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Nanobiotecnologia aplicada à oncologia

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Nanobiotecnologia aplicada à oncologia

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Resumo

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Células cancerosas apresentam habilidade de dividirem incontroladamente e escaparem da morte celular programada. Existem diversas classes de compostos usados no tratamento do câncer e em muitos casos apenas uma pequena fração da dose da droga atinge o sítio tumoral, o que reduz a eficiência terapêutica e aumenta a toxicidade sistêmica. Além disso, células cancerosas apresentam mecanismos de resistência. Em estudos de citotoxicidade, nanopartículas carreadoras são frequentemente mais eficientes contra as células cancerosas que a correspondente droga livre. O Metotrexato (MTX) é um fármaco análogo estrutural do ácido fólico que atua inibindo a ação da enzima dihidrofolato redutase (DHFR), e é comumente utilizado no tratamento de tumores. Porém, pode causar efeitos colaterais e as células tumorais podem apresentar resistência. A obtenção de formulações nanoestruturadas contendo MTX pode diminuir os efeitos indesejados e maximizar o efeito terapêutico. Para aumentar o encapsulamento desse fármaco em suspensões aquosas de nanocápsulas de núcleo lipídico foi sintetizado um derivado esterificado, o éster dietílico de metotrexato ($MTX(OEt)_2$). O presente trabalho teve por objetivo realizar a avaliação *in vitro* da ação de nanocápsulas de núcleo lipídico contendo $MTX(OEt)_2$ e comparação com a ação da substância livre em solução. Para tal avaliação, foram utilizadas cinco linhagens de células tumorais. Verificou-se significativa citotoxicidade e inibição de proliferação celular promovida pelos tratamentos testados. Utilizando-se citometria de fluxo verificou-se aumento de apoptose induzida pela nanoformulação. Ainda, através de PCR em tempo real, pode-se observar alteração no perfil de expressão de genes relacionados a apoptose.Os testes utilizados complementaram-se e foram eficazes em determinar as respostas aos tratamentos. A incorporação do $MTX(OEt)_2$ na nanoformulação demonstrou habilidade em melhorar o efeito antitumoral *in vitro* do composto.

Palavras-chave: Nanobiotecnologia. Metotrexato. nanocápsulas. quimioterapia. toxicidade *in vitro.*

Abstract

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Cancer cells demonstrate the ability for uncontrolled division and to escaping programmed cell death. There are several classes of compounds for the treatment of cancer, and in many cases only a small fraction of the drug dose reaches the tumor site, which reduces therapeutic efficiency and increases systemic toxicity. Furthermore, cancer cells exhibit resistance mechanisms. In cytotoxicity studies, nanoparticle carriers are often more effective against cancer cells than the corresponding free drug. Methotrexate (MTX) is a structural analog of folic acid that inhibits the action of the dihydrofolate reductase (DHFR) enzyme, and is commonly used in the treatment of tumors. However, it can cause side effects and the cancer cells can develop resistance. Obtaining MTX-containing nanostructured formulations can lessen the unwanted effects and maximize the therapeutic effect. To increase the encapsulation of the drug in aqueous suspensions of lipid-core nanocapsules a derivative was synthesized, diethyl ester of methotrexate ($MTX(OEt)_2$). This study aimed to perform the *in vitro* evaluation of the action of lipid-core nanocapsules containing $MTX(OEt)_2$ and the comparison with the action of the free substance in solution. For this evaluation, five tumor cell lines were used. It was observed cytotoxicity and inhibition of cell proliferation associated with treatments. Using flow cytometry analysis, it was observed that apoptosis increased in cells treated with the nanoformulation. Additionaly, by real-time PCR analysis, changes in the expression profile of genes related to apoptosis were detected. The tests used in the study complemented each other and were effective in determining responses to treatments. The $MTX(OEt)_2$ nanoformulation demonstrated the ability to improve the *in vitro* antitumor effect of the compound.

Keywords: Nanobiotechnology. Methotrexate. nanocapsules. chemotherapy. *in vitro* citotoxicity.

Sumário

1 INTRODUÇÃO GERAL

1.1 Câncer

Células cancerosas diferem das células normais do organismo por sua habilidade de dividirem incontroladamente e escaparem da morte celular programada. Embora o número de mutações necessárias para que a transformação maligna ocorra seja variável, todos os tumores dependem de dois mecanismos cruciais: a ativação de oncogenes que promovem proliferação e sobrevivência das celular cancerosas, assim como a inativação de genes supressores de tumor que normalmente reprimiriam o desenvolvimento e crescimento de tumores. Os eventos necessários incluem parada do ciclo celular, senescência e morte celular programada. (HANAHAN; WEINBERG, 2000; HANAHAN; WEINBERG, 2011).

Cerca de 12,7 milhões de novos casos de câncer são diagnosticados globalmente a cada ano. Embora a incidência total de câncer seja mais baixa na América Latina que na Europa ou nos Estados Unidos, o índice de mortalidade é maior. Isto se deve principalmente ao diagnóstico em estágios mais avançados, e em parte está relaconado com o acesso mais restrito ao tratamento (GOSS et al., 2013).

Existem diversas classes de compostos que tratam o câncer, principalmente por serem tóxicos para as células em rápido crescimento e divisão. Drogas citotóxicas são convencionalmente administradas por infusão intravenosa, tipicamente na forma de soluções da droga livre. Apesar da longa história de uso de diversos quimioterápicos e do desenvolvimento de novos regimes terapêuticos para aumento do sucesso clínico, fracasso dos tratamentos são frequentemente observados (LONNING, 2003). Em muitos casos apenas uma pequena fração da dose da droga atinge o sítio tumoral, e este fato reduz a eficiência terapêutica e aumenta a toxicidade sistêmica. Além disso, células cancerosas exercem uma variedade de mecanismos com o

objetivo de enfraquecer a toxicidade dos agentes terapêuticos aos quais elas são expostas. Esses mecanismos de defesa são categorizados como resistência (GIESELER et al., 2003; BAIRD; KAYE, 2003).

Em estudos de citotoxicidade, nanopartículas carreadoras são frequentemente mais eficientes contra as células cancerosas que a correspondente droga livre. A explicação mais provável para esse fenômeno é que nanoparticulas podem carrear a droga para o interior da célula por endocitose, contornando o mecanismo de efluxo da droga (LO, 2000).

1.2 Nanooncologia

Nanotecnologia é a criação, manipulação e utilização de materiais em escala nanométrica. Dada a aproximação, em tamanho, aos componentes funcionais das células vivas, esse campo da ciência apresenta importante papel na biotecnologia. Na área médica, a aplicação da nanotecnologia, dita Nanomedicina, se baseia na utilização dos materiais em nanoescala tanto para o diagnóstico quanto para o tratamento de doenças (SEIGNEURIC et al., 2010). É de enorme interesse empregar essas novas tecnologias no manejo do câncer, já que exibem grande potencial de benefícios na detecção, no diagnóstico, no acompanhamento, e principalmente no tratamento dos pacientes. O que torna a Nanooncologia o mais promissor segmento da Nanomedicina (JAIN, 2010).

Alguns dos obstáculos a serem superados, que impedem a eficiência do tratamento do câncer, incluem: a dificuldade de detecção na fase inicial da doença, para que a terapia possa ser efetiva; a resistência aos esquemas terapêuticos disponíveis; e a grande toxicidade sobre as células saudáveis. Nanopartículas estão sendo desenvolvidas com o objetivo de solucionar esses e outros problemas. A maioria das drogas utilizadas na terapia do câncer é altamente tóxica, um nanocarreador pode reduzir a toxicidade por diminuir a absorção sistêmica inespecífica, sendo determinante para o aumento da efetividade do tratamento (LEE; WONG, 2011).

Nanopartículas são capazes de ultrapassar barreiras biológicas, de incorporar uma grande variedade de agentes quimioterápicos, de entregar

macromoléculas de um medicamento diretamente a um sítio intracelular, e de direcionamento específico ao sítio tumoral. A habilidade de incorporação de fármacos pobremente hidrossolúveis elimina a necessidade de utilização de solventes orgânicos, os quais têm efeitos negativos sobre o organismo. Nanocarreadores possibilitam ainda a sustentação da liberação da droga de maneira controlada, e a co-incorporação e co-liberação de duas ou mais drogas em uma terapia combinada (FERRARI, 2010; ADAIR et al., 2010).

A pesquisa em nanomateriais na terapia do câncer evoluiu rapidamente na última década, seu tamanho controlado e multifuncionalidade são as principais razões para o crescente número de aplicações. A base lógica da utilização da nanobiotecnologia em oncologia é de que as nanopartículas apresentam propriedades não disponíveis em outras moléculas, e fornecem oportunidades para o delineamento de nanoformulações com características até então não conseguidas com agentes terapêuticos e marcadores de imagem tradicionais (FAROKHZAD; LANGER, 2009).

Nanopartículas carreadoras em oncologia devem apresentar o diâmetro de aproximadamente 10 – 100nm. Maiores que 10nm para prevenir filtração renal, ao passo que menores que 100nm para prevenir captura hepática. Podem apresentar a droga encapsulada ou ligada covalentemente a sua superfície, e independente do modo de carreamento devem permitir uma taxa de liberação adequada do agente terapêutico (DAVIS; CHEN; SHIN, 2008). Ou seja, para que o tratamento do câncer seja efetivo, quimioterápicos precisam ser liberados para as células tumorais onde o efeito é esperado e ao mesmo tempo existindo exposição limitada das células normais aos agentes tóxicos. A necessidade dessas características leva à busca pelo direcionamento terapêutico, no qual a aplicação da nanotecnologia tem trazido excelentes resultados.

