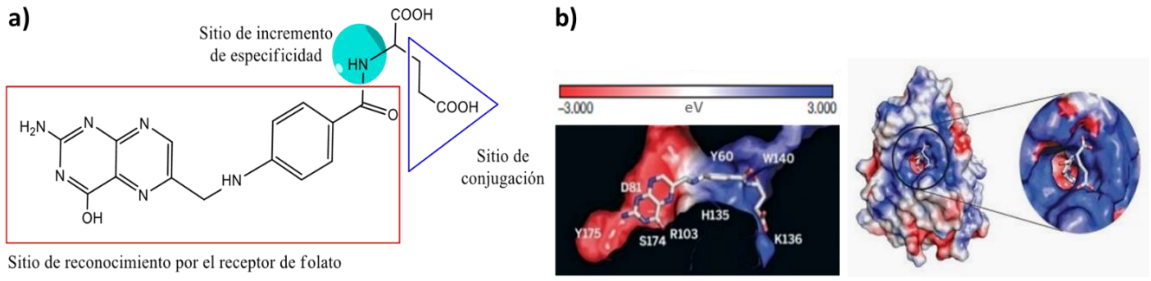

Figura 4. a) Estructura del ácido fólico, mostrando los sitios de reconocimiento; b) representación de densidades electrónicas e interacción con el receptor de folatos. (Chen *et al.*, 2013)

Mansoori *et al.* (2010) realizaron un análisis de la selectividad de algunos conjugados de folato hacía sus receptores, utilizando una línea celular con alta expresión de FR (HeLa) y comparándola con una línea celular con baja expresión de estos. Por otro lado, Werner *et al.*

(2011) diseñaron un sistema de nanopartículas capaces de entregar a las células de cáncer de ovario un agente quimioterapéutico (paclitaxel). Polyák *et al.* (2013) conjugaron ácido fólico con γ-PGA y lo marcaron con ^{99m}Tc como un agente de imagen y diagnóstico precoz para tumores como el carcinoma hepatocelular que, al igual que el cáncer de ovario, sobreexpresa receptores de folato.

Tabla 2. Isoformas del receptor de folato y su expresión en los tejidos.

Isoforma del FR	**Expresión en tejido normal**	**Sobreexpresión en cánceres**	**Localización en la membrana celular**
FR-α (células que proliferan rápidamente)	• Células epiteliales del plexo coroideo • Riñón • Trompas de Falopio • Útero • Epidídimo • Mama • Pulmón • Placenta	• Mama • Cerebro • Pulmón • Colorrectal • Endometrio/uterino • Ovario (su expresión se correlaciona con el estadio y el grado del tumor maligno).	En la capa externa, anclada en el extremo C-terminal de la proteína glicosilfosfatidilinositol (GPI).
FR-β (marcador de linaje neutrofílico)	• Placenta • Bazo • Timo • Células progenitoras hematopoyéticas ($CD34^+$)	Tumores de linaje no epiteliales como: • Sarcomas • Leucemia mieloide	
FR-γ	Tejidos de origen hematopoyético.	Tumores malignos de origen hematopoyético.	Carece del ancla a GPI.
Referencia: Sudimack y Lee, 2000; Ke, Mathias y Green, 2003; Mansoori *et al.*, 2010; Garcia *et al.*, 2011; Walters *et al.*, 2013.			

3.4.3. Funcionalización de nanopartículas de PLGA/γ-PGA con AF.

Las aminas orgánicas como la (1,3)-propil-diamina, (1,4)-dodecil-diamina, dietilendiamina y etilenediamina pueden ser usadas como agentes ligantes (Lu, Gao y Zhao, 2002).

La etilendiamina (EDA) (ver figura 5) es un líquido orgánico que ha sido ampliamente utilizado como agente quelante o entrecruzante debido a los grupos amino que contienen un par de electrones libres en ambos nitrógenos (Tsierkezos *et al.*, 2004). En una reacción tipo carbodiimida para formar enlaces amida, la etilendiamina se ha anclado a diversas nanoestructuras con la finalidad de modificar su superficie para diferentes aplicaciones tales como la remoción de metales y colorantes (Zang *et al.*, 2009; Vuković *et al.*, 2010; Liu *et al.*,

2011; Zhou *et al.*, 2011). Además, este ligante tiene la capacidad de formar enlaces intermoleculares sin modificar significativamente los diámetros hidrodinámicos (Zhou *et al.*, 2010).

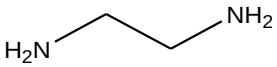

Figura 5. Estructura del ligante etilendiamina ($NH_2CH_2CH_2NH_2$)

La reacción con ácido fólico puede llevarse a cabo mediante una reacción que involucra la activación de los grupos carbonilo con EDC y NHSS, para la formación de un éster de amina el cual reaccionará con los grupos amino de la EDA con la consecuente formación de los grupos amida y del compuesto AF-EDA (ver figura 6). La reacción se realiza con una relación molar 1:1 de AF y EDA, de forma que estequiométricamente se garantiza que un grupo $–NH_2$ esté disponible para ser enlazado a las NPs.

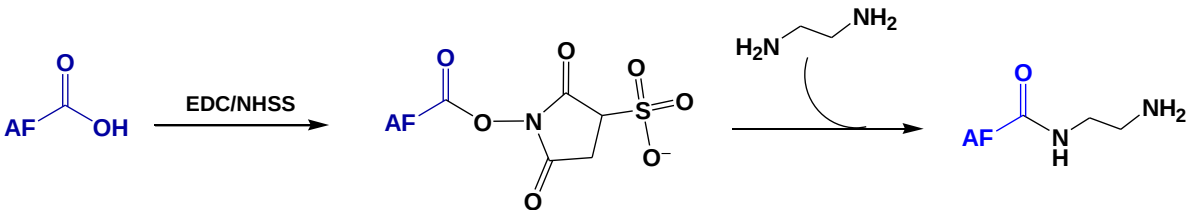

Figura 6. Modificación del ácido fólico con etilendiamina a través de una reacción tipo carbodiimida.

Finalmente, las NPs de PLGA/γ-PGA se someten nuevamente a una reacción tipo carbodiimida, donde el grupo amino libre en el compuesto AF-EDA actúa como nucleófilo uniéndose al centro electrofílico ubicado en el átomo de carbono del grupo acilo activado. La sustitución nucleofílica del acilo activado se lleva a cabo mediante un mecanismo de adición-eliminación obteniendo así las nanopartículas poliméricas funcionalizadas con ácido fólico (PLGA/γ-PGA-EDA-AF) (ver figura 7).

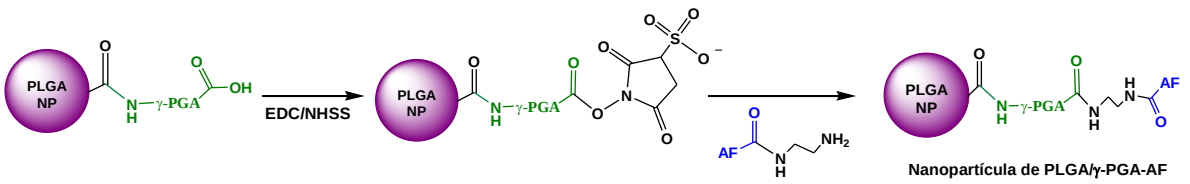

Figura 7. Reacción tipo carbodiimida entre PLGA/γ-PGA y el compuesto EDA-AF.

De acuerdo a los antecedentes presentados, en este proyecto se planteó realizar la obtención y caracterización de nanopartículas poliméricas cargadas con doxorrubicina (ver figura 8), como vehículos adecuados para su transporte y liberación; donde la conjugación con el ácido fólico, molécula de direccionamiento, permitirá su captación selectiva mediada por mecanismos de acumulación pasiva y activa en tejidos tumorales que sobreexpresan receptores de folatos (como en el cáncer cervical), lo que a su vez se reflejará en un incremento de la eficacia terapéutica con un decremento significativo en los mecanismos de toxicidad, comparado con las presentaciones farmacéuticas existentes.

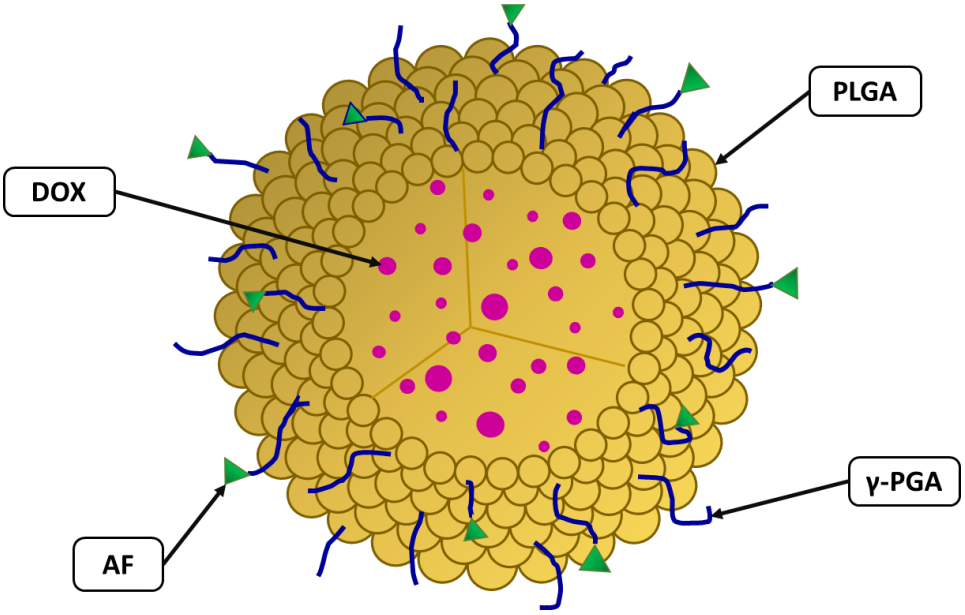

Figura 8. Representación del sistema de nanopartículas de PLGA/γ-PGA-AF para el transporte y liberación de DOX.

4. JUSTIFICACIÓN

Los efectos secundarios de los agentes citotóxicos derivados de la quimioterapia convencional en el tratamiento de cáncer conducen, por lo general, a un éxito clínico limitado. En este contexto, la nanomedicina presenta alternativas en la terapia anticancerosa con el desarrollo de sistemas de administración de fármacos quimioterapéuticos, como son las nanopartículas a base de polímeros.

El sistema polimérico PLGA/γ-PGA conjugado con ácido fólico como ligando de direccionamiento, aún no ha sido estudiado en la administración de fármacos anticancerosos como la doxorrubicina, por lo que representa un sistema innovador. El uso de estos polímeros y ligando de direccionamiento responde a la necesidad de diversificar las estrategias para el tratamiento anticanceroso con DOX, con el fin de combinar las propiedades individuales de cada uno de ellos, reportadas en otros estudios, y conseguir una sinergia que mejore el transporte y liberación de dicho fármaco, para de esta manera lograr un mayor éxito en la terapia.

Con este sistema polimérico (multifuncional y multimérico) se pretende prolongar el tiempo de vida media de la DOX, aumentar las concentraciones terapéuticas en los sitios tumorales por interacciones multivalentes y disminuir los efectos tóxicos por acumulación pasiva en órganos y tejidos sanos.

5. HIPÓTESIS

El sistema conjugado de nanopartículas de ácido poli(láctico-co-glicólico)/ácido poli(gama-glutámico), funcionalizadas con ácido fólico tendrá propiedades fisicoquímicas y biológicas adecuadas para futuras aplicaciones terapéuticas.

6. OBJETIVOS

6.1. Objetivo general

Obtener, caracterizar y evaluar *in vitro*, el sistema multifuncional de nanopartículas de ácido poli(láctico-co-glicólico)/ácido poli(gama-glutámico), funcionalizadas con ácido fólico, para el transporte y liberación de doxorrubicina.

6.2. Objetivos específicos

6.2.1. Obtener y caracterizar nanopartículas poliméricas de:

- Ácido poli(láctico-co-glicólico).
- Ácido poli(láctico-co-glicólico)/ácido poli(γ-glutámico).
- Ácido poli(láctico-co-glicólico)/ácido poli(γ-glutámico)-ácido fólico.
- DOX-ácido poli(láctico-co-glicólico)/ácido poli(γ-glutámico)-ácido fólico.

6.2.2. Determinar la eficiencia de encapsulación y el perfil de liberación de doxorrubicina.

6.2.3. Evaluar *in vitro*, la captación específica y la viabilidad celular.

METODOLOGÍA

7. METODOLOGÍA

7.1. Obtención de nanopartículas poliméricas de:

Ácido poli(láctico-co-glicólico) por el método de emulsión simple/evaporación del solvente (Stevanović et al. 2007).

Se prepara una solución de PLGA en acetona (15 mg/mL), con agitación durante 15 minutos. En seguida, 0.66 mL de metanol se mezclan con 0.5 mL de la solución de PLGA en acetona, por 40 segundos en vórtex. La mezcla anterior se agrega por goteo lento a 3.646 mL de una solución de alcohol polivinílico (PVA) 0.25% (p/v); este paso se realiza en el sonicador durante 10 minutos, con el fin de formar la emulsión. Posteriormente, la acetona y el metanol se evaporan colocando la solución en un evaporador rotatorio durante 15 minutos, con vacío a 67°C y 40 rpm. Finalmente, la solución se centrifuga a 4400 rpm durante 20 minutos y se separa el sobrenadante, el cual se somete al proceso de liofilización, obteniéndose las nanopartículas de PLGA en polvo.

Ácido poli(láctico-co-glicólico)/ácido poli(γ-glutámico), mediante una reacción tipo carbodiimida (Kuo y Yu, 2011).

Se disuelven 10 mg de liofilizado de nanopartículas de PLGA, 96 mg de 1-etil-3-(3-dimetilaminopropil) carbodiimida (EDC) y 23 mg de N-hidroxisulfosuccinimida (NHSS) en 15 mL de agua con agitación durante 4 h, a temperatura ambiente, con el fin de activar los grupos carboxilo presentes en la superficie de las NP-PLGA. A continuación, se agregan 5 mg de γ-PGA a la solución anterior, manteniendo agitación durante 4 h más. Finalmente, las NP de PLGA/γ-PGA se purifican mediante centrifugación con filtros para centrifuga (Amicon® Ultra 30 kDa), y se lavan con agua inyectable. Las NP de PLGA/γ-PGA se resuspenden en 10 mL de agua para su posterior utilización en la funcionalización con ácido fólico.

Ácido poli(láctico-co-glicólico)/ácido poli(γ-glutámico)-ácido fólico, por modificación del AF con EDA y funcionalización por reacción tipo carbodiimida.

En un matraz Erlenmeyer que contiene 10 mL de agua se disuelven 11 mg de ácido fólico, 9.2 mg de NHSS y 72 mg de EDC, con agitación constante por 4h. Por otra parte, 8.32 µL de EDA se disuelven en 5 mL de agua inyectable para obtener una solución con una

concentración de 1.5 mg/mL. Después de las 4 horas de agitación, 1 mL de la solución de EDA se agrega a la solución de ácido fólico (1:1), continuando en agitación por 4 h más.

Mientras tanto, las NP de PLGA/γ-PGA que se resuspenden en 10 mL de agua se hacen reaccionar con 48 mg de EDC y 15 mg de NHSS, con el fin de activar los grupos carboxilo del γ-PGA, mediante agitación por 4 h. Posteriormente, la solución de ácido fólico modificado con EDA, se agrega a la solución que contiene las NP de PLGA/γ-PGA y se continua con agitación durante 4 h más. Después, las NP de PLGA/γ-PGA-AF se purifican de la mezcla de reacción por centrifugación en filtros para centrifuga, y lavadas con agua inyectable. Finalmente, las NP de PLGA/γ-PGA-AF se resuspenden en 4 mL de agua inyectable y se secan por liofilización.

7.2. Carga de DOX en el sistema de nanopartículas.

La carga de doxorrubicina se realiza durante la obtención de las NP de PLGA. Se añaden 200 µL de una solución de doxorrubicina (2 mg/mL) a la solución de PLGA en acetona y se mezclan por agitación durante 25 minutos. El procedimiento continúa como se describió anteriormente. El sobrenadante se ultracentrifuga a 22000 g, por 10 minutos. El sobrenadante resultante se recolecta y se mide por espectroscopia UV-vis a 480 nm, correspondiente a la absorbancia de doxorrubicina, con el fin de calcular la eficiencia de encapsulación y carga, mediante las siguientes fórmulas:

% Eficiencia de encapsulado:

$$\%\mathrm{EE} = \frac{DOX_{\text{añadida}} - DOX_{no\ encapsulada}}{DOX_{\text{añadida}}} \times 100$$

% Eficiencia de carga:

$$\%\mathrm{EC} = \frac{DOX_{\text{añadida}} - DOX_{no\ encapsulada}}{PLGA_{\text{añadido}}} \times 100$$

7.3. Caracterización de los sistemas poliméricos.

- Evaluación del tamaño de partícula por dispersión dinámica de luz (DLS),
- Evaluación de la estabilidad del estado coloidal por potencial zeta.
- Morfología mediante microscopía electrónica de transmisión/microscopía electrónica de barrido (TEM/SEM).
- Evaluación de la formación de los compuestos mediante espectroscopia UV-vis.
- Identificación de grupos funcionales por espectroscopia vibracional (FT-IR).

7.4. Determinación del perfil de liberación de doxorrubicina.

La determinación de liberación de DOX por el sistema de nanopartículas se realiza mediante un método de diálisis, donde 30 mg de nanopartículas cargadas con doxorrubicina (DOX-PLGA-NP o DOX-PLGA/γ-PGA-AF) se dispersan en 2 mL de solución buffer de fosfatos (PBS) y se colocan en una bolsa de diálisis. Posteriormente, la bolsa de diálisis se sumerge en un tubo cónico de 50 mL que contiene 15 mL de PBS como medio de liberación en dos valores diferentes de pH (7.4 y 5.3) para las nanopartículas DOX-PLGA/γ-PGA-AF, y de pH 7.4 para las DOX-PLGA-NP. El tubo cónico se coloca en agitación (110 rpm) a temperatura ambiente por 7 días. A determinados intervalos de tiempo (1 h, 3 h, 6 h, 8 h, 20 h, 1 día, 2 días, 3 días, and 7 días), se toman alícuotas de 0.5 mL y el volumen retirado se reemplaza con PBS. La concentración de doxorrubicina se determina mediante espectroscopia UV-Vis.

7.5. Evaluación *in vitro* de la captación específica de las nanopartículas y la viabilidad celular.

La evaluación *in vitro* de las nanopartículas se realiza en células de adenocarcinoma de cuello uterino humano (HeLa). Las células se cultivan en medio de cultivo RPMI-1640 libre de folatos, suplementado con suero fetal bovino, $NaHCO_3$, penicilina (100 UI/mL), estreptomicina (100 µg/mL), amfotericina (0.25 µg/mL) y ajustado a pH 7.4 - 7.5. Las células se incuban a 37°C, con 5% de CO_2.

Para la evaluación de la captación celular, se siembran $1x10^4$ células por pocillo en una placa de cultivo de 4 pocillos. Después de 24 horas, se remueve el medio de cultivo y se tratan las células con nanopartículas de PLGA/γ-PGA-FA disueltas en PBS durante 3 horas. Por otro

lado, con el fin de evaluar la captación específica por receptores de folato, las células reciben un tratamiento con ácido fólico libre (11 mg/mL) durante 2 horas previas a la exposición a las nanopartículas funcionalizadas con ácido fólico. Se recolectan los sobrenadantes y se miden en fluorescencia/espectroscopia UV-vis.

El ensayo de viabilidad celular se realiza mediante la técnica de cristal violeta. Las células HeLa se siembran en una placa de cultivo de 96 pocillos en una concentración de 4000 células por pocillo con 200 µL de medio de cultivo RPMI-1640 libre de folatos. Posteriormente, el medio de cultivo se reemplaza con muestras de nanopartículas de PLGA/γ-PGA-FA, doxorrubicina libre y nanopartículas de DOX-PLGA/γ-PGA-FA en una concentración de doxorrubicina equivalente de 10 µg/mL. Las células expuestas a las muestras mencionadas se cultivan durante 24 h, 48 h y 72 h, a 37 °C bajo atmósfera de 5% de CO_2, por sextuplicado. Después de cada tiempo, se lleva a cabo el ensayo de cristal violeta. Las células que no reciben tratamiento se consideran como el 100% de viabilidad.

RESULTADOS

8. RESULTADOS

8.1. Resumen de resultados.

Tamaño de partícula y potencial zeta. Utilizando una concentración de PVA de 0.25% p/v, y una concentración de PLGA de 15 mg/mL, se obtuvieron nanopartículas con un tamaño de 185.6 ± 47.2 nm (PDI= 0.16) para PLGA-NP, así como de 597 ± 45.0 nm (PDI= 0.02) para DOX-PLGA/γ-PGA-AF, medido por DLS. El incremento en el tamaño de partícula fue debido a la adición de cadenas pesadas del polímero γ-PGA, que ha mostrado aumentar el diámetro hidrodinámico de partículas e inducir además la formación de aglomerados. Así mismo, se encontró una variación del potencial zeta de -10.3 ± 1.37 mV para PLGA-NP, a 14.2 ± 2.69 mV para DOX-PLGA/γ-PGA-AF. El cambio del potencial zeta fue atribuido a la presencia del ácido fólico y de la etilendiamina utilizada para su modificación, que contienen en su estructura grupos que aportan cargas positivas, además de las presentes en la estructura de la DOX.

