



Development and evaluation of nanoparticles for cancer treatment

Rachel Ouvinha de Oliveira

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**Universidade do Estado do
Rio de Janeiro**
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Du Fondamental à l'Appliqué



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présentée par

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DEVELOPMENT AND EVALUATION OF NANOPARTICLES FOR CANCER TREATMENT

Thèse préparée sous la direction de Gillian Barratt et Luiz Claudio de Santa Maria

soutenue le 2 mai 2014 devant la commission d'examen :

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Publications

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Poster communications

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Ouvinha de Oliveira, R., de Santa Maria, L.C., Vergnaud, J., Astruc D., Barratt, G., XIII èmes Journées de l'École Doctorale, June 2013 - Kremlin-Bicêtre, France. *An in vitro Study on Functionalized AuNPs in Prostate Cancer Therapy.*

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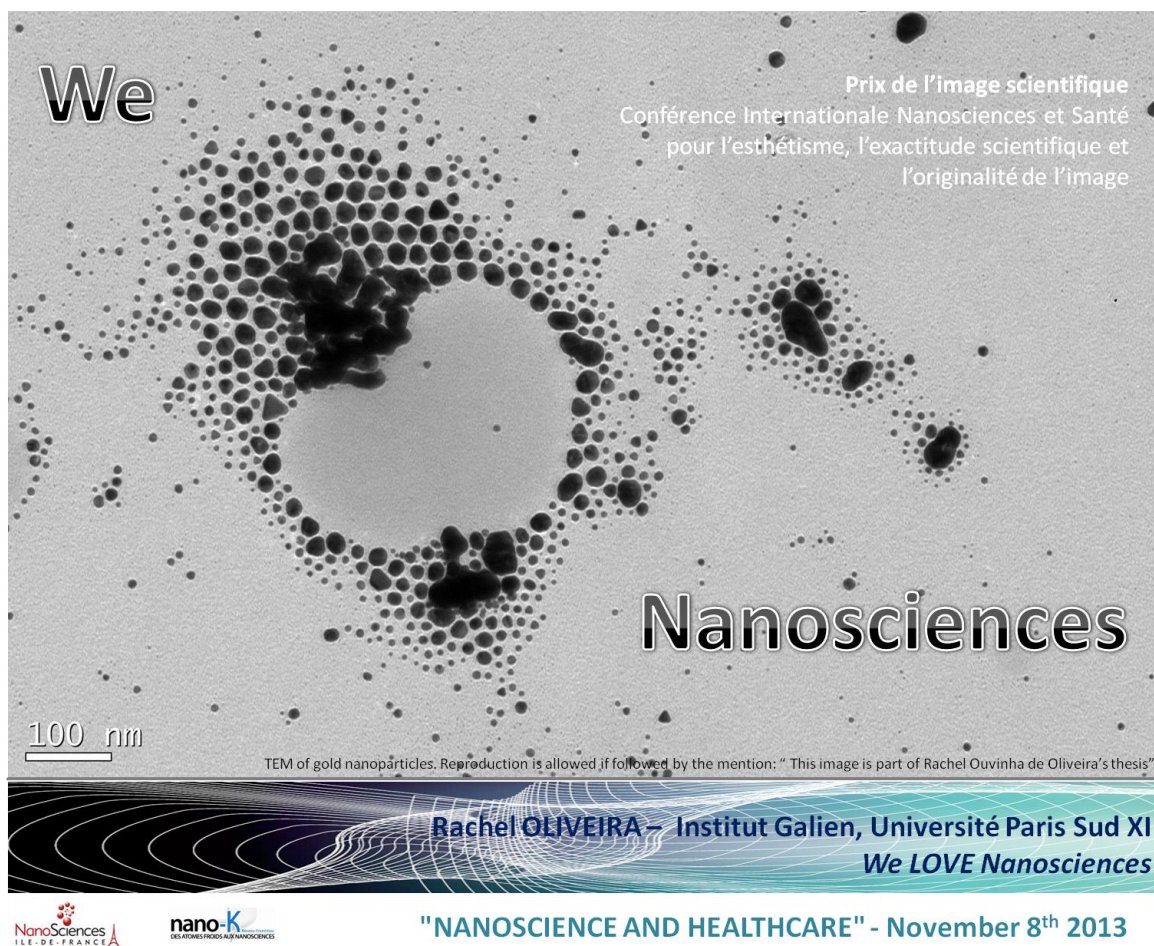
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ABSTRACT

This thesis concerns the development and evaluation of nanoparticles for cancer treatment, and in particular to prostate cancer. The manuscript includes a literature review on the application of nanomedicine to the treatment of prostate cancer. In the first experimental part, functionalized gold nanoparticles were characterized and loaded with docetaxel by non covalent adsorption. These gold nanoparticles showed a sustained cytotoxic effect *in vitro* against prostate cancer cells. The second experimental part of this thesis describes a study of synthesis and nanoprecipitation of polyesters for the co-delivery of two chemotherapeutic drugs, docetaxel (DOC) and mitoxantrone (MIT). Polycaprolactone, poly(lactic acid) and poly(lactide-co-glycolide) were synthesized by ring-opening polymerization with different molecular weights of polyethylene glycol. Monodisperse nanoparticles with diameters of less than 80 nm were produced and were shown to be effective against prostate cancer cells when loaded with MIT and DOC. Moreover, a synergistic effect was observed using combinations of these nanoparticles. Therefore, these polyester based nanoparticles have potential clinical applications.

Keywords: mitoxantrone, docetaxel, gold nanoparticles, polymeric nanoparticles, nanoprecipitation, drug delivery, prostate cancer.

RESUMO

O câncer é uma das doenças mais letais do mundo, sendo a primeira causa de morte nos países desenvolvidos. Mais de 50 medicamentos quimioterápicos clinicamente estabelecidos estão disponíveis e outros estão sendo desenvolvidos para o tratamento do câncer. No entanto, a variedade de terapias disponíveis é acompanhada por uma série de inconvenientes tais como, a difícil diferenciação entre tumores indolentes e agressivos, efeitos colaterais graves, recorrência do câncer, resistência aos tratamentos anteriormente utilizados e a propensão a metástases. Estes fatores não podem ser ignorados e representam grandes desafios a serem superados na terapia do câncer.

Nos últimos anos, a nanotecnologia tem atraído uma atenção considerável devido à facilidade de nanoestruturas de interagir com o organismo na escala molecular. Novas terapias na pesquisa do câncer utilizando a nanomedicina estão sendo desenvolvidas, a fim de conferir propriedades, tais como a melhoria da especificidade, vetorização e eficácia da liberação do fármaco com o objetivo de obter eficácia máxima com efeitos mínimos colaterais.

O presente trabalho foi um estudo multidisciplinar. A química, a biologia e a tecnologia farmacêutica foram combinadas para conceber, sintetizar, caracterizar e avaliar nanopartículas metálicas e poliméricas, a fim de melhorar a vetorização de fármacos quimioterápicos em tecidos neoplásicos.

Esta tese é dedicada ao desenvolvimento e avaliação de nanopartículas para o tratamento do câncer. Embora os nanovetores descritos possuam aplicações potenciais para vários tipos de tumores, este trabalho foi dedicado ao tratamento do câncer da próstata, pois os fármacos encapsulados, docetaxel e mitoxantrona, são quimioterápicos de primeira escolha para câncer de próstata metastático.

O docetaxel é um agente citotóxico bem estabelecido. Tal como paclitaxel, ele é um taxóide de origem natural, no entanto, o docetaxel é sintetizado a partir de um precursor inativo encontrado na árvore *Taxus baccata*. Ele age como um agente

anti-mitótico rompendo microtúbulos que provocam a falha na replicação de células e induzem a morte celular. Apesar da sua comprovada eficácia, os efeitos colaterais associados ao seu uso podem ser incapacitantes. Neutropenia, retenção de líquidos e neuropatia são alguns exemplos de graves reações indesejáveis relacionadas à administração de docetaxel.

Mitoxantrona é uma substância derivada da família das antraquinonas, assim como a Doxorubicina e a Aclarrubicina. Ela foi primeiramente sintetizada em 1979 e, além da sua ação anti-tumoral, possui propriedades antibacterianas, antivirais e imunomoduladoras, sendo também utilizada na terapia da esclerose múltipla. Em contraste com o docetaxel, a mitoxantrona atua como um inibidor da síntese de DNA que interfere na progressão do ciclo celular. Reações adversas são relatadas em pacientes tratados com Mitoxantrona e estão relacionados com o seu acúmulo na tireóide, fígado e coração. A cardiotoxicidade associada à administração deste fármaco parece ser irreversível e dose-dependente o que conduz a uma predisposição à insuficiência cardíaca. Outras reações adversas incluem estomatite, alopecia e mielossupressão.

O uso de nanopartículas metálicas, bem como nanopartículas poliméricas biocompatíveis e biodegradáveis para encapsular estas moléculas é uma abordagem terapêutica original para evitar os efeitos secundários da quimioterapia.

O objetivo deste trabalho foi o desenvolvimento de sistemas de entrega de fármacos multifuncionais baseados em nanopartículas de ouro para a entrega de Docetaxel e em nanopartículas baseadas em copolímeros de poliésteres carregados com Docetaxel e Mitoxantrona.

O primeiro capítulo desta tese é uma revisão da literatura e apresenta-se em forma de publicação sobre as diferentes estratégias de nanomedicina aplicadas ao tratamento do câncer de próstata. O artigo de revisão abrange as bases da vetorização e liberação de medicamentos, bem como os estudos recentes que estão sendo feitos neste campo. Além disso, dois capítulos descrevem o trabalho experimental realizado.

O segundo capítulo, é composto por uma publicação do *International Journal of Pharmaceutics* e diz respeito à síntese de nanopartículas de ouro contendo ou não ácido fólico usando dois comprimentoos de PEG, notadamente, PEG 550 e PEG 2000. Estas nanopartículas foram sintetizadas por nossos parceiros da Universidade Bordeaux 1, seguido de sua caracterização, adição de docetaxel e avaliação do seu efeito antineoplásico contra células de câncer de próstata que foram minhas contribuições pessoais. O núcleo destas nanopartículas apresentou tamanho de 7-10 nm com uma polidispersão estreita. O estudo completo de possíveis interações entre as nanopartículas de ouro contendo folato e as proteínas de ligação do folato foi apresentado como documento suplementar, uma vez que não foi acrescentado no artigo. Posteriormente, o docetaxel foi carregado às nanopartículas de ouro por adsorção e os sistemas finais foram usados diretamente para avaliar a citotoxicidade contra células de cancro da próstata humano *LNCaP*. As nanopartículas de ouro sem o fármaco não apresentaram toxicidade às células *LNCaP* nas concentrações usadas neste estudo. Quando o docetaxel foi adsorvido nas AuNPs, este produziu um efeito citotóxico sustentado *in vitro*. Estas nanopartículas poderiam ser úteis para a concentração de fármaco em tumores sólidos pelo “Efeito de Permeação e Retenção” (EPR effect), e as propriedades do núcleo de ouro podem ser exploradas para a imagiologia de tumores e para a citólise térmica de células tumorais.

O terceiro capítulo descreve a síntese, a caracterização e os ensaios *in vitro* de nanopartículas biodegradáveis e biocompatíveis para a aplicação como sistemas de liberação de princípios ativos, sendo utilizados dois fármacos antineoplásicos diferentes: a Mitoxantrona e o Docetaxel, este previamente estudado. Este capítulo apresenta-se dividido em duas seções.

A primeira seção descreve um estudo feito prévio à síntese polimérica, utilizando um copolímero comercial, o ácido polilático-co-glicólico - polietileno glicol. O copolímero foi nanoprecipitado utilizando diferentes proporções de fase orgânica e aquosa, bem como variando-se a concentração de polímero. Os produtos finais foram caracterizados por espalhamento dinâmico da luz (DSL) e microscopia eletrônica de transmissão (TEM) e mostraram nanopartículas esféricas e polidispersas de 71 a 128 nm.

Uma vez definida a metodologia de obtenção das nanopartículas, uma série de copolímeros do tipo dibloco peguillado foi sintetizada. Policaprolactona, ácido polilático e ácido polilático-co-glicólico foram sintetizados por polimerização por abertura de anel, utilizando-se diferentes pesos moleculares de polietileno glicol monometílico, 2000 e 5000 e octoato de estanho como iniciador. Os copolímeros foram caracterizados por ressonância magnética nuclear de prótons (^1H RMN) e cromatografia por exclusão de tamanho (SEC). Os polímeros obtidos apresentaram pesos moleculares variando de 19000 a 34000 g/mol e o índice de polidispersidade (Pdl) variou entre 1.8 e 2.6. Em seguida, o método de nanoprecipitação foi aplicado a esses copolímeros e forneceu nanopartículas polidispersas de 51 a 80 nm.

A segunda seção é apresentada como um manuscrito que será submetido ao *International Journal of Pharmaceutics*. Este documento refere-se ao uso de um dos copolímeros anteriormente sintetizados, nomeadamente o PLA - PEG 2000 e ao seu encapsulamento usando dois princípios ativos quimioterápicos: docetaxel e mitoxantrona seguidos por testes *in vitro*. Pela primeira vez na literatura, estes agentes antineoplásicos foram encapsulados para administração concomitante. As nanopartículas foram preparadas pelo método de nanoprecipitação e conduziram a nanopartículas monodispersas entre 68 e 82 nm e apresentaram eficiência de encapsulamento (EE) de 58 % para o docetaxel e 6 % para a mitoxantrona. A eficácia antitumoral das nanopartículas contra duas células de cancro da próstata humano PC3 e LNCaP foi avaliada através de sua viabilidade celular (MTS). A eficácia de cada tipo de nanopartículas foi avaliada em separado, bem como misturas dos dois tipos de nanopartículas em proporções diferentes a fim de observar qualquer sinergia, aditividade, ou antagonismo. A absorção das nanopartículas contendo mitoxantrona por células vivas foi estudada qualitativamente por microscopia confocal a laser e confirmado com análise por citometria de fluxo.

O estudo *in vitro* de viabilidade celular (MTS) mostrou que o rendimento de todas as combinações de nanopartículas carregadas diminuíram em até 50 % a atividade das células nas primeiras 48 h. Além disso, um efeito sinérgico foi observado com duas combinações diferentes de MITNPs e DOCNPs e que conduziram a menos de 10 %

da atividade metabólica das células da linhagem PC3, nas primeiras 96 horas de tratamento. Estes resultados indicam que as nanopartículas à base de PLA-PEG desenvolvidas neste trabalho podem ser potencialmente exploradas como veículo para melhorar a solubilidade do Docetaxel e para aumentar a absorção intracelular de Mitoxantrona e, portanto, podem ter aplicações clínicas potenciais.

No final do texto, uma discussão geral resume os resultados experimentais, colocando-os em perspectiva com outros trabalhos descritos na literatura e realçando a sua potencial aplicação na terapia do câncer.

Palavras-chaves: mitoxantrona, docetaxel, nanopartículas de ouro, nanopartículas poliméricas, nanoprecipitação, sistema de liberação, câncer da prostata.

RESUME

Le cancer est l'une des maladies les plus mortelles au monde ; elle est la première cause de décès dans les pays développés. Une cinquantaine de chimiothérapies sont disponibles et d'autres sont encore cours de développement. Cependant, les traitements utilisés actuellement présentent des inconvénients comme de lourds effets secondaires, la distinction entre les différents grades tumoraux, les rechutes, la résistance aux traitements ou encore les phénomènes métastatiques ; ces problèmes ne peuvent être ignorés et représentent un challenge pour les nouvelles thérapies anti-cancéreuses.

Récemment, les nanotechnologies ont attiré une attention considérable par leur capacité d'interaction avec l'organisme à l'échelle moléculaire. De nouvelles thérapies anti-cancéreuses utilisant des nanotechnologies sont en cours de développement et à l'étude pour conférer des propriétés comme une spécificité améliorée, un ciblage du médicament et une efficacité d'administration propres aux nanomatériaux afin d'obtenir un meilleur index thérapeutique.

Ce travail est une étude pluridisciplinaire. Ainsi, chimie, biologie et pharmacotechnie sont alliées pour concevoir, synthétiser, caractériser et évaluer des nanoparticules métalliques et polymériques afin d'améliorer l'administration des chimiothérapies aux tissus néoplasiques.

Cette thèse est consacrée au développement et à l'évaluation des nanoparticules pour le traitement du cancer. Bien que les nanotransporteurs décrits aient des applications potentielles dans de nombreux types de tumeurs, cette étude est dédiée au traitement du cancer de la prostate mais aussi à l'encapsulation de deux médicaments, le docétaxel et la mitoxantrone, qui sont des chimiothérapies de première ligne dans le traitement du cancer de la prostate métastatique.

Le docétaxel est un médicament bien connu. Comme le paclitaxel, il s'agit d'un taxoïde d'origine naturelle; toutefois, le docétaxel est synthétisé à partir d'un

précurseur extrait de l'If (*Taxus Baccata*). Il s'agit d'un agent anti-mitotique qui rompt les microtubules rendant la réplication des cellules défailante et induisant la mort cellulaire. Bien que son efficacité ait été prouvée, les effets secondaires associés à la thérapie peuvent être importants. Neutropénie, œdèmes et neuropathie sont des exemples de sérieuses réactions indésirables rencontrées lors de l'administration de docétaxel.

La mitoxantrone est une anthracycline synthétique comme la doxorubicine ou encore l'aclarubicine. Elle a été synthétisée pour la première fois en 1979 et hormis son activité anti-tumorale, elle possède aussi une activité antibiotique, antivirale et immunomodulatrice et est employée dans le traitement de la sclérose en plaques. En comparaison avec le Docétaxel, la mitoxantrone agit comme un inhibiteur de la synthèse d'ADN qui interfère avec la progression du cycle cellulaire. Des effets secondaires ont été signalés chez des patients traités par la mitoxantrone dus à son accumulation dans la thyroïde, le foie et le cœur. La cardiotoxicité associée à l'administration de ce médicament semble être irréversible et dose-dépendante conduisant à une prédisposition et à des défaillances cardiaques. D'autres effets secondaires incluent la stomatite, l'alopécie et la myelosuppression.

L'utilisation de nanoparticules métalliques ou de nanoparticules polymériques biocompatibles et biodégradables (PNPs) pour l'encapsulation de ces molécules est une approche thérapeutique permettant d'éviter les effets secondaires de la chimiothérapie.

Le but de ce travail est de développer un système de délivrance de médicaments multifonctionnel basé sur des nanoparticules d'or pour le docétaxel et des nanoparticules polymériques de type copolymère à bloc polyéthylène glycol-polyester pour le docétaxel et la mitoxantrone.

Le premier chapitre est une revue de la littérature accepté pour publication dans les *Annales Pharmaceutiques Françaises* sur les différentes stratégies de la nanomédecine pour le traitement du cancer de la prostate. La revue couvre les bases de la délivrance de médicament et de leur ciblage mais aussi les études qui

ont été effectuées dans ce domaine. Suivent deux chapitres qui décrivent le travail expérimental qui a été effectué.

Le second chapitre, une publication de *International Journal of Pharmaceutics*, concerne la synthèse des nanoparticules d'or (AuNPs) avec et sans acide folique utilisant deux longueurs de PEG, le PEG 550 et le PEG 2000 fabriqués par nos partenaires de l'université de Bordeaux I, suivi de leur caractérisation, leur chargement en docétaxel et l'évaluation de leur effet antinéoplasique sur des cellules de cancer de la prostate effectués dans notre équipe. Le cœur des nanoparticules mesure de 7 à 10 nm avec une polydispersité étroite. L'étude complète des interactions possibles entre les nanoparticules exprimant de l'acide folique à leur surface et des Folate Binding Proteins (FBP) sont présentées en annexe comme elles ne sont pas présentées dans l'article. Le Docétaxel a ensuite été chargé dans des nanoparticules d'or par adsorption non covalente et les systèmes finaux ont été utilisés directement afin d'évaluer leur cytotoxicité sur des cellules de cancer de la prostate humaines, les *LNCaP*. Les AuNPs non chargées n'ont pas été toxiques aux concentrations utilisées dans cette étude. Lorsque le docétaxel est adsorbé sur les AuNPs, il se produit un effet cytotoxique prolongé *in vitro*. Ces petites particules pourraient être utiles pour concentrer la substance active dans les tumeurs grâce à l'effet EPR et le cœur de ces nanoparticules d'or pourrait être utilisé pour de l'imagerie des tumeurs ou encore de la cytolysse thermique de cellules cancéreuses.

Le troisième chapitre décrit la synthèse, la caractérisation et les tests *in vitro* de nanoparticules polymériques biodégradables et biocompatibles pégylées comme système d'administration de deux substances actives anti-cancéreuses : la mitoxantrone et le docétaxel étudié précédemment. Ce chapitre est divisé en deux parties.

La première partie concerne une étude réalisée avec un copolymère commercial, le poly(lactide-co-glycolide)-polyéthylène glycol. Ce copolymère a été formulé en nanoparticules par la méthode de nanoprécipitation à différents ratios de phases organique et aqueuse et de concentration en polymère. Les produits finaux ont été caractérisés par diffusion quasi-élastique de la lumière (DLS) et microscopie

électronique à transmission (TEM) et ont montré des nanoparticules sphériques de polydispersité étroite de diamètre allant de 71 nm à 128 nm.

Une fois la méthodologie définie, une série de copolymères diblocs pégylés a été synthétisée. La polycaprolactone, le polylactide et le poly(lactide-co-glycolide) ont été synthétisés par polymérisation par ouverture de cycle à partir du polyéthylène glycol monométhyléther 2000 et 5000 g/mol, jouant le rôle d'amorceur en présence d'étain comme catalyseur. Les copolymères ont été caractérisés par résonance magnétique nucléaire du proton (RMN ^1H) et par chromatographie d'exclusion stérique (SEC). Les polymères obtenus présentent des masses molaires de 19 000 à 34 000 g/mol et un indice de polydispersité (Pdi) variant de 1,8 à 2,6. Ensuite, la méthode de nanoprécipitation a été appliquée à ces copolymères et ont permis d'obtenir des nanoparticules monodisperse de taille variant de 50 à 80 nm.

La deuxième partie est présentée sous forme d'un manuscrit qui sera soumis au *International Journal of Pharmaceutics*. Cette publication concerne l'utilisation du polylactide-co-polyéthylène glycol (PLA-PEG 2000) synthétisé précédemment et son chargement par deux anticancéreux : le docétaxel et la mitoxantrone. Pour la première fois dans la littérature, deux agents anticancéreux ont été encapsulés pour une application concomitante. Les nanoparticules ont été préparées par la méthode de nanoprécipitation, des nanoparticules monodisperses d'un diamètre d'environ 70 nm ont été obtenues, elles présentent un taux d'encapsulation de 58 % en docétaxel et de 6 % en mitoxantrone. L'efficacité anti-tumorale de ces nanoparticules sur des lignées de cancer de la prostate PC3 et LNCaP a été évaluée *in vitro* par MTS. L'efficacité de chaque type de nanoparticule a été évaluée séparément mais aussi sous forme de mélange de ces deux types de nanovecteurs à différents ratios afin d'observer des phénomènes de synergie, d'additivité ou encore d'antagonisme. La capture des nanoparticules contenant de la mitoxantrone par les cellules vivantes a été étudiée qualitativement et confirmée par cytométrie en flux.

L'étude de la viabilité cellulaire *in vitro* a montré une efficacité de toutes les combinaisons de nanoparticules chargées avec au moins une réduction de 50 % de l'activité cellulaire dans les premières 48h. En outre, un effet synergique a été

observé avec deux combinaisons de MITNPs et DOCNPs conduisant à moins de 10 % d'activité cellulaire sur la lignée PC3 après 96 h de traitement. Ces résultats indiquent que les nanoparticules de PLA-PEG 2000 développées dans ce travail peuvent être potentiellement utilisées pour améliorer la solubilité du docétaxel et augmenter la concentration intracellulaire en mitoxantrone et par conséquent avoir des applications cliniques potentielles.

A la fin du manuscrit, une discussion générale résume les résultats expérimentaux, les regroupant avec d'autres travaux décrits dans la littérature et soulignant ainsi leurs potentiels applications en thérapie anti-cancéreuse.

Mots-clés : mitoxantrone, docétaxel, nanoparticules d'or, nanoparticules polymères, nanoprécipitation, vectorisation, cancer de la prostate.

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CHAPTER I

NANOMEDICINE AND ITS APPLICATIONS ON THE TREATMENT OF PROSTATE CANCER

Introduction

The first chapter is written in the form of a literature review which has been published in the *Annales Pharmaceutiques Françaises* in June 2014. Some aspects of nanomedicine, and in particular the drug targeting and drug delivery fields are discussed with special attention to the application of these strategies to prostate cancer therapy.

Fascinating historical cases of prostate cancer in antiquity are presented, as well as the first modern reports. Furthermore, the three main nanoparticle-based drug delivery platforms are described; that is: liposomes, polymeric nanoparticles and metallic nanoparticles. Significant published works, including accounts of therapies in current clinical trials are discussed. Indeed, the small particle-based drugs approved for controlled release of anti-hormones are listed. It is important to note that, although there are still no approved nanoparticle-based formulations for drug delivery in prostate cancer therapy, the use of small polymeric particles plays a important role as they are often employed as the first option for hormonal castration.

However, a critical analysis of the impact of nanomaterials on public health and the environment was made to highlight the need for precise control of the utilization of nanomaterials. Those risks are not yet well established and should be considered.

The article can be found on internet though the following link:

<http://www.sciencedirect.com/science/article/pii/S0003450914000534>

Nanomedicine and its applications on the treatment of prostate cancer

Nanomedicine et ses applications pour le traitement du cancer de la prostate

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Abstract

In recent years, nanotechnology has been the focus of considerable attention in medicine due to the facility which nanostructures interact with the body at the molecular scale. New therapies in cancer research using nanomedicine are being developed in order to improve the specificity and efficacy of drug delivery, thus reaching maximal effectiveness with minimal side effects. This literature review presents cases of prostate cancer in antiquity as well as the first modern reports. Furthermore, the three main nanoparticles based platforms were revised: liposomal, polymeric and metallic. Important published works, including therapies in current clinical trials were discussed. In addition, several formulations of microparticles containing LH-RH analogues approved by the authorities are listed in this document. Whereas, a critical analyses on the health and environmental impact were made to highlight the need of a precise control on the utilization of nanomaterials.

Résumé

Au cours des dernières années, la nanotechnologie a fait l'objet d'une attention considérable en médecine en raison de la facilité avec laquelle les nanostructures interagissent avec le corps à l'échelle moléculaire. Les nouvelles thérapies pour le cancer faisant appel à la nanomédecine sont en cours de développement afin d'améliorer la spécificité et l'efficacité des médicaments, atteignant ainsi une efficacité maximale avec un minimum d'effets indésirables. Cette revue de la littérature présente des cas de cancer de la prostate dans l'antiquité, ainsi que les premiers rapports modernes avant d'exposer l'apport potentiel de la nanotechnologie dans le traitement de cette maladie. Les trois classes principales de nanoparticules sont passées en revue: liposomiale, polymère et métallique. L'ensemble des travaux publiés, y compris des essais cliniques en cours, y ont été discutés. De plus, plusieurs formulations à base de microparticules d'analogues de la LH-RH approuvées par les autorités sont citées dans ce document. Une analyse critique sur la santé et l'impact environnemental est faite pour mettre en évidence la nécessité d'un contrôle précis de l'utilisation des nanomatériaux.

Keywords: nanomedicine, prostate cancer, nanomaterials, drug targeting.

Mots-clés: nanomédecine, cancer de la prostate, nanomatériaux, ciblage.

INTRODUCTION

One of the first scientific descriptions of prostate cancer was made in 1851 [1]. This book presents a number of case studies but defines prostate cancer as a rare disease. There is other historical evidence suggesting that this cancer has been existent since antiquity: an identified case in history of metastasizing prostate carcinoma was found in Russia in a Scythian King skeleton dating from 7th century BC aged 40-50 years at the time of his death [2] while a Ptolemaic Egyptian mummy from 285–300 BC aged 51-60 years old at death had bone and pelvis lesions suggesting metastases originating from a prostate tumor [3].

In contrast, presently more than one million men are diagnosed with prostate cancer every year worldwide and it is the most common non-skin cancer among men, responsible for approximately 307.000 deaths in 2012 [4]. Although this seems to indicate a large increase in incidence that could be classified as an epidemic, the recent rise in life expectancy and advances in medical care would account to a large degree for the growth in the number of cases [5].

Age is a strong risk factor for prostate cancer, leading to more than 80 % of diagnosis being made in men over 65 years old. Moreover, the incidence rises exponentially with age, resulting in an increase of diagnosed men rising with life expectancy. In 1950, men's average life expectancy in developed countries was 64 years old compared with 75 in 2013; the gain is more impressive in less developed countries where it has risen from 40 to 62 years old. By 2050, the number of people over 65 years old is predicted to reach 16 % of the total population. By 2100, total life expectancy is estimated to be between 66 to 97 years, by 2300 from 87 to 106 years and is assumed to continue increasing [6].