Existem dois diferentes mecanismos de liberação direcionada: direcionamento passivo, baseado no tamanho da partícula e propriedades do sítio tumoral; e direcionamento ativo, baseado na utilização de ligantes de superfície capazes de reconhecer e se ligar às células que apresentam a

patologia. Utilizando tanto estratégias de direcionamento passivo ou ativo, os nanocarreadores podem prover concentrações intracelulares aumentadas da droga nas células cancerosas, minimizando a toxicidade nas células normais (DANHIER; FERON; PREAT, 2010).

De forma passiva, o acúmulo do carreador baseia-se no tamanho da nanopartícula, na longevidade do carreador no sangue, na vascularização comprometida da região tumoral, e no microambiente tumoral como um todo. Existem diferenças anatômicas e funcionais entre a vascularização de tecidos normais e tumorais. Vasos sanguíneos originados através do ativo processo de angiogênese, que ocorre em tumores, apresentam lacunas entre as células endoteliais adjacentes, o que leva a uma maior permeabilidade. Essa característica combinada à drenagem linfática comprometida, também presente nos tumores, promovem o chamado efeito de permeabilidade e retenção aumentadas. Todos os nanocarreadores utilizam o efeito de permeabilidades e retenção aumentadas como princípio orientador. Esse efeito permite que nanopartículas extravasem por esses espaços endoteliais e se acumulem no tecido tumoral. O direcionamento se dá porque tal evento não ocorrerá em tecidos com vascularização normal. Já quimioterápicos livres de carreadores geralmente extravasam em tecidos normais levando aos efeitos adversos. Além disso, nanopartículas se mantém mais tempo circulantes, o que facilita o mecanismo de liberação descrito. Enquanto drogas livres podem ser rapidamente excretadas, o que prejudica o índice terapêutico e exige a administração de doses crescentes (JAIN; STYLIANOPOULOS, 2010).

O microambiente tumoral é outro fator que contribui para o direcionamento passivo. As células malignas em hiperproliferação e rápido crescimento apresentam uma alta taxa metabólica, e o fornecimento de oxigênio e nutrientes acaba sendo insuficiente. Em função da glicólise para a obtenção de energia extra, ocorre acidificação do ambiente. Nesse sentido, nanopartículas sensíveis a pH podem ser projetadas de modo a liberarem a droga em pH diferente do fisiológico. Adicionalmente, nanoformulações podem ser desenvolvidas de forma a serem ativadas por atividade enzimática de proteases, já que tais enzimas são ativamente expressas em tumores em

função da capacidade invasiva das células malignas (JAIN; STYLIANOPOULOS, 2010).

Para um direcionamento ativo a nanopartícula deve ser ligada a uma fração com ação direcionadora, com o objetivo de se utilizar mecanismos de reconhecimento molecular para conquistar a liberação específica ou ultrapassagem de barreiras biológicas. Têm sido desenvolvidas estratégias de direcionamento por conjugação com anticorpos específicos ou ligantes como peptídeos ou pequenas moléculas. Os ligantes promovem o reconhecimento de antígenos ou receptores na célula cancerosa, impulsionando a fixação e difusão da nanopartícula para o interior da célula. No interior da célula maligna, a partícula se desintegra fazendo com que haja a instantânea liberação da droga precisamente onde é necessária. A escolha do ligante depende do alvo pretendido e da localização do tumor no organismo. Para tanto, o antígeno ou receptor deve ser expresso exclusivamente nas células tumorais, e devem ser expressos homogeneamente nessas células, além de não estarem presentes na circulação (WANG; THANOU, 2010).

1.3 Antifolatos

Antifolatos foram a primeira classe de agentes antimetabólicos a serem introduzidos na clínica. Folatos são vitaminas B9 que funcionam como doadores de carbono em múltiplas vias biossintéticas cruciais (GONEN; ASSARAF, 2012). Antifolates alcançam seu efeito farmacológico por perturbarem o metabolismo dos folatos (VISENTIN; ZHAO; GOLDMAN, 2012). Nas células, o ácido fólico é reduzido a dihidrofolato pela ação da enzima dihidrofolato redutase (DHFR). Dihidrofolato ainda é uma forma oxidada de folatos intracelulares, e constitui-se substrato da enzima DHFR para a formação de tetrahidrofolato, necessário para a biossíntese de purina e timidina, requeridas para polimerização de DNA (VISENTIN; ZHAO; GOLDMAN, 2012).

Dentre alvos moeculares, a DHFR tem sido explorada no desenvolvimento de agentes anti-cancer. Metotrexato (MTX) é um análogo

estrutural do ácido fólico, e atua principalmente inibindo a ação da DHFR. MTX é um fármaco comumente utilizado no tratamento de diferentes tipos de tumores. Porém, pode causar efeitos colaterais e desenvolver resistência (ASSARAF, 2007). A obtenção de formulações nanoestruturadas contendo MTX pode diminuir os efeitos indesejados e maximizar o efeito terapêutico. Entretanto, o MTX apresenta baixa lipossolubilidade, apresentando baixa incorporação em suspensões de nanocápsulas. Com a finalidade de aumentar o encapsulamento desse fármaco em suspensões aquosas de nanocápsulas de núcleo lipídico, foi sintetizado um derivado esterificado, o éster dietílico de metotrexato ($MTX(OEt)_2$), propiciando maior associação do fármaco com as nanocápsulas, otimizando o preparo da nanoformulação. Até o momento, não tinham sido realizadas avaliações do efeito antitumoral desse derivado e dessa nanoformulação.

O presente trabalho teve por objetivo realizar a avaliação *in vitro* da ação de nanocápsulas de núcleo lipídico contendo $MTX(OEt)_2$ e comparação com a ação da substância livre em solução. Para tal avaliação, foram utilizadas cinco linhagens de células tumorais, e diferentes métodos de avaliação de respostas ao tratamento.

Os dados gerados na presente tese estão apresentados na forma de artigos científicos. Os dois primeiros artigos trazem uma revisão acerca de duas importantes nanopartículas, nanotubos de carbono e nanocápsulas, e suas aplicações em oncologia. Os dois artigos seguintes trazem a avaliação, em diferentes linhagens de células tumorais, dos tratamentos propostos: o derivado do MTX, ($MTX(OEt)_2$), e sua nanoformulação em nanocápsulas de núcleo lipídico.

2 ARTIGO 1

Applications of Carbon Nanotubes in Oncology

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Abstract

Nanooncology is based on the use of nanoscale materials to provide tools for cancer detection, prevention, diagnosis and treatment. Due to their unique physical and chemical properties, Carbon Nanotubes (CNTs) are among newly developed products and are currently of much interest, with a large amount of research dedicated to their novel applications. In cancer research, many advantages of CNTs in drug delivery systems, cellular Imaging, and Cancer Photothermal therapy draw attention. Their physicochemical features enable introduction of several pharmaceutically relevant entities and allow for rational design of novel candidate nanoscale constructs. Thus, a detailed understanding of recent progress in nanooncology, focusing on biomedical research exploring possible application of carbon nanotubes, is required to consider the medical applications of these materials.

Introduction

Nanobiotechnology, the application of nanotechnology in life sciences, is starting to show the promise of a great impact on medicine, especially in cancer. Nanooncology is based on the use of nanoscale materials to provide tools for cancer detection, prevention, diagnosis and treatment. Nanoparticles can be engineered to incorporate a wide variety of chemotherapeutic agents and target the delivery of these agents specifically to the tumor site, providing opportunities for designing properties that are not possible with other types of drugs. Besides showing potential as a new generation of cancer therapeutics, many types of nanoparticle-based technologies are in development for improve diagnostic imaging of a variety of cancer types. Different kinds of nanocarriers are being investigated for medical applications, such as polymeric nanoparticles, lipid-based carriers, dendrimers, inorganic nanoparticles and carbon nanotubes (CNT) (Jain 2010).

Nanoparticles are suitable for two tasks required for targeted drug delivery to pathological sites in the body: passive and active targeting. Passive targeting refers to the accumulation of a drug carrier system at a desired site owing to physico-chemical or pharmacological factors. It benefits from the size of nanoparticles, longevity of the carrier in blood, and the unique properties of tumor vasculature. This approach makes use of the anatomical and functional differences between normal and tumor vasculature, as angiogenic blood vessels in tumor tissues have gaps between adjacent endothelial cells. This characteristic coupled with poor lymphatic drainage induces the enhanced

permeability and retention (EPR) effect, which enables macromolecules, including nanoparticles, to pass through these gaps into extravascular spaces and accumulate inside tumor tissues. Another contributor to passive targeting is the microenvironment surrounding tumor cells, since hyperproliferative cancer cells have a high metabolic rate, and the supply of oxygen and nutrients is usually not sufficient to maintain this growth, the use of glycolysis to obtain extra energy results in an acidic environment. Active targeting involves the attachment of a homing moiety, such as a monoclonal antibody or a ligand, to deliver a drug to pathological sites or to cross biological barriers based on molecular recognition processes (Misra et al. 2010).

Due to their unique physical and chemical properties, carbon nanotubes are among newly developed products and are currently of much interest, with a large amount of research dedicated to their novel applications. Thus, a detailed understanding of recent progress in nanooncology, focusing on biomedical research exploring possible application of carbon nanotubes as drug delivery carriers and diagnostic devices, is required to consider the medical applications of these materials.

Cancer therapy

Cancer arises through a multistep mutagenic process, allowing cancer cells to acquire properties of unlimited proliferation potential and self-sufficiency in growth signals and resistance to both anti-proliferative and apoptotic cues. The current treatments are surgery, radiation therapy and chemotherapy, all of which cause damage to healthy cells (Luo et al. 2009).

The efficacy of chemotherapy is often limited by resistance mechanisms, including decreased uptake into cancer cells, enhanced detoxification and efficient elimination of the drugs from cells. Moreover, severe side-effects can restrict the drug dose or even cause termination of the therapy. If not the discovery of new drugs, the strategy to increase the anticancer activity and minimize resistance should be improving delivery of conventional chemotherapeutics (Arlt et al. 2010).