Eficiencia de encapsulado y eficiencia de carga. Se obtuvo una EC de 0.33% ± 0.012 %, con una EE de 47.97 % ± 1.8 %. Estos porcentajes fueron obtenidos al elegir las concentraciones de polímero, surfactante y fármaco, que no aumentaran el tamaño de partícula y se obtuvieran eficiencias adecuadas.

Espectroscopia FT-IR. El espectro de las NP de PLGA mostraron un patrón de absorción correspondiente a las unidades monoméricas de ácido láctico-láctico a 1453 cm^{-1}, glicólico-glicólico a 1425 cm^{-1}, y láctico-glicólico a 1376 cm^{-1}, del polímero PLGA. Además, una banda en 3414-3240 cm^{-1} perteneciente al grupo -OH de alcohol, y un pico a 1737 cm^{-1}, evidencian la presencia del surfactante PVA, formando la nanopartícula. La modificación con γ-PGA se pudo observar debido a la presencia de dos picos: uno a 1570 cm^{-1} correspondiente a la vibración de amida II, y otro a 1607 cm^{-1}, correspondiente a la vibración de amida I. El espectro de la conjugación del ácido fólico mostró que el esqueleto formado por el anillo de pteridina y el fenilo pudo ser observado en el rango de 1750-1500 cm^{-1}, con una banda amplia en 1600 cm^{-1} correspondiente al solapamiento de las vibraciones de C=C y C=N aromáticos y el enlace amida del γ-PGA. La presencia de DOX en el sistema se evidenció debido al aumento de la intensidad de la señal observada a 1757 cm^{-1} atribuido a la vibración del C=O proveniente de los grupos quinona y cetona de la estructura de la DOX.

Espectroscopia UV-Vis. La presencia de DOX en el sistema de nanopartículas se pudo observar debido a la existencia de una señal a 480 nm. Mientras que la modificación superficial de la NP de PLGA con γ-PGA y la conjugación con AF se evidenciaron al observar una banda centrada a 208 nm y una señal a 280 nm, respectivamente.

Microscopia SEM/TEM. Se observaron estructuras cuasi-esféricas correspondientes a las nanopartículas con diámetros entre 10 y 250 nm. La aparición de regiones con cúmulo de material polimérico puede ser correlacionado con el aumento de tamaño de partícula observado por DLS

Cinética de liberación del fármaco. El perfil de liberación de DOX por los sistemas de nanopartículas se ajustaron a un modelo sigmoideo de Hill (ecuación de Hill). La liberación de DOX mostró una dependencia del pH atribuible a las propiedades pH-sensible de los polímeros utilizados, obteniendo un porcentaje máximo de liberación de 55.6 ± 0.6 %, a pH 5.3 y un tiempo medio de liberación de siete días.

Estudio de captación celular específica. El análisis ANOVA realizado para comparar la captación celular mostró diferencia estadísticamente significativa entre las células cuyos receptores fueron bloqueados y aquellas que no. Las células HeLa con receptores no bloqueados exhibieron 3.4 veces mayor captación de nanopartículas. Esto sugiere un mejoramiento de la captación, mediado por la interacción de las nanopartículas direccionadas con los receptores de folatos.

Ensayo de viabilidad celular. Se utilizaron ANOVA y Test de Bonferroni para el análisis estadístico. El tratamiento con DOX mostró el mayor efecto a todos los tiempos de exposición. El efecto producido por DOX-PLGA/γ-PGA-AF en las células HeLa fue 1.8 veces mayor a las 72 h respecto a 24 h, esto puede ser explicado en términos de la cantidad de fármaco libre disponible para producir daño. Como se demostró en el perfil de liberación, la liberación del fármaco es dependiente del pH, por lo que el tiempo de exposición es un factor a considerar para permitir que el sistema libere la cantidad de DOX necesaria. Finamente, el sistema vació no mostró tener efecto sobre la viabilidad celular a ningún tiempo de exposición.

8.2. Artículo enviado.

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Article type	Research Paper

Abstract

Polymeric nanoparticles are being studied as current drug delivery systems for cancer treatment. These systems are frequently more effective than their freely-delivered counterparts. Targeting of polymeric nanoparticles enhances the effectivity in cancer therapy by acting in targeted cells and decreasing side effects in healthy tissues by passive uptake. A novel targeted drug delivery nanoparticle system based on poly(D,L-lactide-co-glycolide) acid (PLGA) copolymer conjugated with folic acid (FA) for delivery of the anticancer model drug doxorubicin (DOX), was developed. DOX-PLGA nanoparticles were obtained by the oil in water (O/W) emulsification-solvent evaporation technique. Then, their surface was modified with poly(L-γ-glutamic acid) (γ-PGA) and finally conjugated to modified FA as a targeting ligand against folate receptors which are over-expressed in some types of tumour cells, through carbodiimide chemistry. The surface modification and FA conjugation were followed by UV-Vis and FT-IR spectroscopies. The nanoparticle morphology was determined by TEM/SEM. Particle size, PDI and zeta potential were determined using DLS studies. Encapsulation efficiency, loading efficiency and DOX release kinetics were determined. DOX-PLGA/γ-PGA-FA nanoparticles were tested in HeLa cells in order to evaluate cellular uptake and cell viability. Quasi-spherical nanoparticles with an average hydrodynamic particle size lower than 600 nm (DLS) were obtained. Spectroscopic techniques demonstrated the successful surface modification with γ-PGA and FA conjugation. Release profile of DOX-PLGA/γ-PGA-FA nanoparticles responded to acidic conditions, showing a release percentage of 55.4 ± 0.6 % after seven days. HeLa cells exhibited a decrease in viability when treated with DOX-PLGA/γ-PGA-AF nanoparticles, and cellular uptake was attributed to FA receptor-mediated endocytosis. These results suggest that DOX-PLGA/γ-PGA-FA nanoparticles are a potential targeted drug carrier for further applications in cancer therapy.

Keywords	Folic acid; PLGA nanoparticles; γ-PGA; targeted drug delivery; multimeric FA nanoparticles, sustained-release system
Taxonomy	Treatment Delivery Device, Nanoparticle Based Devices, Polymeric Materials, Drug Targeting, Targeted Therapy, Delivery System
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Biodegradable poly(D,L-lactide-co-glycolide)/poly(L-γ-glutamic acid) nanoparticles conjugated to folic acid for doxorubicin targeted delivery

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ABSTRACT

Polymeric nanoparticles are being studied as current drug delivery systems for cancer treatment. These systems are frequently more effective than their freely-delivered counterparts. Targeting of polymeric nanoparticles enhances the effectivity in cancer therapy by acting in targeted cells and decreasing side effects in healthy tissues by passive uptake. A novel targeted drug delivery nanoparticle system based on poly(D,L-lactide-co-glycolide) acid (PLGA) copolymer conjugated with folic acid (FA) for delivery of the anticancer model drug doxorubicin (DOX), was developed. DOX-PLGA nanoparticles were obtained by the oil in water (O/W) emulsification-solvent evaporation technique. Then, their surface was modified with poly(L-γ-glutamic acid) **(**γ-PGA) and finally conjugated to modified FA as a targeting ligand against folate receptors which are over-expressed in some types of tumour cells, through carbodiimide chemistry. The surface modification and FA conjugation were followed by UV-Vis and FT-IR spectroscopies. The nanoparticle morphology was determined by TEM/SEM. Particle size, PDI and zeta potential were determined using DLS studies. Encapsulation efficiency, loading efficiency and DOX release kinetics were determined. DOX-PLGA/γ-PGA-FA nanoparticles were tested in HeLa cells in order to evaluate cellular uptake and cell viability. Quasi-spherical nanoparticles with an average hydrodynamic particle size lower than 600 nm (DLS) were obtained. Spectroscopic techniques demonstrated the successful surface modification with γ-PGA and FA conjugation. Release profile of DOX-PLGA/γ-PGA-FA nanoparticles responded to acidic conditions, showing a release percentage of 55.4 ± 0.6 % after seven days. HeLa cells exhibited a decrease in viability when treated with DOX-PLGA/γ-PGA-AF nanoparticles, and cellular uptake was attributed to FA receptor-mediated endocytosis. These results suggest that DOX-PLGA/γ-PGA-FA nanoparticles are a potential targeted drug carrier for further applications in cancer therapy.

KEY WORDS: Folic acid; PLGA nanoparticles; γ-PGA; targeted drug delivery; multimeric FA nanoparticles, sustained-release system.

1. INTRODUCTION

Nanomedicine comprises the process of diagnosing, treating, curing, and preventing diseases by using nanomaterials. Among the nanomaterials used in the treatment of several diseases, an important fraction corresponds to polymers that form nanoparticles (solid colloidal particles ranging from 1 to 1000 nm in size) [1], in which excipients may alter release time and increase efficacy of active ingredients, being encapsulated in the polymeric matrix or directly conjugated to the polymer [2,3].

These novel drug delivery systems are frequently more effective than their freely-delivered counterparts. In cancer therapy, the advantages of the polymeric nanoparticles include (a) delivery systems that can extend drug circulation half-life, (b) increased drug concentration at the tumour site through the passive Enhanced Permeation and Retention (EPR) effect, and (c) reduced non-specific uptake due to active targeting [4].

One of the most widely used polymers in the fabrication of polymeric nanoparticles is the copolymer poly(D,L-lactide-co-glycolide) acid (PLGA), due to its biocompatible and biodegradable properties [3,4]. Furthermore, PLGA exhibits many of the ideal properties of a nanoscale delivery system, allowing the encapsulation of the drug within the polymer matrix and providing long-term release of the encapsulated agent [5,6].

PLGA-based systems have been used in the treatment of cancer, showing a notable improvement of the therapy [7–11]. Drug release can be modulated by manipulating several parameters related to PLGA degradation, such as pH. PLGA can be degraded into shorter chain alcohols and acids upon exposure to the acidic microenvironment (approximately pH 5.3) around tumour tissues. Accordingly, drugs can be distributed around tumour microenvironment [12].

On the other hand, PLGA lacks reactive main or side chains, making the interaction with biological systems and the modification with biologically-active moieties difficult, restricting its application. Poly(L-γ-glutamic acid) (γ-PGA) is an anionic homopolyamide widely used in nanoparticulate systems because of its hydrophilicity and the improvement in the interaction between polymers (e.g. PLGA) and biological systems [13–19].

Despite the fact that most nanoparticles are expected to accumulate in tumours due to the EPR effect, passive approaches suffer from several limitations. A useful strategy to achieve efficient tumour targeting and to overcome these limitations is to conjugate carriers with specific ligands that recognize and bind to their cognate receptors on the surface of cancer cells through ligand-receptor interactions that induce receptor-mediated endocytosis and drug release inside the cell [20]. Efficient binding and internalization requires that receptors are over-expressed homogenously on target cancer cells with respect to those on normal cells. The cell receptors density and ligands, three-dimensional architecture of nanoparticles, ligand conjugation chemistry and the types of ligands available may affect ligand-receptor interactions, which may be enhanced by the multivalent nature of the nanoparticle (NP), achieving a high targeting specificity and delivery efficiency, while avoiding non-specific binding and possible cell resistance mechanisms [21,22].

One of the most widely studied small molecules as a targeting moiety for the delivery of agents is folic acid (FA). FA receptors are selectively over-expressed in a number of tumour cell types, but present in low or non-detectable levels in most normal cells [20]. Besides, FA has a high binding affinity for the folate receptor (Kd = 10^{-9} M), and is internalised via receptor-mediated endocytosis, making it a good targeting candidate for nanocarriers delivering an active agent into the cells [20,22].

The aim of this work was to develop a novel PLGA/γ-PGA-based drug delivery system conjugated with folic acid for delivery of the anticancer model drug doxorubicin and targeted against folate receptors over-expressed in some types of cancer cells.

2. MATERIALS AND METHODS

2.1. Materials

Poly(D,L-lactide-co-glycolide) acid terminated had a lactide to glycolide ratio of 75:25. Polymer molecular weight was 11300 g/mol. Poly(vinyl alcohol) (Mowiol®4-88), with a 31000 g/mol molecular weight, had 88.0% mol hydrolysis and 10.5% residual content of acetyl. Poly-L-γ-glutamic acid sodium salt had a molecular weight ≥ 750 kDa (MALLS). Folic acid (HPLC purity ≥97%). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), doxorubicin hydrochloride 98.0 – 102.0% (HPLC). RPMI-1640

medium, N-Hydroxysulfosuccinimide sodium salt (NHSS) ≥ 98% (HPLC). These chemicals were all purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without purification. Other chemicals and solvents were of reagent grade. The HeLa cell line was obtained from National Institute of Cancerology, Mexico.

2.2. Methods

2.2.1. Preparation of PLGA nanoparticles

PLGA nanoparticles were prepared using the single emulsification-solvent evaporation method and PVA [poly(vinyl alcohol)] as stabilizer [6]. A preliminary study was performed based on a multifactorial two-tier design. PLGA in acetone solutions (10 mg/mL and 15 mg/mL) and aqueous PVA solutions (0.25, 0.5, 1, 3, 10 [% w/v]), were evaluated. The best combination in terms of the smallest particle size and the highest encapsulation and/or DOX-loading efficiency was chosen to prepare PLGA nanoparticles. Briefly, 0.66 mL of methanol was mixed with 0.5 mL of PLGA acetone solution and vortexed. Immediately after, the solution was slowly dropped over 3.646 mL of aqueous PVA solution and ultra-sonicated for 10 minutes. Afterwards, acetone and methanol were evaporated by rotary vacuum evaporation (65 °C, 40 rpm, 15 min). Finally, the aqueous solution was centrifuged for 20 minutes at 4400 rpm. The supernatant was collected, freeze-dried and stored for further use.

2.2.2. DOX loading and encapsulation efficiency

Once PLGA and PVA concentrations were established, various DOX concentrations (13.6, 21.7, 32.4, 42.9, 53.4, 104, 241.2 [μg/mL]) were evaluated in order to find the saturation point of the nanoparticle system.

The DOX-PLGA nanoparticles were prepared by adding different volumes of 2 mg/mL doxorubicin solution to a solution of PLGA in acetone. The solution was stirred for 25 minutes. After centrifugation, the supernatant was ultra-centrifuged at 22000 x g for 10 minutes. The supernatant was collected, and the pellet was washed and re-suspended in injectable-grade water (Figure 1a). The DOX-loaded amount was determined by UV-Vis spectroscopy. Absorbance of supernatants was measured at 480 nm, according to doxorubicin absorbance. Encapsulation efficiency (%EE) and loading efficiency (%LE) percentages were calculated as follows:

$$\%\text{EE} = \frac{DOX_{added} - DOX_{non\ encapsulated}}{DOX_{added}} \times 100$$

$$\%\text{LE} = \frac{DOX_{added} - DOX_{non\ encapsulated}}{PLGA_{added}} \times 100$$

2.2.3. Modification of DOX-PLGA nanoparticles with γ-PGA

DOX-PLGA/γ-PGA nanoparticles were obtained by the Kuo and Yu method [15]. Briefly, 10 mg of lyophilised DOX-PLGA nanoparticles, 96 mg (0.50 mmol) of EDC and 23 mg (0.19 mmol) of NHSS were dissolved in 15 mL of injectable-grade water by stirring for 4 h. After that, 5 mg of γ-PGA was added and stirred for 4 h at room temperature. The resulting solution was purified by ultracentrifugation in centrifugal filters (Amicon® Ultra 30 kDa) for 25 minutes. DOX-PLGA/γ-PGA nanoparticles were washed and re-suspended in 10 mL of injectable-grade water (Figure 1b).

2.2.4. Functionalization of DOX-PLGA/γ-PGA nanoparticles with folic acid

11 mg of folic acid (FA, 24.42 µmol), 9.2 mg (79.94 µmol) of NHSS and 72 mg (0.37 mmol) of EDC were dissolved in 10 mL of injectable-grade water. The mixture was stirred for 4 hours. After that, 1 mL of 1.5 mg/mL ethylenediamine (EDA, 24.95 mM) solution was added to the FA solution and stirred for 4 h (Figure 1c).

Meanwhile, 48 mg (0.25 mmol) of EDC and 15 mg of NHSS were added to 10 mL of DOX-PLGA/γ-PGA solution and stirred for 4 h. Afterwards, FA solution was added to the DOX-PLGA/γ-PGA solution and stirred for 4 h, in order to obtain DOX-PLGA/γ-PGA-FA conjugated nanoparticles. The mixture was purified through centrifugal filters (25 min). DOX-PLGA/γ-PGA-FA nanoparticles were washed and suspended in 5 mL of injectable-grade water. Finally, DOX-PLGA/γ-PGA-FA nanoparticles were freeze-dried and stored for posterior use (Figure 1d).

2.3. Characterization

2.3.1. Nanoparticle size and zeta potential

Particle size (Dynamic light scattering, DLS) and zeta potential were measured using a Nanotrac analyzer (Nanotrac Wave, Model MN401, Microtract, FL, USA). Lyophilised samples were analysed using injectable-grade water as diluent. All measurements were performed with a wavelength of 657 nm at 20 °C, current of 15.79 mA, electric field of 14.35 V/cm and sampling time of 128 µs.

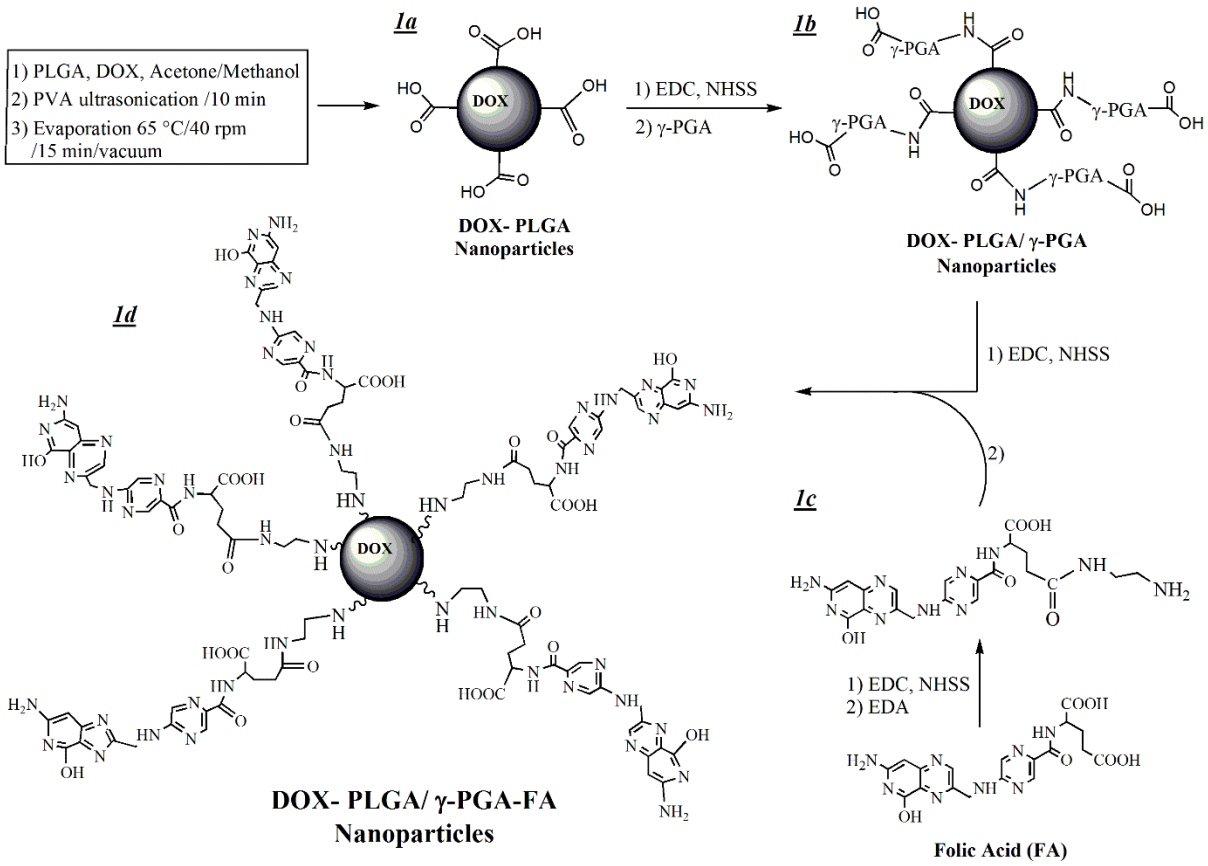


Figure 1. Schematic synthesis of a) DOX-PLGA NPs preparation, b) conjugation of γ-PGA to DOX-PLGA NPs (DOX-PLGA/γ-PGA NPs), c) modification of folic acid and d) DOX-PLGA/γ-PGA-FA NPs.

2.3.2. UV-Vis spectroscopy

Absorption spectra in the 190–800 nm range was obtained with a Thermo Genesys 10S spectrometer using a 1-cm quartz cuvette. Nanoparticles were measured through UV-Vis analysis to monitor the conjugation reactions.