Post mortem studies suggest that most men over 85 years old have histologically identified prostate cancer. Franks concludes that if a man reaches the age of 100 years old, he has almost 100 % probability of developing prostate cancer [7].

Increased incidence can also be linked to better diagnosis, and in particular the prostate-specific antigen (PSA) blood test carried out in asymptomatic men since the early 1990s [8]. This aspect combined with a greater acceptance by the population of the digital rectal examination leads to an estimation of 16 % of men having a diagnosis of prostate cancer during their life as a result of PSA screening [9].

1. Clinical Features of Prostate Cancer

Prostate cancer can be confined in the prostate gland and is then classified as early grade stage. However, it is defined as locally advanced when it breaks through the prostate gland capsule. From this stage, the tissues and lymph nodes are more likely to be reached which may culminate in a metastatic phase. Prostate cancer cells spread mostly by the lymphatic route to bones: especially vertebrae, femur, pelvis and ribs [10].

Prostate cancer cells that become hormone-independent are often highly invasive and more likely to progress to metastasis. A study conducted by Bubendorf *et al.* examining 1589 autopsies of prostate cancer patients over 27 years revealed that more than 90 % of the metastases were located in bone [11].

1.1. Prostate Cancer Treatment

Until the early 1990's, prostate cancer and other types of urinary obstruction usually had the same diagnosis and treatment, namely surgery and endocrine therapy [12]. One of the oldest techniques for androgen deprivation is orchidectomy, which is the total or partial removal of the testes. This practice started to be used for therapeutic applications at the beginning of the 20th century when the role of the testicles in prostatic enlargement began to be understood [13].

Another surgical method is the removal of the prostate gland. The first transpubic prostatectomy was performed in 1867 by Billroth and thereafter

many other surgical procedures were developed to improve this practice. However, complications such as infections, loss of blood, potency and continence remained a challenge [14]. In 1979, Reiner and Walsh described a technique for performing a radical retro-pubic prostatectomy that would become the basis for modern surgical methods [15].

Furthermore, hormonal therapy is now widely used and has become the mainstay of treatment for different stages of the disease, frequently as the first option for non metastatic tumors [16]. Although patient survival is prolonged, the tumor usually becomes androgen-independent after 24-36 months of treatment after which most patients develop more aggressive hormone-refractory cancers [17].

Medical advances have impacted on treatment as well as diagnosis. In the last ten years, new drugs have increased the life expectancy in men with advanced and terminal prostate cancer by a factor of almost three [18].

Until recently, hormone-refractory prostate cancer had only an estimated 1 year relative survival rate [19]. Today, about 83 % of patients survive for ten years [20] as a result of adjuvant therapy such as radiation therapy [21], immunotherapy [22] and chemotherapy [23], used alone or in combination [24].

Despite the variety of treatments available, problems such as distinguishing indolent from aggressive tumors, serious side effects, recurrence of the cancer, resistance to treatment and the propensity to metastasize still represent the major challenges in prostate cancer therapies [25]. Among the different strategies that could be used to overcome those difficulties nanotechnology has emerged as a promising candidate.

2. Nanotechnology and Nanomedicine

In recent years, nanotechnology has been the focus of considerable attention in medicine due to the facility with which nanostructures interact with the body at the molecular scale. New therapies in cancer research using nanomedicine are

being developed in order to improve the specificity and efficacy of drug delivery, thus reaching maximal effectiveness with minimal side effects [26].

A generally accepted definition of nanotechnology is given by The National Nanotechnology Initiative in the United States as the understanding and control of matter at nanoscale dimensions which is accepted to be approximately from 1 to 100 nanometers, where unique phenomena enable novel applications [27]. Nanomedicine is the subdivision of nanotechnology applied to the medicine, defined as the process of diagnosing, treating, and preventing disease and traumatic injury, relieving pain, and preserving and improving human health, using molecular tools and molecular knowledge of the human body [28].

Miniaturization can affect a material's fundamental properties compared with the bulk state. This effect is mainly due to the increase of the specific surface area in inverse proportion to the particle size. Moreover, not only the available area changes but also the arrangement of atoms at the surface which can confer new electronic, optical, thermal and magnetic properties which in turn influence biological interactions. For example, the size and the surface charge of particles can directly affect cellular uptake [29]. Nanoparticles can be tailored to a particular application. For example, decreasing the size of the particles to the nanoscale enables surface modification and favors cell uptake. Despite the small size, nanoparticles can be loaded for instance, with DNA or molecules such as therapeutic and diagnostic agents [30].

2.1. Drug Delivery

Drug delivery has been an important axis of biotechnology research, its goal being to delivery of a specific agent to a precise site of action to produce a desired pharmacological effect [31]. As well as the target, other factors such as the nature of the carrier and the route of administration must be considered when developing a drug delivery strategy [32].

The concept of drug targeting was created over a century ago by Paul Ehrlich, who invented a strategy to direct specific molecules to selectively attack

pathogens which he referred to as a “magic bullet” [33]. The first targeted drug, Salvarsan which was effective against syphilis, was a prodrug in which the active molecule was chemically modified to increase its interaction with the target [34]. However, with advances in nanotechnology, the non covalent association of drugs with particulate carriers has come to the fore.

The challenge of developing drug delivery devices is, most of all, the biocompatibility of the system. This means the ability to overcome the protective mechanisms present in the body without being toxic or triggering any immunological response in the organism. Furthermore, dispersibility, stability, permeability and good interaction with the cell membrane are decisive factors for the design of an effective drug delivery system [35]. Figure 1 illustrates the most important factors for the conception of a drug delivery device. Advances in the understanding of the chemical and biological interactions between drug delivery systems and the surrounding tissues have allowed these systems to be optimized [36].

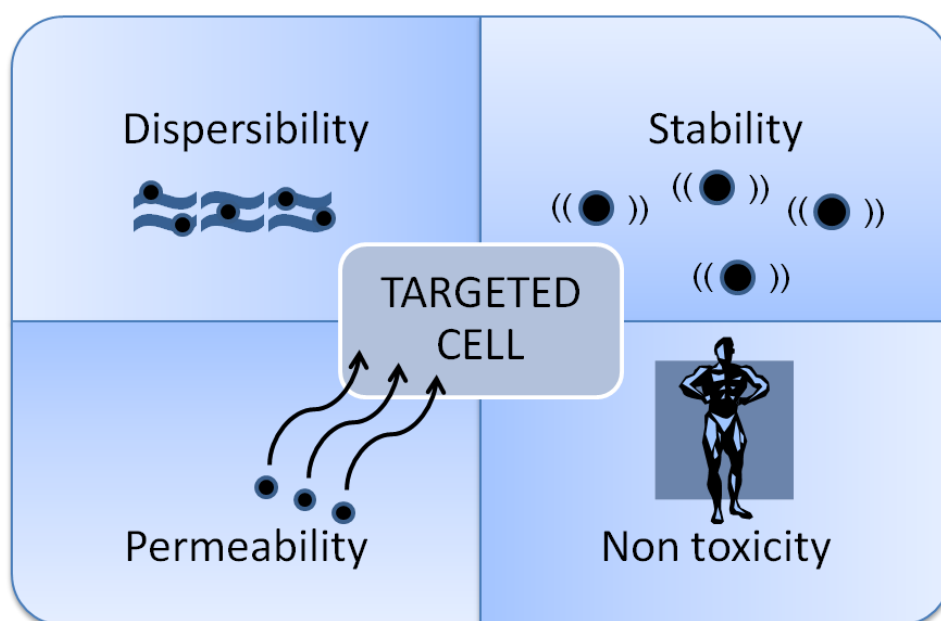


Figure 1: Main factors contributing to the biocompatibility of a drug deliver carrier.

2.1.1. Drug Targeting to Tumors

The concept of drug targeting is particularly applicable to the treatment of cancers. One of the biggest challenges to medical science today is to develop effective antineoplastic therapy. Conventional chemotherapy delivers a cytotoxic agent indiscriminately to neoplastic and normal cells. Drug targeting in cancer treatment is designed to avoid damage to the healthy organs and tissues and still increasing the tumor uptake. Nanotechnology-based chemotherapeutics can be tailored to deliver increased amounts of drug to the target tumor tissues by modifying their distribution [37]. This strategy can also optimize the clinical impact using combination therapies [38]. Important elements for nanoparticles engineering are shown in Figure 2.

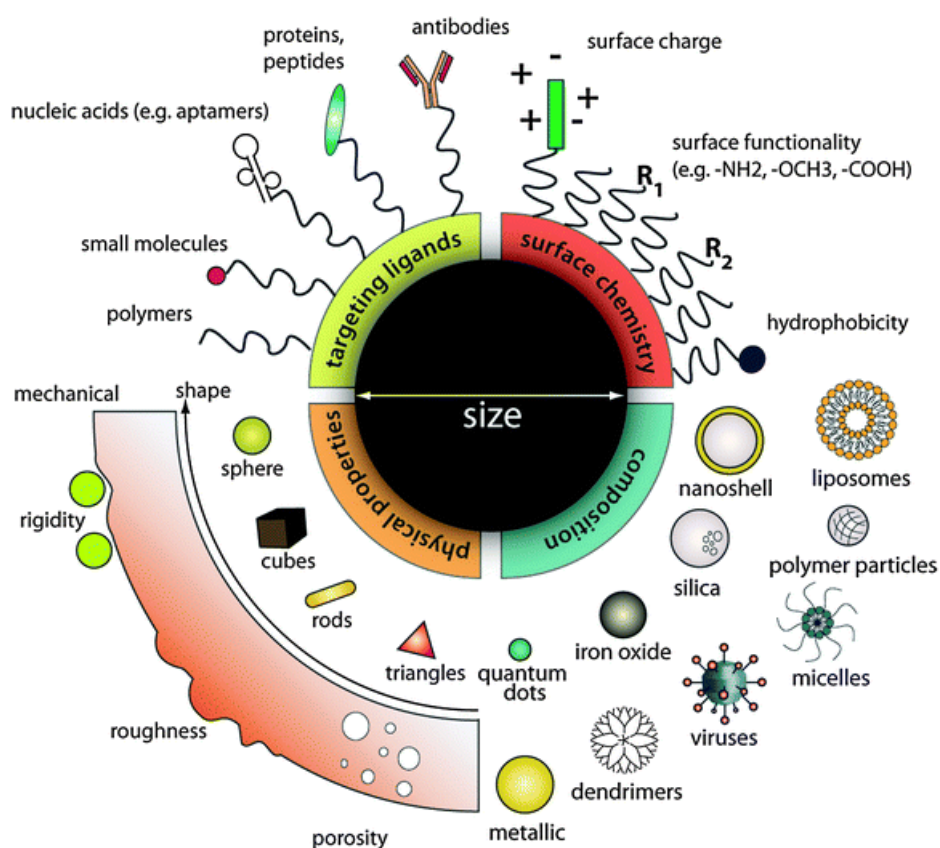


Figure 2: Important elements for nanoparticles design. Reproduced from the reference [39] with permission of The Royal Society of Chemistry.

2.1.2. Nanomedicine for Cancer Applications

Over the past ten years, scientists at the interface between biology and chemistry have been optimizing new strategies based on multifunctional nanoparticles [40]. Recently, the evolution of nanotechnology has allowed a range of new properties to be conferred on drug delivery systems such as delivery of poorly soluble drugs [41]; increase of cell permeability [42]; enhance transmembrane delivery [43]; co-delivery of two or more therapeutic agents with different properties [44]; tracking of the delivery system by imaging [45] and site-specific targeting [46].

Nanosized carriers are particularly appropriate in drug delivery to malignant cells because of some features of the tumor microenvironment and tumor angiogenesis. Solid tumors often have a leaky and irregular vasculature compared with healthy vessels. The endothelial cells that form the inner lining of the vessels do not have a normal monolayer configuration with tight junctions, compromising its barrier function [47].

Scanning electron microscopy has been used to show that the size of openings between defective endothelial cells can be up to 2 μm in diameter, which allows the entrance of small substances and molecules, including nanosized drug delivery systems [48]. On the other hand, the barrier dysfunction may increase the traffic of cancer cells in the bloodstream, increasing the chance of metastasis [49].

The size of the carriers must be carefully controlled because although nanosized systems are able to circulate for longer time, if they are too small, they can be eliminated by renal clearance. On the other hand, if they are large enough to be recognized by the immune system, they can be easily captured by phagocytic cells. In both cases, they will not reach their target [50]. It is known that nanoparticles up to 500 nm can penetrate through cell membranes [51] and nanocarriers smaller than 200 nm have minimal capture by the mononuclear phagocytic system, thus extending the circulation time [52].

If the size is regulated, the carrier-associated drugs tend to have a long circulating time and penetrate into tumor tissues more than free drugs. In addition, there is an impaired function of the lymphatic drainage, which facilitates the accumulation of the nanosized particles within the tumor. This phenomenon was first described by Maeda and Matsumura almost 30 years ago and it is known as the enhanced permeability and retention (EPR) effect [53].

The efficiency of the EPR effect is related to the tumor phenotype. Size, vascularity, perfusion and necrosis can influence the accumulation of nanoparticles inside the tumor. For example, small sized and high vascular nodules are more likely to be subject to the EPR effect. Indeed, in tumors larger than one cm the EPR effect has been demonstrated to be more heterogeneous. The accumulation of the carrier-associated drugs by the EPR effect was demonstrated to be more prominent in metastatic tumors [54]. Studies conducted by Heneweir *et al.* demonstrated that three prostate cancer xenografts showed a relationship between the accumulation of macromolecules and the tumor phenotypes (degree of necrosis) at early time points [55].

Even when tumors show unfavorable EPR properties, some strategies can be employed to optimize the uptake of nanocarriers. Many vascular mediators such as vascular endothelium growth factor (VEGF), bradykinin peptide and nitric oxide can affect the EPR effect in solid tumors. They play an important role in tumor development and probably in also metastasis which depends on vascular permeability. Therefore, the modulation of these factors can increase the EPR effect and thus the accumulation of the targeted drugs into tumors [56]. For example, the use of vasodilators like nitric oxide releasing agent can amplify the drug targeting. Their infusion into the arteries that supply the tumor may enlarge the endothelial fenestrations leading to an enhanced delivery of blood and nanocarriers to the neoplastic tissue [57].

Paradoxically, vasoconstrictors like angiotensin-II can also assist drug delivery through inducing hypertension. The blood flow volume through the defective

tumor blood vessels cannot be regulated in the same way as the normal ones in which the smooth muscle layer can constrict, causing higher blood pressure and flow rate to keep blood flow volume constant. In this way, larger amounts of nanocarriers are able to accumulate within the tumor due to the local increase of blood flow. Suzuki *et al.* demonstrated that the blood flow can increase 5.7 fold in tumor tissue while preserving the regular blood flow in the normal tissues through elevating the blood pressure up to 150 mmHg by the infusion of angiotensin-II [58]. It was showed by Nagamitsu *et al.* that the elevation of blood pressure by angiotensin-II can improve drug delivery and therapeutic efficacy in highly refractory solid tumors [59].

2.1.3. Reaching the Tumor: Active and Passive Targeting

Nanoparticle accumulation within tumors can be achieved by both passive and active targeting.

Passive targeting is based on two physiological phenomena occurring in bloodstream: the convection and diffusion. The convection process occurs by a pressure driven blood flow movement [60] and it is responsible for the transport of large molecules through the wide fenestrations in the tumor endothelium. Diffusion is mainly responsible for the transfer of highly lipophilic and low molecular weight compounds across the cell membrane according to the concentration gradient. The EPR effect can increase the accumulation of nanocarriers within the tumors [61]. This phenomenon was visualized in prostate cancer by Sandanarai *et al.* using a fluorescent nanoprobe and intravital microscopy [62].

In active targeting the surface of the nanoparticles is modified to achieve a specific interaction between the target cell and the carrier by binding to overexpressed receptors in the tumor site. However, to bind the target cells the nanocarriers must first reach the tumor and the EPR effect is still necessary. Active targeting by itself does not improve overall drug accumulation inside the

tumors but it improves cell recognition and uptake [63]. Examples of functional ligands for targeting tumor cells are transferrin [64], folate [65], and galactosamine [66].

2.1.3.1. Long-circulating nanoparticles

The probability of reaching the tumor is enhanced with an increase of the nanocarrier's circulation time in the bloodstream. To achieve this, surface properties can be changed by the addition of end-attached hydrophilic polymers which will confer "stealth" properties on them. In brief, these particles will be "hidden" from the mononuclear phagocytic system, preventing their early elimination [67].

Poly(ethylene glycol), (PEG) is the most widely used polymer for this purpose and it has been demonstrated that the accumulation of PEGylated nanoparticles in tumors was more than doubled when compared with non modified nanoparticles, accompanied by about three-fold reduction in nanoparticle clearance [68]. Other hydrophilic polymers such as dextrans, heparins, and polyvinylpyrrolidone can also be used to the same effect [69].

Despite the few examples of nanoparticle-based clinical treatments for prostate cancer, the number of publications is growing. A recent advanced search of the PubMed database for prostate cancer and nanotechnology or nanoparticles in the field's title plus abstract yielded 248 results against 67733 of prostate cancer itself which decreases to 34 if the term drug delivery is added, as shown in Figure 3.

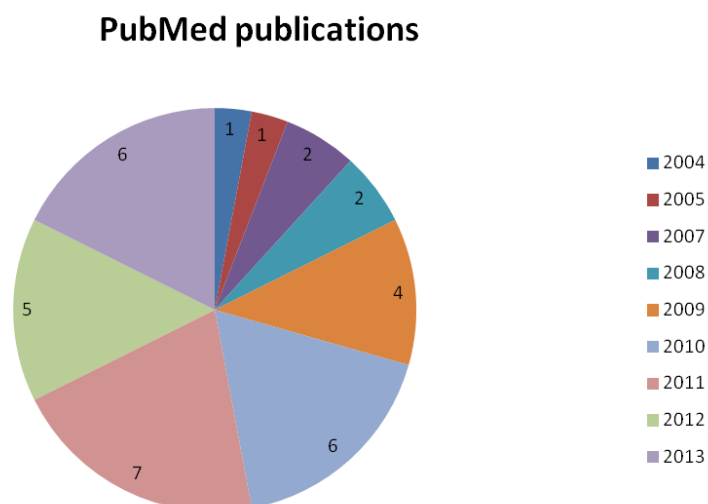


Figure 3: Number of publications per year found in January 2014 an advanced search at PubMed database.

In the rest of this review, advances in nanomedicine applied to the treatment of prostate cancer will be discussed, taking into account the mentioned factors mentioned above that influence drug delivery by nanocarriers. After a brief introduction of the different types of devices employed to this end, results obtained with the three main nanotechnology platforms will be detailed.

3. Liposomal Platform

A large proportion of the research in nanotechnology applied to cancer today concerns liposomal carriers. Liposomes are biodegradable single or multilamellar spherical vesicles which can encapsulate hydrophobic and hydrophilic substances due to their aqueous core surrounded by lipid bilayers. They may differ considerably in terms of structure and size depending on their composition and preparation method. Usually their size ranges from 90 to 150 nm and it can be composed of synthetic or natural lipids. The main component is phospholipids, often supplemented with cholesterol [70].

Liposomes can interact with cells to deliver their content in four different ways: adsorption, endocytosis, lipid exchange and fusion, although endocytosis is the

most important for drug delivery. The size, the composition and the presence of targeting agents will influence the mechanism of interaction, as well as the type of cell and the local microenvironment [71]. Structural features of liposomal drug delivery systems are shown in Figure 4.

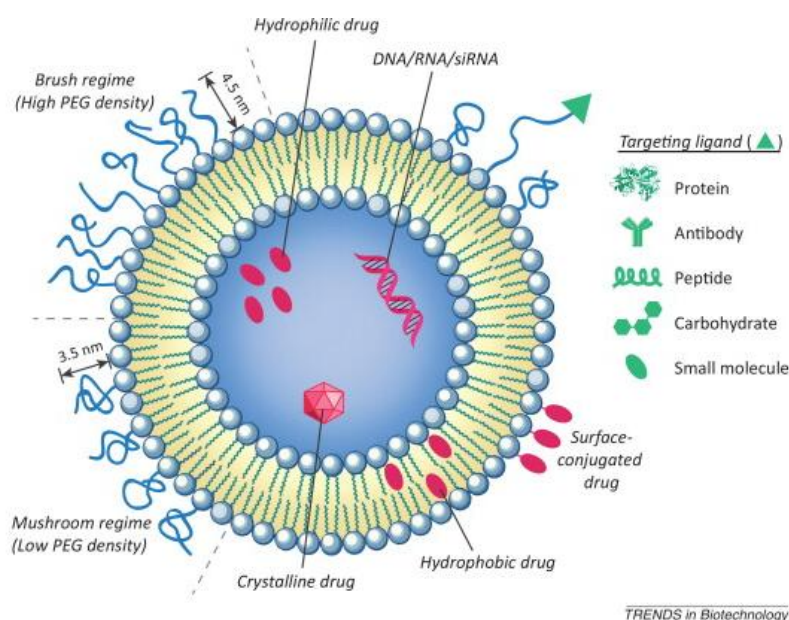


Figure 4: Liposomal drug delivery design considerations. Reprinted from publication [72], with permission from Elsevier.

In 1995 the first nanocarrier-based therapeutic was approved by the FDA: this was Doxil®, a pegylated doxorubicin-loaded liposome approved for the treatment of Kaposi's sarcoma. Despite its potential for prostate tumor treatment, it is approved for ovarian cancers only [73]. Doxil® is marketed as Caelyx® outside the United States.

3.1. Liposomal Nanoparticles in Prostate Cancer

Myocet® is a non-pegylated liposomal doxorubicin formulation approved for treatment of breast cancer and in Phase II trial for prostate therapy. Montanari *et al.* conducted studies to compare the activity of two passively targeting formulations, Myocet® and Caelyx®, against prostate cancer cells. They observed a better efficacy of Myocet® than of the pegylated form, despite the theoretical advantage of a long circulation time [74]. The encapsulation of

doxorubicin was shown to increase the dose of the active molecule that could be administered safely and to decrease the toxicity in non-tumor related tissues, such as myocardium, compared to the free drug [75].

Narayanan *et al.* studied two less well established drugs: curcumin and resveratrol encapsulated separately or together in liposomal carriers. They demonstrated a decrease in prostatic adenocarcinoma in mice and indeed, in vitro studies with the same formulation revealed apoptosis induction and an effective inhibition in cell growth [76].

Thangapazham *et al.* also developed liposomal formulations containing curcumin which were specifically targeted to prostate cancer cells by coating with an antibody to prostate membrane specific antigen (PSMA). This formulation was about 10-fold more efficient at inhibiting cell proliferation than the free drug in LNCaP and C4-2B cell lines [77].

Liposome-based drug delivery platforms have advantages in terms of biocompatibility because of the similarity of their lipid composition to that of the cell membrane. Indeed, they present low toxicity and they are able to incorporate both hydrophobic and hydrophilic drugs protecting them from degradation. Nevertheless, their instability and their short half-life are limiting factors for their application. Indeed, serum proteins can interact with the liposomes, destabilizing the membrane and facilitating their opsonization leading to fast clearance. The major research challenge for liposome use is to find the best functionalization strategies to overcome these issues [78].

4. Polymeric Nanoparticle Platform

Nanoparticles prepared from a wide range of polymers have already showed their efficacy to improve the bioavailability and the pharmacokinetic properties of approved chemotherapy drugs for prostate cancer, leading to a great advance in this field [79].

Natural polysaccharides such as chitosan and albumin as well as polyesters such as poly(D,L-lactic-co-glycolic acid) (PLGA) [80], poly(D,L-lactic acid) (PLA) [81] and poly(ϵ -caprolactone) (PCL) [82] which can be modified with PEG units forming pegylated co-polymers are the most commonly used polymers for drug delivery. They are biodegradable, biocompatible and they are able to encapsulate a variety of drugs [83].

Nanoparticulate carriers can have various morphologies, such as nanocapsules, nanospheres, micelles and dendrimers [84].

Nanocapsules are vesicular systems composed of central aqueous or oily core encircled by a polymeric shell. The main techniques that can be used to form this kind of nanoparticles are interfacial polycondensation, interfacial or emulsion polymerization, nanoprecipitation and emulsification/solvent evaporation [85].

Nanospheres are particles with a matrix structure in which drugs and ligands can be dispersed, encapsulated, chemically bound, adsorbed or entrapped within the whole of the particle or at the surface [86].

Nanocapsules and nanospheres are usually formed from linear polymers or copolymers. Highly branched polymers can also form structures known as dendrimers. The name dendrimer means tree, from the Greek word dendron, referring to its multi-branched architecture. Tomalia *et al.* published the first paper on poly(amidoamine) (PAMAM) dendrimers in 1985 [87] and since then, many studies are being focused in this kind of polymeric conformation for application in the treatment of cancer, among others [88].

Polymeric micelles are colloidal particles formed by amphiphilic copolymers with a high proportion of hydrophilic chain than those used to form nanoparticles, arranged in a core-shell structure. They have attractive properties as drug delivery systems. They have usually have a hydrodynamic diameter between 5 to 100 nm and their ability to incorporate poorly soluble molecules makes them a good candidate for cancer drug delivery [89].

Despite the variety of conformations that polymers can adopt, the properties of nanoparticles which make them suitable for drug delivery are quite similar. Thus, many authors do not specify the exact structure of their nanocarriers, focusing on their composition and their efficacy, while referring to them in a general manner as polymeric nanoparticles.

4.1. Polymer-based Devices in Prostate Cancer

The team of Farokhzad was a pioneer in the development of functionalized targeted aptamer-conjugated polymeric nanoparticles using prostate cancer treatment as a model. The method was to co-precipitate the antineoplastic drug docetaxel with the co-polymer PLGA-PEG followed by surface functionalization with A10 aptamer able to bind to PSMA. A 77-fold increase in binding LNCaP prostate cells was shown compared to the non-targeted NPs [90].

In vivo, these nanoparticles were able to reduce tumor size in LNCaP xenografts in nude mice, leading to 100 % survival during 109 days of studies after a single intratumoral injection, compared with 14 % survival in the group treated with free docetaxel. These results demonstrate the potential of these bioconjugates in prostate cancer therapy [91].

Farokhzad also encapsulated cisplatin in PLGA-PEG-Apt nanoparticles, showing an improvement of 3-fold in the therapeutic index and a decrease of nephrotoxicity in mice bearing LNCaP xenografts, when compared to the free cisplatin [92].

A mixture of docetaxel encapsulated within PLGA-PEG and PLA-PEG containing cisplatin was also tested. This strategy was designed to overcome single drug exposure challenges such as drug resistance. A synergy between the two drugs was found, showing at least 5.5-fold more cytotoxicity than nanoparticles carrying only one of the drugs [93].

Curcumin-loaded nanocarrier was developed by Anand *et al.* for passive targeting. This was prepared from biodegradable PLGA-PEG with a particle

size of about 81 nm. *In vitro* tests showed that the prostate cell line DU-145 had lower viability after 72 h when treated with the encapsulated curcumin than with the free drug [94].

A drug delivery approach has been applied to hormonal treatment of prostate cancer for some time. Indeed, several formulations of PLGA microparticles for LH-RH agonists release are commercially available. In these formulations, the drugs are released progressively from the matrix by a combination of diffusion and polymer degradation [95].

This strategy of controlled release decreases the frequency of injections required for conventional hormonal therapy down to one every 6 months, leading to a better adherence and efficacy of the treatment [96]. Despite being out of the nano-size range and not being targeted therapy, these polymer-based formulations play an important role in prostate therapy nowadays [97]. Therefore, we have summarized some available formulations in Table 1.

Table 1: List of some commercially approved polymer-based formulations used in prostate cancer treatment.

Trade name	Company	Polymer	Active	Administration	Formulation
Lupron Depot [®]	Abbott	PLGA	Leuporelin	intravenous	microspheres
Firmagon [®]	Ferring	PLGA	Degarelix	subcutaneous	powder*
Decapeptyl [®]	Ipsen	PLGA	Triptorelin	intramuscular	microspheres
Trelstar Depot [®]	AndaMeds	PLGA	Triptorelin	intramuscular	microspheres
Enantone Depot [®]	Takeda	PLGA	Leuporelin	subcutaneous	microspheres
Prostap [®]	Wyeth	PLA	Leuporelin	subcutaneous	microspheres
Zoladex [®]	AstraZeneca	PLGA	Goserelin	subcutaneous	implant

* The combination of the powder and the aqueous reconstitution phase forms a gel subcutaneously.