Chemotherapeutics have limitations due to the lack of selectivity and severe toxicity, decreasing the anticancer dose is highly favorable for lowering toxic side effects of drug therapy to the normal organs and tissues. In this sense, Nanotechnology, through nanoscaled carriers for drug transport with the goal to improve the drug efficacy by lower doses and fewer side-effects, is a promising instrument (Arlt et al. 2010; Sobhani et al. 2011). In addition, limited solubility, poor distribution among cells, inability of drugs to cross cellular barriers, and especially a lack of clinical procedures for overcoming multidrug resistant (MDR) cancer, all limit the clinical administration of chemotherapeutic agents (Ji et al. 2010).

The application of nanomaterials as drug carriers can improve anticancer therapeutic efficacy by both passive and active targeting mechanisms (Misra et al. 2010). Passive target is induced by tumor-specific EPR-effect, which warrants the development of nanomedicine and is applicable for any biocompatible macromolecular compounds above 40 kDa, even larger than 800 kDa. The drug concentration in tumor compared to that of the blood can be usually as high as 10-30 times, and it is not just passive targeting for momentary tumor delivery, but it means prolonged drug retention for more than several weeks or longer (Maeda et al. 2009).

Carbon nanotubes (CNTs) have been proposed as multipurpose innovative carriers for drug delivery and diagnostic applications. Their physicochemical features enable introduction of several pharmaceutically relevant entities and allow for rational

design of novel candidate nanoscale constructs for drug development (Prato et al. 2008). CNTs loaded with drugs (which can be regarded as macromolecular agents) can extravasate in tumor tissues over time, the concentration in tumor will reach several folds higher than that of the plasma (Zhang et al. 2010).

Thus, Carbon nanotubes can help greatly in treating the cancer cells by delivering chemotherapeutic agents achieving better uptake by malignant cells without affecting collateral tissues. Consequently, nanotubes potentially lower the dose of drug by localizing its distribution at the tumour site (Beg et al. 2011).

Carbon Nanotubes

Carbon nanotubes (CNTs) are hollow graphitic nanomaterials with very high aspect ratios. Can be described as rolled sheets of graphene with lengths from several hundred nanometers to several micrometers, which can be single-walled (SWNTs) with diameters of 0.4- 2 nm, or multi-walled (MWNTs) consisting of 2–30 concentric tubes positioned within one another, with outer diameters ranging from 2 to 100 nm (Cheung et al. 2010; Liang and Chen 2010) (Fig.1). Several reviews have been published on synthesis and structural conformation of CNTs (Tasis et al. 2006; Beg et al. 2011).

The three main techniques to produce CNTs are electric arc discharge, laser ablation and chemical vapor deposition. These methods involve synthesis at high temperature, pressure and the use of reaction catalysts, leading to fine structures of CNTs along with some synthesis induced impurities like graphitic debris and catalytic particles, and there are various important methods for purification (Prakash et al. 2011).

Recently, efforts have been devoted to exploring the potential biological applications of CNTs motivated by their interesting properties (Cheung et al. 2010). Noticeably, biomedical research is exploring possible application of carbon nanotubes as drug delivery carriers and diagnostic devices (Liang and Chen 2010). These materials can be used for hyperthermic ablation of cancer cells due of their strong optical absorption in the NIR wavelength region, as well as for drug delivery to cancer cells owing to their high surface areas (Huang et al. 2011b). The advantages of carbon nanotubes in biomedical applications rely on the fact that they can be easily internalized into a wide variety of cell types and through several mechanisms (Kostarelos et al. 2007), being able to act as delivery vehicles for a variety of molecules relevant to therapy and diagnosis.

Since cellular internalization of CNTs takes an important role in targeting therapy for cancer, understanding the exact internalization mechanisms is crucial for pharmaceutical application of CNTs (Raffa et al. 2010). The suggested mechanisms by which CNTs cross the cell membrane are still being debated, with two major intracellular uptake mechanisms being proposed: endocytosis/phagocytosis and nanopenetration. Whereas it was demonstrated that the mechanism for the cellular uptake of CNTs is endocitosys (Kam et al. 2006), it was also reported that the CNTs are able to enter the cells by a nanopenetration mechanism (Cai et al. 2005). When caring a chemotherapeutic agent, by virtue of endocytosis, the CNTs can be taken up by the cell before the chemotherapeutic drugs are cleaved off CNTs, and thus, targeting delivery is realized.

Regarding cellular dynamics, Zhou et al. (2010), using confocal laser scanning microscopy, observed SWNTs dispersed in phospholipid-polyethylene glycol (PLPEG) and conjugated with different molecules, in tumor, normal, and macrophage cells, to determine the subcellular localization and to study the transmembranal mechanism of

SWNTs. They found that SWNT-PL-PEG-FITC were localized in the mitochondria of both tumor and normal cells due to mitochondrial transmembrane potential. The mitochondrial SWNT-PL-PEG, when irradiated with a near infrared light, could induce cell apoptosis due to mitochondrial damages. And also, SWNT-PL-PEG could be localized in different subcellular components by conjugations of different molecules. Subcellular localization of surface-modified SWNTs depends on how they enter the cells. When the conjugated molecules can specifically target tumor cells, they are bound to the cell surface, their pathway into the cell is through endocytosis, resulting in the lysosomal distribution. While for non cell-targeting molecules the entry mode depends on the properties of the conjugated molecules (Zhou et al. 2010).

Carbon nanotubes are insoluble in most organic or aqueous solvents, are hardly dispersed in aqueous solutions and have a strong tendency to interact hydrophobically and aggregate (Kostarelos et al. 2009). Therefore, for biological application the nanotube surface needs to be modified. Hence, for biomedical and pharmaceutical applications, various chemical modifications, by different strategies, have been used to solubilize and disperse carbon nanotubes in water, and make then biologically compatible. Such modifications can be performed by covalent attachment of chemical groups, supramolecular adsorption or wrapping of functional molecules, or yet the filling of the inner cavity of the nanotube (Tasis et al. 2006). Furthermore, the edges of the tube holes have oxidized functional groups where covalent attachment of chemicals is possible (Ajima et al. 2008).

The modifications can improve biocompatibility and capability to penetrate cell membranes, enhance effective transport of molecules into the cytoplasm via cell membrane without producing a toxic effect, and also offer a flexible platform for further derivation processes (Bianco et al. 2005). Thereby, modification of carbon nanotubes through covalent or non-covalent functionalization of their external walls is a key step for biomedical applications because a wide variety of active molecules can be linked to a functionalized carbon nanotube (Pastorin et al. 2006) (Fig.2).

The two main methodologies are based on the non-covalent coating of nanotubes with amphiphilic molecules (like polymers or surfactants), and the covalent functionalization of the nanotube surface by grafting various chemical groups directly onto the backbone (Tasis et al. 2006; Tasis et al. 2003; Kostarelos et al. 2009) Covalent functionalizations are strong and offer the possibility of introducing multiple functionalities. By this type of functionalizations, defective carbon atoms on the sidewall or at the end of CNTs can be oxidized by strong oxidants to generate carboxylic acid groups or carboxylated fractions, which can be chemically modified via amidation or esterification, and then various polymers, metals, and biological molecules can be grafted to the surface (Zhang et al. 2010).

The method of introducing carboxylic and other oxygen-containing groups to the inert carbon nanotube allows the covalent attachment of functional molecules, thus offering new opportunities for applications in science (Georgakilas et al. 2002), but it also generates so-called “oxidation debris” by breaking up CNTs during oxidation. Heister et al. (2010) demonstrated that the same acid oxidation protocol leaves some CNT samples nearly unaltered, whereas others are significantly broken up, leading to altered CNT dimensions and surface properties, which influence their dispersion in salt solutions, cellular growth media, and human plasma. A similar diversity was found for the effect of the removal of oxidation debris, which leaves the tubes with a clean surface, but also impairs dispersion stability. However, functionalization of CNTs with appropriate biomolecules allows for tailoring of their surface properties and is shown to improve dispersion (Heister et al. 2010).

Compared to covalent functionalization of the CNT sidewall, the supramolecular non-covalent functionalization approach has the advantage of establishing strong interactions with the nanotubes without altering their electronic nature and peculiar features, like optical properties useful for various biological imaging and sensing applications (Di Crescenzo et al. 2011). Furthermore, another strategy relies on the fact that CNTs have a high affinity for DNA and RNA, and are able to condense DNA to varying degrees, which can grant aqueous solubility and provide gene transfer vector systems (Singh et al. 2005).

Clearly, for a good biomedical performance the degree of aggregation and individualization of nanotube materials in biological fluids has an important role. In addition, the blood clearance is one of the parameters that must be determined for the development of any pharmacological agent, and this parameter also depends on the nanotube surface modification (Liang and Chen 2010).

Polyethylene glycol (PEG) has been a traditional mean of functionalization of carbon nanotubes in drug delivery systems (Foldvari and Bagonluri 2008). The presence of PEGchains endows CNTs with a hydrophilic coating, which also reduces protein adsorption and phagocytosis. Thus, PEGylation is one of the most effective methods to prolong the blood-circulation time, due to the properties of polyethylene glycol to overcome the phagocytic activity of the reticulo-endothelial system (Liang and Chen 2010; Di Crescenzo et al. 2011).

The long circulation time is generally desired for nanovehicles as it allows nanomaterials to repeatedly pass through tumor vasculatures facilitating passive targeting to the cancer cells by the enhanced permeability and retention effect of tumor blood vessels (Maeda et al. 2000). Liu et al. (2011) systematically studied the relationship between polymer surface coatings and *in vivo* behaviors of SWNTs. By controlling the PEGylation degree they achieved an optimal blood circulation half-life of SWNTs at 12 e 13 h, which afforded relatively low RES accumulation, high tumor uptake and low skin retention of SWNTs in mice, ideal for *in vivo* cancer treatment.