2.3.3. FT-IR spectroscopy

The IR spectra of lyophilised samples were acquired through a PerkinElmer System 2000 spectrometer with an ATR platform (Pike Technologies), by applying attenuated total reflection Fourier transform infrared (ATR-FT-IR) spectroscopy. Resolution 0.4 cm^{-1}, 40 scans, and a 4000 – 400 cm^{-1} operating range.

2.3.4. Transmission electron microscopy (TEM)

The morphology of the nanoparticles was analysed through a Jeol JEM 2010 HT microscope operating at 200 kV. Samples were prepared for analysis by evaporating a drop of nanoparticle suspension on a carbon-coated TEM copper grid.

2.3.5. Scanning Electron Microscopy (SEM)

Surface topography was evaluated with a JEOL JSM 6510LV microscope operating at 20 kV, using secondary electron signals. Samples were sputtered with a thin layer of approximately 15 nm of gold using a Denton Vacuum DESK IV system.

2.3.6. In vitro drug release kinetics

Released doxorubicin concentration was determined by UV-Vis spectroscopy after dialysis. Briefly, 30 mg of either DOX-PLGA-NP or DOX-PLGA/γ-PGA-FA nanoparticles were dispersed in 2 mL of phosphate buffered saline (PBS) and placed in a dialysis bag (30,000 Da MWCO). Then, the closed bag was immersed in a 50 mL tube containing 15 mL of PBS as the release medium at two different pH values (pH 7.4, pH 5.3). The tube was then agitated (110 rpm) at room temperature. At different time points (1 h, 3 h, 6 h, 8 h, 20 h, 1 day, 2 days, 3 days, and 7 days), 0.5 mL aliquots were removed for analysis and volume was replaced with fresh PBS.

2.3.7. Cell culture

Human cervix adenocarcinoma cells (HeLa) were cultured in sterile folate-free RPMI medium (Sigma-Aldrich, USA) supplemented with bovine fetal serum, penicillin (100 UI/mL), streptomycin (100 µg/mL), and amphotericin (0.25µg/mL). The cells were incubated at 37 °C in a humidified environment and 5% CO_2.

2.3.8. In vitro cellular uptake study

Nanoparticle uptake by HeLa cells was evaluated. Cells were cultured in a 4-well plate ($1x10^4$ cells per well). After 24 h, medium was removed. To test whether nanoparticle uptake occurs *via* folate receptors, a group of HeLa cells were pre-treated with 500 µL of free folic acid (11 mg/mL) in order to saturate receptors, and incubated at 37°C for 2 h. After receptor saturation, both blocked and non-blocked HeLa cells were treated with PLGA/γ-PGA-FA nanoparticles (675 µg/mL) eluted in PBS for 3 h. Finally, supernatants were collected and measured by UV-Vis spectroscopy at 280 nm, wavelength corresponding to FA. Uptake percentage was calculated with regards to PLGA/γ-PGA-FA nanoparticles in PBS that were not exposed to cells. The baseline was registered as untreated cells in the same conditions. The experiment was performed in triplicate.

2.3.9. Cell viability assay

Cell viability was assessed by the crystal violet staining assay. HeLa cells (4000 cells/well) were seeded on a 96-well plate in 200 µL of folate-free RPMI-1640 medium, and allowed to adhere for 24 h at 37°C. Then, the culture medium was replaced with either PLGA/γ-PGA-FA nanoparticles, free DOX, or DOX-PLGA/γ-PGA-FA nanoparticles at equivalent 10 µg/mL doxorubicin concentration. Cells were incubated for 24 h, 48 h and 72 h, at 37°C under 5% CO_2 atmosphere, in sextuplicate. After a given time, the crystal violet assay was performed and untreated cells were considered as 100% of cell viability.

2.4. Statistical analysis

All data was analysed using OriginPro 8.6 software. Statistical analysis was performed with either one-way or two-way ANOVA and Bonferroni mean comparison at 0.05 significance level.

3. RESULTS

3.1. Preparation of PLGA nanoparticles

The oil in water (O/W) emulsification-solvent evaporation method has been described as a relatively simple process to obtain PLGA-nanoparticles, where surfactant and polymer concentrations were important factors in the final characteristics of the nanoparticles. Based on the evaluated combinations, it was observed that the higher PVA concentration, the higher nanoparticle size. Thus, the lowest PVA concentration (0.25 % w/v) was chosen as the optimal surfactant concentration. Once PVA concentration was established, two PLGA concentrations were analysed in order to achieve the highest doxorubicin loading and encapsulation efficiencies. A concentration-dependant behaviour was observed, since 15 mg/mL PLGA concentration had higher efficiencies compared to 10 mg/mL, remaining the lowest nanoparticle size. Finally, the smallest nanoparticle size was found at PLGA and PVA concentrations of 15 mg/mL and 0.25 % (w/v), respectively. Volume mean diameter of PLGA nanoparticles before functionalization was found to be of 185.6 ± 47.2 nm, showing a wide distribution (polydispersity index, PDI = 0.16) with an apparent monomodal population. Changes in size were observed by DLS when PLGA nanoparticles were conjugated to γ-PGA and FA (Table 1).

Table 1. Characteristics of different nanoparticle systems (Mean ± SD, n=3).

Nanoparticle system	Size (nm)	PDI	Zeta potential (mV)
PLGA-NP	185.6 ± 47.2	0.16 ± 0.05	- 10.3 ± 1.37
PLGA/γ-PGA	501 ± 67.3	0.09 ± 0.03	-16.4 ± 1.99
PLGA/γ-PGA-FA	537 ± 57.1	0.10 ± 0.07	8.1 ± 0.82
DOX-PLGA/γ-PGA-FA	597 ± 45.0	0.02 ± 0.01	14.2 ± 2.69

3.2. Encapsulation efficiency (%EE) and loading efficiency (%LE)

PLGA nanoparticles showed a loading efficiency behaviour dependant on polymer and surfactant concentrations. The highest loading efficiency (0.33 % ± 0.012 %) was taken as a mandatory parameter of system saturation, with an encapsulation efficiency of 47.97 % ± 1.8 %, when 104 µg/mL of doxorubicin concentration was tested.

3.3. Characterization studies

3.3.1. FT-IR spectroscopy

The FT-IR spectrum of PLGA nanoparticles (Figure 2a) showed the peaks corresponding to copolymer characteristic groups: 2942 cm^{-1} and 2918 cm^{-1} (CH bend), 1737 cm^{-1} (C=O of ester), 1376 cm^{-1} (CH_3 from lactide), and 1300-1000 cm^{-1} (ester). A triple-peak absorption pattern is also present corresponding to bonds between monomeric units of lactide-lactide at 1453 cm^{-1}, glycolide-glycolide (G-G) at 1425 cm^{-1}, and lactide-glycolide (L-G) at 1376 cm^{-1}, within the PLGA polymer chains. Also, characteristic PVA peaks were seen at 3414–3240 cm^{-1}, belonging to the -OH group from the alcohol. The peak at 1737 cm^{-1} is due to the presence of non-hydrolysed PVA, ergo acetylated residues from polyvinylacetate that was not totally converted to PVA. The characteristic peaks from the -OH group could be used to identify an interaction between PVA and PLGA in the nanoparticle, since similar peaks are present in the PLGA-NP spectrum [6,23]. The surface modification of PLGA-NP by γ-PGA (Figure 2b) can be observed due to two peaks seen at 1570 cm^{-1}, corresponding to the asymmetric stretching vibration of COO- and the N-H bending vibration for amide II, and 1607 cm^{-1} corresponding to the symmetric stretching vibration of amide I present in the γ-PGA polymer [24].

The PLGA/γ-PGA-FA nanoparticle spectrum (Figure 2c) showed a band centred at 3299 cm^{-1}, corresponding to the OH group and increased by the N-H stretching vibration of the ethylenediamine structure used to modify the folic acid molecule. Also, the pteridine and phenyl ring skeleton of FA can be observed in the range of 1750-1500 cm^{-1} with a broad band at 1600 cm^{-1} corresponding to the overlapping of signals from aromatic C=C and C=N stretching vibrations and amide bond of the homo polyamide γ-PGA. Finally, in the DOX-PLGA/γ-PGA-FA nanoparticle spectrum (Figure 2d), an increase in the intensity of the band at 1757 cm^{-1} was observed, attributed to the C=O stretching vibration from quinone and ketone groups of the doxorubicin structure as well as the pteridine and phenyl ring skeleton from FA. These results allowed to confirm the presence of DOX and FA in PLGA nanoparticles.

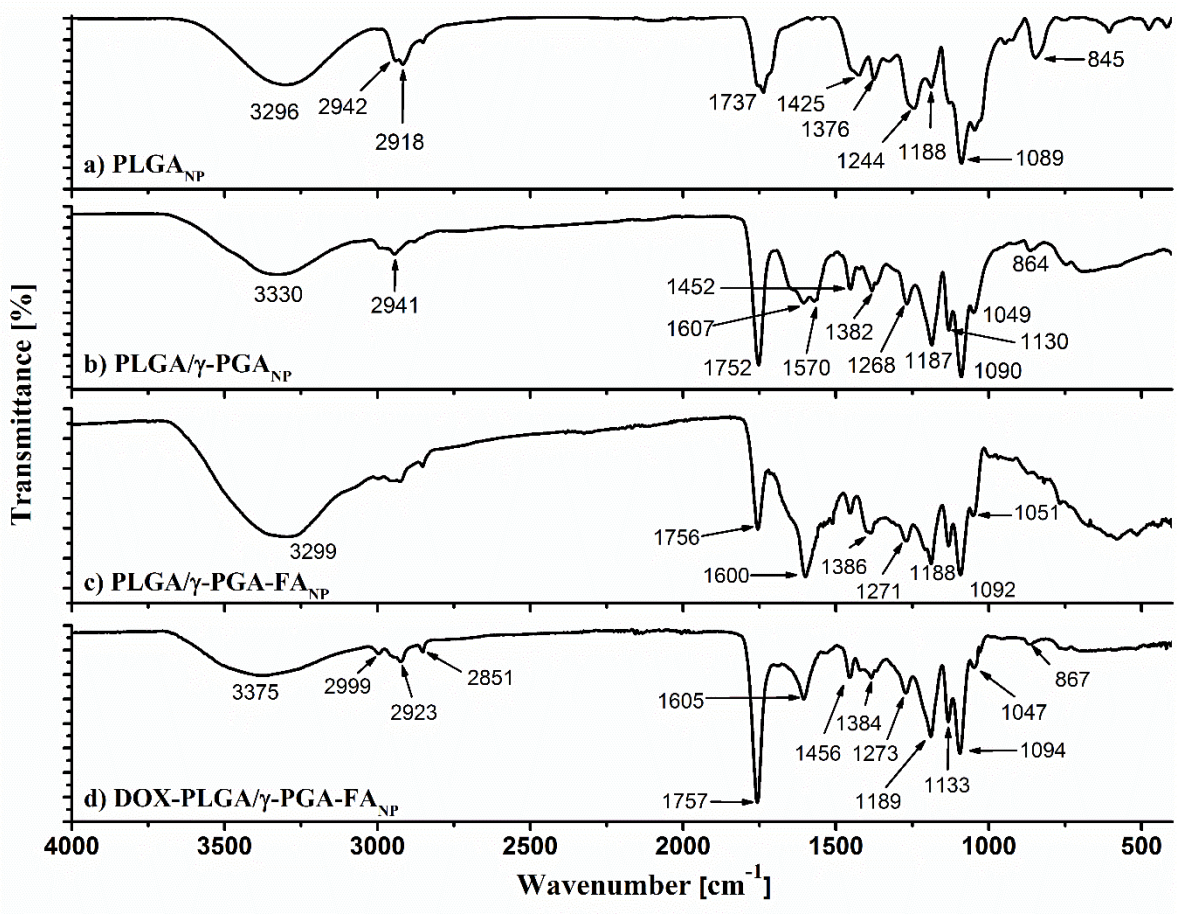


Figure 2. FT-IR spectroscopy of a) PLGA NPs, b) PLGA NPs modified with γ-PGA, c) PLGA/γ-PGA NPs functionalised with FA and d) DOX-loaded PLGA/γ-PGA-FA NPs.

3.3.2. UV-Vis spectroscopy

The reaction steps of PLGA/γ-PGA-FA nanoparticle formation and DOX entrapment were followed by UV-Vis spectroscopy. The PLGA spectrum showed a signal increase in the UV region (200 nm – 400 nm) without defined bands, whereas γ-PGA polymer displayed a well-defined band in the UV region centred at 204 nm (Supplemental figure 1). The conjugation of γ-PGA to PLGA nanoparticles showed an intense and broad band centred at 208 nm.

The doxorubicin spectrum exhibited three narrow and well-defined bands identified at 230 nm, 253 nm and 290 nm, whereas a wide and composed band was observed from 380 nm to 570 nm, centred between 480 and 490 nm (Supplemental figure 2). When doxorubicin was

encapsulated into PLGA nanoparticles, the bands at 230 nm, 253 nm, 290 nm, and 480 nm were found in the UV-Vis spectrum of DOX-loaded PLGA nanoparticles, with significant increase on baseline intensity due to particulate nature of the samples. Once the surface modification with γ-PGA was performed, the characteristic band of DOX at 480 was observed, which demonstrated that DOX remained in the modified nanoparticles. The band at 208 nm, corresponding to the surface modification of PLGA nanoparticles with γ-PGA, was also observed (Figure 3).

Folic acid analysed by UV-Vis spectroscopy showed two characteristic absorbance bands at 280 nm and 348 nm. The successful FA conjugation to PLGA/γ-PGA nanoparticles was demonstrated by means of the bands obtained at the same wavelength when the final PLGA/γ-PGA-FA system was analysed (Supplemental figure 3). Spectra of DOX-PLGA/γ-PGA-FA nanoparticles and PLGA/γ-PGA-FA nanoparticles were indistinguishable. However, signals of folic acid could be clearly seen in both spectra (Figure 3).

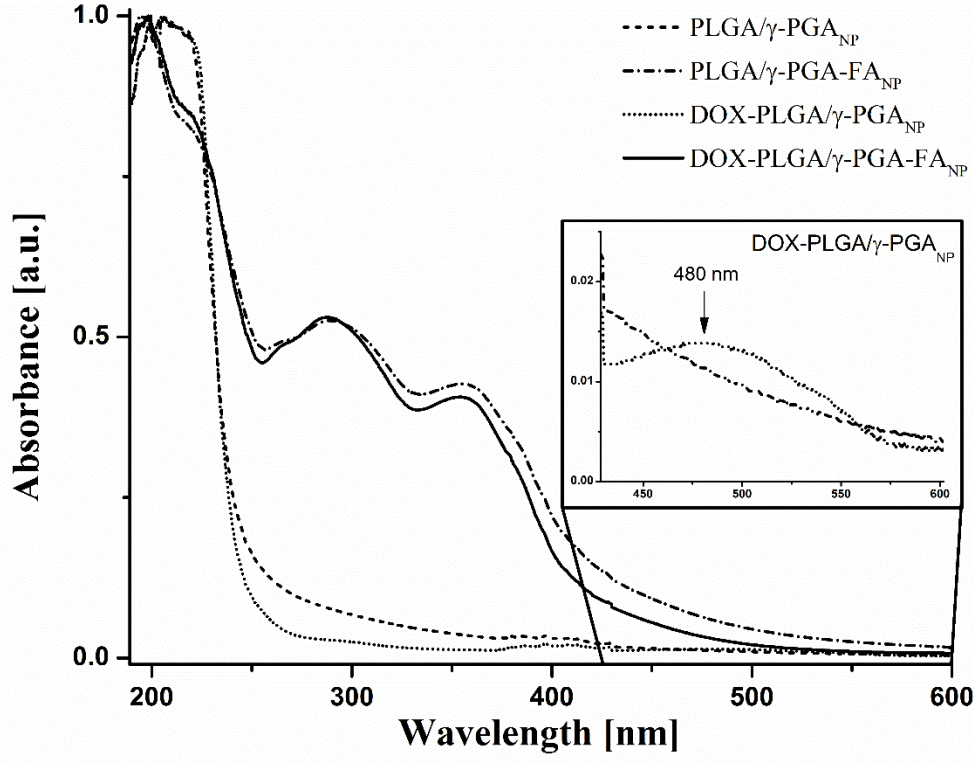
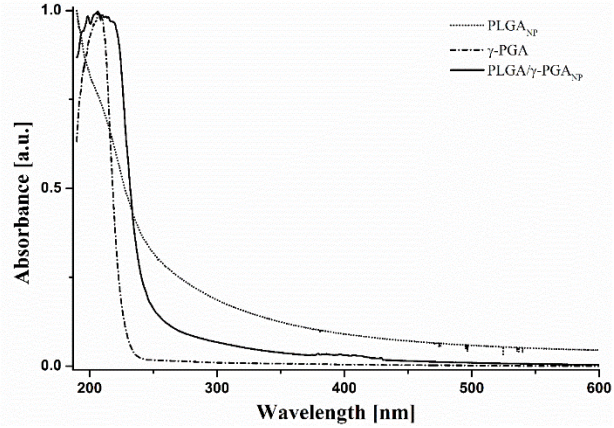
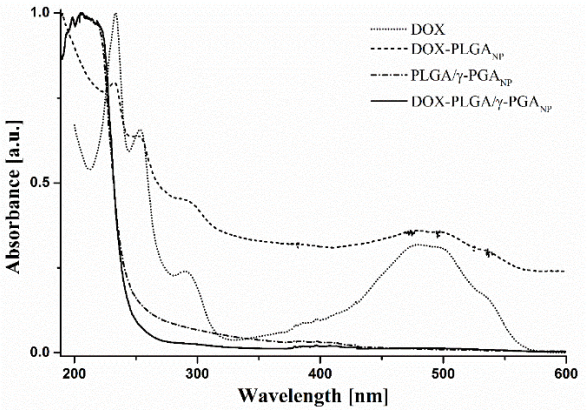


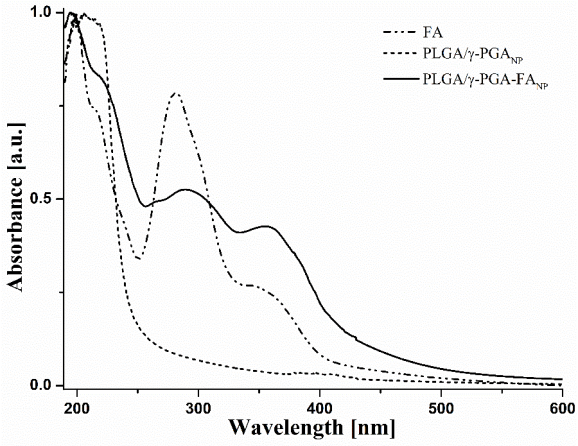
Figure 3. DOX-PLGA/γ-PGA-FA nanoparticles formation by UV-Vis spectroscopy.



Supplemental figure 1. Surface modification of PLGA nanoparticles with γ-PGA by UV-Vis spectroscopy.



Supplemental figure 2. DOX-loading nanoparticles and surface modification of PLGA nanoparticles with γ-PGA by UV-Vis spectroscopy.



Supplemental figure 3. Folic acid conjugation to PLGA/γ-PGA nanoparticles.

3.3.3. SEM/TEM microscopy

TEM/SEM images demonstrate the presence of polymeric structures of PLGA (Figure 4a), with high density cores and distinctive quasi-spherical structures (Figure 4c) with diameters ranging from 10 to 250 nm. Nanoparticle formation was characterised by the maintenance of nanometric structures even when nanoparticles were DOX-loaded and FA-conjugated (Figure 4b). The appearance of PLGA-NP, PLGA/γ-PGA nanoparticles and PLGA/γ-PGA-FA nanoparticles was indistinguishable by electron microscopy techniques.

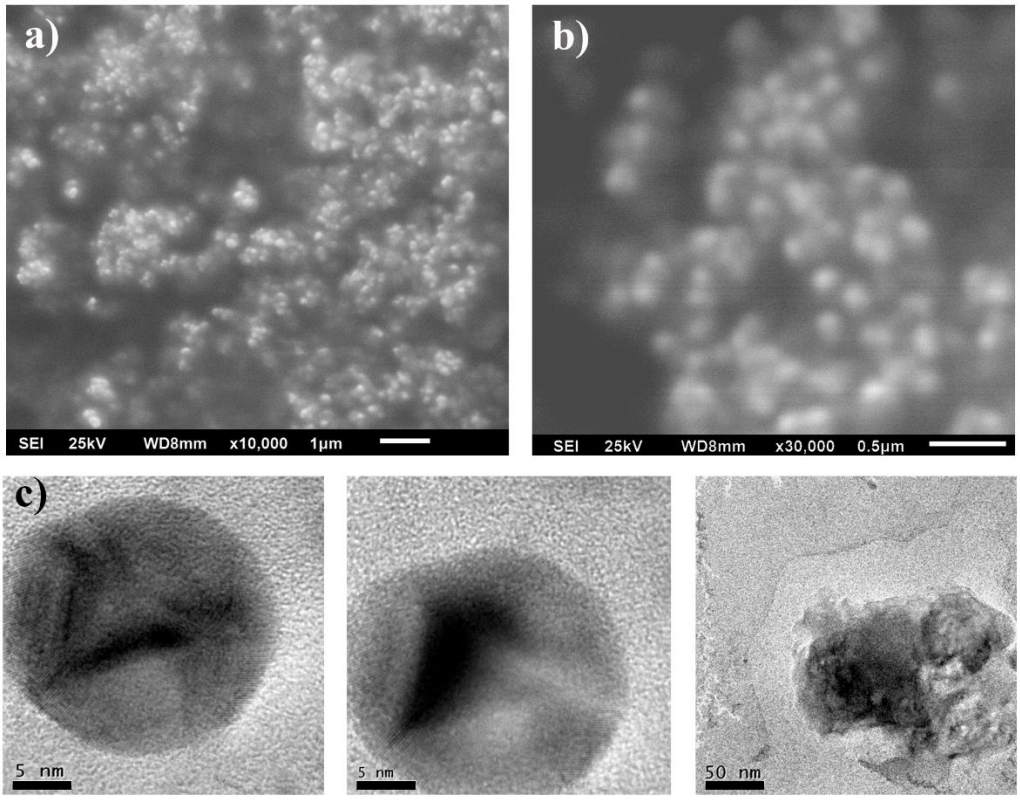


Figure 4. SEM images of a) PLGA NPs; b) DOX-PLGA/γ-PGA-FA NPs and c) TEM of demonstrative shape found in DOX-PLGA/γ-PGA-FA nanoparticles.