Polymeric nanoparticles tend to be more stable than liposomes because of lower interactions with serum proteins, particularly if their surface is functionalized. Size, particle degradation and controlled release are features that can be modified according to the matrix constituents and the targeted tissue. Drug activity is generally preserved because the methods of obtaining polymeric nanoparticles are usually fast and without complex chemical

reactions steps [98]. In spite of the advantages, the wide range of choice in composition and functionalization makes it difficult to predict the pharmacological behavior of polymeric nanoparticles. However, the main challenges for all types are to avoid immune responses and to maximize the payload of active drugs [99].

5. Metallic Nanoparticles Platform

Metallic nanoparticles are versatile tools in biomedical research due to their stability combined with their small size, useful optical properties and easy functionalization. Applications such as thermal ablation [100], radiotherapy enhancement [101], diagnostic assays [102] and drug delivery [103] have been the focus of recent studies. Additionally, the intrinsic metal properties are favorable for the combination of therapy and diagnostics in the same particle, in the method known as theranostics [104].

Besides the proven biocidal effect of silver nanoparticles (AgNPs) [105, 106], they have been also shown to have some specific features for cancer applications. For example, silver nanoparticles may have antiangiogenic properties [107] and inhibit cell proliferation in cancer development [108]. Gopinath *et al.* investigated the AgNPs cytotoxicity in cancer cells and observed induction of apoptosis and a synergic effect with the chemotherapeutic agent 5-fluorouracil [109]. Recently, Firdhouse and Lalitha described a cost-effective “green” synthesis of silver nanoparticles of around 100 nm using an aqueous extract of *Alternanthera sessilis*. The resulting NPs demonstrated to have a significant activity against PC3 prostate cells [110].

Despite these encouraging results obtained with silver NP, gold nanoparticles are at the head of the noble metal nanoparticles for cancer applications. Cai *et al.* described the versatility of AuNPs as the “gold magic bullet” since they present great versatility in terms of surface functionalization, imaging use and multiple therapeutic entities [111].

5.1. Metallic Nanoparticles in Prostate Cancer

The group of Roa has investigated the functionalization of AuNPs to enhance radiation cytotoxicity in different cancer models. For example, they synthesized glucose-bound AuNPs and demonstrated an increase in toxicity and sensitivity in DU-145 prostate cancer cell line [112].

Arnida *et al.* studied the toxicity and cellular uptake of AuNPs in PC3 prostate cell line according to their size, shape and surface properties. They found that nanoparticles of 50 nm without a pegylated surface had a better uptake compared with the other particles evaluated [113].

Gold nanoparticles have been synthesized with different surface functionalization by the Astruc team [114]. Indeed, good results against LnCaP prostate cell lines were found by us when AuNPs were used to encapsulate the antineoplastic drug docetaxel [115].

One important property of gold nanoparticles for cancer applications is their thermal diffusivity. The use of local hyperthermia guided by gold nanoparticles accumulation with the tumor is a recent strategy to provoke cancer cell necrosis [116].

Superparamagnetic compounds such as iron oxide are being used for local delivery. In this case, the nanoparticles are guided to the targeted area by an external magnetic field where the nanocarriers can release antineoplastic drugs [117]. Figure 5 shows different strategies for cancer therapy using metallic nanoparticles.

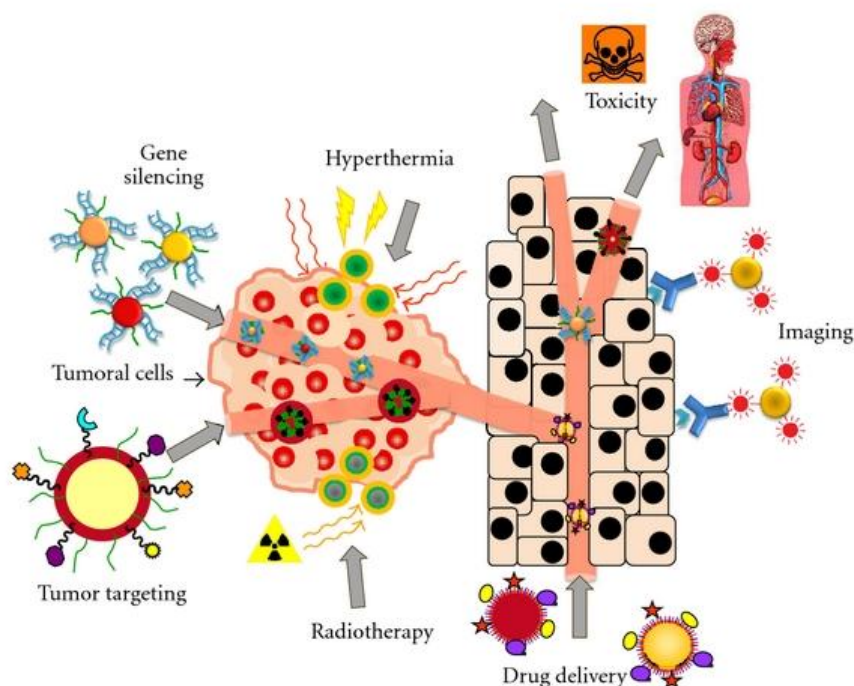


Figure 5: Strategies for cancer therapy using metallic nanoparticles. Copyright© 2012 João Conde *et al.* [118].

Concerns are often raised about the possible toxicity of metallic nanoparticles. In general, the toxicity of nanoparticles is determined by a number of factors. A range of features such as shape, morphology, physical and chemical properties and electronic coordination must be analyzed. Furthermore, toxicity *in vivo* cannot always be predicted from *in vitro* studies.

However, there is one important aspect of metal nanoparticles that cannot be ignored: the toxicity of the ions derived from them. Some studies indicate that the toxicity of the metallic nanoparticles is mainly due to their capacity to release ions. Thus, the ionic form seems to present a higher toxicity than the elemental state [119]. Transition metal ions can undergo redox cycling and generate reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide and superoxide ions. The biological activity of ROS can result in DNA damage, enhanced lipid peroxidation and lipid peroxidation and impaired intracellular calcium homeostasis, disrupting the normal cell cycle [120].

6. Other Nanoparticle-Based Platforms for Prostate Cancer Therapy

In the last ten years, other inorganic compounds have attracted attention in the domain of cancer treatment, including carbon nanotubes and quantum dots. Carbon nanotubes, like gold nanoparticles, can be used for thermal ablation therapeutics together with targeting agents, such as folate. Quantum dots are made from light emitting semiconductor materials, a property which allows their application in bioimaging and biodiagnostics. Thus, quantum dots can be combined with polymeric nanoparticles in order to bind targeting ligands and it was demonstrated to be efficient *in vivo* using mice as a model [121]. Both carbon nanotubes and quantum dots are being studied for cancer therapeutics as carriers of small interfering RNA (siRNA) to tumor cells *in vitro* [122].

Different drug delivery strategies can be combined to create new properties and avoid drawbacks. For example, a strategy to avoid the problem of the poor stability of inorganic nanoparticles in physiological fluids is to encapsulate those particles within a polymeric matrix. A recent review about this strategy used for biomedical applications has been published by Ladj and co-workers [123]. Furthermore, gold can be combined with other materials in order to take advantage of its thermal quality. Nanoshells, for example, can be made of silica or polymer beads surrounded by a gold layer. This composition allows the functionalization of the carrier to enhance the accumulation within tumors. The gold shell absorbs light in the near infrared wavelength region and thus enables the controlled hyperthermic ablation of the cancer cells *in vitro* [124].

Despite the increase in research and publications describing the use of nanomedicine in prostate cancer therapy, the number of clinical trials undertaken for this application remains small. Table 2 summarizes these investigations.

Table 2: An overview of targeted nanocarriers applied to prostate cancer undergoing clinical investigation. Clinical data are extracted from <http://clinicaltrials.gov> and cited references.

Nanocarrier	Product	Functionalization	Therapeutic	Phase	Organization	References
Liposomes	Caelyx/ Doxil	Pegylated	doxorubicin	II	Janssen- Cilag	[125-128]
Liposomes	Myocet	Not applied	doxorubicin	II	Enzon	[74, 129, 130]
Polymeric NPs PLGA	BIND- 014	Peptide	docetaxel	II	BIND Biosciences	[131]
Polymeric NPs Cyclodextrin	CALAA- 01	Pegylated; Transferrin	anti RRM2 siRNA	I	Calando	[132]
Polymeric NPs Poly(L- glutamic acid)	Xyotax	Not applied	paclitaxel	II	Cell Therapeutics	[133, 134]

7. Safety and Environmental Impact of Nanoparticles

Although the nanotechnology is now widely studied in the field of nanomedicine, appropriate safety guidelines must be drawn up based on nanoparticle toxicity to ensure their safe use [135].

The specific properties of the particles and the target tissue both plays role in determining the NPs biocompatibility [136]. Even if some type of nanoparticle may present a health risk, intelligent design of these carriers can increase the benefit-risk ratio [137]. For example, in-vivo studies that showed severe toxicity of naked AuNPs in mice also demonstrated a decrease in this toxicity after functionalization of the NP with immunogenic peptides [138].

The toxicity of nanoparticles to the environment also needs to be considered. As for human cell interaction with nanoparticles, the cell walls of plants, algae and fungi are susceptible to damage. Unfortunately, since NPs persist in the ecosystem for a long time, tracking these particles and undertaking toxicological studies is difficult [139]. Hence, from an environmental point of view, the risks

are still not very well understood and the impact of manufactured nanomaterial represents a new area in toxicology [140]. In particular, the ecotoxicological risks in the aquatic environment and its linked danger to human health have been highlighted based on the uptake and bioavailability of these forms into organisms and cells [141].

CONCLUSION

In this paper, the basis of drug targeting and delivery in nanomedicine has been reviewed. The application of different platforms for potential prostate cancer therapies has been described, showing how the design of the nanocarriers can be tailored to the application. However, the use of nanoparticles for cancer therapy is complex and biological and chemical interactions need to be taken into account.

As well as the efficacy and the biocompatibility of these nanoparticles, their toxicity should be well understood. The benefits of the nanoparticulate form need to outweigh any undesirable effects. Indeed, the environmental risks are still not very well established and need to be considered.

Thus, nanomedicine seems to have great potential for prostate cancer therapy. Many different strategies based on nanoparticles are being studied for this purpose. Some of them are undergoing clinical trials; however no nanoparticle-based drug for prostate cancer is yet approved by the regulatory agencies. Up to now, only small particles being used for prostate tumor therapy are for hormonal release but unfortunately the long-term efficacy is still controversial.

Therefore, the perspectives seem to be positive. The number of publications that demonstrate promising results is increasing and hopefully in the near future prostate cancer patients will be able to benefit from new strategies to replace or to complement the current treatments.

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CHAPTER II

SYNTHESIS AND *IN VITRO* STUDIES OF GOLD NANOPARTICLES LOADED WITH DOCETAXEL

Introduction

Much research is being devoted to the targeting delivery of docetaxel. This taxane is one of the few drugs which are effective in hormone-refractory prostate cancer patients. Combinations involving docetaxel were shown in phase-II studies to bring about a significantly decline in the levels of Prostate Specific Antigen (PSA) rate compared to the current docetaxel therapy. However, toxicity remains a concern and there is a need to develop formulations which could deliver docetaxel more specifically to cancer cells [1].

New therapies in cancer research using nanomedicine are being developed in order to improve the specificity and efficacy of drug delivery, thus reaching maximal effectiveness with minimal side effects [2].

Innovative new forms capable of reaching the tumor are necessary for the development of a suitable carrier system that confers all the characteristics required by a good anti-cancer drug. The effectiveness of docetaxel allied with nanotechnology approaches could be a important strategy to improve prostate cancer therapies.

Prostate Specific Membrane Antigen and Folate

The discovery of PSMA (Prostate Specific Membrane Antigen) is one of the most important findings in the study of prostate cancer. PSMA has an affinity for folic acid and its overexpression has been found to give prostate cancer cells a growth advantage in a physiologically relevant folate environment *in vitro* [3]. Not only can this be used as a diagnostic tool and a means of following the efficacy of therapy but it also provides a good target for therapy. Indeed, the efficacy of drugs can be largely improved by specific targeting to folate receptors. Folate has been used as a ligand for the targeting of proteins [4], liposomes [5] and others molecules [6, 7]. When molecules of folic acid are bound covalently to macromolecules, the conjugate is internalized by endocytosis after binding to PSMA [8].

Poly(ethylene glycol) (PEG)

The ability of colloidal drug delivery systems whose surfaces have been modified by poly(ethylene glycol) (PEG) to accumulate within solid tumours has been amply demonstrated. PEG reduces the opsonization of the particle surface with blood proteins and thereby delays their capture by phagocytic cells. The drug delivery systems can then remain in the circulation and reach the tumor by the so-called “enhanced permeation and retention” (EPR) effect, when the tumour capillary endothelium is leaky and the lymphatic drainage is impaired [9]. Attaching PEG chains to the surface of nanoparticles made from biodegradable polyesters has allowed anticancer drugs, including docetaxel, to be delivered to solid tumours [10].

Gold nanoparticles

Gold nanoparticles (AuNPs) have been known for a long time as assemblies of gold atoms, with diameters of the order of 1 to 100 nm [11]. They have been used with promising results as drug delivery vehicles [12]. More recently, spherical AuNPs have been used for surface photothermal cancer therapy via irradiation of the plasmon band that corresponds to their resonance absorption around 530 nm. One of the major interests in using AuNPs is their easy bioconjugation and biomodification. In particular, the strong affinity of AuNP surface for thiolate ligands has allowed surface conjugation with peptides, proteins and DNA via the naturally available cysteine thiol or a synthetically incorporated thiol as in single-stranded DNA. For example, folate has been grafted for the selective targeting of cancer cells [13]. Thiolated PEG passivates AuNPs and masks them from the intravascular system, allowing increased blood circulation times [14]. AuNPs can be grafted with multiple functionalities, making a powerful drug delivery system [15].

The aims of this part of the study were to formulate gold nanoparticles carrying docetaxel and PEG with and without folic acid, to characterize them and to evaluate their anticancer activity *in vitro*.

The second chapter presents the first part of the experimental work. A paper entitled “Synthesis and *in vitro* studies of gold nanoparticles loaded with docetaxel” published in May 2013 in the *International Journal of Pharmaceutics* describes and discusses the part of the study dedicated to the gold nanoparticles as potential carrier of docetaxel for prostate cancer application. The graphical abstract of the mentioned article is shown in Figure 1.

The synthesis of the nanoparticles was carried out by our partners at the Bordeaux 1 University. The subsequent characterization, encapsulation and *in vitro* tests against prostate cancer cells were my personal contribution. Supplementary data are attached to this chapter presenting the results of the surface plasmon resonance measurements which were not shown in the publication.

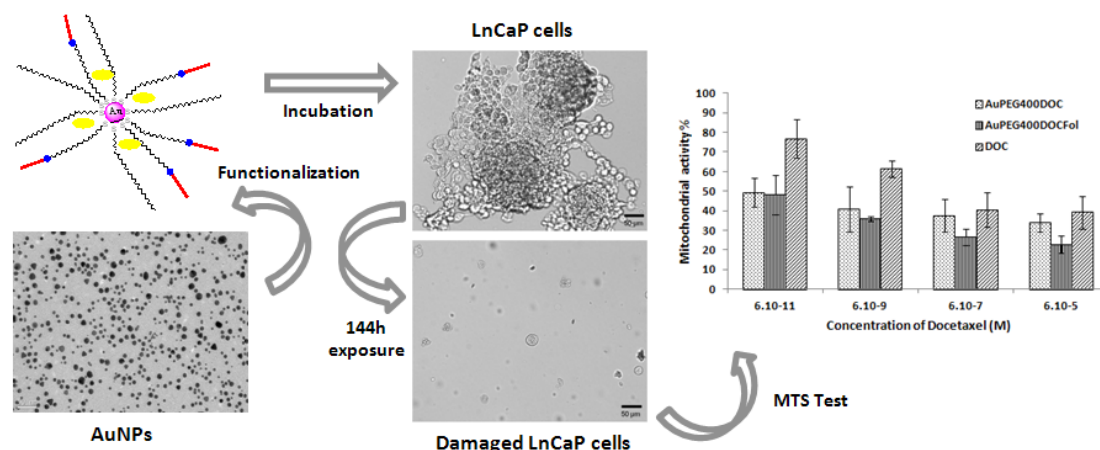


Figure 1: Graphical abstract of the article “Synthesis and *in vitro* studies of gold nanoparticles loaded with docetaxel”.

The article can be found on internet though the following link: <http://www.sciencedirect.com/science/article/pii/S0378517313004341>

1. Synthesis and *in vitro* studies of gold nanoparticles loaded with docetaxel

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Synthesis and *in vitro* studies of gold nanoparticles loaded with docetaxel



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ABSTRACT

The aim of these studies was to synthesize, characterize and evaluate the efficacy of pegylated gold nanoparticles (AuNPs) that differed in their PEG molecular weight, using PEG 550 and PEG 2000. The synthesis of the gold nanoparticles was carried out by modified Brust method with a diameter of 4–15 nm. The targeting agent folic acid was introduced by the covalent linkage. Finally, the anti-cancer drug docetaxel was encapsulated by the AuNPs by non covalent adsorption. The nanoparticles were characterized by transmission electron microscopy and used for *in vitro* studies against a hormone-responsive prostate cancer cell line, LnCaP. The loaded nanoparticles reduced the cell viability in more than 50% at concentrations of 6 nM and above after 144 h of treatment. Moreover, observation of prostate cancer cells by optical microscopy showed damage to the cells after exposure to drug-loaded AuNPs while unloaded AuNPs had much less effect.

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1. Introduction

Prostate cancer is the second most important cancer in terms of incidence in both sexes and the sixth in terms of mortality for men throughout the world according to the International Agency for Research on Cancer (Ferlay et al., 2010). At the moment, this cancer is treated with hormone therapy, but this is usually only effective for 24–36 months before the patient develops resistance (Holzbeierlein et al., 2004). Docetaxel was the first cytotoxic therapy to show a survival benefit in castration-resistant prostate cancer. For this reason, it remains an important part of the treatment against metastatic prostate cancer despite its toxicity and other limitations (Hwang, 2012).

Like paclitaxel, docetaxel belongs to the family of taxanes, which are diterpenes produced by plants of the genus *Taxus* (Zhao and Astruc, 2012). Docetaxel is a semi-synthetic taxane that binds irreversibly to β -actin, thus altering microtubule polymerization dynamics, disrupting cell mitosis and interphase microtubule

function and triggering apoptosis (Kraus et al., 2003). In phase-II studies, combinations of drugs including docetaxel have been shown to result in a significantly higher PSA decline $\geq 50\%$ in hormone-refractory prostate cancer patients (Ferrero et al., 2006; Mezynski et al., 2012; Oudard et al., 2005). However, the toxicity of the drug remains a concern.

Much research is currently being directed toward developing targeted delivery systems for docetaxel, in order to increase the amount of drug reaching the cancer cells and spare normal cells. Lipid nanocapsules (Sánchez-Moreno et al., 2012), nanoparticles piloted by aptamers (Farokhzad et al., 2006) and block copolymer micelles (Gao et al., 2008; Gaucher et al., 2005; Mei et al., 2009; Ungaro et al., 2012) are some examples of nanocarriers containing docetaxel designed to target various types of cancer, such as lung, breast and prostate cancer. The encapsulation of this molecule within gold nanoparticles bearing folate on their surface and thus able to target the prostate cancer cells is an original therapeutic approach to improve its effectiveness.

Gold nanoparticles of various sizes and morphologies have attracted considerable interest for medical applications (Boisselier and Astruc, 2009; Zhao et al., 2013), including photothermal therapy (Lal et al., 2008; Nijdom et al., 2006; Pissuwan et al., 2006), cancer diagnosis (Lee, 2007; Llevot and Astruc, 2012; Zeng et al., 2011), tumor imaging (Copland et al., 2004; Kim et al., 2010), and

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drug delivery (Brown et al., 2010; Ghosh et al., 2008; Paciotti et al., 2006; Llevot and Astruc, 2012). The plasmon resonance properties of these nanoparticles allow their characterization and detection in biological systems (Jain et al., 2007).

There are several synthesis routes for this type of nanoparticles (Daniel and Astruc, 2003). Surface functionalization can impart the required properties to the nanoparticles; for example, specific recognition (Raschke et al., 2003; Wang et al., 2005) or biocompatibility (Mout et al., 2012). The effective use of these nanoparticles requires controlled interactions with biomacromolecules (Mout et al., 2012) and their toxicity must be carefully evaluated (Murphy et al., 2008).

Folic acid has high capacity for targeting cell membrane receptors on a range of cell types, allowing nanoparticle endocytosis (Mansouri et al., 2006). Moreover, folic acid has high affinity for prostate-specific membrane antigen (PSMA), overexpressed on prostate cancer cells (Ghosh and Heston, 2004).

This work concerns the development of a multifunctional drug delivery system (DDS) based on gold nanoparticles (AuNPs). The nanoparticle surface was modified with poly(ethylene glycol) to prevent opsonization, delay their capture by macrophages and thereby allow them to circulate longer in the body and reach the tumor by the EPR (enhanced permeation and retention) effect (Lyer et al., 2006). The targeting agent, folic acid, was introduced by the covalent linkage on the amino-terminus of PEG. Docetaxel was associated with the AuNPs by non covalent adsorption.

2. Materials and methods

2.1. Materials

All solvents and chemicals were used as received. ^1H NMR spectra were recorded at 25 °C with a Bruker 300 (300 MHz) spectrometer. All the chemical shifts are reported in parts per million (δ , ppm) with reference to Me_4Si for the ^1H and ^{13}C NMR spectra. Absorption spectra were measured with Perkin-Elmer Lambda 19 UV–vis. spectrometer. The CellTiter 96[®] Aqueous One Solution Reagent (MTS) reagent was purchased from Promega (Madison, USA). Cell culture reagents were from Lonza (Basel, Belgium). Docetaxel (DOC) and folate-binding protein (FBP) were purchased from Sigma–Aldrich (Illkirch, France) and DMSO was from Carlo Erba (Milan, Italy).

2.2. Synthesis of gold nanoparticles

2.2.1. Synthesis of gold nanoparticles modified with PEG: AuPEG550 and AuPEG2000

Gold nanoparticles containing PEG were synthesized by direct modified (single-phase) Brust type method using a mixture of HAuCl_4 methanol and of two thiol-PEG ligands: one type ligand is thiol-PEG550 or thiol-PEG2000, and the other type is functional thiol-PEG400- $\text{NH}_2\cdot\text{HCl}$. PEG-thiol ligands were described previously (Zhao et al., 2012). Only one PEG chain length was used for the functional thiol-PEG-ammonium: 400 Daltons (PEG 400).

2.2.2. Synthesis of gold nanoparticles modified with PEG and folate: AuPEGFol550 and AuPEGFol2000

2.2.2.1. Synthesis of HS-PEG- $\text{NH}_2\cdot\text{HCl}$. The thiolate-PEG- $\text{NH}_2\cdot\text{HCl}$ (HS-PEG- $\text{NH}_2\cdot\text{HCl}$) was synthesized as described previously by anionic ring-opening polymerization of ethylene oxide using the allyl alcohol/potassium naphthalene initiator system (Yoshimoto et al., 2008). ^1H NMR (CDCl_3 , 300 MHz) 3.58 (40H, $-\text{CH}_2\text{CH}_2\text{O}-$), 3.17 (2H, $-\text{CH}_2\text{NH}_2\text{HCl}$), 2.70 (2H, HSCH_2-).

2.2.2.2. Synthesis of HS-mPEG. The synthesis route of thiolate-mPEG (HS-mPEG) was as described in Zheng et al. (2004). To

summarize, the starting material for synthesizing HS-mPEG is mPEG-Br. After refluxing with thiourea in ethanol, the bromo group was converted to an isothiuronium bromide and then to a $-\text{SNa}$ group after a second refluxing with sodium hydroxide. Neutralization with dilute HCl generated mPEG-SH. Distillation of the extracted HS-mPEG produced the pure compound with 74% yield. ^1H NMR (CDCl_3 , 300 MHz) 3.60 (40H, $-\text{CH}_2\text{CH}_2\text{O}-$), 3.33 (3H, $-\text{OCH}_3$), 2.68 (2H, HSCH_2-).

2.2.2.3. Synthesis of FA-NHS. The synthesis of FA-NHS (Folate-NHS) was carried out as described previously (Zhang et al., 2010). Folic acid (FA) and anhydrous triethylamine were added to anhydrous DMSO. To this solution, *N*-hydroxy succinimide of dicyclohexyl carbodiimide were added and the reaction mixture was stirred for 48 h. After filtration, the filtrate was poured into ethyl acetate. The yellow precipitated powder was collected by filtration and washed twice with ethanol. The crude product was further purified by precipitation in ether, with a yield 77%. ^1H NMR (DMSO- d_6 , 300 MHz) 8.60 (1H, pyrazine ring), 7.61 (1H, arom ring), 6.97 (1H, $-\text{CH}_2\text{NH}-$ arom ring), 6.63 (1H, arom ring), 4.64 (2H, $-\text{CH}_2\text{NH}-$ arom ring), 2.82 (4H, $-\text{NOC}-\text{CH}_2\text{CH}_2-\text{CON}-$), 2.29 (2H, $-\text{CH}_2\text{CH}_2\text{COO}-$), 1.99 (2H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$).

2.2.2.4. Synthesis of AuPEGNPs. Thiolate-mPEG (Mw = 550 mg; 55 mg, 0.1 mmol) and thiolate-PEG- NH_2HCl (Mw = 490; 49 mg, 0.1 mmol) were dissolved in 10 mL MeOH, and this solution added into a solution of HAuCl_4 (100 mg) in 30 mL ($\text{MeOH}:\text{H}_2\text{O}$ 1:1). After stirring for 5 min, 1 mL of a freshly prepared NaBH_4 (100 mg) aqueous solution was added dropwise and stirred vigorously for another hour. Then, MeOH was evaporated under reduced pressure, and the water phase was salted into 30 mL CH_2Cl_2 with a minimum amount of NaCl. The organic phase was separated and dried over Na_2SO_4 . After evaporation of the solvent under vacuum, the crude product was dissolved in 30 mL distilled water followed by dialysis. Yield: 45 mg (61%). ^1H NMR (CDCl_3 , 300 MHz) 3.58 (40H, $-\text{CH}_2\text{CH}_2\text{O}-$), 3.33 (3H, $-\text{OCH}_3$), 3.17 (2H, $-\text{CH}_2\text{NH}_2\text{HCl}$), UV–vis: Plasmon band at 530 nm.

2.2.2.5. Synthesis of AuPEGNPs bearing folate residues. 20 mg of AuPEGNPs from the above synthesis was dissolved in 4 mL CH_2Cl_2 , and 0.02 mmol FA-NHS was dissolved in 4 mL DMSO and added, 0.2 mL triethylamine (TEA) was added to the mixture and stirred overnight at room temperature. After evaporating CH_2Cl_2 under vacuum, 50 mL ether was poured into the solution, and then the precipitate was collected and dissolved into CH_2Cl_2 , and filtered over paper. The filtrate was evaporated to obtain the product as 14 mg of deep red crystals. ^1H NMR (DMSO- d_6 , 300 MHz) 6.97 (1H, $-\text{CH}_2\text{NH}-$ arom ring), 6.63 (1H, arom ring), 3.58 (40H, $-\text{CH}_2\text{CH}_2\text{COO}-$), 3.33 (3H, $-\text{OCH}_3$), 2.29 ($-\text{CH}_2\text{CH}_2\text{COO}-$), 1.99 (2H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$). UV–vis: Plasmon band at 530 nm.

Four different gold nanoparticles were synthesized using the methodology described above:

AuPEG550 – Gold nanoparticles modified with PEG 400 and PEG 550

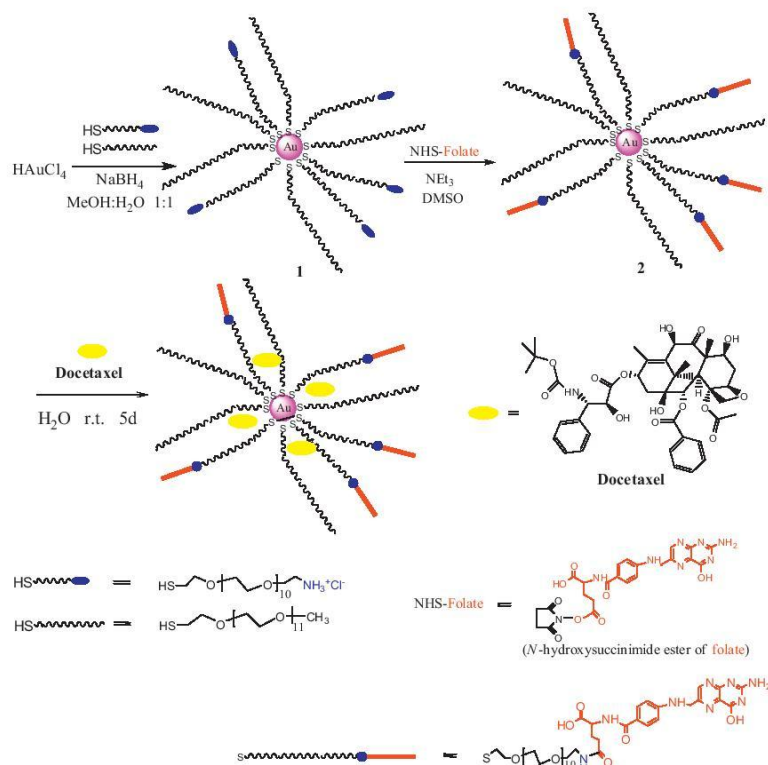
AuPEG550Fol – Gold nanoparticles modified with PEG 400 with folate and PEG 550

AuPEG2000 – Gold nanoparticles modified with PEG 400 and PEG 2000

AuPEG2000Fol – Gold nanoparticles modified with PEG 400 with folate and PEG 2000.