Lately, other strategies have been proposed. The functionalization of multiwalled carbon nanotubes (MWNTs) with highly hydrophilic and biocompatible poly(vinyl alcohol) (PVA) increase their aqueous solubility, and shown no obvious toxicity (Sahoo et al. 2011). Also, hyperbranched poly citric acid (PCA), with a high capacity for conjugation to drug molecules, was used for the functionalization of MWNTs instead of polyethylene glycol with limited arms that is commonly used to conjugate to other molecules for drug delivery systems (Sobhani et al. 2011).

Poly citric acid is a highly biocompatible hydrophilic polymer (Thomas et al. 2009) that beyond decreasing hydrophobicity of CNTs is also a highly functional polymer with a large number of carboxylic functional groups that confer a high loading capacity (Sobhani et al. 2011). As also reported in a study using polyglycerol where MWNTs assumed a circular form (Adeli et al. 2009), this kind of functionalization causes conformational changes from linear toward curved nanotubes, due to the hydrophobicity of CNTs and high hydrophilicity of poly citric acid in aqueous solutions, consequently reducing their size (Sobhani et al. 2011).

Concerning active targeting, researchers have been developing CNTs coupled with cancer cell-specific targeting moieties to enhance CNTs uptake by cancer cells while limiting uptake by normal cells (Bhirde et al. 2009; Kam et al. 2005). To drive CNTs for selective internalization into cancer cells, it is advantageous to functionalize them with antibodies of antigens overexpressed on the cancerous cell surface or with specific ligands that recognize receptors on the cancerous cell. For this purpose, some targeting molecules have been reported, such as Folic acid (Dhar et al. 2008),

monoclonal antibodies (McDevitt et al. 2007), epidermal growth factor (Bhirde et al. 2009), Rituxan (to selectively recognize CD20 cell surface receptor on B-cells) and Herceptin (to recognize HER2/neu receptor positive breast cancer cells) (Welsher et al. 2008).

As for other molecules, to conjugate immunoglobulins (Ig) to CNTs, covalent bonding and noncovalent interactions strategies have been reported, and the Immuno-CNT constructs (antibody (Ab)–CNT conjugates) can modulate immunological functions, provide specific targeting, and enhance the efficacy of antitumor therapies (Venturelli et al. 2011). CNTs combined with the antibody anti- P-glycoprotein (which is overexpressed on multidrug resistant cells) and loaded with an antineoplastic drug, could not only specifically recognize the multidrug resistant cells, but also demonstrated the effective loading and controllable release performance of the drug (Li et al. 2010). It was also reported CNTs constructs appended with a radiotherapeutic agent and the tumor neovascular-targeting antibody E4G10, thus increasing specificity and limiting the cellular damage of healthy tissues (Ruggiero et al. 2010).

Another promising application of CNTs in biology and medicine is the development of advanced biosensor devices (Wang et al. 2004; Valcarcel et al. 2007). Recently, Park et al. (2011) synthesized D-(+)-Galactose-conjugated single-walled carbon nanotubes (SWNTs) for use as biosensors to detect the cancer marker galectin-3. High levels of this circulating marker are correlated with an increased potential for malignancy in several types of cancer, and the electrochemical sensitivity measurements of the D-(+)-galactose-conjugated SWNTs differed significantly between the samples with and without galectin-3, indicating that conjugated SWNTs are potentially useful electrochemical biosensors for marker detection. In attempt to diagnose multi drug resistance (MDR) in cancer, which is responsible for a large portion of chemotherapeutic failures, Zhang et al. (2011) demonstrated a new strategy using an electrochemical sensor based on carbon nanotubes aiming a fast and sensitive method of assessment of cancer MDR, and thus guiding how to reverse it in clinic therapy.

For gene therapy, the generation of CNT- DNA complexes can be especially beneficial because the presence of DNA in this complex stabilizes the carbon nanoparticle in aqueous suspensions and the DNA molecule is protected from unwanted enzymatic cleavage and nucleic acid binding protein interference when attached to the carbon nanotubes (Singh et al. 2005; Wu et al. 2008). A gene delivery concept based on ethylenediamine-functionalized single-walled carbon nanotubes (f-SWNTs) using the oncogene suppressor p53 gene as a model gene was successfully tested *in vitro* in MCF-7 breast cancer cells (Karmakar et al. 2011). They demonstrated the ability of f-SWNT-p53 complexes to act as gene delivery vehicles and facilitate the delivery of p53 plasmid DNA, resulting in the expression of this protein. This system could be the foundation for novel gene delivery platforms based on the unique structural and morphological properties of multi-functional nanomaterials and be a tool for the preliminary screening of different genes for their ability to affect cancer cell growth (Karmakar et al. 2011).

There is evidence that carbon nanotubes can produce immune responses when covalently linked to highly immunogenic peptide sequences (Pantarotto et al. 2003). Moreover, carbon nanotube-peptide constructs can improve the immunogenicity of a weakly immunogenic clinically relevant cancer-associated peptide, and therefore they are promising tools to explore ways to improve vaccine therapy against cancers (Villa et al. 2011).

In conclusion, as other nanomaterials, the biocompatibility and applicability of CNTs depends on controllable properties such as size and morphology, and also additives and surface modifications, since interactions between carbon nanotubes and

living cells are strongly dependent on surface chemistry. In cancer research many advantages of CNTs in drug delivery systems, cellular Imaging and Cancer Photothermal therapy, draw attention.

Drug Delivery

Often, chemotherapeutic agents offer a limitation of solubility and cell-penetration ability. Plus, the systemic toxicity caused by lack of selective limits the clinical applications. Therefore, development of an effective drug delivery system becomes an active area of research. The bioavailability of a drug can be maximized by the attachment to a suitable carrier. The therapeutic efficacy can be improved and side effects reduced because of facilitated transportation of drugs to the desired target.

As mentioned, CNTs are potential drug delivery vectors due to their ability to cross cell membranes easily and their high aspect ratio as well as high surface area, which provides multiple attachment sites for drug targeting. Also, they are stable, inert and present higher surface area-to-volume ratio than spheres (Liu et al. 2007b), which provides higher loading capacity for guest molecules, suggesting the potential utility of these materials as carriers in drug delivery systems that require higher loadings of therapeutic agents (Liu et al. 2009; Matsumura et al. 2007).

Carbon nanotubes offer many potential advantages over other types of nanoparticles for cancer therapy. Their unique physical properties permit efficient electromagnetic stimulation and highly sensitive detection using various imaging modalities. Their large surface area and internal volume also allows drugs and a variety of small molecules, such as contrast agents, to be loaded onto the nanotube. Carbon nanotubes have been used to halt tumor growth in the context of various therapeutic modalities including chemotherapy, hyperthermia and gene silencing (Kostarelos et al. 2009).

SWNTs are promising carriers for drug delivery since they are relatively safe inorganic materials which are capable to penetrate cell membranes, can be covalently functionalized with small molecules, and several anticancer drug molecules have been transported into different types of cells by appropriately functionalized SWNTs (Liang and Chen 2010). However, MWNTs seem to be more suitable for the encapsulation of drugs because of their wider inner diameter, and the outer shells can be functionalized without destroying the side walls (Arlt et al. 2010).

In order to perform the drug load, different approaches can be followed, ranging from covalent or noncovalent attachment of drug molecules to the sidewalls of functionalized CNTs (Liu et al. 2008a; Wu et al. 2009) to the incorporation of drug molecules into their interiors (Ajima et al. 2008; Hampel et al. 2008). CNTs have been proved to be versatile drug carriers, increasing the activity of various drugs used in cancer treatment, such as doxorubicin (Huang et al. 2011a), carboplatin (Arlt et al. 2010) and paclitaxel (Sobhani et al. 2011).

The chemical conjugation changes the chemistry entities of the drugs, especially when the drugs are covalently conjugated via non-biodegradable linkages (Lay et al. 2010), so, physically loading the drugs onto CNTs could be preferred because of no changes in the chemical entities, but this approach may be limited to certain drugs (Liu et al. 2007b; Hampel et al. 2008). Lay et al. (2010) set up an approach to physically load Paclitaxel (PTX) onto the side walls of CNTs by immersing PEG-g-SWNTs and PEG-g-MWNTs in a saturated solution of PTX in methanol. PTX loaded PEG-g-CNTs could be well dispersed in aqueous solution without aggregation. Moreover, PTX could be

sustained released from PEG-g-CNTs faster than free PTX, with a high *in vitro* efficacy to kill cancer cells.

A successfully synthesized MWNT–PVA could form stable complexes with the Camptothecin (CPT) via noncovalent interactions and exhibited higher cytotoxic activity compared to free CPT alone (Sahoo et al. 2011). Therefore, functionalized MWNTs can be load and mediate delivery of poorly water-soluble anticancer drugs, and as a water soluble complex can significantly improve the activities of the drug, enhancing the cellular uptake (Sahoo et al. 2011). Another strategy is functionalization with the safe and nontoxic polysaccharide Chitosan. When the Chitosan is functionalized on the surface of CNTs, the cells become attached to the sidewalls of the nanotubes, resulting in the desired targeted release to the cells, with improved drug absorption (Beg et al. 2011).

Aiming to minimize the disadvantage of systemic administration of the chemotherapeutic drug Doxorubicin (DOX), which kills healthy cells, it was synthesized a nanocarrier by non-covalent attachment of DOX to CNT surface followed by encapsulation of CNTs with folic acid-conjugated Chitosan, resulting in a system with characteristics of both controlled release and specific targeted, given that folate receptors are overexpressed on cancer cells (Huang et al. 2011a). There was an initial burst release and then a gradual release of DOX, which is a desirable characteristic for therapy. Also, the system had a higher drug release at acidic medium, which promotes higher drug release caused by partial dissociation of hydrogen bonding interaction between DOX and SWCNT. The superior controlled release from chitosan-folic acid encapsulated nanocarrier in contrast to non-encapsulated can be attributed to degradation of Chitosan and diffusion through the Chitosan shell, and also folic acid–DOX hydrogen bonding (Huang et al. 2011a).