3.4. In vitro drug release kinetics

In order to evaluate the potential of PLGA/γ-PGA-FA nanoparticles as a drug carrier, *in vitro* release of DOX by DOX-PLGA/γ-PGA-FA nanoparticles at two different pH values (7.4 and 5.3), and DOX-PLGA nanoparticles at pH 7.4 was evaluated. Figure 5 displays release

profiles of encapsulated systems. The release kinetics were fixed to a sigmoid model (Hill equation) to describe the relationship between non-linear drug release and exposure time in media at determined pH. DOX-PLGA nanoparticles showed the highest DOX release percentage throughout the experiment with a burst release in the course of the first day, with a value of 29.91 ± 0.22 %. The Hill model predicted a maximum concentration release of 64 ± 4% after the seventh day, with a mean release time at day 2, approximately. The burst phase was not observed in DOX-PLGA/γ-PGA-FA nanoparticles at pH 5.3 or pH 7.4. DOX-PLGA/γ-PGA-FA nanoparticles at pH 7.4 showed doxorubicin released of 12.05 % ± 0.008 % after 7 days, with a maximum drug release of 18.8 ± 3.6 % obtained by fixing it to the Hill equation. Meanwhile, DOX-PLGA/γ-PGA-FA nanoparticles at pH 5.3 showed a maximum drug release of 55.4 ± 0.6 % and a mean release time of seven days.

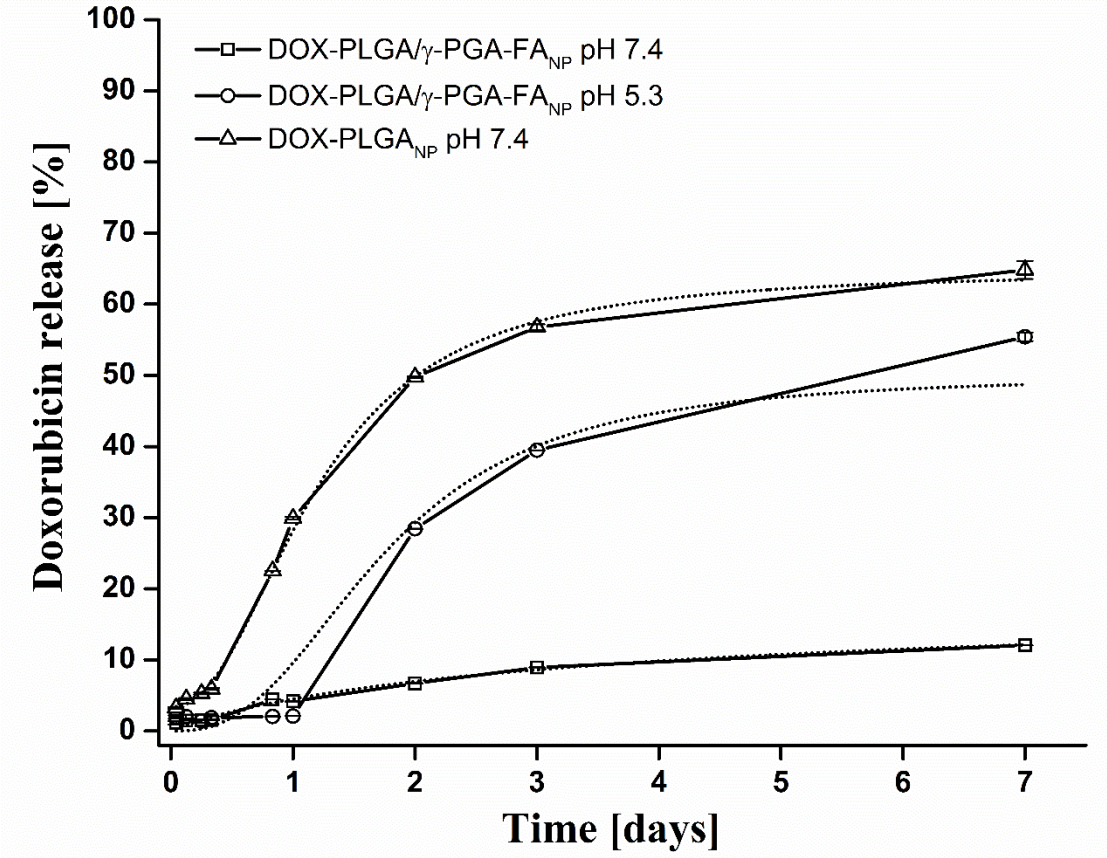


Figure 5. *In vitro* release profiles of doxorubicin-loaded PLGA/γ-PGA-FA nanoparticles at pH 5.3 and 7.4, and DOX-PLGA nanoparticles at pH 7.4.

3.5. In vitro cellular uptake study

To demonstrate that folate can mediate the specific uptake of PLGA/γ-PGA-FA nanoparticles *via* folate receptor, a blocked receptors model was chosen to conduct the study. Figure 6 shows comparative PLGA/γ-PGA-FA nanoparticle uptake by cells whose folate receptors were free or saturated with folic acid. The ANOVA test was conducted to evaluate the cellular uptake showing a statistical difference between blocked and non-blocked cells receptors. Non-blocked FA receptors on HeLa cells exhibited a 3.4-fold higher uptake than cell with blocked receptors.

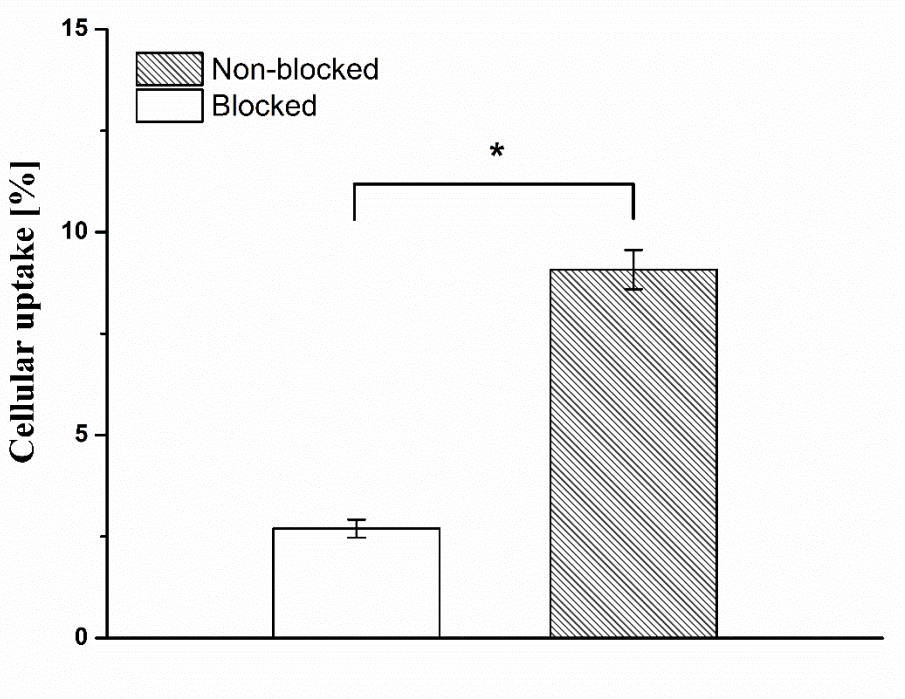


Figure 6. Cellular uptake of PLGA/γ-PGA-FA by blocked FA-receptor HeLa cells and non-blocked receptor HeLa cells. *Significantly difference at the 0.05 level.

3.6. Cell viability assay

The effect of treatment with DOX-PLGA/γ-PGA-FA nanoparticles, PLGA/γ-PGA-FA nanoparticles and free DOX on HeLa cells, was evaluated. ANOVA and Bonferroni tests were used to compare treatments and exposure times ($p \leq 0.05$). As revealed in Figure 7, the cell viability of free DOX with regards to DOX-PLGA/γ-PGA-FA nanoparticles was lower

at all exposure times. The effect on viability produced by DOX-PLGA/γ-PGA-FA nanoparticles on HeLa cells was 1.8-fold higher at 72 h compared to 24 h. Additionally, statistical differences among 24 h, 48 h, and 72 h for PLGA/γ-PGA-FA nanoparticles with respect to non-treated cells, were not found.

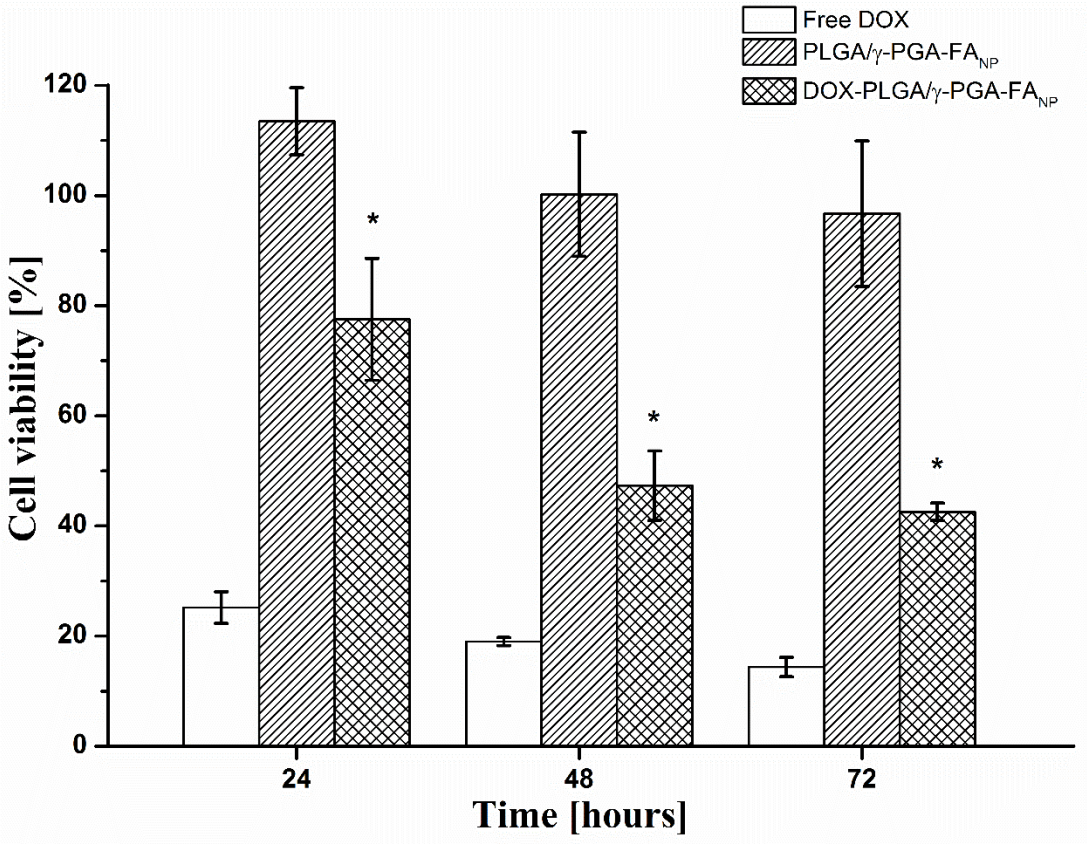


Figure 7. Cell viability of HeLa cells after exposure to free DOX, DOX-loaded PLGA/γ-PGA-FA NPs and PLGA/γ-PGA-FA NPs at 24 h, 48 h and 72 h. *Significantly difference at the 0.05 level.

4. DISCUSSION

Carbodiimide chemistry has been extensively used as a practical way to conjugate carboxylic acids to primary amines to obtain covalently-conjugated systems with more advantages than those materials modified by adsorption processes. The γ-PGA was grafted to carboxylic acids present on PLGA-NP using a carbodiimide reaction and the sulfo-hydroxysuccinimide

analogue in order to increase the yield reaction [22,25]. Prior to FA conjugation to PLGA/γ-PGA nanoparticles, the FA was modified with EDA to obtain FA-NH_2, so that there would be certainty that FA was successfully conjugated through the carboxylic acid. This modification does not affect the possibility of FA binding to folate receptors by specific recognition (pteridine group) [26].

PLGA nanoparticles showed a volume mean diameter of 185.6 ± 47.2 nm. There was a significant increase in size when γ-PGA was anchored to PLGA nanoparticles. These changes are due to the linkage of heavy γ-PGA polymer chains that were grafted to the nanoparticle surface. It has been observed that an increase in molecular weight of γ-PGA leads to an increase in particle size attributed to the extensibility of the hydrophilic γ-PGA chain, which enhances the swolling capability and promotes the space occupation of γ-PGA into nearby surroundings, randomly expanding on the surface of PLGA nanoparticles and leading to a high particle size and a broad distribution [15]. Additionally, the shrinkage of surface γ-PGA produces an adherent force among the polymeric colloids and can form interconnected clusters which could influence the particle size of PLGA/γ-PGA nanoparticles measured by DLS [15,27,28].

Folic acid conjugation to PLGA/γ-PGA nanoparticles produced an increase in particle size measured by DLS (537 nm ± 57.1 nm). This increment was not as evident as that in the superficial modification of PLGA nanoparticles with γ-PGA, although a high particle size remained. SEM and TEM images of the DOX-PLGA/γ-PGA-FA final nanoparticle system displayed some regions with cumulus high density cores corresponding to nanoparticle structures, and the formation of clusters when the nanoparticles were modified with γ-PGA, was observed. These results correlate with the increase in diameter obtained by DLS. DOX-PLGA/γ-PGA-FA nanoparticles displayed particle sizes lower than 250 nm when they were found isolated by TEM or SEM techniques.

Folic acid has been used in different types of nanoparticles such as metallic and polymeric nanoparticles. In this research, when FA was conjugated to PLGA/γ-PGA nanoparticles, the zeta potential value changes from negative to positive, attributable to the modification on FA with ethylenediamine at the carboxyl group, which produced a loss of negative charges. Moreover, folic acid chemical structure contains a pteridine ring with amino groups present

which could contribute to the change toward positive charge [29], so the subsequent binding of EDA-FA to PLGA/γ-PGA nanoparticles leads to modification of zeta potential value. As soon as folic acid had been conjugated to the nanoparticles, a variation in the value of zeta potential occurs. In all cases, zeta potential of nanoparticles had negative values, but once folic acid is conjugated, the value of negative zeta potential shifts towards zero [30–33].

Surface modification, as well as functionalization of nanoparticles, was possible to perform even when nanoparticles were drug-loaded, which indicates that doxorubicin is not involved in the carbodiimide reaction. It was observed that when nanoparticles were loaded with doxorubicin, zeta potential value of the final systems PLGA/γ-PGA-FA and DOX-PLGA/γ-PGA-FA was incremented from 8.08 mV to 14.2 mV, respectively, suggesting that a significant fraction of doxorubicin remained at the nanoparticle surface in a protonated form, influencing the steric hindrance and minimizing the events of coalescence that occur among nanoparticles in a colloidal dispersion. Additionally, superficial DOX was evidenced throughout carbodiimide reactions, where a loss of approximately 43% of DOX was observed, due to the long stirring time required for reaction achievement and thus DOX entrapped superficially in the layer of PVA stabilizer could be released easily.

The UV-Vis and FT-IR measurement throughout the drug-loading, surface modification with γ-PGA, and FA conjugation procedures also demonstrated that DOX was loaded efficiently and nanoparticle modifications were made successfully. On the UV-Vis spectrum, the broad band found at 208 nm, corresponding to the insertion of γ-PGA polymer to PLGA nanoparticles, represents a redshift after γ-PGA attachment, confirming the chromophore conjugation and effective surface modification of nanoparticles. FT-IR analysis showed significant differences in wavenumber within the range of 1760-1400 cm^{-1}, when PLGA nanoparticles surface was modified. The FT-IR spectrum of PLGA/γ-PGA nanoparticles showed two significant peaks at 1570 cm^{-1} and 1607 cm^{-1}, due to the carboxylate groups and amide bounds, respectively, present in the polymeric chain of γ-PGA, which were not observed in PLGA-NP before modification. The γ-PGA polymer spectrum showed signals corresponding to the mentioned groups. However, these signals are shifted to larger wavenumbers in PLGA/γ-PGA nanoparticles, due to covalent bindings (amide formation) as well as intermolecular van der Waals or hydrogen bond interactions between PLGA

nanoparticles and the γ-PGA polymer, related to the three-dimensional arrangement of nanoparticle surface. Similar shifts toward higher energy frequencies were seen by Guan et al. [13] when gold nanoparticles were successfully capped with γ-PGA, modifying the surface.

FT-IR and UV-Vis analysis confirmed the conjugation of folic acid to PLGA/γ-PGA nanoparticles. The UV-Vis spectrum showed the distinctive band of folic acid assigned to the π-π* transition of the pteridine ring around 280 nm, which has been extensively described [34–36]. In the same way, the FT-IR spectrum of PLGA/γ-PGA-FA nanoparticles showed the characteristic peaks corresponding to the structural units of the folic acid molecule. Also, the increased intensity of the broad band at 3299 cm^{-1} evidences the presence of hydrogen bonds, attributable to an increase in local concentration of amino groups from EDA bonded to FA.

The presence of doxorubicin in DOX-PLGA/γ-PGA-FA nanoparticles could also be demonstrated by FT-IR spectroscopy. The increase in the intensity of the band at 1757 cm^{-1}, corresponding to quinone and ketone carbonyl groups indicated that the doxorubicin rings are present in the final nanoparticulate system.

Doxorubicin has been previously encapsulated by PLGA nanosystems for cancer therapy, showing different encapsulation and loading efficiency percentages over 67% and 0.65 %, respectively, depending on the method and materials used for synthesis [37–41]. DOX-PLGA nanoparticles prepared by the single emulsification-solvent evaporation method and the use of PVA as stabilizer showed suitable encapsulation and loading efficiency percentages of 47.97 ± 1.8 % and 0.33 ± 0.012 %, respectively. The DOX-PLGA nanoparticle formulation was performed to achieve the highest loading efficiency and, at the same time, a suitable DOX encapsulation along with an appropriate nanoparticle size. The increase in surfactant and polymer concentrations could allow higher doxorubicin entrapment in function of the PVA layer increase and/or the amount of PLGA polymer available to entrap the drug. However, these changes may give rise to larger nanoparticles. Consequently, these factors should be considered in future experiments in order to optimize and improve the found results under the tested conditions in this study, by analysing different PVA and PLGA concentrations.

Release behaviour of DOX-loaded PLGA nanoparticles exhibited an initial fast release, followed by a sustained phase, which is a similar drug release to that previously reported [42–46] at neutral pH. The first stage is attributed to the amount of DOX entrapped on the PVA layer, followed by a controlled release as a consequence of PLGA degradation where the cleavage of ester bonds in the polymer chain produces lactic and glycolic acids, which induced autocatalysis of PLGA due to pH changes [47], leading to the disintegration of the nanoparticle structure and promoting the release of DOX. DOX release from DOX-PLGA/γ-PGA-FA responded to acidic conditions (pH 5.3), with a significant increase in free doxorubicin compared to physiological conditions, attributable to the PLGA and γ-PGA, which are pH-sensitivity polymers [48,49].

DOX-PLGA/γ-PGA-FA nanoparticles at pH 7.4 exhibited a lower release rate than that of DOX-PLGA nanoparticles. It has been seen that the hydrophilic characteristics of γ-PGA on the nanoparticle surface could allow the penetration of water to the hydrophobic DOX-PLGA nucleus, promoting the erosion and accelerating the release rate. However, a high molecular weight of γ-PGA leads to both a low quantity of the grafted γ-PGA on the particle surface, decreasing the erosion effect produced by water penetration as well as an increase in the level of chain entanglement and steric hindrance for releasing DOX. Additionally, the presence of moieties such as FA on nanoparticle surfaces could reduce the release rate of DOX. This is because FA could intensify the chain entanglements, elongate the diffusion path of DOX, diminish the concentration gradient, and hinder the release [15,50]. This study was carried out in saturation conditions and results indicate that the control on γ-PGA and FA conjugations opens the possibility for controlling the kinetic release by variating the proportion and molecular weight of polymer on the nanoparticle surface as well as the concentration of FA.