2.3. Characterization of the nanoparticles

Transmission electron microscopy (TEM) with a Philips EM208 (1996) instrument equipped with wide-field CCD camera



Scheme 1. Synthesis of gold nanoparticles and encapsulation of docetaxel.

acquisition (AMT) was used to evaluate the size and possible aggregation of nanoparticles containing PEG 550. A 120 kV electronic microscope 1400 equipped with wide-field CCD camera acquisition (Gatan) was used to observe the gold nanoparticles containing PEG 2000.

2.4. Surface plasmon resonance measurement

The interactions between FBP covalently immobilized on the surface of a CM5 sensor chip and the gold nanoparticles modified with folate were analyzed by surface plasmon resonance with a Biacore T100 instrument (GE Healthcare). Functionalization of the different channels of the chip was carried by amine coupling of FBP using EDC and NHS in the presence of ethanolamine.

2.5. Docetaxel loading

DOC was weighed accurately docetaxel (XP105 DeltaRange Analytical Balance – Mettler Toledo, Zurich, Switzerland) and a stock solution in DMSO at 29, 6 mg/mL was prepared.

Docetaxel was associated with the AuNPs by non covalent adsorption (François et al., 2011). 1 mL of a suspension of AuNPs at 10 mg/mL was added to 14.3 mL of an aqueous solution of DOC at

0.28 mg/mL. Both solutions were sterilized using a 0.2 μm cellulose acetate sterile syringe filter and handled in a sterile environment in order to prevent microbial contamination during the association protocol. The mixture was stirred for 5 days at 30 °C. Previous work (François et al., 2011) using a spectroscopic technique showed that this protocol leads to association of all the added DOC. Therefore, the mixtures were used directly to evaluate cytotoxicity against human prostate cancer cells, without any separation step.

The addition of DOC to the different types of nanoparticles described above generated four batches of loaded nanoparticles:

AuPEG550DOC – AuNPs containing DOC, PEG 400 and PEG 550
 AuPEG550DOCFol – AuNPs containing DOC, PEG 400 with folate and PEG 550
 AuPEG2000DOC – AuNPs containing DOC, PEG 400 and PEG 2000
 AuPEG2000DOCFol – AuNPs containing DOC, PEG 400 with folate and PEG 2000.

2.6. Cytotoxicity evaluation

The human prostate cell line LnCap (ECACC Reference 89110211) was obtained from the Institut Bergonié, Bordeaux. Cells were routinely grown in RPMI-1640 medium supplemented

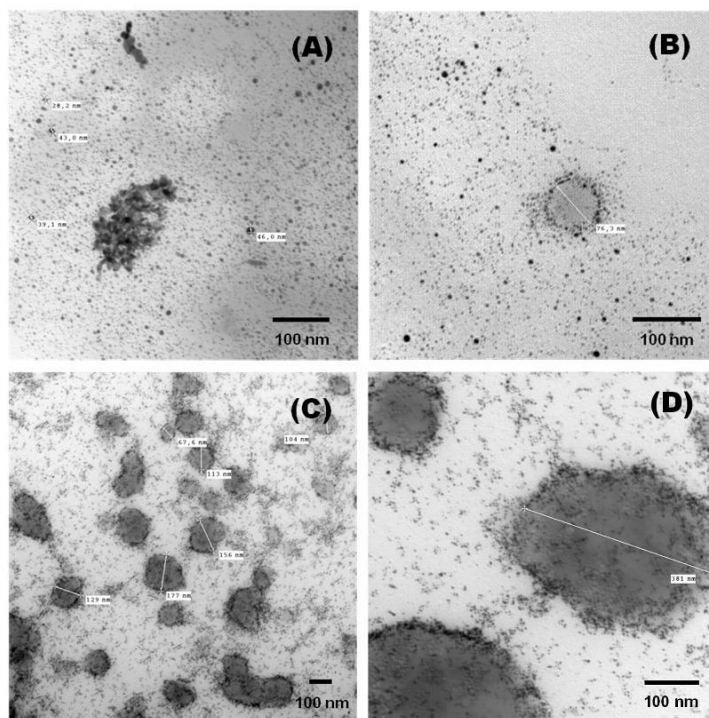


Fig. 1. TEM images of aggregates of AuPEG550 (A–B) and AuPEG550Fol (C–D). Bar = 100 nm.

with L-glutamine, 10% of fetal bovine serum and 1% penicillin–streptomycin. Cells were incubated at 37 °C in an atmosphere containing 5% CO₂ and passaged once a week.

Cell viability was estimated by mitochondrial metabolic activity using the Promega CellTiter 96® Aqueous One Cell Proliferation (MTS) assay (Malich et al., 1997). The absorbance recorded in this colorimetric assay reflects the number of viable cells.

The cells were seeded in 96-well plates (5000 cells in 50 µL per well). Twenty-four hours later, free DOC, unloaded AuNPs and AuNPs loaded with DOC were suspended in culture medium, serially diluted and added 96-well microtiter plates (50 µL/well). The plates were incubated for a further 24, 48 or 144 h. Triplicate wells were used for each condition, and each experiment was performed in triplicate. Ninety minutes prior to the end of each exposure period, the MTS reagent (20 µL/well) was added.

The absorbance of the formazan product was read with a 492 nm high-pass filter in a Multiskan MS microwell plate reader (Lab-system, Ramat-Gan, Israel). Background absorbance due to the non-specific reaction between the test compounds and the MTS reagent or to light scattering was measured in wells without cells and was subtracted from the values measured in the presence of cells (Hayes and Markovic, 2002).

The relative cell viability was calculated with respect to control wells containing cell culture medium without nanoparticles or DOC according to the formula $[A]_{\text{test}}/[A]_{\text{control}} \times 100$, in which $[A]_{\text{test}}$ is

the absorbance of the tested sample and $[A]_{\text{control}}$ is the absorbance of control sample (Braydich-Stolle et al., 2005).

2.7. Statistical analysis

Cell culture data are reported as means of three experiments (each with triplicate wells) \pm SD. Statistical analysis on the raw data was performed by one-way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey's multiple comparison) for comparison between groups. A significance level of 95% ($p < 0.05$) was accepted (Viviani et al., 2005).

3. Results and discussion

As shown in Scheme 1, AuNP were synthesized by modified Brust method (Brust et al., 1994). From the relative integrations of the peaks of the aryl group of the folate in the PEG-400- or PEG-2000-folate ligand and the PEG groups in the ¹H NMR spectrum of AuNP 1 (not shown), it can be seen that the AuNP surface was capped by 50% HS-mPEG and 50% HS-PEG-NH₂·HCl. Indeed, the integration at 3.17 ppm belongs to the –CH₂NH₂·HCl on HS-PEG-NH₂·HCl and the integration at 3.3 ppm belongs to the –OCH₃ on mPEG, compared with the two integrations.

This indicated that 50% of capped ligand on AuNP modified with the two types of PEG (1 on Scheme 1) could be functionalized by

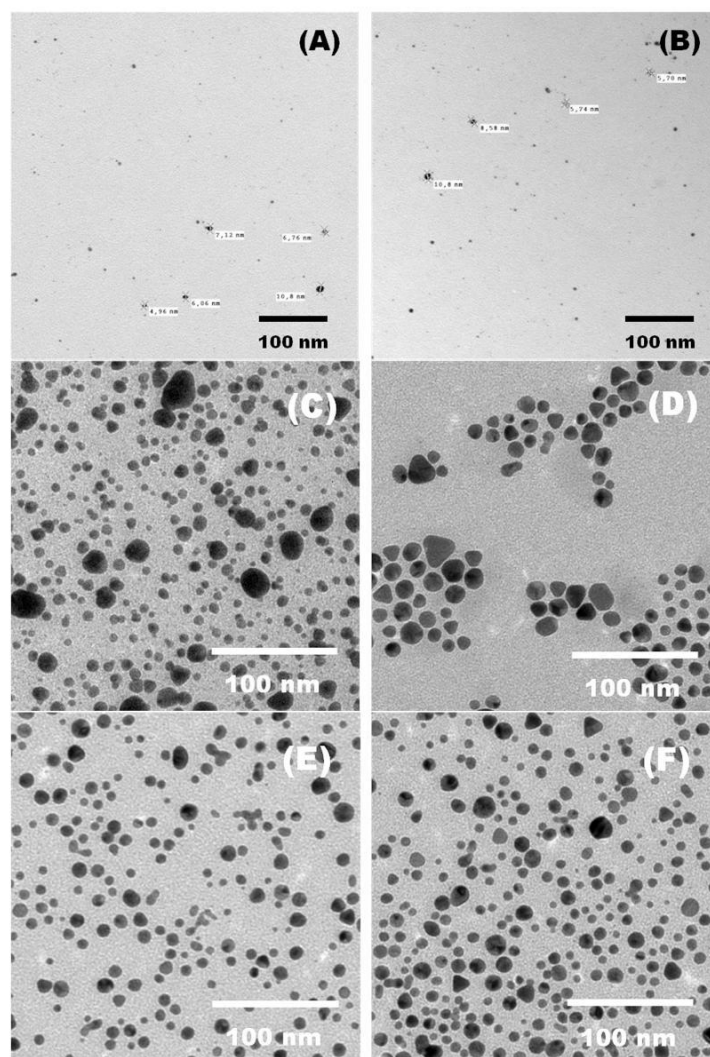


Fig. 2. TEM images showing non-lyophilized AuPEG550Fol (A–B), AuPEG2000 (C–D) and AuPEG2000Fol (E–F). Bar = 100 nm.

NHS-folate through the coupling reaction, leading to AuNPs with bearing folate groups at their surface (2 on Scheme 1).

The folate functionalization at the termini of AuNP is confirmed by the ^1H NMR spectrum (sSI). The plasmon band is observed at 530 nm which also confirms that the size of the AuNPs is more than 3 nm (not shown).

The AuNPs with or without folate were used to encapsulate DOC for the assessment of its *in vitro* activity. During the incubation of DOC with the nanoparticles numerous weak hydrogen bonds form between the OH and NH group of DOC (Scheme 1) and the numerous oxygen atoms of the PEG polymer. In this way, PEG acts as a solvent for the DOC molecules.

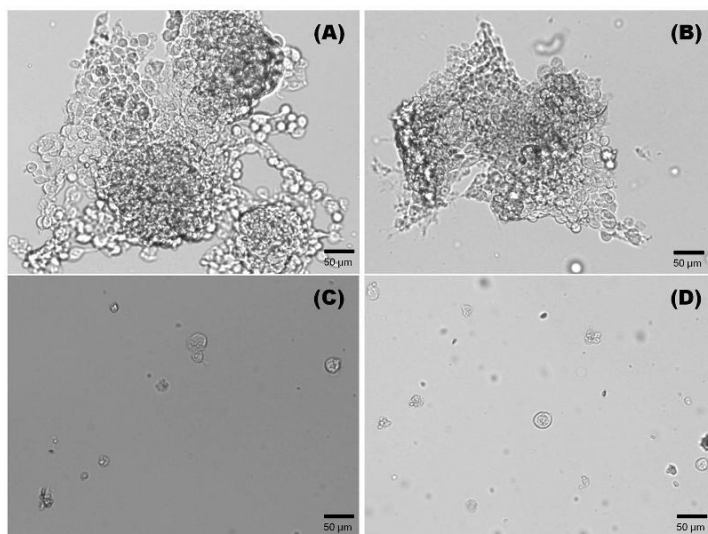


Fig. 3. Optical microscopy images of LnCaP cells after 144 h of contact with: (A) control without treatment; (B) cells incubated with AuPEG550Fol; (C) cells incubated with free DOC; (D) cells incubated with AuPEG550FolDOC. In (C) and (D) the DOC concentration was 60 µM. The bar represents 50 µm.

3.1. Characterization of AuPEG550 and AuPEG550Fol

Fig. 1 shows TEM images of AuPEG550 before click functionalization (A and B) and AuPEG550Fol (C and D), both freeze-dried. Fig. 2 shows AuPEG550Fol, AuPEG2000 and AuPEG2000Fol in aqueous suspension without freeze-drying.

The AuNPs prepared by the Schiffrin–Brust (Brust et al., 1994) method showed individualized particles with a gold core diameter of 7 ± 3 nm for the nanoparticles containing PEG 550 and 10 ± 5 nm for the nanoparticles containing PEG 2000 with a narrow polydispersity, as determined by TEM (Fig. 2). The freeze-drying process seems to provoke the aggregation of some nanoparticles because even after 30 min of treatment in an ultrasound bath, the TEM images showed aggregates with irregular shapes up to 400 nm (Fig. 1). This phenomenon has already been described in the literature (França et al., 2010).

3.2. *In vitro* cytotoxicity toward LnCaP prostate cancer cells

The morphology of the cells was observed after 6 days of treatment using an optical microscope (Leitz Diaplan) at $100\times$ magnification. Typical photomicrographs are shown in Fig. 3. There was no difference in the morphology of untreated cells and that of cells treated with unloaded AuNPs. However, both the nanoparticles loaded with docetaxel and the free drug at the same concentration clearly reduced the number of LnCaP cells remaining in the wells.

The toxicity toward LnCaP cells of the unloaded nanoparticles was estimated using the MTS after 24, 48 and 144 h of contact, as seen in Fig. 4. The concentrations of AuNPs were chosen to correspond to those of the loaded NPs and ranged between 35 pg/ml and 35 µg/ml. The overall difference was not statistically significant for all concentrations and times (one-way ANOVA test).

Fig. 5 summarizes the cell viability measured by the MTS test after treatment with free DOC or the different AuNPs formulations containing DOC. The results are expressed as a percentage of the appropriate control (DMSO or empty nanoparticles).

Fig. 5A shows LnCaP viability after a 24-h exposure to DOC-loaded gold nanoparticles with PEG 550 chains, with or without folate, compared with free DOC. The presence of folate did not affect the cytotoxicity of the loaded nanoparticles at any concentration.

Free DOC produced a statistically significantly greater effect at the two lower concentrations than the loaded NPs. However, above 6.10^{-7} M docetaxel, the difference decreased making the effects of all treatments statistically equivalent.

Fig. 5B shows the results of LnCaP viability after 24 h of exposure to DOC-loaded gold nanoparticles with longer PEG chains (PEG 2000). At the lowest concentration, the AuPEG2000DOC were less effective than AuPEG2000DOC; however this trend was not reproduced at other concentrations, where the two formulations yielded similar results. Unlike AuNPs with PEG 550, the effect of free DOC was statistically significantly greater than that of loaded AuNPs at all concentrations.

Fig. 5C shows the viability after 48 h of exposure to free docetaxel and AuNPs bearing PEG 550. The effect of AuPEG550DOC was concentration-dependent. AuDOCPEG550Fol showed a less evident effect of concentration. There was no statistically significant difference between the effects of free DOC and the loaded AuNPs at any concentration.

Fig. 5D shows cell viability after 48 h of exposure to free DOC and AuNPs (PEG 2000). Like the AuNPs with shorter PEG, AuPEG2000DOCFol demonstrated a statistically significant effect on cell viability for the two most concentrated suspensions compared with the control. The AuNPs without folate only had significant activity at 6.10^{-5} M. At all concentrations, treatment with AuPEG2000DOCFol resulted in significantly lower viability than the same AuNPs without folate, except at 6.10^{-9} M.

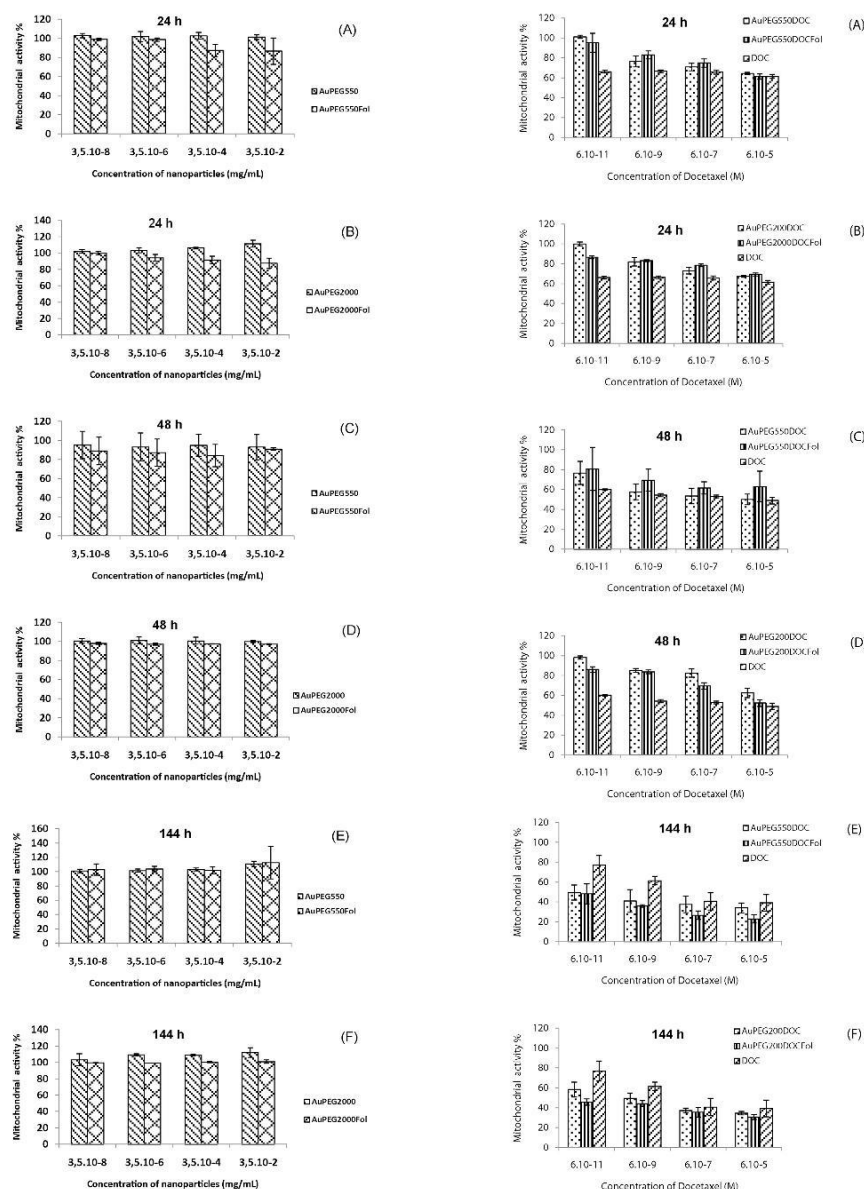


Fig. 4. Mitochondrial enzyme activity of LnCaP after 24 h (A, B), 48 h (C, D) and 144 h (E, F) exposure to free DOC or to unloaded nanoparticles containing PEG 550 (A–C–E) and PEG 2000 (B–D–F). Results are expressed as a percentage of untreated control. Values are the means \pm SD of three experiments.

Fig. 5. Mitochondrial enzyme activity of LnCaP after 24 h (A, B), 48 h (C, D) and 144 h (E, F) exposure to free DOC or to loaded nanoparticles containing PEG 550 (A–C–E) and PEG 2000 (B–D–F). Results are expressed as a percentage of untreated control. Values are the means \pm SD of three experiments.

Fig. 5E shows the cell viability after a longer exposure time (144 h) for the loaded AuNPs with the shorter PEG chains (PEG 550). The DOC-loaded nanoparticles produced a significant reduction in the cell viability at all concentrations. There was no difference between AuNPs with and without folate. Similar results were obtained with the AuNPs with longer PEG chains at 144 h (Fig. 4F). At this exposure time, the free drug showed a marked dose-response, with significant reductions in viability at all concentrations except the lowest concentration tested (6.10^{-11} M).

In general, free DOC showed significant cytotoxicity toward LNCaP cells at all exposure times and concentrations except after 144 h at the lowest concentration, confirming the sensitivity of this cell line to DOC. The decrease in the efficacy of the lower concentrations of DOC at long incubation times could be due to precipitation of this very hydrophobic molecule in the culture medium.

Compared with free DOC, the loaded nanoparticles were less effective against LNCaP cells over the first 48 h. After 144 h of exposure the nanoparticles containing DOC were in general more effective than the free drug suggesting that there is a slower release of the incorporated drug and that its association with the AuNPs prevented its precipitation in the medium. An effect of the PEG chain length could only be observed after 48 h of exposure, when the AuNPs bearing shorter chains (PEG 550) had more effect on viability than those with longer chains (PEG 2000). This may indicate that the higher molecular weight PEG chains retained the DOC in the AuNPs longer and it was therefore less available to cells.

The DOC-loaded nanoparticles reduced the mitochondrial activity by 50% or more at concentrations of 6.10^{-9} M and above after 144 h of treatment (Fig. 4E and F). However, no significant difference was observed between folate-bearing AuNPs and non modified ones. This might indicate that the folate residues are not orientated correctly for binding to PSMA, or that the non modified PEG chains cause steric hindrance, especially since these chains were longer than those carrying the folate. This hypothesis was confirmed by surface plasmon resonance measurements made with the Biacore instrument (data not shown). AuNPs bearing folate showed no specific interactions with folate-binding protein (FBP). A similar observation had been made concerning liposomes bearing PEG terminated with folate residues and was considered to indicate steric hindrance from the PEG chains (Botosoa et al., 2011).

The decrease in the number of cells after contact with DOC-loaded nanoparticles was confirmed by observation with an optical microscope. Fig. 3 shows far fewer cells after 144 h of treatment with free or AuNP-associated DOC compared with control cultures or cultures treated with unloaded nanoparticles, where the cells can be seen to be growing as aggregates typical for this line. Cells incubated with AuPEG2000DOC and AuPEG2000FolDOC (data not shown) showed similar changes in the appearance of the cell monolayer to AuPEG550DOC (Fig. 3B) and AuPEG550FolDOC (Fig. 3D), respectively.

4. Conclusion

In this study, gold nanoparticles have been functionalized with PEG 550 and PEG 2000 by “click” chemistry in order to encapsulate docetaxel by non covalent interactions. The nanoparticles were also functionalized with folate. The unloaded nanoparticles were not cytotoxic to LNCaP cells at the concentrations used in this study (between 35 pg/ml and 35 µg/ml). When DOC was adsorbed onto the AuNPs it produced a sustained cytotoxic effect on LNCaP cells in an *in vitro* test. These small particles could be useful for concentrating drug in solid tumors by the EPR effect, and the properties of the gold core could be exploited for tumor imaging and for thermal cytotoxicity of the tumor cells.

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Supplementary Information

1.1. Surface Plasmon Resonance Measurements – Biacore Data

Biacore analysis uses the optical phenomenon of surface plasmon resonance to detect and measure the interactions between one immobilized component and another in solution or suspension. This method was used to analyse the possible interaction between the synthesized gold nanoparticles functionalized with folate and Folate Binding Proteins (FBP) covalently linked to a sensor chip with a carboxymethylated dextran surface. The affinity of the FBP for the folate is measured by the binding kinetics of its interaction [16]. A sensorgram of an interaction curve is shown in Figure 2.

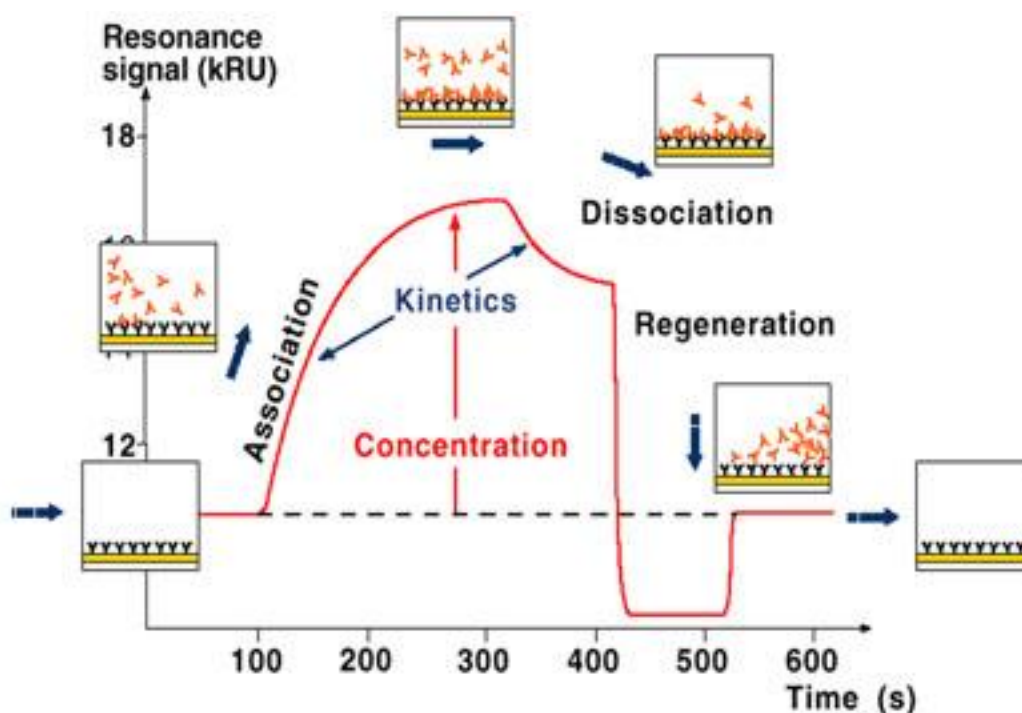


Figure 2: Typical sensorgram of an interaction's kinetics (Courtesy of GE Healthcare).

Figure 3 shows the sensorgram that illustrates the passage of the gold nanoparticles containing PEG 550 and folate (AuPEG550Fol) through the non-specific interaction channel (red curve) and the specific interaction channel (green curve) with the folate binding proteins coated onto the gold chip. The sample was run at a concentration of 1 mg/ml with 300 s of contact and flow rate 30 $\mu\text{L}/\text{min}$.

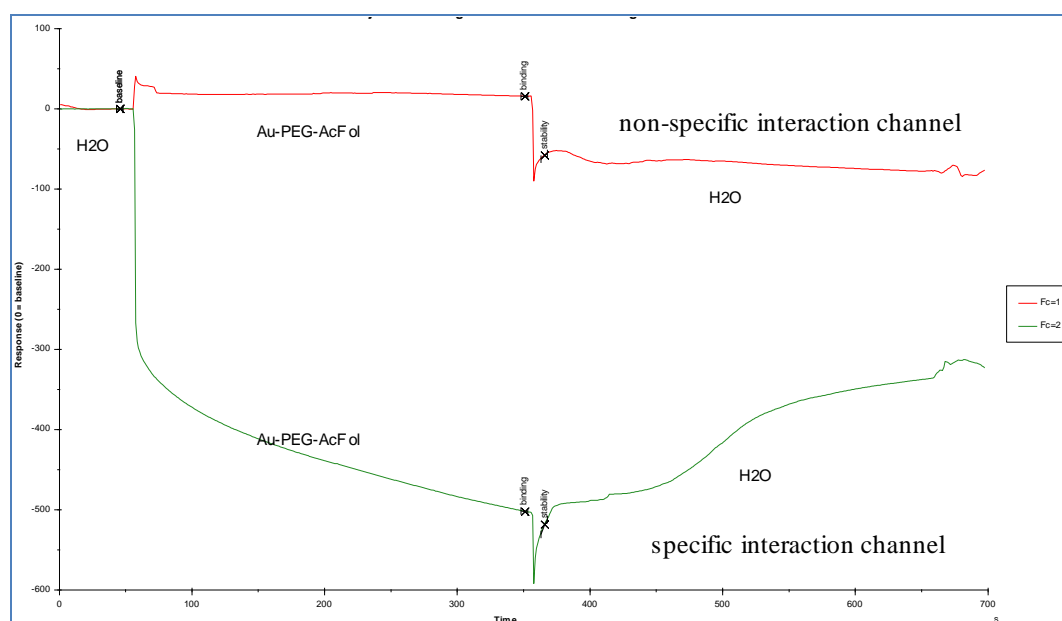


Figure 3: AuPEG550Fol 1 mg/ml; time of contact 300 s and flow rate 30 $\mu\text{L}/\text{min}$.