Nanoparticles can also be explored as nanocontainer-based drug depots, which constantly provide the active drug, being very useful in case of unstable drugs that become inactivated over time. CNTs loaded with the alkylating chemotherapeutic drug Carboplatin (CP), which is a second- generation platinum agent, showed stronger activity than free Carboplatin (Arlt et al. 2010). The capability of carboplatin to induce apoptosis was doubled when the drug was transported by CNT–CP, while no significant toxicity was found after cell exposure to unloaded CNTs. Furthermore, CNT–CP continuously released their payload during the observation period, showing a maximum release of platinum of 68% at day 14 without reaching a plateau (Arlt et al. 2010).

Recently, Sobhani et al. (2011) proposed a novel drug delivery system for cancer chemotherapy based on multiwalled carbon nanotubes functionalized with hyperbranched poly citric acid (MWNT-*g*-PCA) and covalently attached to the commonly used potent chemotherapy drug Paclitaxel (PTX), to produce a MWNT-*g*-PCA-PTX conjugate. This complex is taken up by cells through endocytosis, where the cleavable ester bond between Paclitaxel and poly citric acid is hydrolyzed, and Paclitaxel is released into the cytoplasm, showing a higher cytotoxic effect than free Paclitaxel. That could be related to the increased cell penetration of Paclitaxel when conjugated. Moreover, the MWNT-*g*-PCA alone had no significant effect on cell viability, suggesting that the cytotoxicity was caused by the conjugated Paclitaxel only. The improved cytotoxic effect on the cancer cell lines may be due to higher cell penetration of the conjugated nanotubes, a behavior that could be associated to amphiphilicity of the MWNT-*g*-PCA for improved cell wall interaction compared with absolute hydrophobic Paclitaxel, also to the unique conformation of the carbon nanotube-based nanocarrier, which can penetrate into various cells. Furthermore, the

release of the drug from the conjugate was higher at an acid pH, which is suitable for the release of drugs in tumor sites (Sobhani et al. 2011).

Intending to fight against metastases, Yang et al. (2011) used magnetic carbon nanotubes mMWNT as chemotherapeutic agent vehicles to targeted cancer metastatic lymph nodes under the guide of implanted magnet, and showed successful application of intra-lymphatic delivery of Gemcitabine (GEM) using mMWNTs. Di Crescenzo et al. (2011) proposed the use the biocompatible amphiphilic diblock copolymer poly (ethylene glycol-b-propylene sulfide) ($PEG_{44}PPS_{20}$), in order to assess the ability of MWNTs dispersed with $PEG_{44}PPS_{20}$ to assist and direct the entry of the desired amount of Doxorubicin (DOX) in cancer cells and enhance its cytotoxic activity. The authors demonstrated that $PEG_{44}PPS_{20}$ was capable of efficiently and stably dispersing CNTs and of finely tuning the DOX loading onto the nanotube surface. The $PEG_{44}PPS_{20}$ coated MWNT/DOX complex exhibit efficient cell internalization and enhanced cytotoxic activity compared to both DOX alone and DOX-loaded copolymer micelles.

Zhang et al. (2009) described a system employing two different polysaccharides, sodium alginate (ALG) and chitosan (CHI), and further functionalization with targeting group folic acid (FA) and an anticancer drug DOX. The complete system displayed excellent stability under physiological conditions, but at reduced pH typical of the tumor environment, intracellular lysosomes and endosomes, the DOX was efficiently released and enters the cell nucleus inducing cell death.

Using the antibody (Ab) target approach, Venturelli et al. (2011) designed and prepared new Ab–CNT constructs with anti-MUC1 Ab covalently conjugated to carbon nanotubes. Anti-MUC1 Ab recognizes the mucin 1 (MUC1) receptor which is overexpressed on a series of human cancer cells, and it was chosen as an active targeting molecule in view of the development of a multimodal CNT-based hybrid with therapeutic properties. They obtained conjugates that formed stable homogeneous dispersions under physiological conditions and can be useful for biomedical investigations.

In summary, the transporting capabilities of carbon nanotubes combined with appropriate surface modifications and their unique physicochemical properties can lead to a new kind of nanomaterials for cancer treatment (Ji et al. 2010). An overview of recent research on applications of CNTs as drug delivery systems in oncology is shown in Table 1.

Cellular Imaging and Cancer Photothermal Therapy

Carbon nanotubes have unique electrical, thermal and spectroscopic properties that offer advances in the detection and monitoring of diseases (Kostarelos et al. 2009). Besides having a surface area that allows efficient loading of multiple molecules along the length of the nanotube sidewall (Liu et al. 2007b), the intrinsic optical and electrical properties of CNTs can be utilized for multimodality imaging and therapy.

For therapeutic hyperthermia, heating of an anatomical area without damaging the surrounding tissue is difficult. The potential approach to solving this problem involves the use of nano-sized biocompatible particles that convert absorbed electromagnetic energy to heat after they localize in the tumor (Marches et al. 2011). CNTs have the ability to absorb near-infrared (NIR) radiation (700–1100 nm) and then convert it into heat, which provides an opportunity to new strategies for cancer thermal therapy (Kam et al. 2005). Also, biological systems are known to be transparent to the same spectral window, so, this can be used for optical imaging of nanotubes inside

living cells, with low autofluorescence background of cells and tissues (Welsher et al. 2008).

Nanotubes play an important role in diagnostic procedures by helping in the imaging of organs, by identifying the site of action of drugs in targeted delivery systems, and by having great potential to act as a contrast agent in imaging and identification of cancer cells (Pramanik et al. 2009). Thus, intrinsic fluorescence properties of carbon nanotubes enable their application as biological imaging agents (Liu et al. 2008c), their NIR photoluminescence property can be used for *in vitro* cell imaging (Welsher et al. 2008), they can be used for photothermal therapy (Chakravarty et al. 2008; Kam et al. 2005), photoacoustic imaging (De la Zerda et al. 2008), and to deliver therapeutic drugs with externally controlled release capabilities (Kam et al. 2005).

Magnetic CNTs have shown promising results as a Magnetic resonance imaging (MRI) contrast agent with high nuclear magnetic resonance relativities, little cytotoxicity and high cell-labelling efficiency (Bai et al. 2008). It was demonstrated that poly(-acrylic acid) functionalized multi-walled carbon nanotubes (PAA-g-MWNTs) decorated with magnetite nanoparticles (Fe3O4) can be efficiently taken up by lymphatic vessels and delivered to regional lymph nodes *in vivo* with little toxicities (Yang et al. 2009).

Carbon nanotubes are capable of photo-excitation. Since they are photoabsorbers, combining laser irradiation can enhance thermal deposition and targeted destruction of cancer cells. Selectively target CNTs, by the use of antibodies, associated with NIR radiation showed success in treating cancer cells both *in vitro* and *in vivo* (Zhou et al. 2009; Burke et al. 2009). To destroy cancer cells using hyperthermia, target CNTs uptake only by cancerous cells provide an opportunity to locally heat the carbon nanotubes using infrared laser radiation, resulting in thermal destruction of cancer cells without harming surrounding cells (Kam et al. 2005).

For laser cancer therapy and improved diagnostic imaging, it has been suggested that a nanomaterial known as a single walled carbon nanohorn (SWNH) offer many advantages. SWNHs are much like SWNTs, but short and conically-shaped sealed structures, with an overall diameter ranging between 50 and 100 nm (Murakami et al. 2004). One important aspect is that SWNHs are typically produced by laser ablation of pure graphite target samples, eliminating toxicity associated with the presence of metal catalysts (Miyawaki et al. 2008). Inclusion of SWNHs may allow the treated tissue to absorb more therapeutic laser light, enhancing the ability to produce lethal temperature elevations necessary for tumor destruction and inhibition of tumor recurrence, selectively increasing the temperature while minimizing thermal impact on locations where NIR laser light is directed without SWNHs (Whitney et al. 2011).

Since highly proliferative tumors have the capacity to create albumin deposits, cancer cells overexpress specific human serum albumin (HSA) receptors and are able to internalize large amounts of albumin, Iancu et al. (2011) proposed a method for the noncovalent functionalization of multiwalled carbon nanotubes with HSA for the selective targeting and laser-mediated necrosis of liver cancer cells, showing interesting results. Innovatively, Mocan et al. (2011) presented a new method of selective nanophotothermolisys of pancreatic cancer (PC) using MWNTs functionalized with HSA in an original designed model of living PC, using ex-vivo-perfused pancreatic specimens that were surgically removed from patients with ductal adenocarcinoma. They obtained a selective photothermal ablation of the malign tissue based on the selective internalization of MWCNTs with HSA inside the pancreatic adenocarcinoma after the ex-vivo intra-arterial perfusion.

Through the attachment of a MRI contrast agent onto CNTs in a suitable way, the obtained CNT-based hybrids can be used as an MRI contrast agent and a drug carrier simultaneously. In this sense Wu et al. (2011) develop a solvothermal method to synthesize MWCNT/CoFe2O4, and *in vitro* experiments revealed that the hybrids displayed low cytotoxicity and an excellent MRI enhancement effect on cancer cells. In addition, the hybrids showed a high loading capacity for the anticancer drug DOX, were stable, and allowed fast drug release in acidic environments.

Toxicity

Whereas the potential multifunctionality of carbon nanotubes has been shown sufficiently, their toxicity is still controversially discussed. The rapid progress in the development and use of nanomaterials is not yet matched by adequate toxicological investigations. Growth in manufacturing of nanomaterials requires knowledge and control of the possible adverse health effects that may take place throughout the production and use (Shvedova et al. 2009). Thus, the potential toxic effects for the environment and for health have become an issue of concern. The published toxicity data of CNTs are not solid and even conflicting (Liu et al. 2008b; Liu et al. 2007a; Singh et al. 2006; Cherukuri et al. 2006; Dumortier et al. 2006; Warheit et al. 2004; Worle-Knirsch et al. 2006; Wang et al. 2011; Pacurari et al. 2011).The variation may be due to a wide range of tube diameters, lengths, chiralities, catalyst content, and also different functionalizations, bringing the need of standardization of protocols to establish accurately the toxicity and long term fate of the CNTs.