HeLa cells have been demonstrated that they over-express folate receptors [51,52]. To confirm the receptor specificity of cellular uptake, HeLa cells were incubated with free folic acid to block or reduce folate receptors on the surface of cancer cells prior to treatment with PLGA/γ-PGA-FA nanoparticles [20]. FA-treated cells served as a model of cells with a low number of folate receptors. HeLa cells overexpressing folate receptors exposed to nanoparticles containing FA as a targeting ligand showed a notably higher uptake than those

cells pre-treated with free FA. PLGA/γ-PGA-FA nanoparticles also exhibited a non-specific uptake by non-FA-treated HeLa cells. However, results suggest that folic acid present on the PLGA/γ-PGA-FA nanoparticle surface significantly improves the uptake by HeLa cells, supporting a receptor-mediated uptake mechanism.

The cell viability study was performed on HeLa cells in order to evaluate PLGA/γ-PGA-FA nanoparticles as a potential drug carrier for cancer therapy. Doxorubicin concentration was homogenised for all treatments. Free DOX showed the highest cell death percentage at all exposure times with statistically significant difference ($p \leq 0.05$). However, the effect of DOX-PLGA/γ-PGA-FA was more evident after 72 h of treatment. This could be explained in terms of the DOX available to act against cancer cells. First, culture medium must possess an acidic pH, mimicking the acidic microenvironment around tumours [12,53], where DOX-PLGA/γ-PGA nanoparticles could start releasing DOX. Additionally, drug-loaded nanoparticles should also be internalised into cells, where the acidic conditions of lysosomes could finally degrade the polymeric system and thus achieve the amount of drug necessary to reach the desirable effect. This pH-dependant behaviour was observed in the profile release of DOX-PLGA/γ-PGA-FA nanoparticles when these were evaluated at pH 5.3.

PLGA/γ-PGA-FA nanoparticles did not demonstrate an effect on HeLa cells. The effect displayed by DOX-PLGA/γ-PGA-FA was significantly higher compared to PLGA/γ-PGA-FA nanoparticles without doxorubicin, suggesting that the effect observed was due to the presence of the drug in nanoparticles.

In order to assess their therapeutic potential, DOX-PLGA/γ-PGA-FA nanoparticles need to be tested on *in vivo* models. PLGA/γ-PGA conjugated to folic acid may provide an alternative therapy for chronic degenerative diseases such as cancer, where the overexpression of folate receptors offers a therapeutic target.

5. CONCLUSION

In this study, PLGA nanoparticles were properly prepared. The spectroscopy techniques demonstrate the correct surface modification and the successful conjugation with folic acid, resulting in an active drug targeting device. The cytotoxic effect against HeLa cells showed a dependence on drug release over the exposure time, due to the pH-sensitive characteristics

of DOX-PLGA/γ-PGA-FA nanoparticles. The enhancement of cellular uptake by a receptor-mediated uptake mechanism of nanoparticles into HeLa cells was demonstrated. Therefore, the PLGA/γ-PGA-FA system is a potential target-specific drug delivery system with molecular recognition of over-expressed folate receptors.

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8.3. Artículo publicado.

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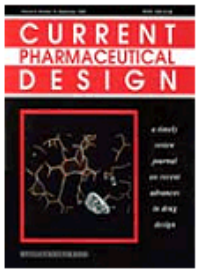

Polymer-Based Drug Delivery Systems, Development and Pre-Clinical Status

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Polymer-Based Drug Delivery Systems, Development and Pre-Clinical Status

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Abstract: ***Background***: The nanomedicine is considered as the application of nanotechnology in the medical field where nanoparticles are sized in the nanoscale range. Drug delivery technologies are becoming increasingly important as a scientific area of investigation. Controlled-release systems and drug-targeting systems represents an alternative to traditional delivery nanoparticles, and the use of polymers is increasing nowadays. Although polymers could be classified as excipients, they are capable of modifying the biopharmaceutical and biokinetic behaviour of the transported active molecule increasing its efficacy and stability, and reduced cytotoxicity on healthy peripheral tissues. ***Methods***: The goal of this work is to collect and analyse the most current polymeric nanoparticles development as controlled-release and drug-targeting systems in cancer, infectious diseases and immunomodulation areas, as alternatives to conventional therapies. ***Results***: This review provides an update on the polymeric nanoparticles development analysing the trend of polymeric-based drug delivery systems, future opportunities and challenges of this fast-growing area. ***Conclusion***: With the thorough comprehension of biological effects depending on structure, it is possible to design specific systems for specific diseases, treatments and patients. The ability of polymer-based nanoparticles to modify and improve pharmacokinetics and pharmacodynamics, associated to techniques for enhancement of the therapeutic efficiency with minimal side effects, demonstrate the advantages of these systems.

Keywords: Nanomedicine, polymeric nanoparticles, drug delivery systems, controlled release, drug targeting.

1. INTRODUCTION

The definition of "nanotechnology" remains a subject of controversy, with no universally accepted classification. The criteria for a particle to be considered within the scope of nanotechnology has been traditionally determined on the basis of size, shape, physicochemical properties, quantum properties, as well as function and final application [1, 2].

Nanomedicine is considered as the application of nanotechnology in the medical field, *i.e.* the application of nanotechnology-based therapeutics and imaging agents for diagnosis, monitoring, prevention and treatment of chronic-degenerative or infectious diseases [3]. The convergence of nanotechnology and medicine is currently at an early stage, but is expected to have a revolutionary impact on healthcare. Advances in genetics, proteomics, molecular and cellular biology, material sciences and bioengineering have all contributed to the development of this field [4].

Most nano-based pharmaceuticals can be found in sizes well below 100 nm. However, this cut-off does not contemplate the use of excipients that alter release time and efficacy, as well as resulting in an increase of the particle size.

Unique physicochemical behaviour often emerges from nanomaterials with defining features of greater than 100 nm. Thus, a construct is loosely classified as a nanomedicine if it has at least one dimension in the nanoscale range (*i.e.*, measured in nanometers, <1000 nm) and exhibits novel properties based upon those dimensions [4, 5].

Novel properties arise more than just from their quantum-mechanical nature. In medicine, unique properties of nanoparticles are more notable when dealing with drug bioavailability, which is often increased due to the high relative surface area, controlled release of transported drugs as well as active or passive uptake. One clear example is the passive uptake of nanoparticles with sizes typically in the aforementioned range by target sites through the enhanced permeability and retention (EPR) effect, which is better explained in another section of this review. Moreover, nanoparticle characterization and *in vivo*/*in vitro* evaluation in pre-clinical trials are important to predict the further physiological behaviour, in absence or presence of disease.

1.1. Polymers in Medicine

The most exciting field in nanomedical research has been the design and development of multifunctional nanoparticles (NP). There is an impressive number of possible design combinations around single elements to develop personalized medicine that can have diagnostic/therapeutic agents (theranostics) incorporated into their structure simultaneously. Some of those elements may also serve as vectors with high specific recognition for target molecules, opening up the possibility for targeted therapy.

Polymers have a highly relevant role in medicine. In recent decades, polymers have been widely used in biomedicine due to their excellent properties such as biodegradability and biocompatibility. Since the interaction between polymeric materials and biological systems occurs in a wide range of molecular processes, there is no a precise definition for a biocompatible material or an accurate measurement of biocompatibility. Nevertheless, the polymeric materials will be considered as biocompatible as long as they perform their function with an appropriate host response, without eliciting any undesirable local or systemic effects [6, 7].

In general, biomaterials with medical applications must (1) not evoke a sustained inflammatory response; (2) possess a degradation time coinciding with their function; (3) have appropriate mechanical properties for their intended use; (4) produce non-toxic degradation by-products that can be readily reabsorbed or excreted; and (5)

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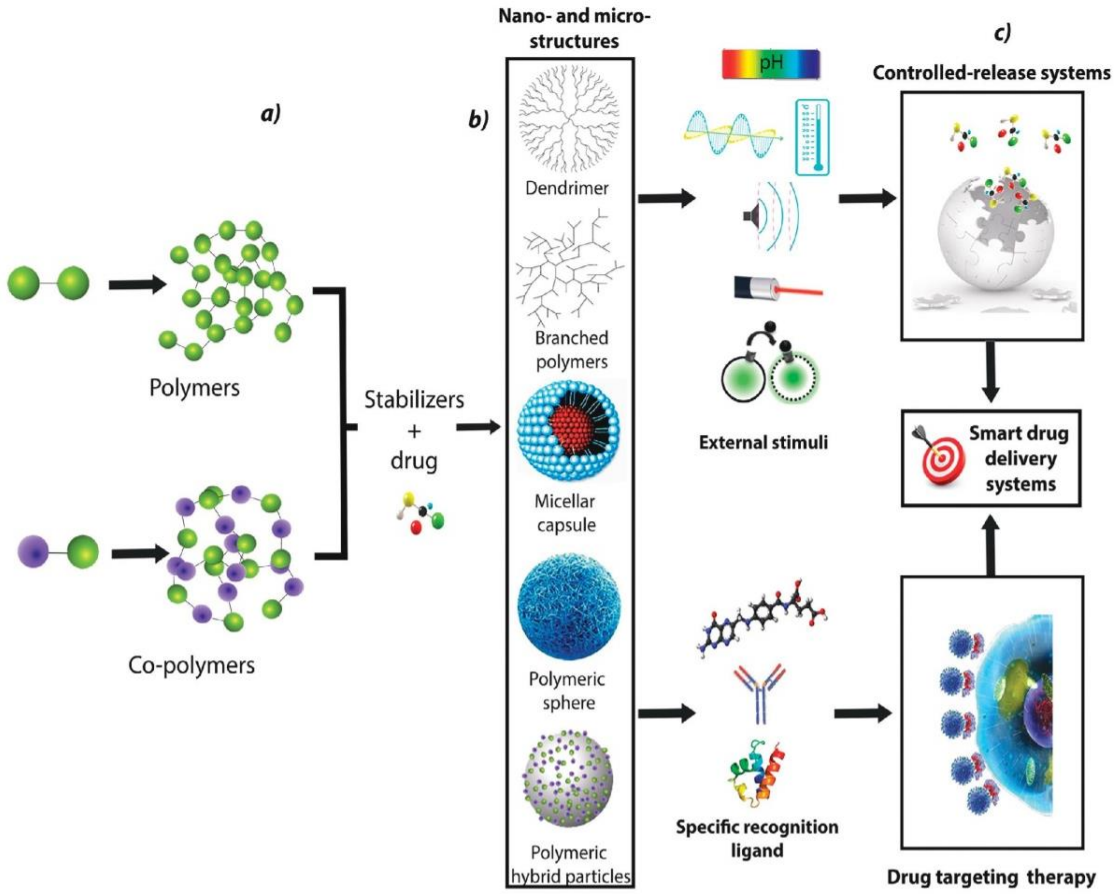

Fig. (1). Schematic representation of polymeric drug delivery systems showing examples of **a**) main components, **b**) representative structures and **c**) ideal smart drug delivery system built from controlled-release and drug targeting systems.

have appropriate permeability and processability for its intended application [8, 9].

These so-called biopolymers are promising in some fields such as regenerative medicine, tissue engineering and therapeutic applications. Table **1** presents examples of some polymers and their medical applications in drug delivery system development and tissue engineering.

Despite the progress achieved in medical fields, the complexity of the biological systems and their components limit advances in the understanding of the interactions between polymers and biological systems [10]. However, over the past 2 decades, more than 40 nanomedicines have been approved for routine human use, and many others are currently in pre-clinical and clinical development stages [11].

1.1.1. Polymers in Drug Delivery

Drug delivery systems have become one of the most important and active applications of these nanomedicines, used to transport drugs to a target organ or molecule for therapeutic intervention, including liposomes, organic and inorganic nanoparticles, polymer-based therapeutics, micelles, nanocrystals, carbon nanotubes, etc. [12].

Polymeric controlled-release and drug-targeting systems represent an alternative in the enhancement of the therapeutic benefit of current drugs. Although polymers are considered excipients in pharmaceutics because they do not have a specific therapeutic activity, they are able to modify the biopharmaceutical and biokinetic behaviour of the transported molecule. New formulations claim to improve drug effects by preventing the drug from interacting with other molecules that could sequester it or alter its chemical structure, thereby limiting its pharmaceutical action. Besides, they have the ability to transport and deliver drugs to target cells with greater efficacy, reduced cytotoxicity on peripheral healthy tissues and, in some cases, increase the stability, solubility and biodistribution of the transported molecule [13-15].

In drug delivery systems, active biomolecules can be embedded or covalently bonded to the polymeric carriers to facilitate drug transportation to the target site. Furthermore, in order to improve drug delivery characteristics, stimuli-responsive polymers may be used to develop "smart" drug delivery systems which are able to respond to different stimuli such as temperature, pH, magnetic fields, ultrasonic waves, light, feed-back regulated release, etc. (Fig. **1**). The goal of these systems is to lead the drug inside the target organ or tissue, independently of site and administration methods, with controlled release in a specifically selected location and time, with minimal or absent concentration in other non-target organs and tissues, reducing side effects by passive accumulation [16, 17].

Table 1. Medical application of polymeric nanosystems.

Polymer	Application	Example	Refs.
Poly(L-lactic acid) (PLLA)	Promising in drug delivery.	A self-assembled bovine serum albumin–PLLA NP for delivery of betulinic acid, as a model drug.	[18]
Poly(lactide-co-glycolide) (PLGA)	Drug delivery and tissue engineering scaffolds.	Delivery of chemotherapeutics such as docetaxel and doxorubicin, and biomolecules such as siRNA, insulin, and other drugs.	[19-21]
		Nanostructured anti-bacterial films for skin regeneration with alkaline treatment of the PLGA membrane.	[22]
Poly(ortho-esters) (POE)	Drug delivery	Nanoparticles that contain vancomycin hydrochloride, a hydrophilic antibiotic.	[23]
		Oral delivery of chemotherapeutics with high efficiency.	[24]
Polyurethanes (PU)	Drug delivery	Doxorubicin-loaded NP with thermo- and pH-sensitive properties.	[25]
Polyphospha-zenes	Drug delivery	Nano/microfibers containing antibiotics such as fluoroquinolone, ciprofloxacin or norfloxacin.	[26]
Poly (anhydride ester) (PAE)	Creation of prodrug polymers.	A salicylate-based PAE microsphere employed as a carrier to encapsulate and deliver insulin. In addition, anti-inflammatory activity was observed when salicylic acid was released from the microspheres.	[27]
		A PAE composed of EDTA and antimicrobial agent (*i.e.*, carvacrol, thymol, or eugenol) with antioxidant and antibacterial activity.	[28]
Poly(ethylene glycol) (PEG)	Drug delivery and tissue engineering.	PEG is commonly used to cap (PEGylation) or coat other degradable polymers to convey steric stabilization, limiting the interactions between the device and the host.	[9]
Collagen	Drug delivery.	Collagen-coated PLA microspheres for the delivery of imatinib mesylate in cancer treatment.	[29]
Poly(γ-glutamic acid) (γPGA)	Drug delivery and anti-cancer treatment.	Chitosan/γ-PGA NPs as vehicles for diclofenac to stifle local inflammatory reactions.	[30]
		Low molecular weight γ-PGA as a novel adjuvant material for use in cancer vaccines when it is immunized with ovalbumin as a model antigen.	[31]
		γ-PGA treatment enhances apoptosis in colorectal cancer cells, in part by modulating the activity of the COX-2 and AMPK signalling pathways.	[32]
Chondroitin sulphate (CS)	Drug delivery and treatment of cartilage tissue engineering	Doxorubicin-loaded CS NP in combination with deoxycholic acid –a bile acid- or chitosan for anticancer treatment.	[33, 34]
		Terbinafine- loaded hydroxyapatite nanowhiskers were encapsulated with CS microspheres as a colon targeted drug delivery system.	[35]
		Use of AuNPs for enhancing the delivery of CS in osteoarthritis treatment.	[36]

1.2. Polymeric Nanoparticle Characterization

1.2.1. Physicochemical Characterization

The complexity of nanoparticles as a multi-component three-dimensional construct requires careful design and engineering, detailed orthogonal analysis methods and a reproducible scale-up and manufacturing process to achieve a consistent product with the intended physicochemical characteristics, biological behaviours, and pharmacological profiles. The desired product is heavily dependent upon the biological application of the nanoparticles in keeping with the objectives of its development [37].

There are a wide variety of methods for determining nanoparticle size distributions, including light scattering, differential mobility analysis, time of flight mass spectroscopy (TOF-MS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), among many others. Both dynamic light scattering (DLS) and static light scattering (SLS) have been used extensively to analyse size and the polydispersity index (PI). Although these techniques provide excellent information about size, microscopy is used as a complementary method to evaluate not only size, but also shape and morphology. For nanoparticles, electron microscopy is normally required to capture images and is the only technique that provides reliable information regarding shape at this scale. However, optical techniques and atomic force microscopy (AFM) can be employed to investigate particle morphology as well (see Fig. **2**) [37, 38].

Complementary tools and analytical procedures have been used to obtain reliable information on engineered systems. For example, Francis *et al.* set up an analytical method based on an ion-milling system for sample preparation followed by particle imaging with a field emission scanning electron microscope (IM-FESEM). This last approach made it possible to visualize the internal morphology of drug-loaded polymeric nanoparticles [39].

Classically, the surface charges of particle systems are approximated through zeta potential measurements. Xu R. reported three methods for zeta potential determination of suspended particles, viz., electrophoretic light scattering (ELS) (acoustic and electroacoustic), concluding ELS is the best choice for many applications due to its sensitivity, accuracy and versatility [40].

Surface area is normally analysed through gas adsorption using the Brunauer-Emmett- and Teller (BET) method or surface titrations (wet chemical) and aerosol diffusion chargers. On the other hand, compositional surface analysis techniques of nanoparticles include Auger electron spectroscopy (AES), electron energy loss spectroscopy (EELS), X-ray photoelectron spectroscopy (XPS), electron spectroscopy for chemical analysis (ESCA) and secondary ion mass spectroscopy (SIMS) [38, 41]. Fig. (**2**) summarizes the main characteristics of particle size measurement and common surface analysis techniques.

Other techniques have also been used in polymeric nanoparticle characterization, including differential scanning calorimetry and X-ray diffraction, to reveal the amorphous state of the drug in nanoparticles, and Fourier-transform infrared spectroscopy to detect interactions between the drug and polymer at a molecular level [42].

1.2.2. Biological Characterization

Another important and challenging area of nanoparticle characterization is its biological behaviour. Despite the fact that there is a lot of interest and effort being put into the development of nano-based biomedical applications, the level of clinical output remains

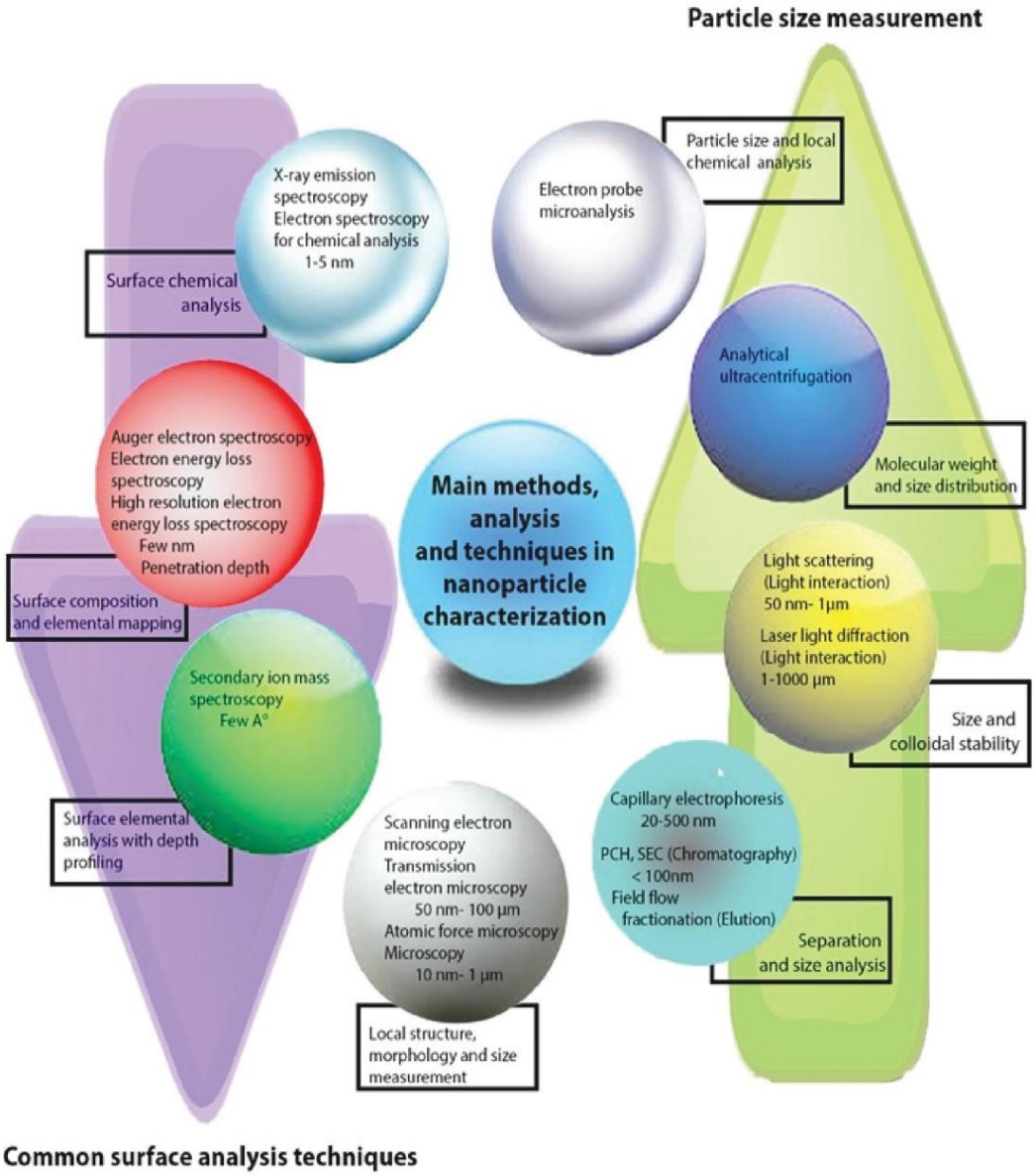

Fig. (2). Main methods, analysis and techniques for particle size measurement and common surface composition of polymeric nanoparticles; common surface techniques show the range of adequate resolution.

limited due to uncertainty in the toxicological profiles of nanoparticles. The initial screening for toxicity is preferably performed *in vitro*. However, extrapolation to the *in vivo* outcome remains very challenging [43].