The red line corresponds to a typical non-specific interaction curve. However, the appearance of the green is the opposite of what is expected for folate interactions which would be a similar curve to the one shown in Figure 2, appearing above the red curve. It was decided to run the same sample with a longer contact time with the surface (600 s) at a lower flow rate 5 $\mu\text{L}/\text{min}$ to maximize interactions between the nanoparticles and the channel surface. The sensorgram is shown in Figure 4.

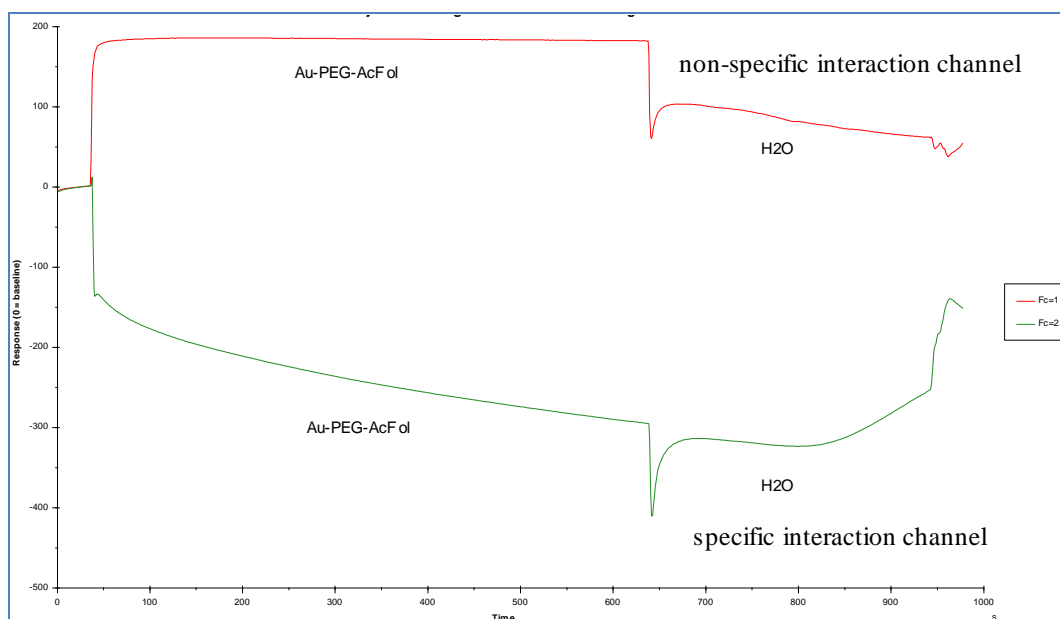


Figure 4: AuPEG550Fol 1 mg/ml; time of contact 600 s and flow rate 5 μ L/min.

We observed that a similar phenomenon occurred in the specific interaction (green) channel .

The second batch of gold nanoparticles was also analysed. Particles bearing folate and a longer PEG chain (AuPEG2000Fol) were passed through the specific and non-specific channels. The sample concentration used was 0.5mg/mL with 600s contact time and flow rate of 20 μ L/min as shown in Figure 5.

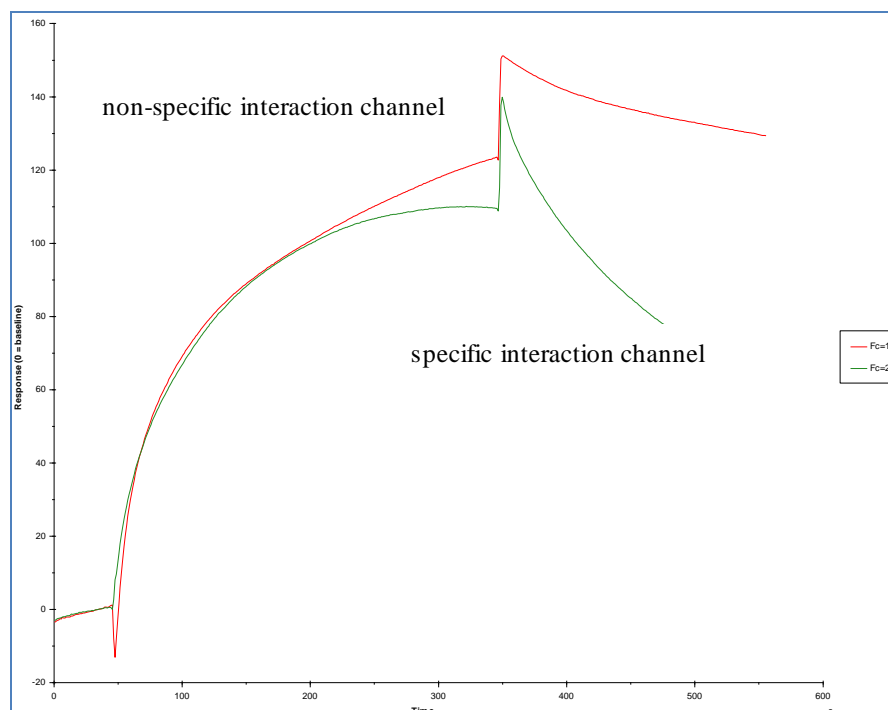


Figure 5: AuPEG2000Fol 0.5 mg/ml; time of contact 600 s and flow rate 20 μ L/min.

The shape of the curves is representative of specific interactions. However, the two channels, nonspecific and specific, show the same profile. The non-specific signal is even greater than the specific signal.

Based on the results obtained with the two particle types, AuPEG550Fol and AuPEG2000Fol, it is not possible to confirm that there is an interaction between the gold nanoparticles and the channels functionalized with folate binding proteins.

The results obtained from nanoparticles with shorter PEG (AuPEG550Fol) might be explained though the ability of PEG to influence the

microthermodynamics between proteins and surface interfaces [17]. The mobility and flexibility of the PEG chains may prevent the adhesion of the gold nanoparticles on the FBP layer avoiding the interaction between the folate and the proteins. The schematic Figure 6A illustrates this hypothesis.

Although, the two curves resulted from nanoparticles containing longer PEG (AuPEG2000Fol) show interactions between the particles and both channels, specific (FBP) and non-specific (ethanolamine). The longer PEG tends to be more extended than the PEG550 in aqueous suspension. Thus, the availability of the polar groups is higher in the longer PEG, therefore, the interactions can be more effective (Figure 6B).

Polysaccharides like dextran, used as base of the sensor chip in this Biacore analysis can provide a confusing result. The presence of non-specific binding can lead to a false positive effect, or either increases the positive reading result [18].

For instance, the unexpected interaction between the AuPEG2000Fol and the non-specific channel can be explained by inspection of the available polar groups of the nanoparticles that could interact with the ethanolamine surface forming strong and weak bonds with the chain, for example the NH group derived from the N-hydroxy succinimide and the OH group of the ethanolamine could form a hydrogen bond as shown in the Figure 6C.

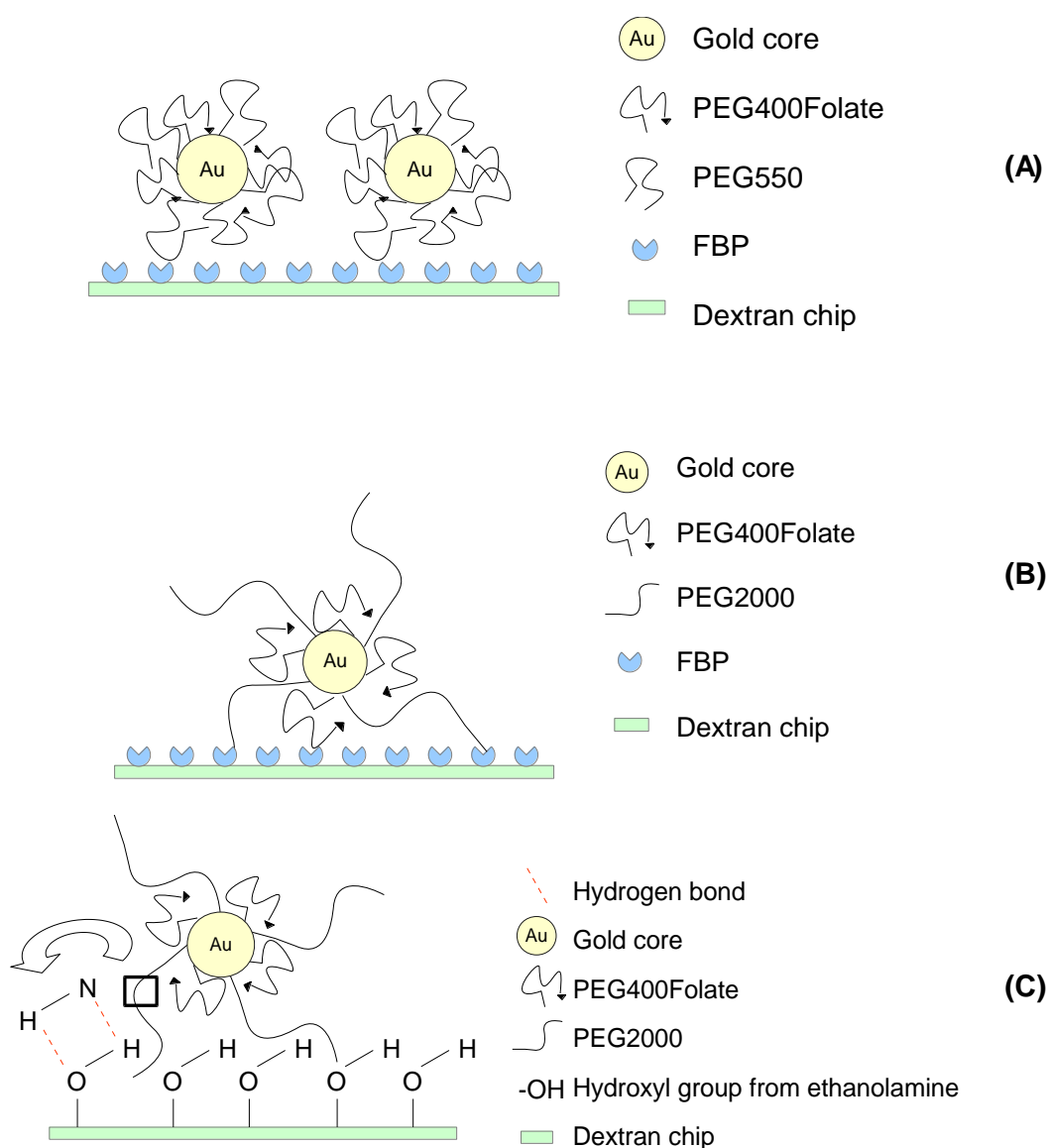


Figure 6: Schematic hypothesis of three possibilities of interaction between AuNPs and the Biacore channels: (A) AuPEG550Fol and specific channel; (B) AuPEG2000Fol and specific channel; (C) AuPEG2000Fol and non-specific channel.

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CHAPTER III

BIODEGRADABLE AND BIOCOMPATIBLE NANOPARTICLES FOR PROSTATE CANCER TREATMENT

Introduction

Biodegradable and biocompatible polymeric nanoparticles have been attracting a lot of interest over the past decades as drug delivery devices. The goal of this strategy is to increase the amount of active drug that is delivered to a target tissue. This approach could minimize the side effects while improving the therapeutic effect.

Among the antineoplastic agents that have been investigated as encapsulated drugs for cancer therapeutics are mitoxantrone and docetaxel. Those drugs have a proven cytotoxicity against different kinds of tumors. However, they induce significant side effects that can lead to disability or even death.

The encapsulation of these two molecules with different mechanisms of action against tumor cells into polymeric nanoparticles is an original therapeutic approach to avoid the side effects of chemotherapy. The aim of this work was to develop a multifunctional drug delivery system based on polyester based nanoparticles loaded with mitoxantrone and docetaxel.

This chapter is divided into two sections:

The first section first describes a preliminary study of nanoparticle preparation using a commercial copolymer, poly(lactide-co-glycolide)–poly(ethylene glycol). The copolymer was nanoprecipitated using different ratios of organic and aqueous phase, as well as varying polymer concentrations. The final product was characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

Once the methodology was defined, a series of diblock pegylated copolymers was synthesized. Polycaprolactone, poly(lactic acid) and poly(lactide-co-glycolide) were made by ring-opening polymerization with different molecular weights of polyethylene glycol monomethylether, 2000 and 5000 using Tin (II) 2-ethylhexanoate as initiator. The copolymers were characterized by proton nuclear magnetic resonance (^1H NMR) and size exclusion chromatography (SEC) after which each copolymer was

nanoprecipitated. The nanoparticle size, size distribution and zeta potential were determined by DLS at intervals in order to follow their stability.

The second section is presented as a manuscript submitted to the *Special Journal Issue on Advances in Nanoscience and Nanotechnology*. This paper concerns the use of one of the poly(lactic acid)–poly(ethylene glycol) copolymers previously synthesized, PLA-PEG 2000, and its loading by nanoprecipitation with two anti-cancer drugs: docetaxel and mitoxantrone. The drug encapsulation efficiency (EE) was measured by ultraviolet-visible spectrophotometry (UV-Vis). The cytotoxicity of the loaded nanoparticles and the corresponding free drug against two different human prostate cancer cell lines, PC3 and *LNCaP*, were evaluated by the MTS assay. The effectiveness of each type of nanoparticle was evaluated separately as well as in mixtures of both at different ratios in order to observe any synergy, additivity or antagonism effect.

CHAPTER III

BIODEGRADABLE AND BIOCOMPATIBLE NANOPARTICLES FOR PROSTATE CANCER TREATMENT

SECTION 1: Synthesis and Nanoprecipitation of Biodegradable and Biocompatible Copolymers

INTRODUCTION

Drug delivery is a therapeutic strategy that aims to modulate the distribution of active drugs. The drug carrier must be able to deliver a biologically relevant molecule to its target, to protect it from degradation and to allow a high concentration of drug to accumulate in a specific target. In this way, drug efficacy can be increased while reducing the risk of side effects by reducing the amount reaching healthy tissues [1].

Nanoparticles prepared from a wide range of polymers have already shown their efficacy to improve the bioavailability and the pharmacokinetic properties of approved chemotherapy drugs, leading to a great advance in this field [2].

Natural polysaccharides such as chitosan and albumin as well as polyesters such as poly(D,L-lactic-co-glycolic acid) (PLGA) [3], poly(D,L-lactic acid) (PLA) [4] and polycaprolactone (PCL) [5] which can be modified with PEG units to form pegylated copolymers are the most commonly used polymers for drug delivery. They are biodegradable, biocompatible and they are able to encapsulate a variety of drugs [6].

This section is devoted to the synthesis and nanoprecipitation of nanoparticles from six different polymers: PLA-PEG, PLGA-PEG and PCL-PEG, each with two PEG chain lengths. Each synthesis was performed by ring-opening polymerization of the polyester with either PEG Mw 2000 or PEG Mw 5000. A preliminary study was made prior to polymer synthesis using a commercial copolymer (PLGA-PEG) in order to optimize the methodology for nanoparticle preparation. This part was carried with the aid of M. Ferid Tatietse Tewane during his Master 1 research internship.

1. Materials

All solvents and chemicals were used as received. Poly(D,L-lactic-co-glycolic acid) polyethylene glycol (PLGA-PEG) Resomer® type RGP d 50105 was purchased from Boehringer-Ingelheim (Germany); D,L-lactide was purchased from Polysciences (USA). Polyethylene glycol monomethylether 2000 g/mol (PEG 2000) and polyethylene glycol monomethylether 5000 g/mol (PEG 5000) were purchased from Fluka (Switzerland). Stannous 2-ethylhexanoate, caprolactone and glycolide were purchased from Sigma–Aldrich (France). Acetone, diethyl ether, petroleum ether and dichloromethane (DCM) were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Deuterated chloroform (CDCl_3) and dimethyl sulfoxide (DMSO) were obtained from Carlo Erba (France). Ultra pure water was produced by reverse osmosis (MilliQ®, Millipore).

2. Nanoprecipitation of commercial PLGA-PEG

The nanoparticles were prepared following the nanoprecipitation method with modifications [7]. This technique consists in dissolving the copolymer in an organic solvent which is miscible with water; acetone was used in this study. The organic solution was weighed as was the ultrapure water into which the organic mixture was added dropwise through a 0.50 x 16 mm needle (Braun, Germany) with a perfusion pump (Ealing, France) at a rate of 2 mL/min with magnetic stirring of the water phase at room temperature.

The nanoparticles were formed spontaneously in water by rapid diffusion of acetone. The suspension of nanoparticles was stirred for a further 20 min after which the acetone was removed under reduced pressure (rotary evaporator, Büchi, Swiss).

The suspension was then purified by centrifugation (10 min, 10 000 g). Polymer agglomerates precipitated while the desired smaller nanoparticles remained suspended in the supernatant. The supernatant was removed with a micropipette and then characterized.

In this study, two experimental parameters of the preparation of polymeric nanoparticles were varied: the concentration of the polymer (5 mg/mL - 20 mg/mL) and the organic phase to aqueous phase ratio (1:2 and 1:4).

2.1. Nanoparticle characterization

Nanoparticle size, size distribution (Pdl) and zeta potential were determined by dynamic light scattering (DSL) at 25 °C (Zetasizer® 4, Malvern Instruments) using the undiluted final aqueous stock suspension of nanoparticles. Results were expressed as the average of three measurements. The particle size is presented as Z-average.

Transmission electron microscopy (TEM) with a Philips EM208 (1996) instrument equipped with wide-field CCD camera acquisition (AMT) was used to evaluate the size and morphology of the nanoparticles. Phosphotungstic acid (PTA) 2 % at pH 7.0 was used to stain the nanoparticles.

2.2. Results and discussion

Nanoparticles were first prepared from a commercial PEG-PLGA copolymer at a concentration of 10 mg/mL and ratios of organic phase/water (O:W) of 1:2 and 1:4 in order to evaluate the influence of the O:W ratios on the size distribution of the particles. Nanoprecipitation was performed without using surfactants. Acetone was chosen as the organic solvent because it is readily miscible with water and dissolves the commercial copolymer (PLGA-PEG) rapidly and well. Table 1 shows the size and polydispersity (Pdl) of the nanoparticles prepared using the concentration of 10 mg/mL of copolymer.

Table 1: Size and polydispersity index (Pdl) of the nanoparticles as a function of the ratio of organic phase/water (O:W).

Ratio O:W	1:4	1:2
Size (nm)	85	80
Polydispersity index	0.18	0.22

It can be observed that the ratio O:W does not have a great influence on the size and polydispersity of the nanoparticles. This result is similar to that of Cheng *et al.* which showed that the ratios O:W, when less than 0.5, had no influence on the size distribution of PLGA-PEG nanoparticles formed using acetone as the solvent for the polymer [8].

The polymer concentration in the organic phase was varied in order to verify its influence on the size and polydispersity of the nanoparticles. The ratio O:W was maintained at 1:2. The results are summarized in Table 2.

Table 2: Size and polydispersity index of nanoparticles as a function of the concentration of copolymer in the organic phase.

Concentration of PLGA-PEG (mg/mL)	5	10	20
Size (nm)	71	80	128
Polydispersity index	0.15	0.22	0.23

It can be seen that the size and polydispersity of the nanoparticles decline with the decrease of the polymer concentration in the organic phase. These results are similar to those of Legrand *et al.* which showed that an increase of the poly(lactic acid) homopolymer concentration in the organic phase led to an augmentation in the size of nanoparticles formed by nanoprecipitation [9].

The concentration of 5 mg/mL was chosen for the subsequent nanoprecipitations due to the small size and good polydispersity obtained with under these conditions. According to the literature, polymeric nanoparticles of slightly above 70 nm have reduced liver uptake and have prolonged systemic circulation [10]. The results of dynamic light scattering were confirmed by the transmission electron microscopy images that show small and spherical nanoparticles (Figure 1).

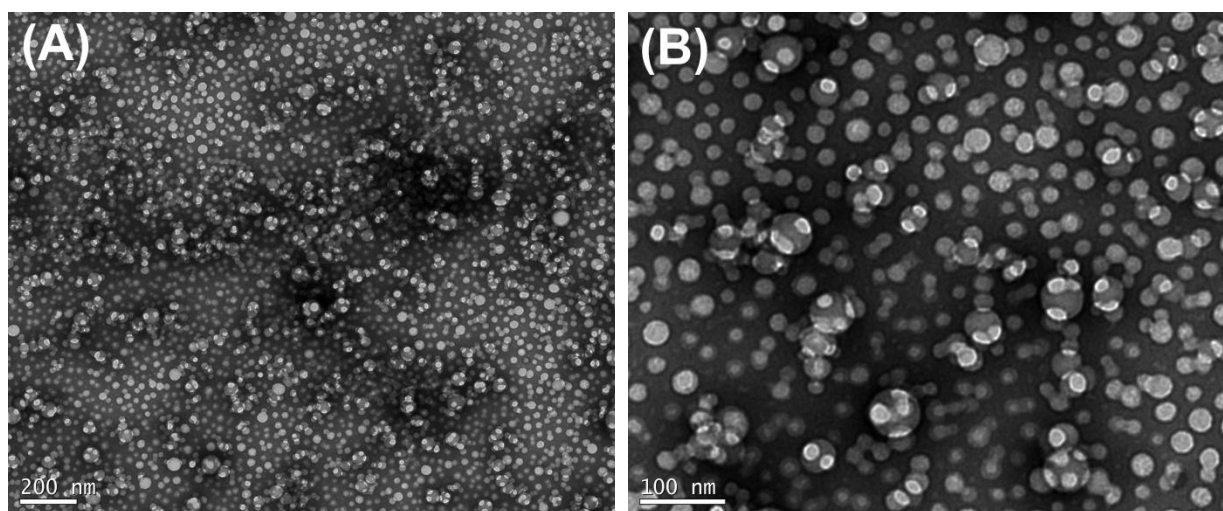


Figure 1: Transmission electron microscopy (TEM) images of PLGA-PEG obtained by nanoprecipitation using 5 mg/ml of polymer and ratio O:W = 1:2. (A) image magnification of 10,000 X; (B) image magnification of 80,000 X.

3. Synthesis of copolymers

Six different copolymers based on PLA-PEG, PLGA-PEG and PCL-PEG were synthesized for further nanoprecipitation experiments. Each synthesis was performed by ring-opening polymerization with two different lengths of polyethylene glycol, PEG Mw 2000 and PEG Mw 5000.

3.1. Polycaprolactone-poly(ethylene glycol) – (PCL-PEG)

Polycaprolactone (PCL) is biodegradable polyester which has received much attention for the use in biomedical applications, especially for the controlled delivery systems. PCL may be degraded in physiological conditions by hydrolysis and this process permits the release of entrapped drugs. Additionally, enzymes and microorganisms may help in the degradation process of this polymer. However, the degradation of PCL is significantly slower compared with PLA and PGA [11]. Pure PCL is degraded very slowly due to its high crystallinity, but when it is copolymerized with more hydrophilic segments its properties can be tailored to particular applications [12]. Thus, many researchers have used PCL-based systems for drug delivery [13-16].

The biodegradable diblock copolymers PCL-PEG 2000 and PCL-PEG 5000 were synthesized by ring-opening polymerization of caprolactone initiated by PEG and using SnOct_2 as catalyst as previously described with some modifications [17]. In brief, for the synthesis of PCL-PEG 2000, 4.5 g of caprolactone, 7 wt % PEG 2000 and 1 wt % SnOct_2 were added to a tube for polymerization. The tube was left under vacuum for 1 h and sealed. The polymerization was carried out in an oil bath at 115 °C for 20 h. After the conclusion of the reaction, the resulting product was purified by the following procedure that was repeated twice: the product was dissolved in DCM and then precipitated in petroleum ether. The final purified product was dried in a dynamic vacuum. The same procedure was used for the synthesis of PCL-PEG 5000 using 17 wt % of PEG 5000.

3.2. Poly(lactic acid)-poly(ethylene glycol) – (PLA-PEG)

PLA is an amorphous polymer widely applied for pharmaceutical and medical applications because of its biocompatibility and low toxicity [18-20].

PLA-PEG copolymer was synthesized by ring-opening polymerization as described before with modifications [21]. In brief, for the synthesis of PLA-PEG 2000, 5 g of D,L-lactide, 11 wt % PEG 2000 and 1 wt % SnOct_2 were added to a tube for polymerization. The tube was left under vacuum for 1 h and sealed. The

polymerization was carried out in an oil bath at 115 °C for 20 h. After the conclusion of the reaction, the resulting product was purified by the following procedure that was repeated twice: the product was dissolved in DCM and then precipitated in mix of petroleum ether and diethyl ether 1:1. The final purified product was dried in a dynamic vacuum. The same procedure was used for the synthesis of PLA-PEG 5000 using 27 wt % of PEG 5000.

3.3. Poly(lactide-co-glycolide)-poly(ethylene glycol) – (PLGA-PEG)

Like PCL and PLA, PLGA copolymer has been extensively used for drug encapsulation and controlled delivery. It is able to form nanoparticles entrapping small molecules. Despite its biodegradability and biocompatibility, challenges as poor stability in aqueous suspension and the higher diameter compared with PCL and PLA nanoparticles still remain to be overcome [22].

PLGA-PEG was also synthesized by ring-opening polymerization as previously described with modifications [23]. For the synthesis of PLGA-PEG 2000, 2.3 g of D,L-lactide, 80 wt % of glycolide, 22 wt % PEG 2000 and 1 wt % SnOct₂ were added to a tube for polymerization. The tube was left under vacuum for 1 h and sealed. The polymerization was carried out in an oil bath at 115 °C for 20 h. After the conclusion of the reaction, the resulting product was purified through the following procedure: the product was dissolved in DCM and then precipitated in mix of petroleum ether and diethyl ether 1:1. It was re-dissolved in DCM and re-precipitated in diethyl ether. The final purified product was dried in a dynamic vacuum. The same procedure was adopted for the synthesis of PLGA-PEG 5000 using 55 wt % of PEG 5000.

3.4. Proton nuclear magnetic resonance (¹H NMR)

The structure, number average molecular weight, the PEG content of the copolymer and the number of monomeric units in the polymer chain were determined after ¹H NMR analysis in a Bruker AC 300 Hz spectrometer. Figures 2, 3 and 4 represent number of monomeric units as n, m and w. The sample was solubilized in deuterated chloroform (CDCl₃) and analysed at 25 °C.

3.5. Size exclusion chromatography (SEC)

SEC was used to determine the polydispersity index (Pdl) of the copolymer. It was performed at 30 °C with two columns from Polymer Laboratories (PL-gel MIXED-D; 300x7.5mm; bead diameter: 5 µm; linear part: 400-4 x 10⁵ g.mol⁻¹) and a differential refractive index detector (Spectrasystem RI-150 from Thermo Electron Corp.), using chloroform (CHCl₃) as eluent, at a flow rate of 1 ml.min⁻¹, and toluene as a flow-rate marker. The calibration curve was based on poly-(methylmethacrylate) (PMMA) standards (peak molar masses, $M_p = 625-625\ 500\ \text{g.mol}^{-1}$) from Polymer Laboratories.

3.6. Nanoparticles preparation

The nanoparticles were prepared using the methodology previously described for the commercial PLGA-PEG polymer. Identical conditions were applied for all batches: 5 mg/ml of copolymer and ratios of acetone / water (O:W) of 1:2.

3.7. Results and Discussion

All the polymers were synthesized by the ring-opening polymerization method using SnOct₂ as catalyst. The molecular weight and the weight content of PEG of each copolymer were calculated from the ¹H NMR spectrum. The polydispersity index (Pdl) was measured by size exclusion chromatography. The results are summarized in Table 3.

The ¹H NMR spectra are shown in the Supplementary Information.

3.7.1. PCL-PEG synthesis

PCL-PEG copolymers were synthesized from caprolactone and poly(ethylene glycol) as shown in Figure 2. The hydroxyl group of PEG initiated the ring-opening polymerization. In the ¹H NMR spectrum the methylenic protons (–CH₂–) of the PEG segment appear at 3.65 ppm. The peaks at 4.06 ppm, 2.31, 1.66 and 1.39 ppm characterize the PCL chain [24]. The results confirm that the copolymer could be synthesized reliably.