The diameter of tubular nanomaterials is an important determinant for their loading capacities (Hilder and Hill 2009) and an association of the toxicity of multiwalled nanotubes and their diameter was reported (Wang et al. 2009). Several studies evaluated toxicology utilizing pristine nanotubes, mostly poor-quality aqueous dispersions, which logically are the most difficult to handle biologically (Boczkowski and Lanone 2007). Moreover, residual metal catalyst may play an important role in the cytotoxicity (Firme and Bandaru 2010; Vittorio et al. 2009), since oxidative stress is proposed as a key mechanism of CNT-induced toxicity, and it is usually linked to the metallic impurities (Pulskamp et al. 2007). Then, designed functionalized CNTs without residual heavy metals, tend to be biocompatible and nontoxic at cellular level (Firme and Bandaru 2010; Dumortier et al. 2006; Nimmagadda et al. 2006; Murugesan et al. 2006), offering the potential exploitation of nanotubes for drug administration.

Lately, Burke et al. (2011) assessed the potential thrombogenic effects of functionalized MWCNTs *in vitro* and *in vivo*, and found that the thrombogenic potential can be substantially moderated through covalent functionalization. The systemic administration of covalently functionalized MWCNTs does not initiate a strongly pro-coagulant state, which is an important factor for their clinical use.

Considering interference with dye-based viability assays, agglomeration issues related to the method of dispersion, and oxidant stress due to metal contamination of CNT, the data available favor the conclusion that well-dispersed, purified CNTs exhibit relatively low cytotoxicity *in vitro* (Shvedova et al. 2009). So, to consider clinical applications it is important to understand pharmacological and toxicological properties, evaluate risk/benefit ratios, identify which physico-chemical characteristics of carbon nanotubes are capable of driving the toxic responses, and design of carbon nanotubes that are biocompatible and safe.

Conclusions

Nanosystems hold great potential to overcome many of the present obstacles of therapy and diagnoses in oncology, and carbon nanotubes have been actively explored as multipurpose innovative versatile carriers because of their great material properties. The uses of CNTs as bioactive molecules are still at an early research stage and face some challenges, but their unique physical and chemical properties hold great hope for cancer management. Overall, the results clearly indicate potential applications of CNTs in oncology, making important steps towards safe medical applications of CNTs.

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Table 1 Overview of recent research on carbon nanotubes in drug delivery systems

Type of CNT	Functionalization	Drug	Biological system	Reference
SWNT	phospholipid-branched polyethylene glycol	paclitaxel	*in vivo*	Liu et al., 2008a
SWNT	epidermal growth factor	cisplatin	*in vitro/in vivo*	Bhirde et al., 2009
MWNT	hydrophilic diaminotriethylene glycol	hydroxycamptothecin	*in vitro/in vivo*	Wu et al., 2009
SWNT	Polysaccharide materials and Folic acid	doxorubicin	*in vitro*	Zhang et al., 2009
SWNT/MWNT	polyethylene glycol	paclitaxel	*in vitro*	Lay et al., 2010
SWNT	Antibody of P-glycoprotein	doxorubicin	*in vitro*	Li et al., 2010
MWNT	ethylene glycol-b-propylene sulfide	doxorubicin	*in vitro*	Di Crescenzo et al., 2011
SWNT	folate-chitosan conjugate	doxorubicin hydrochloride	*in vitro*	Huang et al., 2011a
MWNT	poly(vinyl alcohol)	camptothecin	*in vitro*	Sahoo et al., 2011
MWNT	hyperbranched poly citric acid	paclitaxel	*in vitro*	Sobhani et al., 2011
MWNT	CoFe2O4	doxorubicin	*in vitro*	Wu et al., 2011
MWNT	poly(acrylic acid) and Fe3O4	gemcitabine	*in vitro/in vivo*	Yang et al., 2011

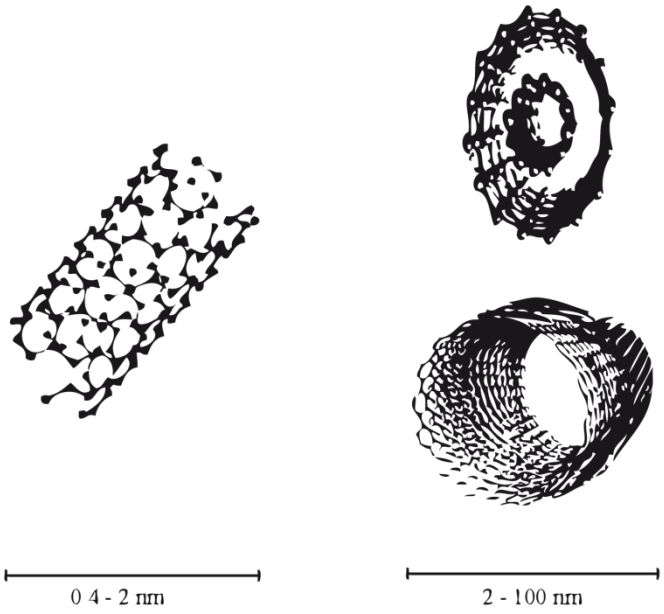

Fig. 1 Single-walled nanotubes and multi-walled nanotubes showing typical diameters

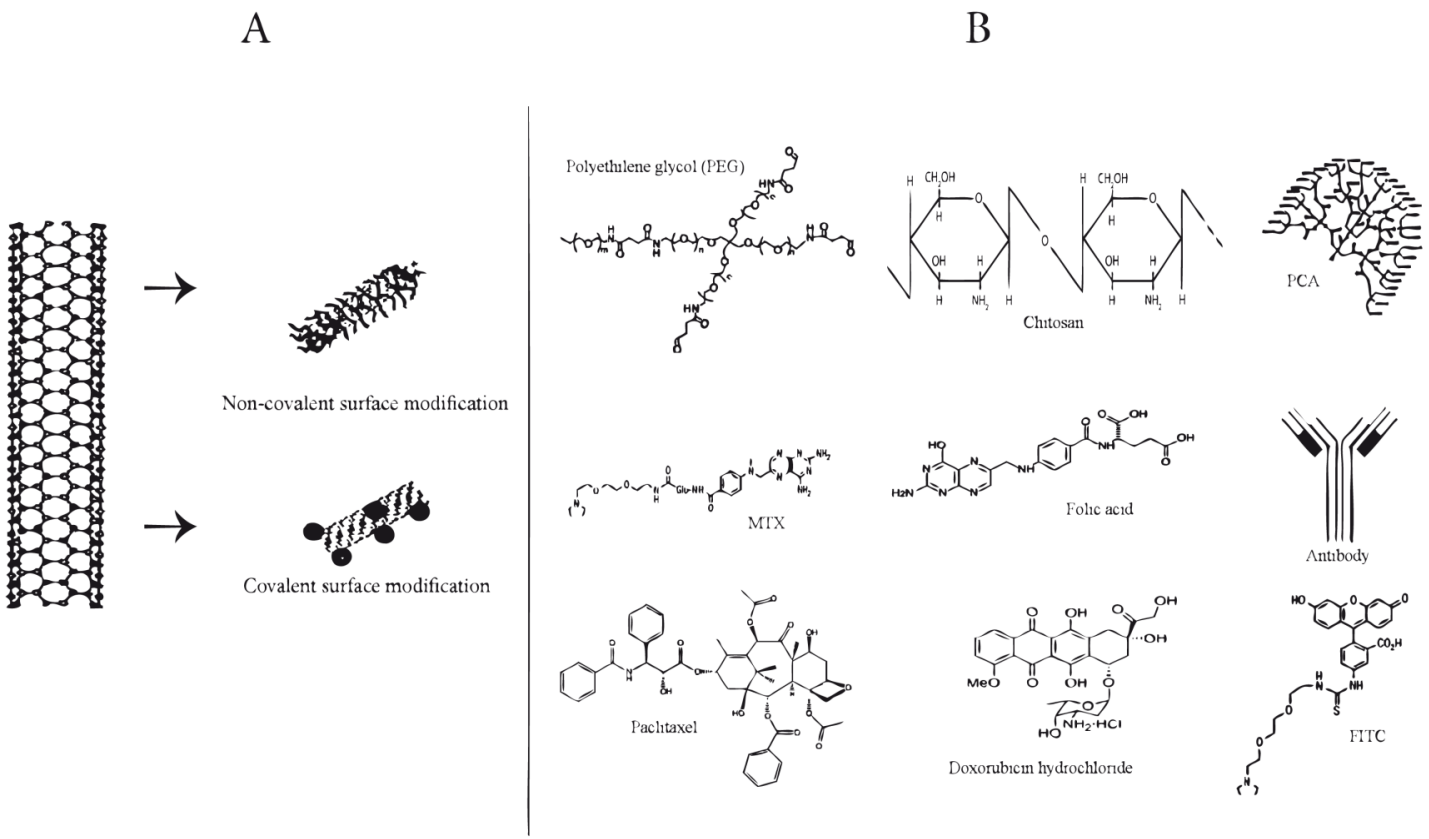

Fig. 2 Functionalization schemes: (A) schematic illustration for covalent and non-covalent modifications of carbon nanotubes; (B) some molecules that can be used for engineering carbon nanotubes formulations

3 ARTIGO 2

Developments in the use of nanocapsules in oncology

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Developments in the use of nanocapsules in oncology

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Abstract

The application of nanotechnology to medicine can provide important benefits, especially in oncology, a fact that has resulted in the emergence of a new field called Nanooncology. Nanoparticles can be engineered to incorporate a wide variety of chemotherapeutic or diagnostic agents. A nanocapsule is a vesicular system that exhibits a typical core-shell structure in which active molecules are confined to a reservoir or within a cavity that is surrounded by a polymer membrane or coating. Delivery systems based on nanocapsules are usually transported to a targeted tumor site and then release their contents upon change in environmental conditions. An effective delivery of the therapeutic agent to the tumor site and to the infiltrating tumor cells is difficult to achieve in many cancer treatments. Therefore, new devices are being developed to facilitate intratumoral distribution, to protect the active agent from premature degradation and to allow its sustained and controlled release. This review focuses on recent studies on the use of nanocapsules for cancer therapy and diagnosis.