In early development stages, verification that nanoparticles are able to release incorporated drugs in order to achieve a biological effect is needed. Thus, *in vitro* drug release analyses are necessary, in which consideration of the environment in which the nanoparticles will be released is important.

Most researches have simulated physiological fluid using phosphate buffer solution (PBS) at different levels of pH. The released drug has been analysed using different analytical techniques, such as HPLC and fluorescence, across various sample times whilst undergoing continuous mechanical stirring, in order to provide simulated physiological conditions. It is also possible to simulate other conditions, such as tumour microenvironment, where pH levels are often lower than the rest of the body, and in this manner, evaluate nanoparticle drug release behaviour. This is especially the case for polymers with pH-sensitive properties [44-46].

The new formulation must also be evaluated in certain types of cells in order to analyse its specific action (*e.g.* toxicity, cellular uptake and viability). Grabowski *et al.* investigated *in vitro* PLGA-based nanoparticle toxicity using a variety of endpoints, such as impact on mitochondrial activity, induction of oxidative stress (production of reactive oxygen species), induction of apoptosis/necrosis and inflammatory response (secretion of pro-inflammatory cytokines), towards macrophages [47].

Cellular uptake of nanoparticles has been extensively studied due to its relevance in achieving the desired effect. Use of a specific cell type to evaluate uptake depends upon the aim of the analysis. For example, cancer cell linessuch as HeLa and Caco-2 have been used to assess nanoparticles for chemotherapy applications. However, a microbiological approach must also be considered due to the recent efforts to enhance antibacterial activity. Thus, microorganisms such as *Salmonella typhi* have been studied *in vitro* for cellular uptake and nanoparticle activity. Techniques to study cellular uptake and intracellular localization of nanoparticles include the measurement of fluorescence intensity using confocal laser scanning microscopy imaging, flow cytometer, cryo-SEM, TEM and optical density measurement [42, 44-46].

Measurement of cell viability and proliferation forms is the basis for numerous *in vitro* assays of a cell population's response to external factors. Cell viability is assessed by methods that measure loss of membrane integrity, as well as loss of metabolic activity, anti-tumour activity, cytotoxicity and mitochondrial activity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is an example of a widely accepted test used to examine cell proliferation [41, 45, 48, 47].

Other important *in vitro* assessments are required for conventional pharmaceuticals such as haemolysis, complement activation, immune response activation and thrombogenicity. Neun and Dobrovolskaia described a simple and reliable method for analysis of nanoparticle haemolytic properties *in vitro* [47].

Nanoparticle interaction with the immune system can be analysed in cell-based assays on dendritic cells or immune organs such as lymph nodes. Thrombogenicity is examined initially through induced platelet aggregation and effects on blood coagulation in a series of biochemical tests, such as prothrombin time, activated partial thromboplastin time, thrombin time or whole-blood cell clotting time [45-46].

Early *in vivo* studies should include examination of nanoparticle effects on organs (such as the liver, lungs, heart and kidney) and the immune system. The most recent FDA guidance for immunotoxicity assessment of human pharmaceuticals, ICH S8, recommends an initial *in vivo* study evaluating haematology, clinical chemistry, gross pathology, immune organ weights and histology. This is to be followed by examination of immune cell function for those cases in which the preliminary study indicates potential immunotoxicity. It is important to mention that *in vivo* biodistribution and toxicity studies establish safety for clinical trials, and all pre-clinical characterization schemes must necessarily include *in vivo* determination of nanoparticle pharmacokinetics, ADME process (absorption, distribution, metabolism and elimination) and toxicity in animal tests [41].

1.3. Passive and Active Drug Targeting

The ideal conception of the use of drugs is limited by its ability to selectively accumulate in the pathological compartment. Actually, only a small portion of the administered dose reaches the target site and promotes its therapeutic effect. Therefore, researchers have resorted to finding strategies for a selective accumulation of the drug in the target site (see Fig. **3**). In this sense, "drug targeting" can provide a high concentration specifically at the site of action without passive accumulation in healthy tissues [49, 50].

The concept of "drug targeting" can be viewed as the recognition and binding of the drugs with their target site, providing a therapeutic effect or diagnostic use. In certain cases, several physicochemical and physiological features of the target area may be utilized for successful drug targeting (*e.g.*, pathological sites have an affected and leaky vasculature).

Two basic requirements should be considered in the design of nanocarriers to achieve effective drug delivery. First, drugs should be able to reach the desired action sites after administration with minimal loss of their concentration and activity in blood circulation. Second, drugs should only kill damaged cells without harmful effects to healthy tissue [51].

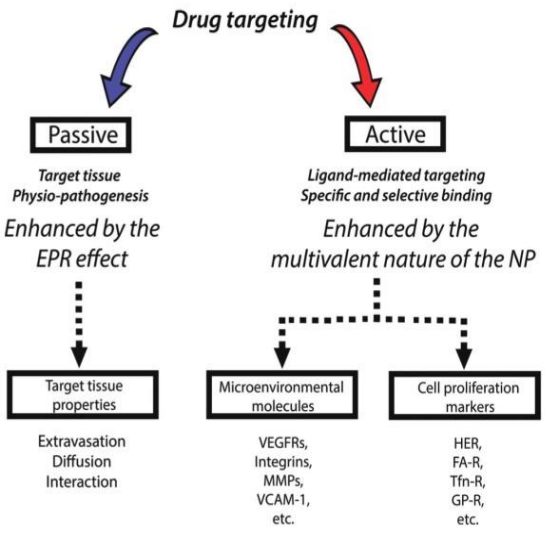

Fig. (3). Strategies for a selective accumulation of drugs in a specific site.

1.3.1. Passive Targeting

Passive targeting is well illustrated by the singularities that occur in tumours. When a solid tumour reaches a given size, the surrounding vasculature is not sufficient to provide all the oxygen supply required for its further proliferation. Angiogenesis promotes the rapid development of new, unorganized and fenestrated blood vessels that offers little resistance to the extravasation of nanoparticles to the tumour space, allowing the enhanced permeability and retention (EPR) effect. Passive targeting takes advantage only from the physio-pathological properties of the target tissue. The distribu-

tion of molecules in a tumour is governed by at least three phenomena: (1) the extravasation of colloids from the blood vessels, (2) their further diffusion through the extravascular tissue, and (3) their interaction with intracellular and/or extracellular targets within the tumour environment [52, 53].

However, the passive strategy is further limited because certain tumours do not exhibit an EPR effect and the permeability of vessels may not be the same throughout a single tumour.

1.3.2. Active Targeting

Active targeting, also called ligand-mediated targeting, relies on the use of target ligands (usually referred as a targeting moiety of a vector molecule such as antibodies, proteins, peptides, nucleic acids, sugars or small molecules such as vitamins) on the surface of nanomedicines. These recognize and bind selectively and specifically to target cells through ligand–receptor interactions involving the receptors or epitopes on certain organs, tissue, and cell or subcellular domains [52, 54].

In order to achieve high specificity, those receptors should be overexpressed in unhealthy cells, but not in normal cells. Furthermore, the receptors should be homogeneously expressed in a specific site and should not be circulating in the bloodstream. Additionally, for intracellular drug release, targeting conjugates must be internalized. The transport can also occur through by receptor-mediated mechanism or by means of internalized molecules linked to drugs, analogous of the "Trojan horse". For example, internalizing TAT-peptide [55, 56].

The interactions of ligand- functionalized NP systems with their target receptor are enhanced by the multivalent nature of the NP. However, specific density of cell receptors and ligands, three-dimensional architecture of nanoparticles, the ligand conjugation chemistry and the types of ligand available are determinant factors that define the adequate and complementary strategy for *in vivo* efficacy of the actively targeted systems [52].

2. DEVELOPMENT AND PRE-CLINICAL EVALUATION

2.1. Polymeric Nanoparticles in Cancer Chemotherapy

Every cancer type requires a specific treatment regimen such as surgery, and/or radiotherapy, and/or chemotherapy. The small size, the ability to encapsulate various drugs, the sustained release mechanism provided by the polymer characteristics, the efficient navigation of the complex *in vivo* environment, the increased uptake given by the EPR effect and the possibility to modify the nanoparticle surface with ligands, could represent great advantages of polymeric nanoparticles, making them a different manner of treatment, potentially superior to conventional cancer therapies, by increasing the therapeutic benefits and diminishing the chemotherapy-related severe side effects.

Most of the targets are over-expressed in cancer processes and this condition could be exploited by using targeting molecules. Tumour-induced angiogenesis allows tumour survival, growth and eventually, metastasis. Anti-angiogenesis approaches are based on the identification of microenvironmental molecules developed in tumour neovasculature that are highly inhibited or absent in epithelial cells from pre-formed blood vessels. The main angiogenic targets explored include: (1) the vascular endothelial growth factor receptors (VEGFRs), (2) integrins (endothelial cell receptors for extracellular matrix proteins harbouring the RGD sequence); (3) matrix metalloproteinase receptors (MMPs) and (4) vascular cell adhesion molecule-1 (VCAM-1), expressed on the surface of endothelial cells during inflammation and cancer. On the other hand, cell proliferation markers are significant targets due to the fact that many of them play an important role in dysregulation of pathway networks. These are overexpressed on certain tumour cells and include: (1) human endothelial receptors (HER), including the epidermal growth factor receptor (EGFR) and HER2; (2) transferrin receptors and folate receptors; (3) glycoproteins, lectins and carbohydrates from cellular surfaces [57, 58].

Overexpressed receptors in breast cancer have allowed the development of different systems targeted to different receptors. Vivek *et al.*, reported a PVP-PLGA nanoparticle conjugate with herceptin promoting the site-specific intracellular delivery of Tamoxifen through the HER2-receptor. This system could improve therapeutic efficiency by enhancing the cancer cell targeted delivery and sustained release of the therapeutic agent [59]. Kulhari *et al.* [60], described the bombesin peptide (BBN) conjugated, docetaxel-loaded PLGA-nanocarrier as a promising carrier for active targeting of the gastrin-releasing peptide (GRP) receptor and finally, Tavassolian and co-workers created a folate (FA)-conjugated poly (L-γ-glutamyl glutamine) (PGG) nanoparticle loaded with DTX, showing that this system was highly effective and successfully localized at the tumour site [61].

Besides, Gao *et al*., utilizing a PEG-PCL delivery system, and Guo *et al*., using a PEG-PLGA system, both reported functionalised nanoparticles using the aptamer AS1411, a DNA aptamer that specifically binds to nucleolin, which is highly expressed on the plasma membrane of both cancer and endothelial cells in angiogenic blood vessels, as the targeting ligand to facilitate anti-glioma drug delivery [62, 63]. Meanwhile, Gao *et al*., used PEG-PCL functionalised nanoparticles with another aptamer (GMT8), which enhanced tumour penetration and served as a targeted glioblastoma therapy [64]. Added to this, the targeting towards these types of cells can also be obtained by using the peptide sequence RGD, as reported by Gao *et al*. who functionalised PEG-PCL nanoparticles with RGD and interleukin-13 peptide to construct a neovasculature and tumour cell dual targeting delivery system in which the RGD sequence could target $\alpha_v\beta_3$ on neovasculature and the interleukin-13 peptide could target IL13Rα2 [65].

The peptide sequence RGD has also been reported in the targeting of PLGA-PEG/Paclitaxel nanoparticles [66], PLGA-PEG/ Cisplatin nanoparticles [67], PLGA-mPEG-PEG-cholesterol/Curcumin nanoparticles [68], PCB-b-PDPA/Doxorubicin nanoparticles [69], among others, representing excellent formulations targeted to different types of cancer (tumour endothelium, breast cancer, melanoma, hepatocellular carcinoma and cervical cancer).

Despite the extensive research on nanoparticle systems for cancer therapeutics (see Table **2**), there are only a few nanoparticle drug delivery systems approved by the U.S. Federal Drug Administration and European Medicines Agency to treat cancer, and among these approved systems, a lesser number are polymeric nanoparticles [57].

Various combinations of different schemes, such as nanoparticle surface modification with targeting ligands and encapsulation (or conjugation) of one or more existing therapeutic agents in polymers, has permitted the rise of more specific and improved polymeric nanoparticle systems to treat cancer with excellent perspectives. Diverse polymeric nanoparticle systems are currently being developed (see Table **3**) and are anticipated to be tested in clinical trials in order to assess their effectiveness in human cancer therapy.

Besides, new approaches have begun to take place in cancer, such as theranostic agents. Du *et al,* described the research of a novel theranostic agent composed of poly (lactic acid) (PLA) nanoparticles that is targeted to tumour vasculature endothelium (TVE) with the peptide GX1, a TVE-specific ligand. At the same time, Endostar was used as an anti-angiogenesis agent and the near-infrared dye IRDye 800CW, as a diagnostic agent. Data showed a specific target delivery and an improved anti-tumour efficacy viewed through bioluminescence imaging in a subcutaneous colorectal tumour-bearing mouse model. However, to fully exploit the targeting potential of these NPs, their uptake mechanism in various cell systems needs to be fully understood and their biodistribution

Table 2. Examples of current polymeric nanoparticles for cancer therapy, in early studies.

Polymeric NP	Therapeutic	*In vitro*/*In vivo* cancer test	Refs.
Dextran-poly(ethylene imine)	DOX	MCF-7 cells	[70]
mPEG–PLGA and mPEG–PLA	OA	A549 and HepG2 cells	[71]
PEG-PHB	L-glutaminase	HeLa cells	[72]
(PLG-*g*-mPEG5K)	Cisplatin	*In vitro*: HeLa cells; *in vivo*: LLC model	[73]
CTS/CS	Curcumin	A549 cells	[74]
CTS	siRNAs	Breast cancer cells	[75]
BSA	Albendazole	*In vitro*: OVCAR3 and SKOV3; *in vivo*: OVCAR3 ovarian cancer xenograft model	[76]
NA GNs-FA (targeted)	Cisplatin	HeLa cells	[77]
ANG-PLGA (brain-targeted)	DOX and EGFR-siRNA	*In vitro*: U87MG cells; *in vivo*: brain orthotopic U87MG glioma xenograft model	[78]
Protamine-PLGA-*b*-PEG-*b*-PLGA	PTX	HepG2	[79]
HA-PLGA	DTX and tanespimycin	*In vitro*: MCF-7, MDA-MB-231, and SCC-7 cells; *in vivo*: SCC-7 xenograft model	[80]
Glycosaminoglycan-PLGA (TME-targeted)	None	*In vitro* cellular uptake: A549 and HPMEC cells	[81]
CTS-coated PCL	Curcumin	*In vitro*: B16F10 melanoma cells; *in vivo*: murine model of experimental metastasis	[82]
IRDye 800CW loaded-GX1-PLA (theranostic agent TVE-tageted)	Endostar	*In vitro*: HUVEC cells; *in vivo*: subcutaneous colorectal tumour-bearing mouse model	[83]
PLH–PLGA–TPGS	DOX	MCF-7 and MCF-7/ADR cells	[84]
mPEG-DOX (self-assemble NP)	Verapamil	MCF-7/ADR cells	[85]
PLGA	DDX3 helicase inhibitor RK-33	MCF-7 Cells	[86]
mPEG-PLA - apigenin/ apigenin PbAE polymers	Flavonoid (Apigenin)	Long-term release of flavone-based polymer, produces inhibition of tumour and cell adhesion.	[87]
Zwitterionic polyphosphoesters–PLA NPs	DOX	Systemic delivery of DOX by zwitterionic polyphosphoester-stabilized NP significantly inhibited tumour growth in a MDA-MB-231 tumour model.	[88]

in both the absence and presence of a tumour burden needs to be evaluated [11].

On the other hand, in cancer immunotherapy, a safe and effective therapeutic development has been described. In particular, vaccination with a dendritic cell (DC) platform has been used as a promising cell-based therapeutic modulator, which successfully delivers tumour specific antigens to lymphatic organs and enhances the immune response for cytotoxic T cells. Polymeric nanoparticle delivery systems containing antigens with immunostimulatory molecules (adjuvants) not only have been proposed to increase antigen-presentation by DCs, but also to provide immune activation and rescue impaired DCs from tumour-induced immunosuppression, allowing the development of a broad armamentarium of targeted drugs against specific immune cells [89, 90].

Meanwhile, Roy *et al.* developed a combined chemo-immunotherapeutic formulation, composed by PLGA-based NPs containing Paclitaxel (PTX) and SP-LPS (non-toxic derivative of lipopolysaccharide), which could directly kill cancer cells as well as activate the immunosuppressed tumour microenvironment to mount a robust anti-tumour immune response [94]. *In vitro* studies suggested that nanoparticles containing PTX and SP-LPS had both direct cytotoxicity and immunostimulatory activity. Hence, this might have potential as a chemo-immunotherapeutic formulation against cancer with advantages over actual chemotherapy in terms of tumour targeting, less toxicity and immunostimulation.

In the same context, Zang *et al.,* evaluated PLGA-nanoparticles containing murine melanoma antigenic peptides (hgp10025e33 and TRP2180e188) and reported to induce cytotoxic T lymphocyte responses efficiently against tumour-associated self-antigens in mouse models. An efficient uptake of PLGA-NP by murine dendritic cells was shown. Furthermore, the anti-tumour activity of NP-mediated peptide vaccination was significantly augmented by combined treatment with interferon-γ, which may prevent tumour escape through up-regulation of MHC class I expression on tumour cells. This system demonstrated the feasibility of NP-mediated antigen delivery in cancer immunotherapy [91].

Finally, Yao *et al.* reported a novel anti-tumour activity nanopolymer for interleukin-2 (IL-2) delivery, consisting of low molecular weight polyethylenimine linked by b-cyclodextrin and conjugated with folate (named H1). Peritumoral injection of these nanoparticles suppressed the tumour growth and prolonged the survival of C57/BL6 mice bearing B16–F1 melanoma grafts. Additionally, they showed that this system stimulated the activation and proliferation of CD8+, CD4+, T cell and natural killer cells in peripheral blood, and increased their infiltration into the tumour environment. Authors suggested that this treatment could be an effective and safe gene therapy strategy for melanoma treatment [92].

2.2. Polymeric Nanoparticles in Infectious Diseases

According to the World Health Organization, infectious diseases are disorders caused by pathogenic microorganisms [101]. The treatment must be specifically dependent upon the type of microorganism that causes the disease and must include antibiotics, antivirals, antiparasitics and antifungals. Existing treatments have shown effectiveness against certain microorganisms. However, issues related with drug toxicity and multidrug resistance can lead to therapy failure; therefore, the development of new therapy strategies are needed in order to enhance drug efficiency and reduce side effects. Polymeric nanoparticles present an alternative to resolve these antibiotic drug drawbacks.

2.2.1. Antifungal NPs

Fungi became important human pathogens in the late 20th century, when an increase in the frequency of infection was recorded as a consequence of medical interventions or related to HIV infections. Additionally, antifungal resistance has been the primary concern in invasive infections with the fungus *Candida* [102].

One of the most promising drugs for the treatment of opportunistic fungal infections is itraconazole (ITZ). However, its low oral bioavailability and the side effects found in commercial intravenous formulations have lead to the development of new formulations. Qiu et al., formulated biodegradable, ITZ-loaded D-α-tocopheryl polyethylene glycol 1000 succinate-b-poly(e-caprolactone-ran-glycolide) (TPGS-b-(PCL-ran-PGA); TPP) nanoparticles (designed as ITZ-loaded TPP NPs) to improve the antifungal efficacy. The *in vivo* and *in vitro* antifungal activity of ITZ-loaded-TPP NPs was evaluated using *Candida albicans*, and in both cases, ITZ-loaded-TPP NPs achieved a high level of antifungal activity, as well as an improvement in the ITZ bioavailability by increasing its aqueous dispensability and extending the duration of drug release [103].