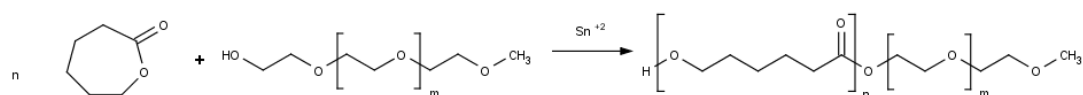


Figure 2: Synthesis of PCL-PEG copolymer

3.7.2. PLA-PEG synthesis

PLA-PEG copolymers were synthesized from D,L-lactide and poly(ethylene glycol) as shown on Figure 3. In the ^1H NMR spectrum the methylenic protons ($-\text{CH}_2-$) of the PEG segment appear at 3.65 ppm. The peaks at 5.20 ppm and 1.65 ppm belong, respectively, to methyl proton ($-\text{CH}_3$) and methine ($-\text{CH}$) of PLA segment. The results confirm that the copolymer could be synthesized reliably as previously demonstrated [25].

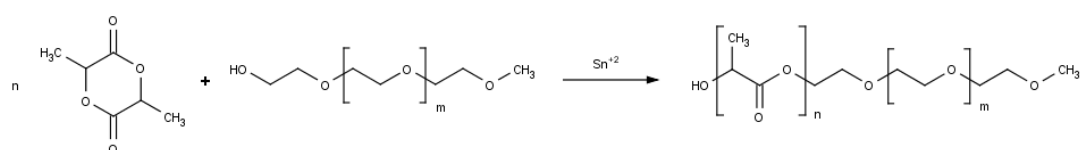


Figure 3: Synthesis of PLA-PEG copolymer

3.7.3. PLGA-PEG synthesis

PLGA-PEG copolymers were synthesized from D,L-lactide (LA), glycolide (GA) and poly(ethylene glycol) as shown on Figure 4. In the ^1H NMR spectrum the methylenic protons ($-\text{CH}_2-$) of the PEG segment appear at 3.65 ppm. The peaks at 5.20 ppm and 1.65 ppm belong, respectively, to methyl proton ($-\text{CH}_3$) and methine ($-\text{CH}$) of PLA segment with additional methylene protons of the glycolide appearing at 4.80 ppm. The results confirm that the copolymer could be satisfactorily synthesized.

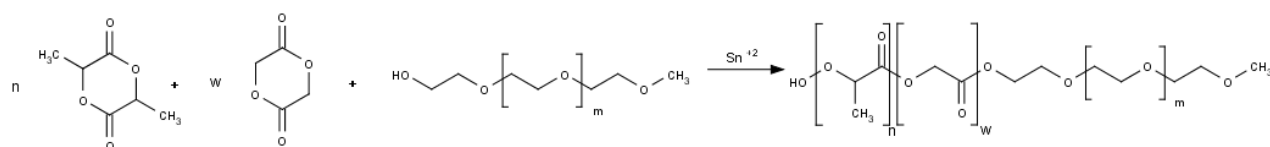


Figure 4: Synthesis of PLGA-PEG copolymer

Table 3: Molecular weight, PEG content and polydispersity index of the copolymers.

Copolymer	Molecular weight (g/mol)	Pdl	PEG content (%)	n	m	w
PCL-PEG 2000	32,000	2.2	6.3	261	45	-
PCL-PEG 5000	34,000	2.2	14.7	254	113	-
PLA-PEG 2000	21,000	2.6	9.4	265	45	-
PLA-PEG 5000	23,000	1.9	21.8	250	113	-
PLGA-PEG 2000	22,000	2.0	9.1	158	45	150
PLGA-PEG 5000	19,000	1.8	26	120	113	98

The copolymers were synthesized by ring-opening polymerization (ROP) which is a chain-growth mechanism enabling the formation of high molecular weight polymers. ROP applied to the polyesters used in this study allows the polymerization of the cyclic monomers, that is LA, CL, and GA through a coordination-insertion mechanism using Tin (II) 2-ethylhexanoate (SnO_2) as catalyst and PEG as co-initiator as shown in Figure 5. The catalyst SnO_2 is widely used for ROP of lactones because of its versatility. Indeed, it is approved by the Food and Drug Administration (FDA) as a food additive. However, it may decompose to octanoic acid in temperatures above 100 °C, leading to side reactions that influence the rate of ROP. The polydispersity

index was found to be around 2 and this can be explained by the intra- and intermolecular transesterification reactions (Figure 6) known to enhance the polydispersity of polyesters during long or high temperature reactions [26, 27]. In this study, copolymers from 19,000 to 34,000 g/mol were synthesized. According to Legrand *et al.*, small calibrated nanoparticles can be produced at high yields using polymers above 40,000 g/mol using acetone as solvent in a dilute regime [9]. Therefore, the copolymers were used for the nanoprecipitation process.

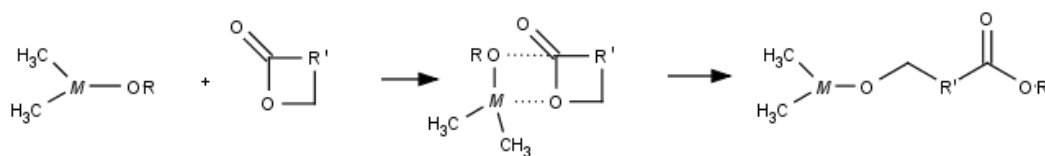


Figure 5: Polymerization mechanism of coordination insertion of lactones. Adapted with permission from reference [26]. Copyright (2014) American Chemical Society.

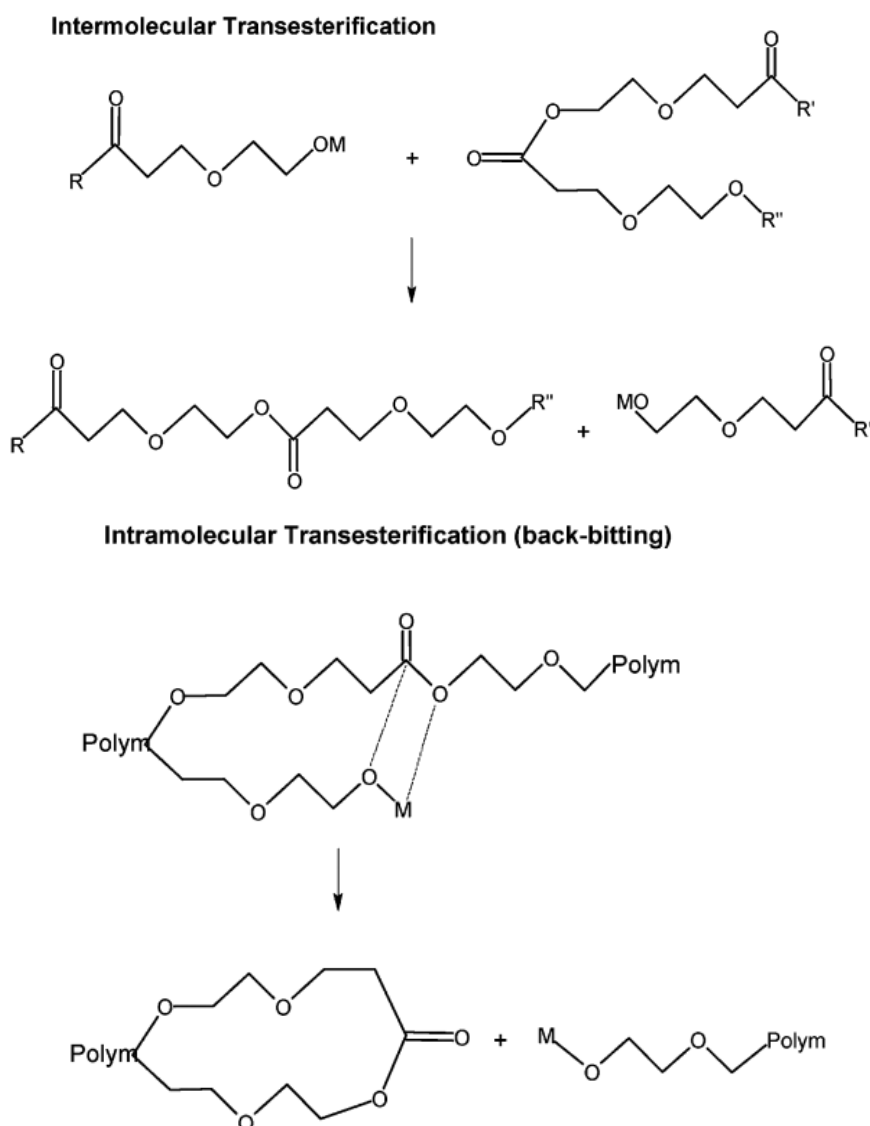


Figure 6: Intermolecular and intramolecular transesterification mechanisms. Reprinted with permission from reference [26]. Copyright (2014) American Chemical Society.

3.8. Nanoparticles preparation and characterization

The z-average size, the polydispersity index and the zeta potential of the nanoparticles are shown in Table 4.

Table 4: Characterization of the nanoparticles

Co-polymer	Z-average size (nm)			Polydispersity index (Pdl)			Zeta Potential (mV)		
	Day 1	Day 5	Day 30	Day 1	Day 5	Day 30	Day 1	Day 5	Day 30
PCL-PEG 2000	77	129	150	0.13	0.18	0.17	-13	-11	-11
PCL-PEG 5000	71	72	126	0.13	0.15	0.34	-4	-6	-7
PLA-PEG 2000	80	81	119	0.12	0.10	0.26	-15	-15	-16
PLA-PEG 5000	51	52	96	0.10	0.14	0.27	-11	-13	-14
PLGA-PEG 2000	78	95	152	0.14	0.25	0.25	-19	-25	-15
PLGA-PEG 5000	69	84	139	0.33	0.39	0.39	-14	-13	-10

The nanoprecipitation method is shown to be efficient to prepare small and quite highly charged particles with diameters of less than 80 nm from each of the copolymers used in this study. In all cases, except for PLGA-PEG 5000, the polydispersity index of the nanoparticles was under 0.15, indicating nearly monodisperse systems. Although the starting material contained relatively polydisperse polymeric chains, this feature did not seem to influence the final Pdl of the nanoparticles in suspension. A previous work carried by Matsumoto *et al.* reported a similar observation. In this work, nanoparticles made of pegylated poly(lactic acid) with a Pdl of 2.0 formed nanoparticles with similar size, size distribution and drug trapping properties as nanoparticles made of monodisperse copolymers [28]. Another work carried out recently by Martin *et al.* demystifies the need to use monodisperse chain polymers to form monodisperse nanoparticles. On the contrary, his findings support the use of controlled polydispersity in polymeric chains as a design tool to enhance the macroscopic properties of nanoparticles, thus stabilizing the dispersions. This phenomenon may be due to the reduction of the strength of repulsion by polydisperse graft length at intermediate inter-particle distances [29].

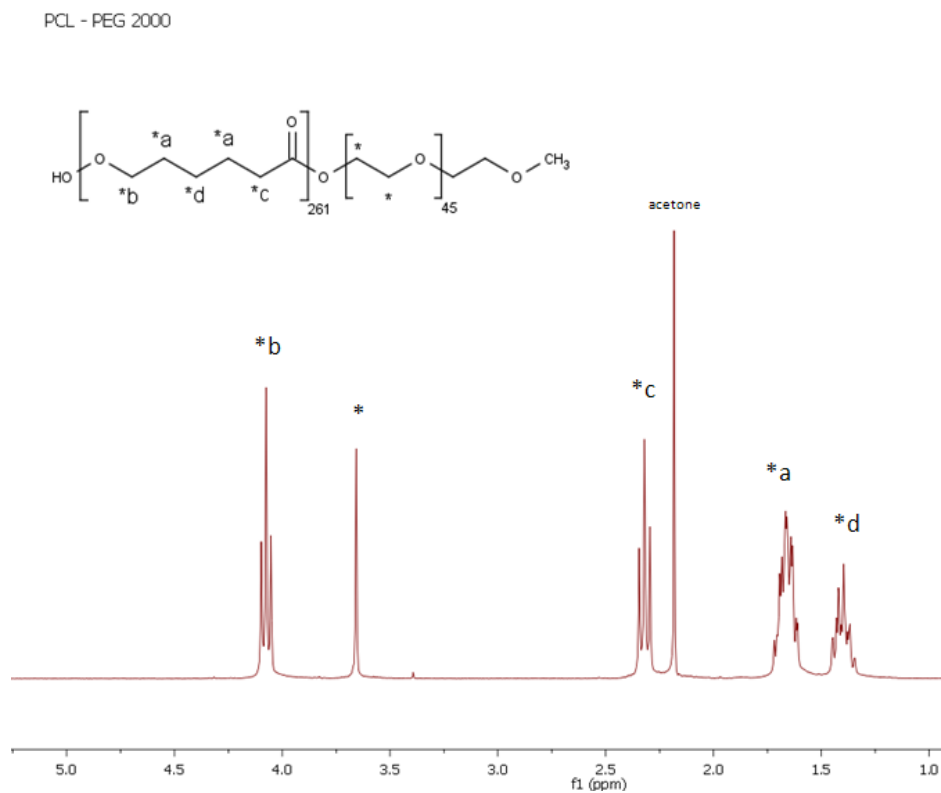
In terms of zeta potential, all the nanoparticles presented negative charge when in suspension in pure water which would reduce their tendency to aggregate. However, after 30 days in aqueous suspension, the PDI of the particles increased up to 0.4 but no precipitation was visible to the naked eye. PEG tends to mask the negative charge of polyesters but stabilizes the nanoparticles by a steric effect.

CONCLUSION

All the nanoparticles formed by nanoprecipitation of the pegylated polyesters copolymers used in this study present potential as drug delivery systems, except PLGA-PEG 5000 due to the high polydispersity of the final suspension. The nanoparticles formed from polylactide showed a smaller size and PDI with a favorable zeta potential, and were therefore chosen for further studies of drug encapsulation.

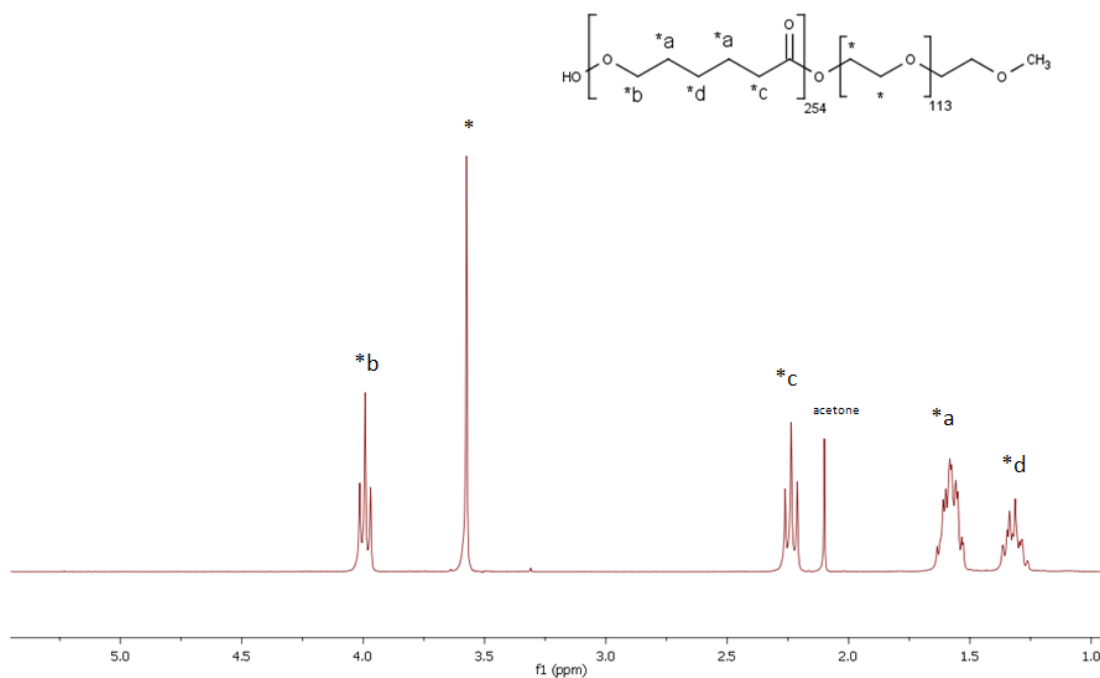
Supplementary Information

^1H NMR spectrum of PCL-PEG 2000

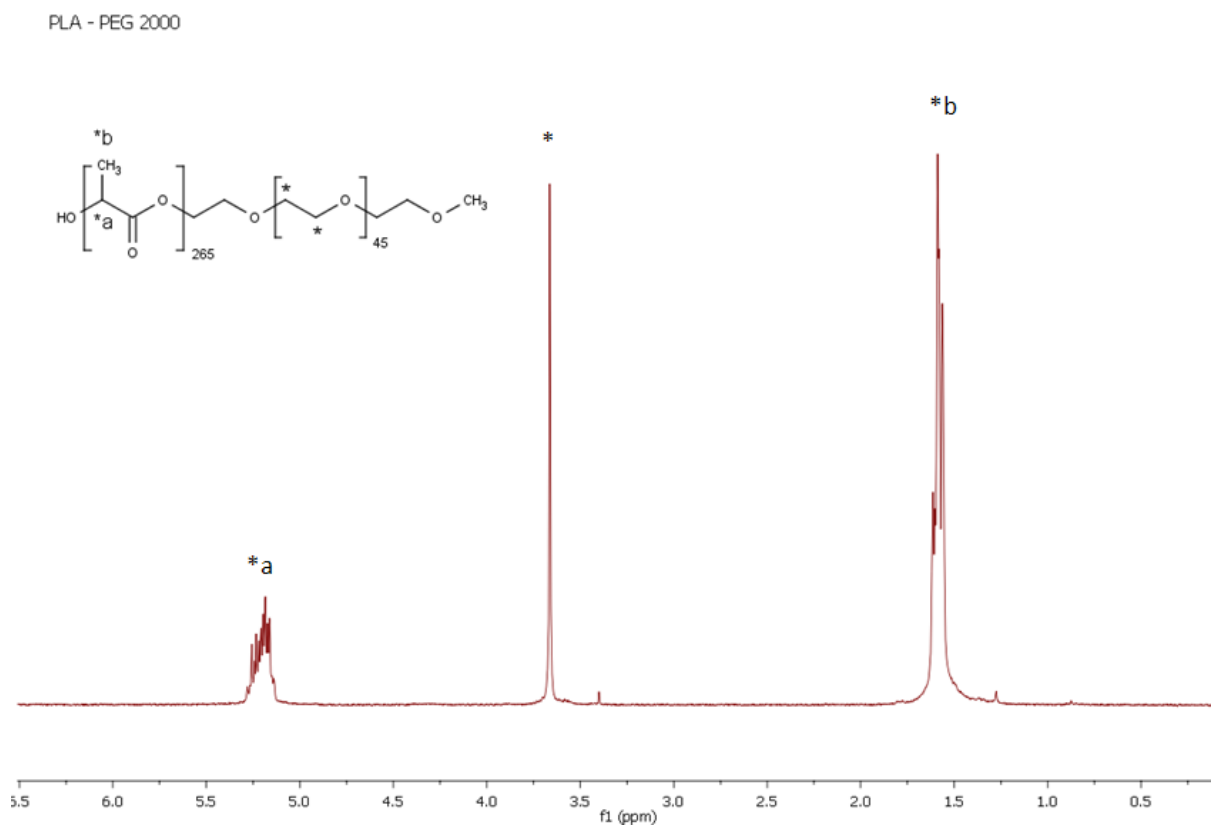


¹H NMR spectrum of PCL-PEG 5000

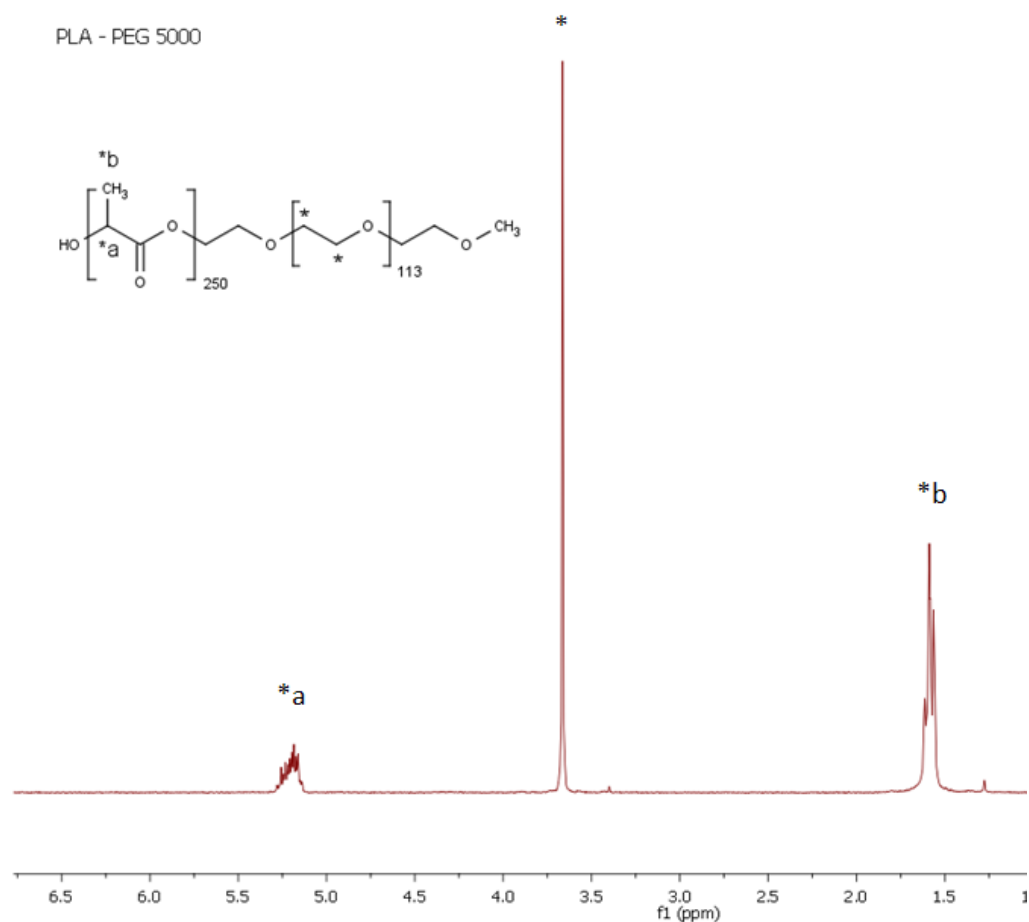
PCL - PEG 5000



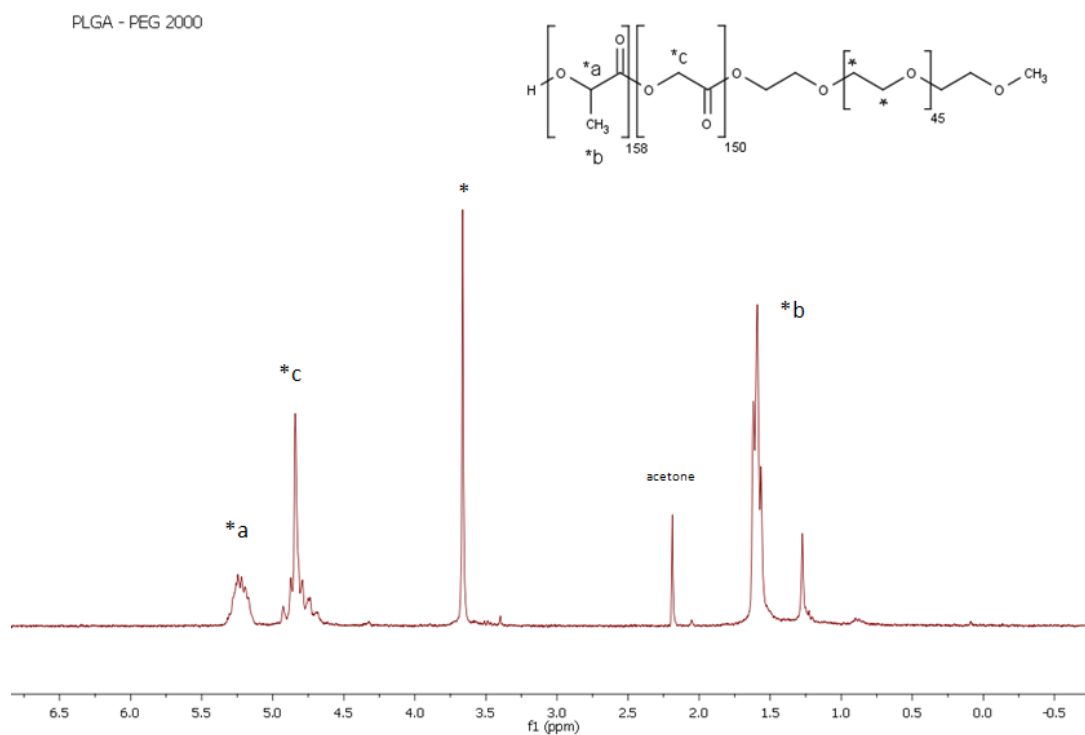
¹H NMR spectrum of PLA-PEG 2000



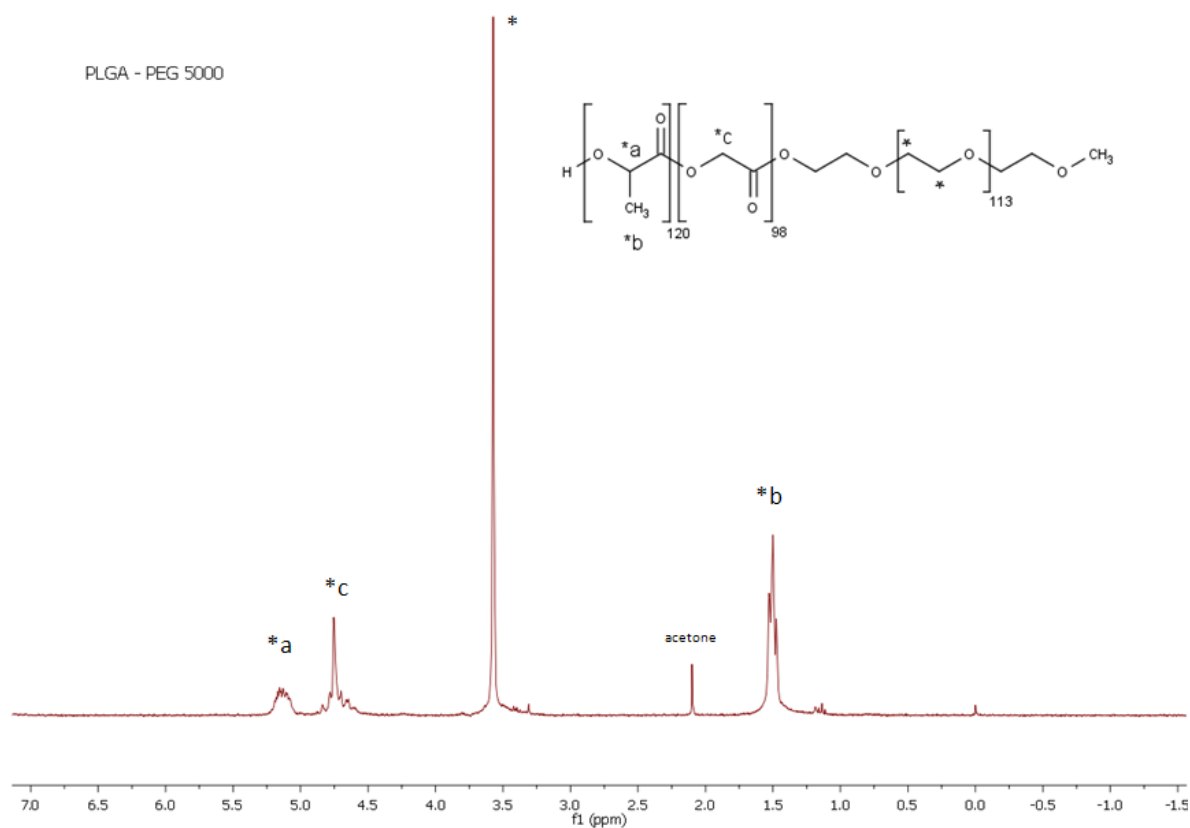
¹H NMR spectrum of PLA-PEG 5000



¹H NMR spectrum of PLGA-PEG 2000



^1H NMR spectrum of PLGA-PEG 5000



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CHAPTER III

BIODEGRADABLE AND BIOCOMPATIBLE NANOPARTICLES FOR PROSTATE CANCER TREATMENT

SECTION 2: New Combination Drug Therapy for Prostate Cancer Based on Nanoparticles

New combination drug therapy for prostate cancer based on nanoparticles

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Keywords: nanoparticles; prostate cancer, encapsulation, mitoxantrone, docetaxel, polymer, PEG

Abstract

Two of the few cytotoxic molecules active against metastatic hormone-refractory prostate cancer are docetaxel (DOC) and mitoxantrone (MIT). The encapsulation of these two molecules with different mechanisms of action against tumor cells within polymeric nanoparticles is an original therapeutic approach to avoid the side effects of chemotherapy. The aim of this work was to develop a multifunctional drug delivery system based on polylactic acid-polyethylene glycol block copolymer nanoparticles loaded with DOC and MIT.