Key words: Nanotechnology; Nanocapsules; Nanocarriers; Cancer

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Introduction

The application of nanotechnology to medicine can provide several benefits, especially in oncology and this has resulted in the emergence of a new field called Nanooncology (1). Drug-loaded nanoparticles provide a promising solution by selectively targeting tumor cells, thereby preventing damage to healthy cells (2). Nanoparticles can be engineered to incorporate a wide variety of chemotherapeutic or diagnostic agents, creating a flexibility in their design that is not possible with other types of drug delivery systems (3).

Active therapeutic molecules are often degraded or opsonized and removed from the bloodstream by macrophages. Therefore, in order to increase blood circulation time and to enhance the probability of the molecule to extravasate in the tumor, a promising alternative is to protect the active molecule inside nanoparticles (4). Colloidal drug carriers can also be rapidly cleared from the systemic circulation due to their recognition as foreign bodies by Kupffer cells in the liver and macrophages in the spleen. Clearance is enhanced by opsonization and activation of the complement system by plasma proteins (5). The elimination and subsequent loss of effectiveness of colloidal systems is strongly influenced by their size, nature, surface charge and hydrophilicity. Modifications of the carrier surface have been used to minimize opsonization and clearance by macrophages (6).

The longevity of circulating drug carriers improves drug delivery to their targets, particularly solid tumors (7). This approach makes use of the anatomical and functional differences between normal and tumor vasculature, with angiogenic blood vessels in tumor tissues containing gaps between adjacent endothelial cells. This characteristic coupled with poor lymphatic drainage characterizes the enhanced permeability and retention (EPR) effect. The EPR effect arises from the fact that nanoparticles can easily extravasate through the defective vasculature system in tumors and subsequently

accumulate in the tumor microenvironment in the presence of ineffective lymphatic clearance, allowing passive targeting to the tumor region (2). While passive targeting takes advantage of physicochemical and physiological factors, active targeting depends on the attachment of a homing moiety, such as a monoclonal antibody or a ligand, for drug delivery to pathological sites or to cross biological barriers based on molecular recognition processes (8). Active targeting should be based on specific cell surface receptors that are overexpressed on cancerous cells, thereby distinguishing the malignant cell from other organ cells.

The success of current cancer therapeutic strategies, such as surgery, radiation therapy and chemotherapy is variable (9). The effectiveness of chemotherapy is limited by poor uptake into cancer cells, enhanced detoxification and efficient elimination of the drugs. Moreover, severe side effects in patients can lead to a reduction of the drug dose or even termination of the therapy. In the absence of new drugs, anticancer therapy can be improved by the targeted delivery of conventional chemotherapeutics. These agents usually have limited solubility, poor distribution among cells, and the inability to cross cellular barriers, thereby limiting their clinical use (10). One of the most relevant processes involved in chemoresistance is the overexpression of P-glycoprotein (P-gp). P-gp can transport lipophilic and cationic substrates, and prevent the accumulation of anticancer drugs in cells by pumping out drug molecules from the cytosol. The uptake of drug-loaded nanocarriers by cancer cells has the potential to overcome these chemoresistant processes (11).

Nanoparticle-based therapy offers important advantages over the traditional approaches, allowing targeted delivery of high payloads of multiple drugs, controlled drug release, and bypassing multidrug-resistance mechanisms (12). Nanocapsules (NC) are potential nanocarriers for several strategies in oncology. A NC is a vesicular system that exhibits a typical core-shell structure in which active molecules are confined to a reservoir or cavity that is surrounded by a polymer membrane or coating. The cavity can contain an active substance in liquid or solid form, or as a molecular dispersion. NC zeta-potential (ZP) is an important index for the stability of a nanoparticle suspension, representing the electric potential at the NC shear plane, that depends on the chemical nature of the polymer and stabilizing agent, and the

medium pH (13). A high absolute value of ZP indicates a high surface electrical charge, which can cause strong repellent forces among the particles and prevent aggregation in solution. It is also important in determining the *in vivo* interaction of the NC with the cell membrane, which is usually negatively charged (14). Some of the current strategies employing NC for cancer treatment research are summarized in Table 1.

NCs can be produced by two approaches: self-assembly and template based. In self-assembly, lipid molecules or amphiphilic block copolymers can aggregate in aqueous solution into vesicular structures and the hollow sphere morphology of these aggregates makes them suitable precursors for the preparation of stable NC formulations by using different reactions. In the template approach, a polymer shell is formed around a template particle that can subsequently be removed, leaving an empty polymeric shell (15). Alternatively, core particles can be synthesized by conventional emulsion polymerization, followed by the addition of a different monomer that results in the formation of a cross-linked shell around a core particle, followed by the removal of the core particles (15).Illustrations of nanocapsular structures are presented in Figure 1.

In the process of the polyelectrolyte (PE) self-assembly on charged surfaces, hollow capsules are prepared via the layer-by-layer (LbL) technique, typically formed by the consecutive deposition of complementary/interacting polymers onto colloidal particles, followed by removal of the sacrificial template (16). However, to avoid PE-induced particle flocculation it is necessary to use low particle concentrations and unadsorbed PE has to be carefully removed after each step (15).

The principle of LbL coating is to sequentially expose the substrate to be coated to solutions containing either negatively or positively charged PEs. Adsorption of the PEs is based on electrostatic interactions originating from the charged nature of the substrate and the PEs. Thickness and properties of the coating film can be controlled by the number and composition of the layers and the process conditions. After each coating layer, removal of excess PE can be achieved by different methods. Tangential flow filtration has been proposed for the intermediate purification of the NC dispersion during the LbL process and is a convenient method that retains NC integrity throughout successive purification

steps, and does not involve the use of toxic solvents or harsh conditions (17). In addition, a versatile technique for drug encapsulation involves the adsorption of the drug within a porous particle that can subsequently be LbL-coated, and the template (e.g., silica) can be removed, resulting in the formation of NCs with high loading capacities (16).

In many cancer treatments, particularly for brain tumors, it is difficult to deliver the therapeutic agents to the tumor site and to the infiltrating tumor cells. Therefore, new devices are being developed to facilitate intratumoral distribution, to protect the active agent from premature degradation and to allow its sustained and controlled release. In the following sections, recent studies on the use of NCs in cancer are presented according to their particular NC formulations. However, this division is somewhat arbitrary since the NCs can be assigned to more than one session.

Lipid nanocapsules

Lipid nanocapsules (LNCs) are nanovectors with biomimetic properties that are produced through a phase inversion temperature process following the formation of an oil/water microemulsion. The process relies on at least three main components: an oily phase, an aqueous phase and a nonionic surfactant. LNC structure consists of an oily liquid triglyceride core surrounded by a tensioactive cohesive interface (18). Changes in the proportions of triglycerides, lecithin, salt-water and polyethylene glycol (PEG) hydroxystearate lead to different sized formulations of lipid nanocapsules, ranging from 20 to 100 nm, depending on the quantity of excipients, with a narrow distribution (19). No organic solvent is used during the formulation and all the excipients are FDA approved.

LNCs are characterized as a hybrid structure between polymer NCs and liposomes, with the advantages of greater stability and a solvent-free manufacturing process compared to liposomes. Moreover, the temperature cycling process crossing the phase inversion zone plays a relatively important role in LNC formulation. Reducing the amount of surfactant increases the number of temperature cycles required to stabilize dispersion (20). In general, LNCs have a negative surface charge due to the phospholipid molecules (21)

and the presence of PEG dipoles in their shell, which are able to reduce the surface charge in proportion to their concentration (22). The efficient encapsulation of drugs in the oily core of LNCs can be denoted by no significant modification of the average particle size and ZP.

In addition to their efficient drug-loading capacity, LNCs show adjuvant effects, such as P-gp inhibition properties, which can favor higher anticancer drug concentrations inside cancer cells (23). The *in vitro* gastrointestinal stability of LNCs was studied to evaluate the applicability of oral administration of anticancer drugs. The LNCs were stable in simulated gastric medium and fasted state intestinal medium (24).

LNCs appear to satisfy the properties required for weak complement activation and low macrophage uptake, resulting in an increased bloodstream circulation time due to their nanoscale size range and their high density PEG surfactant shell (20). Introducing surfactants in the post-insertion process alters the surface properties of the nanoparticles. Grafting longer PEG chains, such as PEG 1500 instead of PEG 660, prolonged their circulation time (25). Although the long-circulating properties of LNCs coated with DSPE-mPEG2000 (1,2-Distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy-(polyethyleneglycol)-2000]) have been demonstrated (26), the abundant presence of PEG, especially with long chains on the nanoparticulate surface, can inhibit the internalization of drug-loaded NCs, possibly by preventing interactions between the NCs and the tumor cell surface (27).

Experimental results obtained by Perrier et al. (28) indicate that DSPE-PEGs are efficiently transferred from micelles to LNCs during the process by a temperature-dependent molecular transfer. The hydrodynamic diameter (HD) increased from 55 to 70 nm at 5% DSPE-PEG2000-OCH3. Concentrations greater than 5% had no effect on increasing the HD and an upper limit of 70 nm was observed. In addition, the ZP decreased with the amount of DSPE-PEG2000-OCH3, suggesting that additional DSPE-PEG molecules were still interacting with the LNC. The HD increased with the length of the PEG chain, although less than predicted by an assumption of linear conformation, indicating that the PEG chains interact strongly in a compact conformation. The variation of PEG chain length and subsequent purification by dialysis determined the limits for post-insertion in terms of the nature of transferred amphiphiles. In case

of highly hydrophilic molecules, post-insertion can be reversible and incomplete (28).