Another important antifungal agent commonly used in therapy against severe systemic infections is amphotericin B (AmB). Nevertheless, dose-limiting adverse events are associated with its use, principally renal insufficiency and hematologic toxicity. Recently, polymeric formulations have beenproposed. For example, Casa *et al.* proposed poly(lactide) nanoparticles containing AmB, and Souza *et al.* developed a new formulation for AmB entrapped within PLGA-dimercaptosuccinic acid nanoparticles (NANO-D-AmB). The formulation of PLGA-AmB-loaded nanoparticles (225 nm) was as effective as free AmB against strains of *Candida spp*, with a more sustained release profile (30 % of AmB over 30 days), considerable reduction in toxicity and a very low index of haemolysis, all characterstics associated to the polymeric NP formulation [104, 105].

The NANO-D-AmB particles revealed the presence of a high amount of ^{99m}Tc-NANO-D-AmB in the spleen and lung of healthy mice models, with suitable antifungal efficiency against paracoccidioidomycosis. Furthermore, the entrapment of AmB within nanoparticles reduced its presence in kidneys, which is a relevant fact as it is expected to reduce the nephrotoxicity. Additionally, NANO-D-AmB treatment induced higher levels of IL-12, a pro-inflammatory cytokine that is known to present a protective property against fungal infection, suggesting a potential role of the nanoparticles in modulating the immune response for the host's benefit.

Tang *et al.,* meanwhile, reported on the preparation of AmB-loaded, biodegradable random copolymer nanoparticles of TPGS and PGA (PLGA-TPGS-AMB NPs) for fungal infection treatment. The formulation showed significantly higher levels of antifungal effects than water-suspended AmB, as well as an increase in bioavailability through enhanced aqueous dispersity. *In vitro* and *in vivo* analyses demonstrated a stronger protective effect against candidiasis and a gain in prolonged antifungal efficacy. Authors reported suitable drug delivery systems; however, future clinical trials must be carried out in order to ensure adequate security and effectiveness of drug-polymeric formulations [106].

Recently, pH-responsive, AmB-loaded and surface charge-switching nanoparticle systems were developed by Tang *et al*. Synthetized poly(D,L-lactic-co-glycolic acid)-b-poly(L-histidine)-b-poly(ethylene glycol) (PLGA-PLH-PEG) nanoparticles were modified with anti-*Candida albicans* antibody (CDA) (CDA-AmB-NPs) to increase the targetability. The results demonstrated a significant improvement of bioavailability, targetability and a considerable reduction of the toxicity of AmB with consequent improvement in its antifungal activity. Authors suggested further experimentation in order to establish the specific molecular mechanism, targetability, release mechanism and efficiency of the loaded drug in fungi-infected lesions [107].

As can be noticed, amphotericin B has been extensively studied and there is a wide variety of systems developed in order to increase its efficacy and diminish side effects. Table **4** summarizes some nanoparticle systems developed for Amphotericin B with antifungal or antiparasitic purposes.

2.2.2. Antiparasitic NPs

Some parasitic diseases are not easily treated due to the low bioavailability and high toxicity of current drugs. A clear example are drugs for the treatment of Human African Trypanosomiasis (HAT). Currently, there are only five licensed drugs for the treatment of HAT, namely pentamidine, suramin, melarsoprol and eflornithine.

Arias *et al.* have taken advantage of the highly active endocytosis process in *Trypanosome brucei* and formulated a novel HAT treatment based on PLGA drug delivery NPs. The NPs (145 nm) were conjugated to a nanobody (NbAn33) that specifically recognizes conserved cryptic epitopes on the parasite surface, leading to an active targeting by the model drug pentamidine. The effectiveness was evaluated *in vitro* using *T. brucei*. A 7-fold reduction in the half inhibitory concentration (IC_{50}) of NbAn33-pentamidine-PLGA NPs, was observed. The *in vivo* efficacy of NbAn33-pentamidine-PLGA NPs was assayed in an acute murine model of African trypanosomiasis showing total recuperation of all infected mice with the curative dose diminished to a 100-fold of the minimal full curative dose of free pentamidine (2.5 mg kg^{-1} for free pentamidine). The NbAn33-pentamidine-PLGA NP formulation still showed curative activity in mice (60% survival). Authors concluded that nanobody conjugation was essential for the effectiveness of the formulations in both *in vitro* and *in vivo* experiments, representing a potential alternative therapeutic approach in anti-trypanosome therapy [116].

Amphotericin B has been also used against protozoan infections (see Table **4**), *e.g*. leishmaniosis. In this context, Asthana *et al.* developed two AmB nanoparticle devices. The first, AmB-lipid–polymer hybrid nanoparticles (AmB-LPNPs), by utilizing PLGA as a biocompatible polymer and stearylamine (Sta) as a lipid. The *in vivo* antileishmanial efficacy of AmB-LPNPs was studied against *L. donovani* amastigote-infected hamsters and the results clearly manifested significant parasite growth inhibition (89.4 ± 6.03% inhibition) with AmB-LPNPs. Data demonstrated a considerable amount of AmB in tissues (MRT= 57.2±10.6 h) and consequent slow elimi-

Table 3. Polymeric nanoparticles in cancer, clinical status.

Name, description	Indication	Clinical status	Refs.
Abraxane® (paclitaxel), NP albumin-bound (nab) platform	Breast cancer	Approved in 2005 by the FDA	[93]
Genexol-PM (paclitaxel), Polymeric micelle formulation composed of methoxy-PEG- PDLLA.	Breast and lung Cancers, metastatic breast cancer.	Approved in Korea. Phase II in US and Russia.	[58, 94, 96]
Oncaspar (asparaginase), Polymer–protein conjugate of PEG	Acute lymphoblastic leukemia	Approved and in clinical use.	[58]
SP1049C (doxorubicin), Pluronic L61 and F127 micelle	Adenocarcinoma of esophagus, gastroesophageal junction and stomach	Phase III	
CT-2103; Xyotax™; Opaxio® (paclitaxel), Poly(glutamic acid-conjugate)	Ovarian and other cancers and combinations	Phase III	[95, 96]
NK105 (paclitaxel), PEG-poly(aspartate)	Gastric (G) and breast (B) cancer	Phase I (G), Phase III (B)	
NKTR-102 (irinotecan), PEG-conjugate	Cancer-metastatic breast, ovarian, breast and colorectal cancer	Phase II/III	[58]
BIND-014 (docetaxel), PEG-PLGA, target to prostate specific membrane antigen	Castration-resistant prostate cancer and prostate cancer	Phase II	[97]
CRLX 101 (camptothecin), Cyclodextrin-PEG-NP	Non-small-cell lung cancer and various others	Phase II	[98]
NK911 (doxorubicin), PEG-poly aspartate micelle	Various solid tumours	Phase II	[96]
PEG-Intron (IFNα 2b), Polymer–protein conjugate of PEG	Melanoma, MM, and renal cell carcinoma	Phase I/II	[58]
PK1; FCE28068 (doxorubicin), HPMA copolymer-conjugate	Lung and breast cancer	Phase II	
NC-6004 (cisplatin), PEG–poly(γ-benzyl-L-glutamate) micelle	Solid tumours	Phase I/II	
CALAA-01 (Anti-RRM2 siRNA), Cyclodextrin-PEG-transferrin NP	Solid tumours	Phase I	[94, 99, 100]
Docetaxel-PNP, Polymeric nanoparticle	Various solid malignancies	Phase I	[99]
XMT-1001 (Fleximer technology) ® (camptothecin), polyacetal-conjugate	Gastric cancer, lung cancer	Phase I	[11, 99]
PNU166945 (paclitaxel), HPMA copolymer-conjugate	Various cancers	Phase I	[58]
AD-70, DOX-OXD (doxorubicin), Dextran-conjugate	Various cancers	Phase I	

nation from the body. The *in vivo* biodistribution study showed a low kidney distribution of AmB-LPNPs, demonstrating their low nephrotoxicity. A comparison of the *in vitro* macrophage uptake data and the *in vivo* spleen and liver distribution data obtained, clearly revealed that the higher the phagocytosis *in vitro*, the higher the accumulation in spleen and liver *in vivo*. Elevated macrophage uptake (by the mononuclear phagocytic system) of AmB-LPNPs, rapid plasma clearance and higher drug localization in macrophage-abundant liver and spleen illustrated their anti-leishmanial efficacy *in vitro* and *in vivo*, making them as a promising alternative to commercial AmB-formulations for the eradication of intra-macrophage diseases [117].

The second case refers to the cost-effective, reliable and safe system of the lactoferrin-appended amphotericin B-bearing nanoreservoir (LcfPGNP-AmB), useful in targeted eradication of *Leishmania donovani*, which showed reduced toxicity, increased protective pro-inflammatory mediator expression and down-regulation of disease-promoting cytokines. LcfPGNP-AmB showed augmented anti-leishmanial activity by significantly reducing (~88%) splenic parasite burden of infected hamsters, compared with commercial formulations [118].

Another relevant concern in parasitic disease is malaria, an illness caused by a parasite of the genus *Plasmodium*, taking into consideration the emergence of drug-resistant strains of *P. falciparum* and the prospective advantages of combined therapy, new antiparasitic nanosystem has been developed, based on two antimalarial drugs with different action mechanisms attached to biodegradable polymeric backbone. Kumar *et al.,* conjugated primaquine and dihydroartemisinin (two antimalarial drugs) to polyphosphazene with the purpose of obtaining a linked, combined drug-polymer conjugate and then develop them into nanoparticle formulations to increase their uptake and to achieve targeted drug deliv-

Table 4. Polymeric nanoparticles for drug delivery of Amphotericin B with antifungal or antiparasitic purposes.

Name	Description	Indication	Refs.
PLGA-PEG-AmB	NPs of AmB prepared by a modified emulsification-diffusion method with a vitamin E derivative as a stabilizer.	Effective anti-fungal and anti-leishmanialagent	[108]
PMA-AmB	Very low MWt amphotericin B-polymethacrylic acid nanoparticle.	Nebulised suspension for the prevention of invasive aspergillosis (profilaxis)	[109]
PGA-AmB	Self-assembled, biodegradable polyglutamic acid (PGA)-based formulation of amphotericin B (AmB).	Potent antimicrobial activity similar to that of Fungizone, against *C. albicans*.	[110]
Lecithin/ chitosan-AmB	Amphotericin-B-loaded nanoparticles for prolonged ocular application.	Antifungal susceptibility against *Candida albicans* and *Aspergillus fumigatus*.	[111]
AmB-SA-GCS-NP	AmB encapsulated by self-assembly sodium alginate-glycol chitosan stearate nanoparticles.	Delivery platform against leishmaniasis.	[112]
PLGA-PEG-AmB	Optimization of preparation by study of factors involved in AmB-containing NPs.	Antifungal activity.	[113]
IEO-AmB	Formulation incorporating AmB in poly(isoprene-b-ethylene oxide) amphiphilic block copolymer.	Possible antiparasitic and antifungal activity.	[114]
NQC-AmB	NP delivery system for AmB using a polyelectrolyte complexation technique with chitosan-chondroitin sulphate.	Anti-leishmanial drug-delivery system.	[115]

ery. *In vivo* anti-malarial efficacy was tested in Swiss albino mice infected with *Plasmodium berghei*. Complete elimination of the parasite and prolonged survival of animals was observed at the dose of 0.07+0.07 mmol kg^{-1}, administered for 4 days. It is interesting to note that this combination therapy (primaquine and dihydroartemisinin) in low dose, provided protection over 35 days without any recrudescence [119].

The results revealed that the addition of dihydroartemisinin could have resulted in a rapid decrease in parasitaemia. In summary, this polymer-drug conjugate combination regimen could be a novel drug delivery system effective in the radical cure of malaria.

2.2.3. Antimicrobial NPs

Just as previously described for polymeric nanoparticles, their antimicrobial applications are engineered based on existent drugs, biologicals or chemical compounds and the intrinsic antimicrobial activity of polymers. The goals of these new formulations are: diminishing non-specific toxicity, maximizing drug efficacy, optimizing pharmacokinetics and selectively targeting pathogens in a particular structure or in the whole body. The state of development of some polymeric nanoparticles suggested for antimicrobial applications are summarized in Table **5**.

For example, Water and co-workers explored the use of PLGA nanoparticles to improve the cellular uptake of plectasin, a defensin-class peptide, to treat *Staphylococcus aureus* infections in epithelial cells, with a focus on potential application in drug delivery to the epithelia in airways. *In vitro* dose-response was assessed in infected Calu-3 monolayers. The EC_{50} value was reduced significantly from 1.24 ± 0.15 uM for free plectasin to 0.80 ± 0.12 uM for the nanoparticles loaded with plectasin, showing an improved efficacy [120].

Authors suggested that encapsulation promoted the delivery of plectasin to bacteria, increasing bacterial death. Furthermore, uptake and intracellular localization of nanoparticles was studied *in vitro* in different human airway epithelial cell lines, *i.e*. bronchial cells (Calu-3), alveolar cells (A549), as well as THP-1 macrophages, showing significant uptake in both Calu-3 and THP-1 cells. These results demonstrated that plectasin-loaded PLGA nanoparticles may be taken up by human lung epithelial cells as well as by macrophages in the case of treatment of airway *S. aureus* infections.

On the other hand, Deacon *et al.* developed to bramycin-loaded alginate/chitosan NPs and demonstrated their antimicrobial efficacy using *in vitro* and *in vivo* models of the *Pseudomonas aeruginosa* infection. They investigated a strategy for the utilisation of this nano-platform to deliver the antibiotic more efficiently to the site of infection in the cystic fibrosis lung model by conjugation of dornase alfa (recombinant human deoxyribonuclease I, DNAse), which reduces mucus viscoelasticity by cleavage of DNA and enhances drug penetration through the mucus network. The *in vivo* antimicrobial effects of NPs were evaluated with regards to a *P. aeruginosa* (PA01) infection using a *Galleria mellonella* model. This model has been shown to have positive correlation with mammalian models in determining the virulence of *P. aeruginosa*. The ability of the drug formulations to treat infection-induced mortality was measured by treatment post-infection. NPs offered a protective effect, providing 80% survival, whereas the free drug showed only half this value at 40% survival. The tobramycin polymeric NPs have the desirable properties for such a delivery system in cystic fibrosis pulmonary infection therapies [121].

2.2.4. Antiviral NPs

At the present time, viruses produce a variety of diseases in humans, but only a few antiviral drugs have been developed with effectiveness in a restricted number of clinical situations. Furthermore, most antiviral drugs do not selectively inhibit virus replication without simultaneously injuring the host cell and are therefore accompanied by serious side effects, such as myelotoxicity and immunosuppression. Nanomedicines, particularly polymer-based drug delivery systems are highly promising in the diagnosis, prevention and treatment of intracellular infections such as hepatitis and HIV/AIDS [131]. Some antiviral polymeric-formulations are summarized in Table **6**. However, other antiviral drug-loaded nanosystems are described below.

An important antiviral drug is phosponoformate (foscarnet), an antiviral agent which inhibits the activity of herpes virus DNA polymerase. It is characterized by a low oral bioavailability and a

Table 5. Polymeric nanoparticles proposed for antimicrobial applications.

Name	Description	Indication	Refs.
CTS- micro /nanoparticles	Cationic polysaccharide micro/NP by polyelectrolyte complexformation or encapsulation of antibiotic, phenolics, etc.	Gastric delivery, *i.e. H. pylory*.	[122, 123]
PLGA-NPs	Carvacrol.	*S. epidermidis*	[124]
	Gentamycin.	*P. aeruginosa*	[125]
PLGA-PC	Levofoxacin.	*P. aeruginosa*	[126, 127]
	Calcein.	*P. aeruginosa*	
PLGA-PCL	Levofloxacin.	*E. coli*	[128, 129]
CMC-EDBE-FA (Chitosan derivate)	Vancomycine.	*S. aureus*	[130]

lymerase. It is characterized by a low oral bioavailability and a marked toxicity, such as renal dysfunction and fluctuations in serum calcium levels. Russo *et al.* developed an encapsulated formulation of foscarnet in chitosan nanoparticles with the aim of reducing the toxic effects and extending its activity. Foscarnet released from nanoparticles maintained the antiviral activity of the free drug when tested *in vitro* against human embryonic lung fibroblast (HELF) cells infected with the human cytomegalovirus strain AD-169, and the nanoparticles showed no toxicity on non-infected HELF cells [132].

On the other hand, Gandhi *et al.* reported a new formulation of acyclovir-loaded nanoparticles based on a copolymer with high permeability, prepared with ethyl acrylate, methyl methacrylate and a low content of methacrylic acid ester with quaternary ammonium groups (Eudragit RLPO®). Preliminary results showed that acyclovir-loaded Eudragit RLPO® nanoparticles could be effective in sustaining drug release for a prolonged period (71 % at 24 h), which represents an excellent advantage because the mean plasma half-life of acyclovir is 2.5 h and a five time-a-day administration is required. Thus, these nanoparticles may represent a delivery system that could improve the therapeutic effect of current anti-herpes virus therapy [133].

The HIV/AIDS scene represents a challenging area. Statistics presented up to 2014 by the World Health Organization indicated that 36.9 million people are infected by the Human Immunodeficiency Virus (HIV) and 1.2 million died of AIDS over the last year. The drug Nelfinavir mesylate (NFV) is a non-peptidic HIV-1 protease inhibitor used in the treatment of the Acquired Immunodeficiency Syndrome (AIDS). Poor oral bioavailability and a short half-life (3.5–5 h) remain a major clinical limitation of NFV, leading to unpredictable drug bioavailability and frequent dosing.

In this context, Venkatesh and co-workers reported a new NFV-loaded PLGA NP formulation, which could increase the solubility and oral bioavailability with sustained release of the drug. These results clearly demonstrated that the PLGA-NPs greatly improve bioavailability for NFV. Furthermore, NFV PLGA-NPs showed a longer half-life (19.8 ± 2.09 h) compared to the NFV suspension (6.66 ± 1.25 h), due to the prolonged absorption phase and sustained release of PLGA-NPs. The relative bioavailability of NFV-PLGA NPs was calculated to be 494.4% (improving 4.94-fold) which indicates that PLGA-NPs achieved markedly better absorption of NFV compared with the suspension. In summary, NFV PLGA-NPs showed an increase in oral bioavailability, high plasma concentration, low clearance, long half-life and were capable of exhibiting sustained release over a period of time as compared to pure drug in suspension form [134].

Finally, from a clinical point of view, Neuro-AIDS is a difficult target since the blood-brain barrier (BBB) is poorly crossable by most antiretroviral drugs. Recently, Roy *et al.,* [135] and Kuo and Yu [136], performed two separated studies to evaluate the loading of saquinavir in polymer-based nanoparticles. The second study evaluated the ability of SQV-loaded PLGA NPs with a surface of poly-(γ-glutamic acid) (γ-PGA) to enhance the transport of SQV across a monolayer of human brain microvascular endothelial cells (HBMECs) regulated by human astrocytes, mimicking the BBB. The γ-PGA/SQV-PLGA NPs increased the permeability of SQV across the BBB about 3–6 times. The average grafting of γ-PGA was the determinant factor in efficiency increase of permeability in general, due to the uptake of carriers via the γ-PGA-specific receptor-mediated pathway. Authors suggested that this nanosystem represents a great step for anti-HIV therapy in the central nervous system.

2.3. Applications of Nanoparticles in Immunology

The immune system safeguards the host from infections and malignancies. Generally, as a natural process, any compound that enters the body is recognized as a foreign element and merits the activation of the components of the immune system, whose mission will be to withdraw it from the body through different mechanisms [141].

Current and successful application of drug delivery systems in the field of immunology is focused on the creation of new generations of vaccines, adjuvants and immunomodulatory drugs that aim to improve clinical outcomes in response to a range of infectious and non-infectious diseases such as inflammatory and autoimmune disorders, asthma and cancer, among others [142].

The type of polymer and the route of administration might influence whether there is an enhanced or suppressed immune response [2]. In addition to stimulating and directing the immune response, studies have shown that nanoparticles can be therapeutically used to inhibit detrimental immune responses that occur in allergies, transplant rejection and autoimmunity [141].

2.3.1. Rheumatoid Arthritis and Osteoarthritis

Rheumatoid arthritis (RA) and Osteoarthritis (OsA) are the most common forms of chronic autoimmune disorder, characterized by progressive degradation of the extracellular matrix (ECM), systemic inflammation of synovial joints leading to erosion and cartilage destruction and penetration of the pannus by immune cells which potentialise the inflammatory process. In conjunction, immune cells and fibroblasts located in synovial fluid also increase inflammation by production of cytokines and chemokines. The frequent and long-term duration of RA therapeutics can cause unde-

Table 6. Polymeric nanoparticles proposed for antiviral applications.