The results showed the efficiency of all loaded nanoparticle combinations with at least 50 % of cell activity reduction in the first 48 h. Moreover, a synergistic effect was observed in two different combinations of nanoparticles containing MIT and DOC leading to less than 10% of cell activity against PC3 prostate cells in the first 96 h of treatment. Thus, the proposed combination of chemotherapeutic agents within nanosized delivery systems was shown to be an effective therapeutic strategy.

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INTRODUCTION

Prostate cancer is the most common non-skin cancer among men throughout the world according to the International Agency for Research on Cancer [1]. Hormone therapy usually represents the first-line treatment for this kind of tumor [2]. However, patients generally develop resistance within 36 months and become prone to more aggressive forms of cancer [3]. Two of the few cytotoxic molecules known to be active against metastatic hormone-refractory prostate cancer are docetaxel (DOC) [4] and mitoxantrone (MIT) [5].

The encapsulation of these two molecules with different mechanisms of action against tumor cells within biocompatible and biodegradable polymeric nanoparticles would be an original therapeutic approach to avoid the side effects of chemotherapy.

The aim of this work was to develop a multifunctional drug delivery system based on polylactic acid-polyethylene glycol (PLA-PEG) block copolymer nanoparticles loaded with these two different anti-cancer agents.

In this study, the PLA-PEG copolymer was synthesized by ring-opening polymerization [6]. PLA was chosen as the hydrophobic part of the copolymer because it is an amorphous polymer widely applied for pharmaceutical and medical applications due to its biocompatibility and low toxicity. The anti-cancer drugs docetaxel and mitoxantrone were encapsulated by the nanoprecipitation technique [7]. The drug encapsulation efficiency (EE) was measured by ultraviolet-visible spectrophotometry (UV-Vis) after separation of non encapsulated drug by ultrafiltration. The antitumor efficacy of the NPs against two different human prostate cancer cells PC3 and *LNCaP* was evaluated by MTS method. The effectiveness of each type of nanoparticle was evaluated separately as well as mixtures of the two types of nanoparticle in different ratios in order to observe any synergy, additivity or antagonism effect.

1. Materials and Methods

1.1. Materials

All solvents and chemicals were used as received. The CellTiter 96® AQueous One Solution Reagent (MTS) reagent was purchased from Promega (Madison, USA). Cell culture reagents were from Lonza (Basel, Belgium). Docetaxel (DOC) and mitoxantrone (MIT) and stannous 2-ethylhexanoate were purchased from Sigma–Aldrich (Illkirch, France).

D,L-lactide was purchased from Polysciences (Warrington, USA). Polyethylene glycol 2000 monomethylether (PEG 2000) was purchased from Fluka (Buchs, Switzerland). Acetone, acetonitrile (ACN) and dichloromethane (DCM) were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Deuterated chloroform (CDCl_3) and dimethyl sulfoxide (DMSO) were obtained from Carlo Erba (Peypin, France). Ultra pure water was produced by reverse osmosis (MilliQ®, Millipore).

1.2. Synthesis of PLA-PEG 2000

PLA-PEG copolymer was synthesized by ring-opening polymerization as described previously, with modifications [8]. In brief, 5 g of D,L-lactide, 10 wt % PEG 2000 and 1 wt % stannous 2-ethylhexanoate were added to a dry Schlenk tube for polymerization. The tube was subjected to several vacuum–argon cycles and then the polymerization was carried out in an oil bath at 115 °C for 20 h. After the conclusion of the reaction, the resulting product was purified by repeating the following procedure twice: the product was dissolved in DCM and then precipitated by a mixture of petroleum ether and diethyl ether 1:1. The final purified product was dried in a dynamic vacuum in order to eliminate solvent traces.

1.3. Proton nuclear magnetic resonance (^1H NMR)

The structure, number average molecular weight and the PEG content of the copolymer were determined after ^1H NMR analysis in a Bruker AC 300 Hz spectrometer. The sample was solubilized in deuterated chloroform (CDCl_3) and analysed.

1.4. Nanoparticle preparation

The nanoparticles were prepared by the nanoprecipitation method [7]. Three different types of nanoparticles were made: empty nanoparticles without any added drug (ENPs), mitoxantrone-loaded nanoparticles (MITNPs) and docetaxel-loaded nanoparticles (DOCNPs).

For all preparations, 20 mg of the copolymer was dissolved in 4 mL of acetone. The acetone phase was added using a syringe coupled with a 0.50 x 16 mm needle (Braun, Germany) to 8 mL of distilled water under magnetic stirring with an infusion pump (Ealing, France) at infusion rate of 2 mL/min. The suspension of nanoparticles was stirred for more 20 min after which the acetone was removed under reduced pressure (Büchi, Swiss).

The suspension of nanoparticles was separated from non encapsulated drug using a centrifugal ultrafiltration device with a cut-off of 30 kDa (Amicon® Ultra 30K, Millipore, Darmstadt, Germany) by centrifugation at 12000 g for 20 min.

The nanoparticles were rinsed three times using the same device and resuspended in 8 mL of ultra pure water. The 8 mL final stock suspension of nanoparticles was stored at 4 °C for characterization assays performed in the following hours to avoid polymer degradation or drug release.

To prepare MITNPs, 4 mg of mitoxantrone was added to the aqueous phase as previously described by Wang *et al.* [9]. For DOCNPs, 4 mg of docetaxel was added to the acetone/copolymer phase. 0.1 % of DMSO was added during the two washing steps of DOCNPs in order to remove the non-encapsulated

docetaxel which might have precipitated in pure water. The last rinsing step was carried out with pure distilled water to avoid any interference of DMSO in the subsequent analysis.

1.5. Nanoparticle characterization

Nanoparticle size, size distribution (Pdl) and zeta potential were determined by dynamic light scattering (DSL) at 25 °C (Zetasizer® 4, Malvern Instruments) using the undiluted final aqueous stock suspension of nanoparticles. Results were expressed as the average of three measurements. The particle size is presented as Z-average.

1.6. Encapsulation Efficiency (EE)

The amount of docetaxel and mitoxantrone associated with the nanoparticles was determined by ultraviolet-visible spectrophotometry (UV-VIS) analysis. Spectrophotometric measurements were made on a PerkinElmer Lambda 25 UV-VIS spectrophotometer coupled with a computer loaded with PerkinElmer UV Winlab software. The nanoparticles were separated from non encapsulated drug by ultrafiltration as described before and re-suspended in an appropriate solvent with vortexing for 2 min. The centrifugal filter was pre-rinsed with 0.1 N NaOH followed by two washing steps with purified water in order to avoid any interference from the glycerin content of the filter.

Spectrophotometric detection of docetaxel [10] was carried out at a wavelength of 229 nm using ACN as solvent. A calibration curve was prepared using docetaxel concentrations in the range 5 - 55 $\mu\text{g mL}^{-1}$.

Spectrophotometric detection of mitoxantrone was carried out at a wavelength of 620 nm. Two different curves were prepared with mitoxantrone concentrations in the range 5 - 45 $\mu\text{g mL}^{-1}$, one using DMF and another curve using H_2O .

The solvents, ACN and DMF were used to dissolve the nanoparticles before assay. An additional curve of mitoxantrone was made in water in order to determine the non-encapsulated drug present in the filtrate.

The encapsulation efficiencies (%) of the nanoparticles were calculated based on the follow equation:

$$\text{Encapsulation efficiency (EE) (\%)} = \text{mass of drug in nanoparticles} / \text{initial amount of drug} \times 100\%$$

Data are reported as means of the percentage of the encapsulation efficiencies of three experiments \pm SD.

1.7. Cytotoxicity evaluation

The human prostate cell lines *LNCaP* (ECACC Reference 89110211) and PC-3 (ATCC® CRL-1435™) were obtained from the Institut Bergonié, Bordeaux, and Institut Marie Curie, Paris, respectively. These two cell lines are widely used for *in vitro* studies of drug development for prostate cancer. They are derived from different metastatic sites and present particular features as shown in Table 1.

Table 1: Characteristics of the prostate cell lines used for the *in vitro* studies.

	PC3	LNCaP
Stage of the disease	Late stage	Early stage
Phenotype	More aggressive [11]	Less aggressive
Hormone dependence	Androgen-independent [12]	Androgen-dependent
Site of metastasis	Bone metastasis	Left supraclavicular lymph node
PSMA	Non- expressed [13]	Expressed

The cells were routinely grown in complete RPMI-1640 medium supplemented with 10 % of fetal bovine serum and 1 % penicillin-streptomycin. Cells were incubated at 37 °C in an atmosphere containing 5 % CO₂ and subcultured when the cells reached 80 % confluence.

Cell viability was estimated based on mitochondrial metabolic activity using the Promega CellTiter 96® Aqueous One Cell Proliferation (MTS) assay [14]. The absorbance recorded in this colorimetric assay reflects the number of viable cells.

The cells were seeded in 96-well plates. *LNCaP* cells were seeded at 5000 cells in 50 µL per well and PC3 cells were seeded at 3500 cells in 50 µL per well. Twenty-four hours later the nanoparticles were suspended in culture medium, serially diluted and 50 µL of the nanoparticle suspension were added to each well. The plates were incubated for a further 24 h, 48 h or 144 h. Ninety minutes prior to the end of each exposure period, the MTS reagent (20 µL/well) was added. The cytotoxicity of the unloaded nanoparticles (ENPs) was analyzed after 2, 3 and 7 days of exposure to a concentration range from 19 to 312 µg/mL of polymer.

Cells were treated with different combinations of nanoparticles. The quantity of polymer was kept constant at 125 µg/mL in all test wells by adding empty nanoparticles as necessary. The same combinations were added as free drugs. The drug ratio and concentrations are given in Table 2. The drug concentrations were calculated from the encapsulation efficiencies.

Table 2: Different combinations of nanoparticles used in the cytotoxicity evaluation

Combinations	DOCNPs (%)	Docetaxel (µg/ml)	MITNPs (%)	Mitoxantrone (µg/ml)	ENPs (%)
DOC	50	7.25	0	0	50
MIT	0	0	50	0.78	50
DOC MIT 50:50	50	7.25	50	0.78	0
MIT DOC 50:25	25	3.63	50	0.78	25
DOC MIT 50:25	50	7.25	25	0.39	25

The absorbance of the formazan product was read with a 492 nm high-pass filter in a Multiskan MS microwell plate reader (Labsystem, Ramat-Gan, Israel). The mean of triplicate wells from one of two representative experiments are shown.

Background absorbance due to the non-specific reaction between the test compounds and the MTS reagent was measured in wells without cells and was subtracted from the values measured in the presence of cells [15].

The mitochondrial metabolic activity (%) was calculated using the follow equation: $[A] \text{ test} / [A] \text{ control} \times 100$ where $[A] \text{ test}$ is the mean absorbance of the treated wells for each condition and $[A] \text{ control}$ is the mean absorbance of

the control. The non treated wells used as control for the free drugs and the wells treated with empty NPs were used as control for the loaded nanoparticles [16]. Error bars indicate the range of data for the triplicates.

1.8. Statistical method

Data are reported as means of percentage of the absorbance of three experiments (triplicate) \pm SD. The Student's t-test was used to compare groups. $P < 0.05$ was considered to be statistically significant.

1.9. Confocal microscopy

Live cell imaging and particules observations were done with an inverted confocal laser scanning microscope LSM 510-Meta (Carl Zeiss, Germany) equipped with a helium neon laser (633 nm excitation wavelength), using a Plan-Apochromat 63X/1.4 objective lens, Mitoxantrone fluorescence was collected with a 649-798 nm band pass emission filter. Differential interferential contrast images were acquired with the same laser. The pinhole was set at 1.0 Airy unit. 12 bit numerical images were acquired with LSM 510 software version 3.2.

The cells were seeded in 6-well plates. *LNCaP* cells were seeded with 150000 cells in 1 mL per well and PC3 cells were seeded with 120000 cells in 1 mL per well. Twenty-four hours later the MITNPs were suspended in culture medium, serially diluted to a nanoparticle concentration of 125 μm / mL and added to the cells in 6-well plates (1 mL/well). The plates were incubated for further 10 min, 1h and 4h, when images were acquired in a 37 °C chamber.

1.10. Flow cytometry

Nanoparticles uptake was analyzed by fluorescence-activated cell sorting (FACS) analysis. Cells were seeded in 12-well plates at a density of 50,000 cells/mL (PC3) and 100,000 cells/mL (*LNCaP*) and allowed to adhere 24 h. MITNPs were diluted in RPMI medium to a final nanoparticle concentration of

156 µg/mL of polymer, thus, 9.4 µg/mL of mitoxantrone content. 0.5 mL of nanoparticles suspension were added into each well and incubated at 37 °C. After 10 min, the supernatant was discarded, cells rinsed with PBS and harvested by 350 µl of Trypsin/EDTA (170,000 U Trypsin/L). Cell suspensions were analyzed by flow cytometry (Accuri C6, BD Biosciences), and mean fluorescence intensities were collected analysing 10,000 cells on detector FL-3 using λ excitation of 488 nm and λ excitation of 670 nm. All measurements were performed in triplicate.

2. Results and Discussion

2.1. PLA-PEG 2000 synthesis

PLA-PEG copolymer was synthesized from D,L-lactide and PEG 2000 using stannous 2-ethylhexanoate as catalyst as shown on Figure 1. The hydroxyl group initiated the ring-opening polymerization. In the ^1H NMR spectrum the methylenic protons ($-\text{CH}_2-$) of PEG segment appear at 3.65 ppm. The peaks at 5.20 ppm and 1.65 ppm belong, respectively, to methyl proton ($-\text{CH}_3$) and methine ($-\text{CH}$) of PLA segment (NMR spectrum not shown). The molecular weight was calculated from the ^1H NMR spectrum as 21300 g/mol and the weight content of PEG was 9.4 %. The results confirm that the copolymer could be synthesized reliably as previously demonstrated [17].

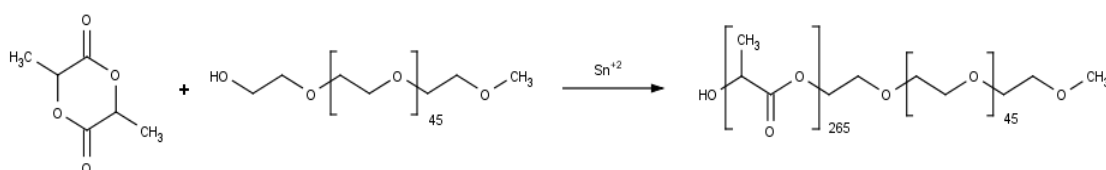


Figure 1: Synthesis of PLA-PEG di-block copolymer.

2.2. Nanoparticle characterization

The concentrations of the drugs within the nanoparticles were determined spectrophotometrically with reference to calibration curves established from standard solutions of docetaxel and mitoxantrone.

ENPs were used as control and showed no interference with mitoxantrone or docetaxel absorption of visible light at the wavelengths used. The calibration curves were linear in the concentration range 5 to 55 $\mu\text{g mL}^{-1}$ of docetaxel in ACN and 5 to 55 $\mu\text{g mL}^{-1}$ in DMF and water.

The encapsulation efficiency, as well as, the size, the polydispersity index and the zeta potential, are shown in Table 3.

Table 3: Characterization of the nanoparticles

Sample	Encapsulation efficiency (%)	Z-average size (nm)	Polydispersity index	Zeta Potential (mV)
ENPs	-	70 ± 6	0.17	-11 ± 2
DOCNPs	58 ± 3	68 ± 3	0.15	-17 ± 3
MITNPs	6 ± 1	83 ± 2	0.12	-17 ± 5

Nanoprecipitation is a simple and efficient method to encapsulate hydrophilic and hydrophobic drugs within polymeric nanoparticles [18]. The concentration of 5 mg/mL of polymer was shown previously by Legrand *et al.* to generate small nanoparticles by this technique [19]. In all our cases, the size of the nanoparticles were lower than 90 nm with a narrow size distribution, thus these particles are likely to be able to reach the tumor tissues by passing through the openings between cells in defective endothelium [20]. The negative zeta potential of between 10 and 17 mV may avoid agglomeration by electrostatic repulsion. Moreover, it will prevent interaction with negatively charged serum components which could form aggregates that could be easily recognized by macrophages and cleared from the circulation [21]. While positively charged nanoparticles can show higher levels of internalization due the electrostatic

attraction to the cell membrane which has an overall negative charge, the internalization of negatively charged NPs may occur by endocytosis after nonspecific binding of the particles to cationic sites on the cell membrane [22]. The Figure 2 illustrates these mechanisms.

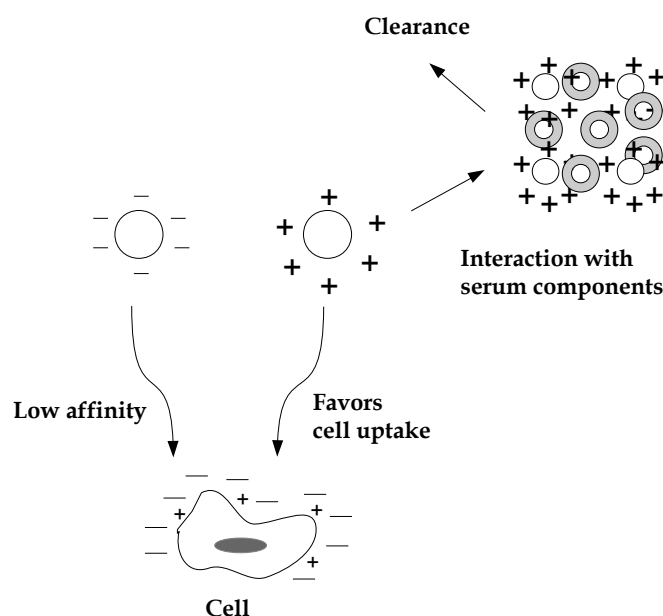


Figure 2: Illustration of the interactions of positive and negative surface charged nanoparticles.

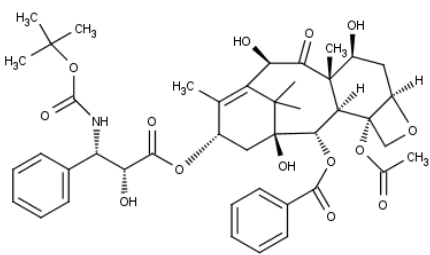
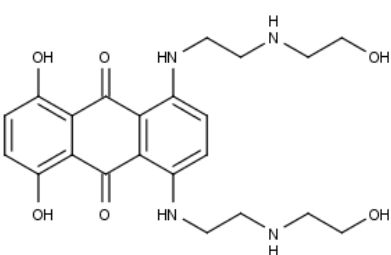
We obtained satisfactory encapsulation efficiencies for both drugs, with values similar to those already reported in the literature, as shown in Table 4.

Table 4: Examples of encapsulation efficiencies (EE%) of docetaxel and mitoxantrone found in literature.

Polymer base	Encapsulated drug	Size (nm)	EE (%)	Reference
PLGA-PEG	docetaxel	422	58	[23]
PLGA	docetaxel	334	65	[24]
PLGA	docetaxel	204	61	[25]
PLGA	docetaxel	130 - 150	41	[26]
PBCA	mitoxantrone	-	8	[27]

The different encapsulation efficacies of the two drugs are to be expected given that mitoxantrone is much more hydrophilic than docetaxel and would therefore have less affinity for the polymer. This did not pose a problem for the biological evaluation because mitoxantrone is generally used in lower doses for prostate cancer treatment than docetaxel, as summarized in Table 5.

Table 5: Characteristics of Taxotere® and Mitoxantrone®

Trade name	Taxotere®	Novantrone®
Generic name	Docetaxel	Mitoxantrone
Recommended dose *	75 –100 mg/m ² [28]	12 mg/m ² [29]
Chemical structure		
Classification	Taxane	Anthracycline
Charge	Non-ionic [30]	Cationic [31]
Solubility in water	Poorly water-soluble	Water-soluble
Administration	Intravenous infusion (I.V.)	Intravenous infusion (I.V.)
Diluents for I.V infusion	Aqueous suspension containing ethanol et polysorbate 80	Aqueous suspension containing sodium chloride, sodium acetate and acetic acid
Indications	Lung, prostate and breast cancers; gastric adenocarcinoma; squamous cell carcinoma of the head and neck [32].	Multiple sclerosis, prostate cancer, non lymphocytic leukemia
Main mechanism of action	Anti-mitotic chemotherapy. It promotes tubulin assembly in microtubules and inhibits their depolymerization [33, 34]	DNA intercalator. It prevents the DNA synthesis, inhibits the transcription and induces mutations [35]
Common side-effects	Neutropenia, mucositis, asthenia, hypersensitivity reactions, cutaneous reactions, alopecia [36]	Cardiotoxicity, alopecia, leukopenia, menstrual disorder, amenorrhea [37]

* Recommended dose for prostate cancer treatment

2.3. Confocal imaging

The cellular uptake of the nanoparticles containing mitoxantrone (MITNPs) by the PC3 and *LNCaP* prostate cell lines was qualitatively studied with confocal laser scanning microscopy thanks to the intrinsic fluorescence of this molecule. The internalization of the nanoparticles was confirmed by flow cytometry analysis (data not shown). The confocal microscopy images (Figure 3) show that fluorescence from the mitoxantrone-loaded nanoparticles could be seen within the cells after only 10 min of exposure. The free drug did not show the same profile (data not shown) and was internalized more slowly. This result could be due to the faster internalization of a negative charged vehicle, which is not likely to form complexes with serum components present in the culture medium in the same way as the positively charged free mitoxantrone as illustrated in the Figure 2. Similar charge effects on cellular uptake have been previously described in the literature [38].

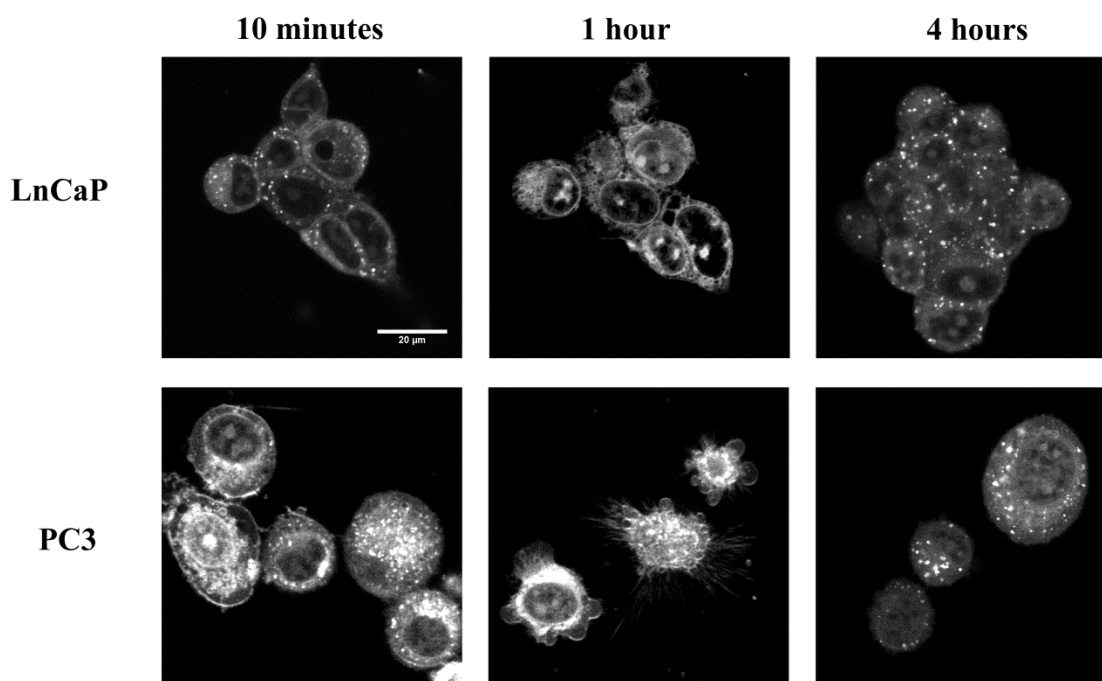


Figure 3: Confocal microscopy images of prostate cancer cells *LNCaP* and PC3, after 10 min, 1 hour and 4 hours exposure to mitoxantrone-loaded nanoparticles.

2.4. Mitochondrial activity

Before determining the cytotoxic effect of the encapsulated drugs on the prostate cancer cell lines, it was necessary to verify that the nanoparticles themselves were not toxic under the test conditions. Figure 4 shows the results of incubation with different concentrations of polymer as ENPS for 2, 3 or 7 days.

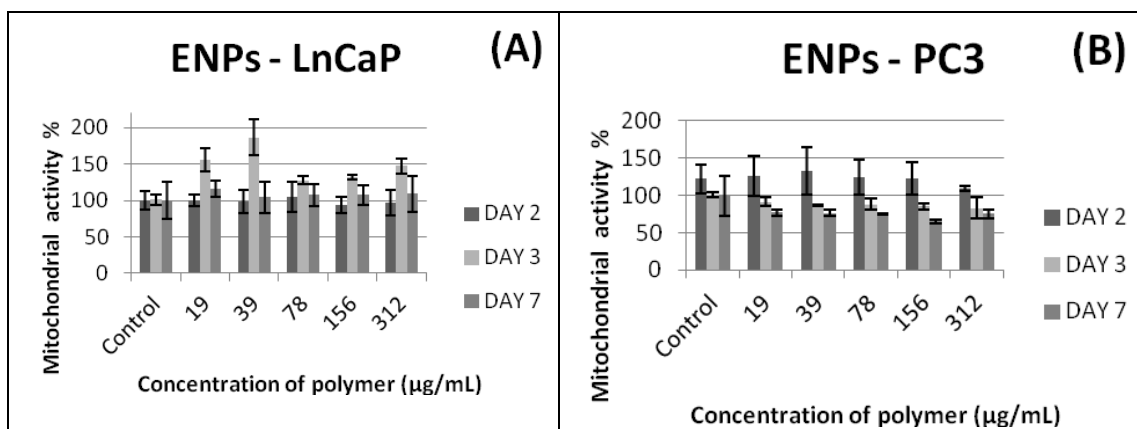


Figure 4: Mitochondrial activity of *LNCaP* (A) and *PC3* (B) cells treated with unloaded nanoparticles.

More than 80 % of the *LNCaP* cells were still viable after treatment with the unloaded PLA-PEG in a concentration range of 19 to 312 µg/mL, whatever the incubation time. The *PC3* cells showed viability of more than 80 % until the third day; however the viability decreased to 60-80 % at the seventh day. These results are in good accordance with the literature [39]. The enhanced mitochondrial activity observed in both cell lines at the shorter incubation times may be due to the oxidative stress caused by the presence of nanoparticles. The *PC3* cell line seems to be more sensitive to this effect and showed a peak of mitochondrial activity after two days followed by a decrease which could be related to mitochondrial damage or a reduction in the number of cells [40]. Although the *PC3* cells were more sensitive to unloaded PLA-PEG nanoparticles than *LNCaP* cells, it is possible to say that the nanoparticles were well tolerated by both cell lines.

Based on these results, a total polymer concentration of 125 µg/ml of DOCNPs and MITNPs and treatment times of 2, 4 and 5 days were chosen. The results are shown in the Figure 5. The corresponding docetaxel and mitoxantrone concentrations were calculated after the encapsulation efficiency analysis and it was previously shown in Table 2.

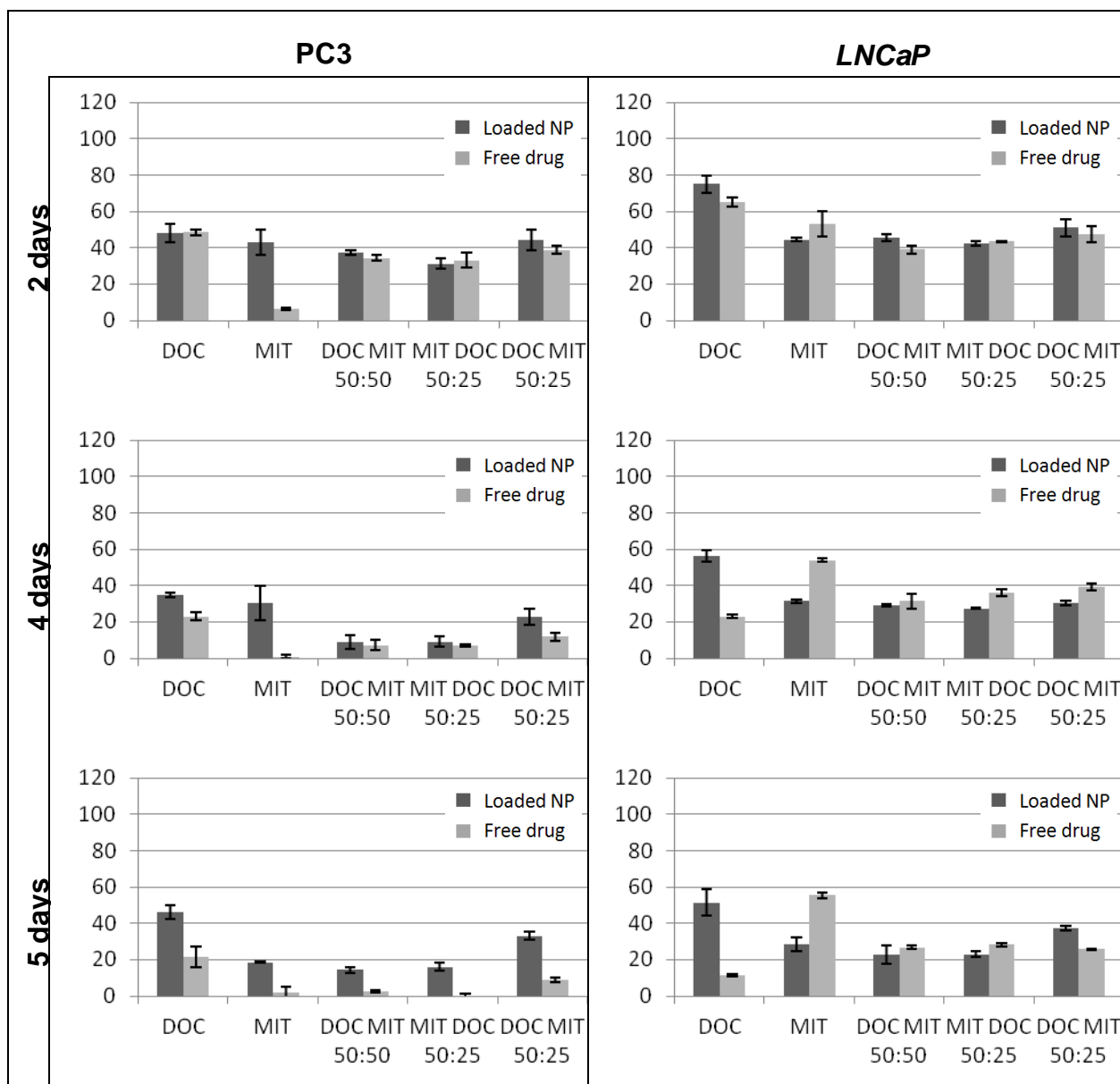


Figure 5: Mitochondrial activity of PC3 and *LNCaP* cells treated with loaded nanoparticles or with free drug. Results are expressed as % of the control (100 %).

All the measured absorbencies were statistically significant from its related control, except PC3 when treated with DOCNPs in the first 48h.

2.4.1. Comparison of free and encapsulated drug

In the PC3 cell line the free drugs were equally or more toxic than the same concentrations loaded in nanoparticles, particularly in the case of mitoxantrone added alone. Thus, although the confocal microscopy results showed a more rapid uptake of nanoparticles, in the longer incubation times used in this experiment, the free drugs were accessible to the cells, as a result of interactions between the hydrophobic docetaxel and the cationic mitoxantrone and the hydrophilic and negatively charged cell membrane of the PC3 cells.

In contrast, nanoparticles loaded with mitoxantrone alone were generally more toxic to the *LNCaP* cells than the free drug. *LNCaP* cells express PSMA proteins which present aromatic amino acid residues [41], that can acquire positive charges when protonated; for example, the amino acid histidine [42, 43]. This interface may favor the internalization of negative charged molecules or particles through electrostatic attraction. Furthermore, free mitoxantrone, a cationic molecule, showed lower efficacy against this PSMA expressing cell line than against PC-3 cells in this experiment. Thus, the encapsulation of this drug within negative charged nanoparticles maximizes its internalization and efficiency. The results obtained from confocal microscopy correlate with this cytotoxic analysis.

The encapsulation of docetaxel does not seem to improve the effectiveness towards either cell line. However, the use of polymeric nanoparticles for docetaxel delivery could have several advantages *in vivo*. For example, the conventional formulation of Taxotere® contains polysorbate 80, a surfactant which increases the apparent solubility of docetaxel in aqueous suspension, but can it can cause serious side effects such as fluid retention and hypersensitivity reactions [44]. The encapsulation of this drug within biodegradable and biocompatible nanoparticles is a potential strategy to increase the dispersibility of docetaxel and allow its application by intravenous route without the need of polysorbate 80. Additionally, these pegylated nanoparticles would improve the

accumulation both docetaxel and mitoxantrone in the tumor tissues by the EPR effect.

2.4.2. Simultaneous delivery of docetaxel and mitoxantrone

The simultaneous delivery of the two antineoplastic agents encapsulated in PLA-PEG nanoparticles was shown to be more efficient than the delivery of the encapsulated drugs separately. The results indicate that all the mixtures of DOCNPs and MITNPs reduced the cellular mitochondrial activity by least 50 % in the first 48 h.

In general, the mitochondrial activity was decreased by treatment with mixtures compared with MITNPS and DOCNPs alone. The least effective mixture was DOC MIT (50:25) which was more effective than DOCNPs but similarly or less toxic than MITNPs whatever the cell line and the incubation time.

The *LNCaP* cell line was more sensitive to treatment of nanoparticles containing MITNPs than to DOCNPs alone. Despite the good efficacy of the MITNPs alone, combinations containing at least 50 % of MITNPs reduced the mitochondrial activity more than the single treatment after 96h.

The combined treatment appeared to be more toxic towards the PC3 cell line than to the *LNCaP* cells. The mixtures containing 50 % of MITNPs, regardless of the DOCNPs content, showed a synergistic effect, being more toxic than MITNPS and DOCNPs alone towards this cell line. The combinations MIT DOC 50:50 and MIT DOC 50:25 led to a reduction of more than 60% in the mitochondrial activity in the first 48 h, and more 90% after 96 h of treatment.

As shown in Table 5 and illustrated in Figure 6, docetaxel and mitoxantrone act at different stages of the cell cycle and are therefore good candidates for combination therapy. However, although both are extensively used for prostate cancer therapy [45] their combination for this type of cancer has never been reported in clinical trials. A mixture of mitoxantrone with prednisone and ixabepilone was described to be effective as second-line therapy for docetaxel-

resistant prostate cancer patients in a Phase-II study [46]. Furthermore, the combination of these two drugs has already been applied to metastatic breast cancer patients and was revealed as a highly effective chemotherapy regimen. Nevertheless, the hematological toxicity was found to be an important challenge to be overcome [47, 48].

The use of nanoparticles to encapsulate those anticancer drugs could minimize their side effects and in addition, it could reduce the multidrug resistance, a common drawback of chemotherapy. The different pathways of drug internalization and accumulation by the EPR effect would avoid the efflux of the drugs from the cancer cells mediated by the P-glycoprotein [49]. For example, polymeric nanoparticles containing PLA are associated with the improvement of docetaxel pharmacokinetics and effectiveness [50]. In addition, the administration of the two drugs in similar nanoparticles would assure that they had similar pharmacokinetic profiles and accumulated within the tumors at the same rate.

Therefore, combinations of nanoparticles containing docetaxel and mitoxantrone (DOC MIT in ratios 50:50 and 50:25) that have been demonstrated in this study to have improved effectiveness *in vitro* towards two different prostate cancer cell lines may have potential clinical applications.

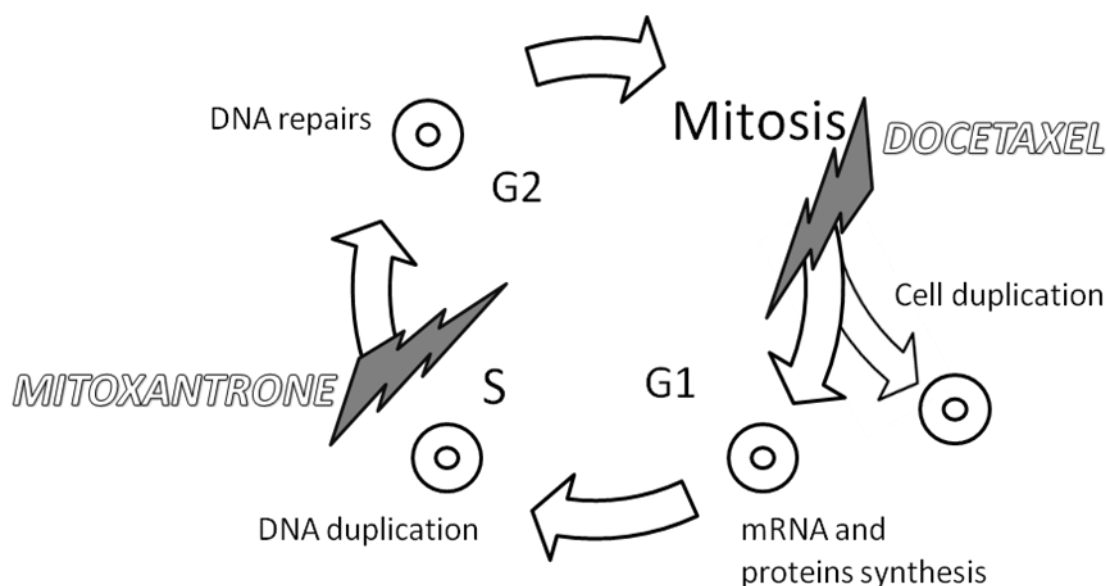


Figure 6: Interference of docetaxel and mitoxantrone in cell cycle.

CONCLUSION

The purpose of this study was to synthesize, characterize and evaluate the *in vitro* effectiveness of nanoparticles prepared from a poly(lactic acid)-polyethylene glycol (PLA-PEG) copolymer and loaded with two cytotoxic drugs, docetaxel and mitoxantrone, that are active against prostate cancer. For the first time in the literature these antineoplastic agents were encapsulated for concomitant application. The nanoparticles were prepared by the nanoprecipitation method, leading to small and monodisperse NPs of around 70 nm in diameter.

The encapsulation efficiencies for docetaxel and mitoxantrone were 58 % and 6 % respectively. *In vitro* studies were carried out with combinations of these particles in different ratios in order to observe any synergy, additivity or antagonism. The uptake of the nanoparticles containing mitoxantrone by living cells was studied qualitatively by confocal laser scanning microscopy and confirmed with flow cytometry analysis.

The *in vitro* study of cell viability showed the efficiency of all loaded nanoparticle combinations with at least 50 % reduction of cell activity in the first 48 h. Moreover, a synergistic effect was observed with two different combinations of MITNPs and DOCNPs leading to less than 10 % of cell activity against PC3 prostate cells in the first 96 h of treatment. These results indicate that the PLA-PEG-based nanoparticles developed in this work could potentially be exploited as carriers to improve the solubility of docetaxel and to increase the intracellular uptake of mitoxantrone and therefore have potential clinical applications.

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GENERAL DISCUSSION

This thesis was devoted to the development and evaluation of nanoparticles for cancer treatment. Although the nanocarriers described have potential applications in various types of tumours, this study was dedicated to prostate cancer therapy.

Firstly, a comprehensive literature survey was made based on the different strategies of nanomedicine applied to the prostate cancer therapies. The review paper covers the basis of drug delivery and drug targeting as well as the recent studies being carried in this field. The three main nanoparticle-based drug delivery platforms are considered; that is: liposomes, polymeric nanoparticles and metallic nanoparticles. Indeed, a critical analysis of the impact of nanomaterials on public health and the environment was made to highlight the need for precise control of the utilization of nanomaterials. The aim of this review was to summarize and provide an update on important work and results concerning research on nanoparticles for drug delivery and targeting for prostate cancer treatment.

The experimental part describes two main platforms: a metallic platform, using gold nanoparticles and a polymeric platform, using biodegradable and biocompatible pegylated nanoparticles. The use of metallic nanoparticles as well as polymeric nanoparticles to encapsulate antineoplastic molecules is a suitable therapeutic approach to avoid the side effects of chemotherapy.

The first part of the experimental study was dedicated to formulating gold nanoparticles carrying docetaxel and polyethylene glycol with and without folic acid, to characterize them and to evaluate their anticancer activity *in vitro*. The synthesis of the nanoparticles was carried by the group of Prof. Didier Astruc at the Bordeaux I University. The characterization, loading with docetaxel and evaluation of their antineoplastic effect against prostate cancer cells was my personal contribution and it was carried out at the Institut Galien Paris Sud XI, in Châtenay Malabry, France, under the supervision of Prof. Gillian Barratt.

Therapies using gold nanoparticles were used as medicinal agents many years before the modern concept of nanoparticles. The Arabic, Chinese and Indian literature reports the use of colloidal gold since 2500 BC. At this time, the nanoparticles were produced following ancient alchemy methods [1].

In modern medicine, gold nanoparticles have become versatile tools in biomedical research due to their stability combined with their small size, useful optical properties and easy functionalization. Furthermore, gold nanoparticles are the most frequently used noble metal nanoparticles for cancer applications. The versatility of gold nanoparticles leads to them being described as the “gold magic bullet” since they present great flexibility in terms of surface functionalization, imaging use and multiple therapeutic opportunities [2]. Moreover, the properties of gold nanoparticles are suitable for the combination of therapy and diagnostics in the same particle, in the method known as theranostics [3].

Gold nanoparticles have properties that render them ideal for drug delivery systems. For example, increases of stability, biodistribution, pharmacokinetics and drug solubility have been reported with the utilization of gold nanoparticles as a drug carrier [4]. Indeed, active and passive targeting mechanisms can be used for enhancement of the nanoparticle specificity and accumulation. Figure 1 shows the different strategies that can be applied for the use of gold nanoparticles in cancer therapy.

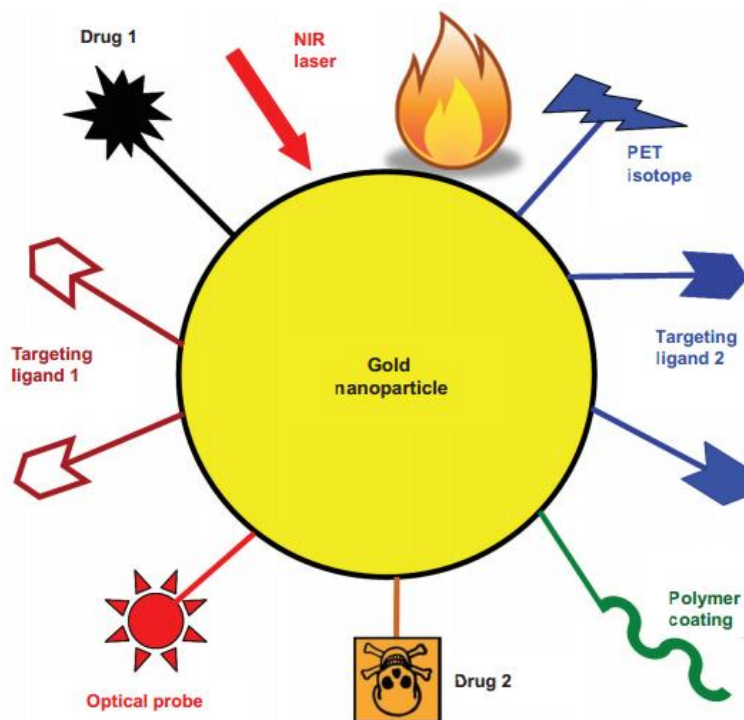


Figure 1: Different functionalizations that may be used on gold-based nanoparticles in cancer therapy. Reproduced from the reference [2] with permission.

Antineoplastic drug as paclitaxel [5], doxorubicin [6], methotrexate [7], oxaliplatin [8] and docetaxel [9] are some examples of anticancer drugs that have been shown to have improved therapeutical potential when conjugated with gold nanoparticles.

Docetaxel is a well established cytotoxic agent. Like paclitaxel, it is a taxoid from natural origin; however, docetaxel is synthesized from an inactive precursor found in the *Taxus baccata* tree. It works as an antimitotic agent disrupting microtubules which causes failure in cell replication and inducing cell death [10]. Although its efficacy has been proven, the side effects associated with therapy can be disabling. Neutropenia, fluid retention and neuropathy are examples of serious undesirable reactions related to docetaxel administration [11, 12].

For this study gold nanoparticles were chosen for docetaxel delivery because of the possibility of synthesizing small gold nanoparticles under 5 nm and their high functionalization potential using molecules for active and passive targeting such as folic acid and poly (ethylene glycol).

Prostate tumour cells overexpress Prostate Specific Membrane Antigen (PSMA) which has an affinity for folic acid. Not only can this be used as a diagnostic tool and a means of following the efficacy of therapy but it also provides a good target for therapy. When molecules of folic acid are bound covalently to macromolecules, the conjugate is internalized by endocytosis after binding to PSMA [13].

On the other hand, poly(ethylene glycol) (PEG) is widely used as passive targeting strategy. PEG reduces the opsonization of the particle surface with blood proteins and thereby delays their capture by phagocytic cells. The drug delivery systems can then remain in the circulation and reach the tumor by the so-called “enhanced permeation and retention” (EPR) effect, when the tumour capillary endothelium is leaky and the lymphatic drainage is impaired [14].

These two strategies can be combined to provide efficient drug delivery to cancer cells. Attaching PEG chains to the surface of nanoparticles made from biodegradable polyesters and including elements targeting PSMA has allowed anticancer drugs, including mitoxantrone and docetaxel, to be delivered to and accumulated within solid tumours [15, 16]. The gold nanoparticles were satisfactorily synthesized with and without folic acid using two PEG lengths, PEG 550 and PEG 2000. After characterization of the nanoparticles by transmission electron microscopy (TEM) it was necessary to adapt the method of storage as the freeze-drying process seemed to form aggregates difficult to disperse. Different batches of nanoparticles were provided in aqueous suspension and these were characterized by TEM to confirm the absence of aggregates and to verify the size and morphology of the particles. The core of the nanoparticles was shown to be 7 – 10 nm in diameter with a narrow polydispersity.

Surface plasmon resonance measurements were carried with a Biacore instrument to analyse the interaction between the AuNPs functionalized with folate and folate binding proteins (FBP). However, it was not possible to confirm that there were interactions between the gold nanoparticles and the channels functionalized with the FBP. The results can be explained by possible non-specific binding between the nanoparticles and the Biacore channels. This effect was explained in the Supplementary Information in chapter II of this thesis.

The association of the anticancer drug, docetaxel, with the AuNPs by non covalent adsorption was carried by stirring for 5 days at 30 °C and the final systems were used directly to evaluate cytotoxicity against *LNCaP* human prostate cancer cells using MTS test.

The docetaxel-loaded nanoparticles reduced the mitochondrial activity by 50 % or more at concentrations of $6 \cdot 10^{-9}$ M and above after 144 h of treatment. However, no significant difference was observed between folate-bearing AuNPs and non modified ones. The decrease in number of cells after contact with docetaxel-loaded nanoparticles was confirmed by observation with an optical microscope.

The unloaded AuNPs were not toxic to *LNCaP* cells at the concentrations used in this study. When docetaxel was adsorbed onto the AuNPs it produced a sustained cytotoxic effect *in vitro*. These small particles could be useful for concentration drug in solid tumors by the EPR effect and the properties of the gold core could be exploited for tumor imaging and for thermal cytolysis of tumor cells. The details of this work are discussed in depth in the chapter II of this thesis.

The second experimental part of this thesis concerns the synthesis, characterization and *in vitro* tests of biodegradable and biocompatible pegylated nanoparticles for the use as drug delivery systems for two different antineoplastic drugs: the mitoxantrone and the previously studied docetaxel.

Mitoxantrone is a synthetic anthraquinone derivative in the same family as doxorubicin and aclarubicin. It was firstly synthesized in 1979 and apart its antitumor action, it also has antibacterial, antiviral and immunomodulatory activities and is employed in multiple sclerosis therapy [17]. In contrast to docetaxel, the mitoxantrone acts as a DNA synthesis inhibitor which interferes with cell cycle progression. Adverse effects are reported in patients treated with mitoxantrone which are related to its accumulation in thyroid, liver and heart [18]. The cardiotoxicity associated with the administration of this drug seems to be irreversible and dose-dependent leading to a predisposition to heart failure. Other side effects include stomatitis, alopecia and myelosuppression [19, 20].

Nanoparticles prepared from a wide range of polymers have already shown their efficacy to improve the bioavailability and the pharmacokinetic properties of approved chemotherapy drugs for prostate cancer, leading to a great advance in this field [21].

Natural polysaccharides such as chitosan and albumin as well as polyesters such as poly(D,L-lactic-co-glycolic acid) (PLGA) [22], poly (D,L-lactic acid) (PLA) [23] and poly (caprolactone) (PCL) [24] which can be modified with PEG units forming pegylated co-polymers are the most commonly used polymers for drug delivery.

For the first time in the literature mitoxantrone and docetaxel were encapsulated for concomitant administration. The use of biocompatible and biodegradable polymeric nanoparticles to encapsulate these molecules is an original therapeutic approach to avoid the side effects of chemotherapy and drug resistance.

The study was initiated by the application of different nanoprecipitation conditions using a commercial poly (lactide-co-glycolide) - polyethylene glycol in order to optimize the methodology for subsequent experiments. The copolymer was nanoprecipitated using different ratios of organic and water phase, as well as polymer concentration. The final product was characterized by dynamic light

scattering (DLS) and transmission electron microscopy (TEM). Nanoparticles from 71 to 128 nm were produced depending on the polymer concentration in the organic phase. A less concentrated solution led to smaller and less polydisperse nanoparticles as previously related [25]. TEM images confirmed that small, spherical and polydisperse nanoparticles could be prepared by this technique.

Subsequently, the synthesis of three different biocompatible and biodegradable block copolymers was performed: polylactic acid - polyethylene glycol (PLA-PEG), poly (lactic-co-glycolic) acid - polyethyleneglycol (PLGA-PEG) and poly (caprolactone) - polyethylene glycol (PCL-PEG). Each copolymer was made with two different PEG lengths, PEG 2000 and PEG 5000, resulting in six block copolymers at the end of the synthesis part. The copolymers were characterized by proton nuclear magnetic resonance (^1H NMR) and size exclusion chromatography (SEC). The results show that the polymers were synthesized reliably. The molecular weight was found to be between 19,000 and 34,000 g/mol and the weight content of PEG of the copolymers varied from 6 to 26. The Polydispersity index (Pdl) varied from 1.8 to 2.6 which in accordance with this kind of ring-opening polymerization, as previously reported [26].

The six synthesized copolymers were nanoprecipitated. The nanoparticle size, size distribution and zeta potential were determined by DLS at predetermined times in order to follow their stability and to select the most convenient polymer for encapsulating chemotherapeutic drugs.

Taking into account the nanoparticle properties, PLA-PEG based nanoparticles were more appropriate for the following studies. Docetaxel and mitoxantrone were loaded onto nanoparticles made from PLA-PEG 2000 and characterized by dynamic light scattering (DLS). The amount of docetaxel and mitoxantrone associated with the nanoparticles was determined by ultraviolet-visible spectrophotometry (UV-VIS) analysis and it was found to be 58 % and 6 % respectively.

The mitoxantrone and docetaxel loaded nanoparticles as well as the free drug was evaluated *in vitro* against human prostate cancer cell lines. The uptake of the nanoparticles containing mitoxantrone by living cells was studied qualitatively by confocal laser scanning microscopy and confirmed with flow cytometry analysis. The results showed that loaded NPs are rapidly internalized by both cell lines, PC3 and *LNCaP*, starting from 10 minutes of contact.

In vitro studies of viability based on mitochondrial activity were carried out using MTS test with combinations of docetaxel- and mitoxantrone-loaded particles, as well as the free drugs in different ratios in order to observe any synergy, additivity or antagonism.

The cell viability study showed the efficiency of all loaded nanoparticle combinations with at least 50 % reduction of cell activity in the first 48 h. Moreover, a synergistic effect was observed with two different combinations of MITNPs and DOCNPs leading to less than 10 % of cell activity against PC3 prostate cells in the first 96 h of treatment. These results indicate that the PLA-PEG-based nanoparticles developed in this work could potentially be exploited as carriers to improve the solubility of docetaxel and to increase the intracellular uptake of mitoxantrone and therefore have potential clinical applications.

The two parts of this thesis presents a common aim: to deliver effective antineoplastic agents to tumor cells using drug delivery devices to avoid the side effects of traditional chemotherapy. Polyethyleneglycol (PEG) was used in all nanoparticles, both gold and polymeric, to provide a hydrophilic shell at the surface, thus increasing their dispersibility in physiological media. In addition, the presence of PEG would increase the nanocarrier's circulation time in the bloodstream and its accumulation inside the tumors by the enhanced permeability and retention (EPR) effect [27, 28]. Hence, the strategy of passive targeting was used in all nanoparticles. However, some gold nanoparticles were decorated with folic acid to benefit from an additional active targeting.

The possible toxicity of gold nanoparticles is a question that is usually raised when considering their applicability. However, pegylated AuNPs devices have been approved by the Food and Drug Administration (FDA) for photothermal therapy [29]. Medical imaging represents the highest impact and growth in nanotechnology at the moment. Gold nanoparticles have conquered a \$ 959 million market and the reports predict a continuous growth [30]. The gold nanoparticles studied in this thesis may need to be adapted for further cancer applications. The addition of functionalities which could facilitate the use of these devices in cancer theranostics would be a promising strategy.

Moreover, the polymeric nanoparticles synthesized in this work showed favorable features for their application as drug delivery systems for cancer therapy. The biocompatibility and biodegradability of the polymers chosen make them an important tool in the design and functionalization strategies in nanomedicine. Indeed, they have already obtained FDA approval for pharmaceutical applications such as biomedical implant devices and drug delivery systems, which makes them a suitable starting material for preparing polymeric nanoparticles [31, 32]. Scale up of the applied nanoprecipitation method has already been demonstrated to increase the production while maintaining the reproducibility of the particles [33, 34]. Nevertheless, further work needs to be done on both carrier platforms in order to characterize them more thoroughly and to optimize their therapeutic applications.

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CONCLUSION AND PERSPECTIVES

The incidence of cancer is increasing worldwide. In 2012, there were 14.1 million new cases including 1.1 million diagnoses of prostate cancer.

Despite the variety of treatments available, problems such as distinguishing indolent from aggressive tumors, serious side effects, recurrence of the cancer, resistance to treatment and the propensity to metastasize still represent the major challenges in prostate cancer therapies. Among the different strategies that could be used to overcome those difficulties nanotechnology has emerged as a promising candidate. New therapies in cancer research using nanomedicine are being developed in order to improve the specificity and efficacy of drug delivery, thus reaching maximal effectiveness with minimal side effects.

The aim of this thesis was to develop two different drug delivery systems for prostate cancer applications: one metallic, using pegylated gold nanoparticles and the other polymeric, using biodegradable and biocompatible pegylated nanoparticles based on polyesters. Those devices were applied to deliver a well established chemotherapy drug, docetaxel, a taxoid that acts as antimitotic agent. In addition, mitoxantrone, a potent anthraquinone which acts as DNA synthesis inhibitor was loaded onto the polymeric based nanoparticles.

Gold nanoparticles were able to entrap docetaxel by non covalent adsorption, thereby increasing the solubility of this hydrophobic drug. It was demonstrated that the unloaded gold nanoparticles were not toxic to the prostate cells line used in this study at the applied concentrations. In contrast, when docetaxel was adsorbed onto the gold nanoparticles it produced a sustained cytotoxic effect *in vitro*. These small particles could be useful to improve the dispersibility of docetaxel in physiological media, thus avoiding the use of the surfactants added for this purpose in the commercial formulation of docetaxel, Taxotere®. Moreover, the pegylated gold nanoparticles could be useful for concentrating the drug in solid tumors by the EPR effect and the properties of the gold core could be exploited for tumor imaging and for thermal cytolysis of tumor cells.

The nanoprecipitation method was shown to be efficient to load both hydrophobic and hydrophilic drugs, docetaxel and mitoxantrone, onto polyester based nanoparticles of around 70 nm in diameter. The cell viability study showed the efficiency of all loaded nanoparticle combinations with at least 50 % reduction of cell activity in the first 48 h. Moreover, a synergistic effect was observed with two different combinations of mitoxantrone and docetaxel loaded nanoparticles leading to less than 10 % of cell activity against PC3 prostate cells in the first 96 h of treatment. These results indicate that the polymeric nanoparticles developed in this work could potentially be exploited as carriers to improve the solubility of docetaxel and to increase the intracellular uptake of mitoxantrone and therefore have potential clinical applications.

However, more studies need to be done on both systems. As far as the gold nanoparticles are concerned, the determination of the encapsulation efficiency needs to be determined more accurately and the storage method should be optimized. The polymeric nanoparticles should be studied in more detail *in vitro* tests to determine whether induction of apoptosis is associated with the uptake of those particles. The drug release profiles and the pharmacokinetics of both drug delivery platforms should be measured.

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