Name	Description	Indication	Refs.
PLGA-(PEG-CsA)-LTP	Sustained drug delivery system by conjugating the liver-targeting peptide (LTP) to PEGylated cyclosporine A (CsA)-encapsulated poly (lactic-co-glycolic) acid (PLGA) NP.	Targeted NP were able to effectively inhibit viral replication *in vitro* and in an HCV mouse model.	[137]
PCL-DAP	Poly(ε-caprolactone) (PCL) nanoparticles containing the antiretroviral drug dapivirine.	Promising next generation of microbicides, intracellular delivery, anti-retroviral activity.	[138]
PVM/MA-GA	Mucoadhesive nanoparticles based on poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA) - glycyrrhizic acid (GA).	Intended for vaginal delivery of GA, a drug with well-known antiviral properties.	[139]
PL/AG/PLL-RMP	Poly(D,L-lactic acid) homopolymer and arabinogalactan (AG)-poly(l-lysine) (PLL) conjugate containing ribavirin monophosphate (RMP).	Nanoparticles for targeting of the liver and sustained release of ribavirin.	[140]

sirable systemic side effects and safety complications that reduce patient compliance. Actual treatment strategies focus on pain relief, reduction of inflammation and prevention of joint destruction rather than complete disease remission. Symptoms can be alleviated by oral analgesics, systemic non-steroidal anti-inflammatory drugs, glucocorticosteroids and disease-modifying anti-rheumatic drugs (DMARDs) (*e.g*. methotrexate, MTX). However, due to its multifactorial aetiology and complex pathogenesis, there are currently no satisfactory treatments [143-149].

Various systems engineered on polymeric nanoparticles have been focused on the treatment of RA and OsA. Table **7** provides some complementary examples of polymeric-engineered nanoparticles for rheumatoid arthritis and osteoarthritis treatments.

The PLGA-based nanoparticles have been the most described in these chronic autoimmune disorders. Costa and Reis designed a novel smart polymeric PEG-PLGA nanosphere platform to carry MTX and an imaging agent, allowing photoacoustic imaging and NIR photothermal applications. MTX-loaded nanospheres hampered the viability of monocytes and macrophages at a higher level than free MTX. These MTX-loaded multifunctional nanospheres led to a significant reduction of IL-1β, IL-6 and TNF-α inflammatory cytokines produced by monocytes and macrophages upon *in vitro* inflammatory stimulation, suggesting a favorable anti-inflammatory activity. The results confirmed that the multifunctional nanospheres represent a promising theranostic platform for RA, by combining MTX and gold nanoparticles for a highly effective targeted chemo-photothermal therapy [150].

Similarly, Kim *et al*. developed methotrexate (MTX)-loaded PLGA-(gold/iron/gold) half-shell nanoparticles conjugated with arginine-glycine-aspartic acid (RGD), which can be applied for magnetic targeted chemo-photothermal treatment and *in vivo* multimodal imaging of RA. The combination of near-infrared (NIR) irradiation and external magnetic field application allowed an accelerated and focused release [151]. These two PLGA-based nanoparticles provided enhanced therapeutic effects compared to the free MTX therapy for the treatment of RA based on chemo-photothermal therapy.

Another interesting formulation was developed by Te Boekhorst and co-workers [148]. They evaluated the anti-inflammatory effects of PLGA nanoparticles loaded with small interfering RNA (siRNA) directed against TNF-α. The siRNA-loaded PLGA nanoparticles mediated a dose-dependent TNF-α silencing in lipopolysaccharide-activated RAW 264.7 cells *in vitro*. Nanoparticles loaded with TNF- α siRNA resulted in a reduction of disease activity, evidenced by a significant decrease of the paw scores and joint effusions, compared to treatment with PLGA nanoparticles loaded with non-specific control siRNA. In addition, proper siRNA dosing seemed to be important for a positive therapeutic outcome *in vivo*. However, further studies are needed to fully clarify the mechanism(s) underlying the observed anti-inflammatory effects of the siRNA-loaded nanoparticles. Additionally, Park *et al.*, using the same siRNA coupled with glucocorticoids (dexamethasone) encapsulated in PLGA nanoparticles, showed inhibition of expression of unnecessary genes and proteins involved in arthritis [152].

Lu *et al*. described the use of hybrid hyaluronic acid (HA) /chitosan (CTS) nanoparticles as a novel non-viral gene delivery vector, which was able to transfer exogenous genes into primary chondrocytes for the treatment of joint diseases. The transfection efficiency of HA/CTS-plasmid nanoparticles was significantly higher than that of CTS-plasmid nanoparticles under the same conditions. The average viability of cells transfected with HA/CTS-plasmid nanoparticles was over 90%. These results suggested that HA/CTS-plasmid nanoparticles could be an effective non-viral vector suitable for gene delivery to chondrocytes [146].

2.3.2. Polymeric Nanoparticles in Vaccine Development

The effectiveness of a vaccine is measured by its ability to interact and stimulate the immune system. Nanoparticles provide an effective method for delivering antigens (inactivated vaccines with incorporated adjuvants). Currently, polymeric antigen delivery systems have been developing as a vaccine strategy and it has been demonstrated that particles with a high antigen payload and optimized attributes could be designed for expected adjuvant purposes, leading to the development of highly efficient vaccine candidates. The use of nanoparticle systems as vaccine carriers result in an increased antigen delivery efficiency to target immune cells, which can play a feasible role in increasing immune responses [90, 156].

In addition, vaccine efficacy is strongly enhanced by antibody-mediated targeting of vaccine components to dendritic cells (DCs), which are professional antigen presenting cells (APCs). Different types of nanoparticles have been used to entrap antigens, or with other agents, such as humanized DC-specific antibodies or immunomodulators, to enable targeted antigen delivery and the activation of APCs [2, 157]. Thus, the antigen may be encapsulated in polymers such as PLGA or polylactide, because of their demonstrated biodegradability and biocompatibility. For example, PEGylated-PLGA (150-200 nm size) has been used to encapsulate hepatitis B surface antigen (HBsAg). Polymer nanoparticles promoted the rapid uptake and the endosomal localization of vaccine antigens in DCs, as well as the subsequent production of high titres of antigen-specific antibodies [2, 141].

Meanwhile, Cruz *et al.* also worked on PLGA-PEG nanoparticles, functionalizing them with the antibody hD1, which does not interact with the complement or Fc receptors, but recognizes the human C-type lectin receptor DC-SIGN on DCs. Targeted delivery

Table 7. Polymeric nanoparticles proposed for Rheumatoid Arthritis and Osteoarthritis treatment.

Name/Description	Indication	Refs.
CTS-BBR / Isoquinoline alkaloid called Berberine chloride (BBR).	Nanoparticles for sustained release of BBR. Possesses promising protective efficacies against OA.	[153]
m-RAFT-TEGM-CHM-IL-1Ra. Self-assembly nanoparticles of the co-polymer (1-(4-nitrophenoxy)-1-oxo-2,5,8,11,14,17-hexaoxanonadecan-19-yl 2-((phenylcarbonothioyl)thio)acetate) - Tetraethylene glycol methacrylate- cyclohexyl methacrylate, for delivery of interleukin-1 receptor antagonist.	Inhibition of IL-1-mediated signaling to equivalent levels as soluble IL-1Ra, for the prevention of degenerative changes in cartilage structure or composition.	[147]
Poly(THPMA)-hP2X7 / 3,5-dichloropyridine derivative (hP2X7) encapsulated in poly(tetrahydropyran-2-yl methacrylate) (poly(THPMA)).	Controlled release of anti-inflammatory therapeutics for the P2X7 receptor antagonist, encapsulated in a pH-sensitive polymer.	[154]
Poly(ethylene glycol) (PEG)-functionalized poly(D,L-lactic-co-glycolic acid) (PLGA)/polyethylenimine (PEI)/siRNA NP, decorated with anti-HLA-DR antibody (siRNA-NP-Ab).	Targeting delivery to HLA-DR+ dendritic cells (DCs) and homogeneously dispersed in a biodegradable film consisting of poly vinyl alcohol (PVA) and λ-carrageenan. Novel siRNA-NP-Ab-film may be a promising platform for preventing HIV infection within the female genital tract.	[155]

improved antigen presentation of NPs and induced antigen-dependent T cell responses at 10–100 fold lower concentrations than non-targeted NPs [157].

Otherwise, amphiphilic poly(γ-glutamic acid)-graf-L-phenylalanine ethyl ester (γ-PGA-Phe) nanoparticles developed by Shima *et al.* were prepared with various grafting degrees of hydrophobic side chains used to evaluate the effect of vaccine carriers on the antigen encapsulation behaviour, cellular uptake, activation of dendritic cells and induction of antigen-specific cellular immunity-based immune responses [158].

Finally, Reddy *et al*. developed pluronic-stabilized polypropylene sulphide nanoparticles, which activated the complement cascade, generating a danger signal *in situ* and potently activating DCs. Nanoparticles could provide direct intracellular access, facilitating engagement of the intracellular Toll-like receptor (TLR) 3, 7, 8 and 9 by their ligands, improving their efficacy as vaccine adjuvants [159].

2.3.3. Miscellaneous Nanoparticles for Modulation of Immune Response

Immunomodulation comprises the therapeutic intervention for decrease or increase of immune system response and has been widely employed in infectious diseases, It has been demonstrated that the incorporation of allergens or adjuvants in nanoparticle-based delivery systems plays an important role in the enhancement of immunomodulatory responses, not only in infectious disease, but also in cancer, autoimmune disorders and inflammatory processes. Table **8** resumes some important investigations focused on polymeric nanoparticles engineered for different immune applications.

To conclude, despite the fact that vaccination is the most effective immunomodulatory technique, new approaches to modulate responses have been built based on the release of cytokines, autoantigens, antibodies, immunomodulatory drugs, viral antigens, naked DNA, anti-inflammatories, etc., by nanoparticle systems, showing an increase in the application of nanotechnology to resolve medical issues.

3. PERPECTIVES

As it has been described throughout this review, nanotechnology has a promising application in medicine. Novel smart drug delivery systems constituted by biocompatible and biodegradable polymers offer the ability to control the drug release from a polymeric matrix as well as the capability of targeting molecular receptors present on cells or tissues by surface nanoparticle modification. Thus, polymeric nanoparticles could be employed as alternative weapons for personalized therapy.

The advantages of polymeric nanoparticles are based on a) improvement of pharmacokinetic behaviour, b) increase of bioavailability, c) optimization of dosage, d) drug transport to specific target sites, e) decrease of health tissue toxicity, f) personalized therapy, g) building of theranostic devices, etc. Although many of these properties are desirable in pharmaceutical nanoparticle development. Biological processes at a molecular level are a strong driving force behind the development of nanotechnology.

The current understanding of the nature of interactions between nanoparticles and biological systems has provided robust evidence for changing the paradigm on treatment of human diseases, including chronic, degenerative, infectious and metabolic disorders. The real therapeutic value of nanomedicine has been defined on particular properties and desirable effects. It is well-known that there is no magic bullet; however, an adequate development of drug-carriers could enhance the efficacy and safety of therapy. The biochemical characteristics of the disease and host responses comprise intricate pathways defined by particular genotype and phenotype, which is important for a better selection of patients with suitable characteristics for nanomedicine treatment, in order to maximize therapeutic response.

Extrapolation from animal models to humans results in complicated and extensive research. The more comprehensive data sets become available, the better the clinical advantages of using active nanomaterials. An adequate physicochemical and biological behaviour of active nanoparticles help build the bridge between preclinical development and clinical use. Nanomedicine engineering is a winding road; however, the growing knowledge in this area could ensure the development of better alternatives to conventional treatment and help to improve life quality.

CONCLUSION

In this article, we described the state of the art on nanoparticle development as a new generation of drug delivery systems. With the thorough comprehension of biological effects depending on structure, it is possible to design specific systems for specific diseases, treatments and patients. Advances in material sciences as

Table 8. Polymeric nanoparticles proposed for modulation of immune response.

Name/Description	Indication	Refs.
Polypropylene sulfide core, block copolymer Pluronic corona NPs.	Lymph node-targeting, complement-activating NP (NPs) as a vaccine platform. Nucleophile-containing NP surfaces activated complement (functionalization *in situ* with C3).	[160]
ALG-CTS-PLGA-Peptide.	Colloidal gel from alginate−chitosan and PLGA NP to deliver Ac-PLP-BPI-NH_2-2 peptide (designed to bind to MHC-II and ICAM-1) in a controlled release manner as a vaccine-like therapeutic to suppress experimental autoimmune encephalomyelitis (EAE) by reducing Th17 proliferation (experimental in the mouse model).	[161]
Leukemia inhibitory factor (LIF) encapsulated in avidin-coated PLGA/PVA NPs.	Targeted nanoparticle to harness endogenous immune-regulatory pathways, powerful new method to modulating T cell developmental plasticity in immune-mediated disease indications.	[149]
Ovalbumin (OVA)-loaded N-trimethyl CTS (TMC), PLGA and TMC/PLGA based NPs.	Useful for increase of immune response by nasal vaccination; TMC NPs were shown to be superior over PLGA NP and PLGA/TMC NP in antigenic (OVA) release, preparation was evaluated in Balb/c mice.	[162]
Erythrocyte membrane-enveloped PLGA NPs for antigenic peptide (hgp10025-33) and toll-like receptor 4 agonist, monophosphoryl lipid (MPLA).	Potential in applying an erythrocyte membrane-enveloped polymeric nanoplatform for an antigen delivery system in cancer immunotherapy. This nanovaccine effectively enhanced IFN-γ secretion and CD 8(+) T cell response.	[163]
Nanoemulsion and porous polymeric PLGA NPs - CHrPfs25 (malaria transmission blocking vaccine Ag).	CHrPfs25 delivered in various adjuvants /nanoparticles elicited strong functional immunogenicity to prevent malaria transmission, pre-clinical studies were carried out in mice.	[164]
FA-CS-NPs (Folate-conjugated chitosan NP's loaded with mouse interferon-γ-inducible protein-10 (IP-10) plasmid.	Experimental data suggested that the gene delivery system of folate-conjugated chitosan nanoparticle loaded with IP-10 plasmid may be a promising strategy for immunotherapy of hepatocellular carcinoma, NP's inhibit angiogenesis and promoted IP-10 expression and induced apoptosis in the tumour.	[165]
Tacrolimus-loaded nanoparticles based on poly(ethylenglycol)-PLGA NPs.	Better pharmacokinetic characteristics and lymphatic targeting efficiency with respect to free tacrolimus.	[166]
Tacrolimus-loaded NP's based on galactosylated PLGA.	Actively targeting the reticuloendothelial system, the *in vitro* release and pharmacokinetics showed sustained release of tacrolimus from nanoparticles promising carrier, for liver targeting of tacrolimus.	[167]
PLGA-NP's for delivery of plasmid DNA encoding mouse IL-10.	The NP's were successful in the suppression of autoimmune diabetes in mice model.	[168]
Poly(D,l-lactic-co-hydroxymethyl glycolic acid) (pLHMGA)).	Vaccine formulation for treatment of human papillomavirus (HPV), using the synthetic long peptides (SLPs) derived from HPV16 E6 and E7 oncoproteins.	[169]

well as molecular biology techniques allow engineering of a great variety of single nanoparticles with controlled components and behaviour, ligand grafts, surface modifications and action mechanisms.

The ability of polymer-based nanoparticles to modify and improve pharmacokinetics and pharmacodynamics, associated to techniques for enhancement of the therapeutic efficiency with minimal side effects, demonstrate the advantages of these systems. Most of the cited studies are still in research stages and the clinical trials fairly lagged behind the overwhelming research work.

Only few were translated from the bench to the clinical phase. However, the understanding of material behaviour in pre-clinical studies ensures the manufacture of optimized drug delivery systems and smart drug delivery systems that, due to their characteristics, promise to be the future of medicine.

Although our understanding of polymeric nanoparticles grows exponentially, further mechanistic studies must be carried out in order to elucidate the biological effects of nanomaterials in biological entities and their interaction at a molecular level under "live" conditions. Functionality and safety of whole new polymeric formulations in the human body, taking into account physiological complexity and the advantages that these drug delivery systems, showed in early *in vivo* and *in vitro* studies result in promising novel strategies for future use in cancer, infectious diseases and immunotherapy.

It is important to note that the increase of therapeutic response and safety requires taking advantage of the biological, physical and/or chemical characteristics of nanomaterials. There is no "magic bullet". However, with a suitable growth in knowledge, the better therapeutic responses will be.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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9. CONCLUSIONES GENERALES

Esta investigación se basó en el objetivo de construir un sistema de nanopartículas poliméricas, con carácter multimérico y multivalente, capaz de transportar fármacos de interés hacia sitios diana, mediante el uso de ligandos de direccionamiento en la superficie de las nanopartículas.

Se prepararon y caracterizaron nanopartículas de PLGA empleando un método de emulsificación-evaporación de solvente, método sencillo y ampliamente utilizado para la obtención de nanopartículas poliméricas. Así mismo, permite controlar el tamaño de las partículas en función de las condiciones de la reacción y la concentración de los reactantes. Se obtuvieron nanopartículas de morfología cuasi-esférica con tamaños adecuados (menores a 600 nm por DLS y menores a 250 nm por microscopía electrónica) y distribuciones homogéneas. Adicionalmente, esta técnica permite la adición de los fármacos a encapsular al momento de la preparación de la matriz polimérica de PLGA, conduciendo a la obtención de eficiencias de encapsulado adecuadas (47.97 ± 1.8 %, para este estudio).

La modificación con γ-PGA sobre la superficie de la nanopartícula de DOX-PLGA, así como la conjugación con ácido fólico, se realizaron exitosamente, siendo comprobadas por las vibraciones observadas en FT-IR de los enlaces amida correspondientes al γ-PGA, y de las estructuras del esqueleto del ácido fólico como los anillos de pteridina y fenilo. De igual manera, las absorbancias detectadas en UV-Vis, principalmente las localizadas en 208 nm, 280 nm and 480 nm, correspondientes a γ-PGA, ácido fólico y doxorrubicina, respectivamente, demostraron la formación del sistema final DOX-PLGA/γ-PGA-FA. Es importante señalar que las modificaciones se realizaron a través de una reacción tipo carbodiimida, la cual permite la formación de enlaces amida, a partir de ácidos carboxílicos activados y aminas primarias, obteniendo sistemas conjugados unidos covalentemente, con mayores ventajas que aquellos obtenidos por procesos de adsorción.

La cinética de liberación del sistema de nanopartículas DOX-PLGA/γ-PGA-AF mostró un comportamiento dependiente del pH. Este comportamiento responde a las características propias de los polímeros utilizados, ya que tanto el PLGA como el γ-PGA, son polímeros pH-sensibles, y al encontrarse en un ambiente ácido, la liberación del fármaco se promueve

a partir de la degradación del material polimérico. Así, la cinética de liberación más adecuada para el sistema de nanopartículas de DOX-PLGA/γ-PGA-AF fue observada a pH 5.3.

La evaluación biológica indicó que el efecto citotóxico sobre las células HeLa fue dependiente de la liberación del fármaco durante el tiempo de exposición, derivado de las características pH-sensible de las nanopartículas de DOX-PLGA/γ-PGA-AF, encontrando un mayor efecto después de 72 h. Además, se demostró el mejoramiento de la captación celular del sistema polimérico de nanopartículas a través de un mecanismo de endocitosis mediado por receptores de folato, al comparar células cuyos receptores fueron bloqueados con aquellas donde los receptores se encontraban libres, teniendo estas últimas una captación 3.4 veces mayor.

Con base a los resultados obtenidos, se concluye que las nanopartículas de PLGA/γ-PGA-AF son un sistema potencial de transporte y entrega direccionada de fármacos, como la DOX, a través del reconocimiento molecular de receptores de folato sobreexpresados en algunos tipos de cáncer, lo que lo hace un candidato adecuado para futuras aplicaciones terapéuticas.

10. PERSPECTIVAS

El tamaño de la nanopartícula tiene especial trascendencia biológica en la mejora de parámetros farmacocinéticos o sobre los mecanismos EPR. Si bien la preparación de las partículas nanométricas se logró exitosamente, se pudo observar al tamaño de partícula como una función dependiente de la concentración del polímero y del surfactante. En función de la metodología de preparación es posible modificar el tamaño de partícula, sin alterar la estabilidad del sistema coloidal, a partir de la experimentación con diversas concentraciones de reactantes, además de las evaluadas en este trabajo.

Se observó además que tanto el γ-PGA como el AF tienen influencia en la estabilidad coloidal del sistema en suspensión, haciéndolo menos estable y conduciendo a la formación de agregados, que derivan en la acumulación de material polimérico. Por ello, se propone la optimización de las reacciones de modificación superficial en función de la variación de las concentraciones de γ-PGA y AF, con el fin de disminuir las interacciones inter-partícula que resultan en la inestabilidad del sistema.

Los sistemas poliméricos como el propuesto en este trabajo, abren la posibilidad de encapsular otros fármacos, o sus combinaciones, mejorando con ello la efectividad del tratamiento al disminuir los efectos secundarios no deseados, optimizando la entrega de los agentes quimioterapéuticos a sitios específicos y estableciendo así mecanismos de daño independiente que maximicen la respuesta terapéutica.

Finalmente, el siguiente paso en el estudio de este sistema de nanopartículas, una vez optimizado, es la evaluación *in vivo*, con el fin de establecer los parámetros farmacocinéticos como la distribución y destino de las partículas en el organismo, teniendo especial atención en los sitios de acumulación por mecanismos pasivos. Adicionalmente se podrán establecer las concentraciones de nanopartículas que proporcionen las dosis de fármaco con las que se obtengan las respuestas terapéuticas deseadas.

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