In the gene delivery field, the most commonly used polynucleotide lipid-based carriers are lipid-DNA complexes, called lipoplexes. These are obtained by mixing cationic liposomes with DNA. However, an excess of cationic lipids can lead to high cytotoxicity (29). Viral vectors are still used for gene expression, despite having several drawbacks. Therefore, LNCs are good candidates for an efficient DNA delivery system, as NCs are an alternative for the maintenance of nucleic acid integrity required for efficient gene delivery in cancer therapy. LNCs provide protection by entrapping plasmid DNA molecules via lipoplexes in their lipid core, and DNA-coated LNCs have been shown to be stable. DNA molecules can be encapsulated and protected against degradation, as demonstrated by Morille et al. (26). Analysis by agarose gel electrophoresis showed that there was no DNA migration after NC formulation followed by a post-insertion process, whereas when incubated with a detergent (Triton), DNA molecules were released, migrating unaltered into the gel.

To increase the stability for efficient *in vivo* DNA transfection, the surface of the LNCs was modified by inserting longer PEG chains into the surface of DNA LNCs by post-insertion of two kinds of amphiphilic and flexible polymers. The first was F108, a block copolymer, and the second was the lipid PEG derivative DSPE-mPEG2000. Measurements of the ZP found that DNA-LNCs were able to partially mask the positive charge due to the presence of the lipoplexes (30). The two polymers DSPE-PEG2000-gal and F108-gal were obtained by chemical and enzymatic galactosylation, respectively. The DNA-LNCs were tested for *in vitro* transfection efficiency on primary hepatocytes. This study confirmed that galactose moieties were accessible on the surface of galactosylated DNA-LNCs coated with F108, and this effect was less pronounced in galactosylated DNA-LNCs coated with DSPE-PEG2000. The F108-gal coating improved gene delivery compared to F108 without galactose. The addition of galactose to DSPE-PEG2000 did not improve transfection efficiency, probably due to the insufficient accessibility of galactose when attached to this polymer. Therefore DNA-LNCs coated with F108-gal can be used for targeted-gene expression based on a cell-specific, receptor-mediated, endocytosis process.

The *in vivo* tumoral transfection potential of amphiphilic DSPE-mPEG2000- and poloxamer F108-coated NCs was tested in a mouse tumor model, demonstrating that DNA LNCs can be efficient in transfecting tumor tissues *in vivo* if coated with DSPEmPEG2000 in association with a special PEG chain conformation (31). Coating with F108 or DSPE-mPEG2000 increased the HD, evidence of an association between the DNA-LNCs and the post-inserted polymers. The size increase, when adding DSPEmPEG2000 at any concentration to the DNA-LNC surface, ranged from 11 to 20 nm, indicating a probable extended chain conformation. By contrast, the size increase due to F108 post-insertion was smaller (about 15 nm), even though the F108 polyethylene oxide chains are 3 times longer than those of the DSPE-mPEG2000. These results suggest a probable coiled configuration of the F108 chains that allowed some accessibility of hydrophobic moieties on the DNA-LNC surface. The decreased surface-charge density was observed for both polymers, but was more marked for the DSPEmPEG2000 coating, with a charge decrease from positive to negative values. Two surface layer parameter measurements (spatial charge density and softness) indicated that the DSPE-mPEG2000 coating was organized in a brush conformation, while F108 had adopted a super-coiled conformation (31).

F108-coated nanocapsules had a dramatically increased transfection rate *in vitro* (30); however, the F108 coating did not expose any transfection in tumor tissues *in vivo* (31). This can be linked to its interaction with other organs before acceding to the tumor site. As attested by Morille et al. (26), F108 has a shorter half-life in blood than DSPE-mPEG2000-coated DNA-LNCs. A major difference between gene expression in tumor tissue and in other organs was observed for DNA-LNCs coated with DSPE-mPEG2000, with only minor transfection observed in other organs (including elimination organs). The *in vivo* chain conformation of DSPE-mPEG2000 probably allowed sufficient accumulation via the EPR effect. At the tumor site *in vivo* gene expression was possible despite the low transfection levels observed *in vitro* (31).

LNCs are suitable for local treatment, including direct intracranial drug delivery, thereby eliminating the need for a chemotherapeutic agent to bypass the blood-brain barrier (BBB). The convection-enhanced delivery (CED) technique, using an external positive pressure gradient injecting therapeutic

fluid in the brain, allows a greater volume distribution to be achieved in comparison to diffusion alone (32). Therefore, LNCs with drug-loaded core delivered by the CED technique appear to be a promising approach for the treatment of brain tumors (27). Overcoming problems related to short drug tissue retention times or heterogeneous distribution, nanoencapsulation may offer advantages such as protection of the active species, reduced brain toxicity, a better drug distribution, and a longer half-life. Alternatively, the route of administration by intracarotid injection may also represent a promising path for drug delivery to brain tumors by giving the nanoparticles a greater chance to cross the BBB (27).

Ferrocifen-type molecules, obtained by attaching a ferrocenyl unit onto a tamoxifen skeleton in order to enhance its cytotoxicity, are anticancer drug candidates (33), although they suffer from poor bioavailability due to their highly hydrophobic phenol groups. These hydrophobic bioorganometallic molecules, which contain at least one carbon directly bound to a metal or metalloid, can be active in glioblastoma multiform therapy, but their water insolubility can impede their potential biological activity. Allard et al. (34) have shown that it is possible to formulate these molecules in lipid NCs. LNCs loaded with the ferrocenyl diphenol compound called "ferrociphenol" (Fc-diOH), one of the most active molecules in this new class of organometallic drugs, proved to be effective against 9L-glioma cells and in a 9L animal model and exhibited advantages over swollen micelles, which are smaller sized particles produced by a similar process with the same excipients (34).

LNCs were rapidly taken up by 9L cells, while micelles were taken up at much lower levels. Although smaller than the LNCs, the presence of a high density PEG coating likely decreased the interaction of the swollen micelles with cells, resulting in low internalization. This suggested that the LNCs improved the intracellular bioavailability of the Fc-diOH, which had a much higher cytotoxic activity than empty LNCs, and also demonstrated a higher cytotoxic effect in glioma cells than in astrocytes. In the *in vivo* glioma model, Fc-diOH-loaded LNC significantly inhibited tumor growth and this was not observed in rats treated with Fc-diOH-loaded micelles (34).

The activity of the Fc-diOH-LNCs was evaluated in 9L cells in association with radiotherapy (RT) and a combined treatment using the CED of Fc-diOH-

LNCs with external beam RT was evaluated in 9L glioma-bearing rats (35). For the combined RT, sucrose was dissolved in the aqueous phase of the LNC suspension after formulation, to enhance the viscosity of the infusion, and its presence did not affect the LNC size and had no toxic effect on cells *in vitro*. The treatment was more efficient when cells were first treated with Fc-diOH followed by RT, improving survival time compared to all other treatments and the control groups. The treatment combination in such order showed synergy and not only an additive effect, and the radiosensitizing effect of Fc-diOH may explain this enhanced efficacy. The synergy between the organometallic compounds and RT indicated a potential therapeutic application for this class of molecules, which often suffer from bioavailability problems. In the intracranial *in vivo* model, the Fc-diOH-loaded LNCs with the highest dose entrapped were administered to 9L glioma-bearing rats by the CED, followed by external RT. This resulted in a significant increase in median survival time compared to the chemotherapy group, to the group treated with empty LNCs followed by the same irradiation protocol, and to the group treated only with RT (35).

The identification of a subpopulation of human mesenchymal stromal cells, called "marrow-isolated adult multilineage inducible" (MIAMI) cells that could be used as delivery vehicles was used to evaluate the therapeutic efficacy of MIAMI cells carrying Fc-diOH-LNCs (36). Internalization of the drug-loaded LNCs did not affect MIAMI cell viability, and MIAMI cells loaded with Fc-diOH-LNCs were cytotoxic to glioma cells. The Fc-diOH-LNC-loaded MIAMI cells induced a significant, dose-dependent, inhibition of U87MG cell proliferation, which suggested that the MIAMI cells were able to deliver the Fc-diOH or Fc-diOH-LNCs. Furthermore, this cytotoxic effect was confirmed *in vivo* in a heterotopic U87MG glioma model. Although the authors successfully demonstrated that the MIAMI cells were able to efficiently internalize the drug-loaded LNCs while remaining viable, and deliver the drug-loaded LNCs into tumors, the effects of Fc-diOH-LNC-loaded MIAMI cells and those of Fc-diOH-LNCs alone were not compared (36).

Tumor-targeting nanoscale vectors able to deliver radionuclides have been recently developed. The LNCs permit the encapsulation of a lipophilic complex of Rhenium-188, and Vanpouille-Box et al. (37) reported the LNC^{188}Re-SSS as a radiopharmaceutical carrier for internal RT in rats with

hepatocellular carcinoma. No intolerance following LNC^{188}Re-SSS intra-arterial injection was observed, and therapeutic efficiency was evidenced by a reduction in tumor progression. This could be combined with an altered angiogenesis process as indicated by lower vascular endothelial growth factor levels. In addition, an increase in the median survival and preservation of healthy liver tissue compared to control groups was observed.

Novel LNC formulations towards the development of new mitochondria-targeted medicines have been proposed to improve the administration and biological activity of an analogue of the pro-apoptotic molecule HA14-1 (38). HA14-1 was designed to inhibit Bcl-2/Bax interactions and thus stimulate apoptosis (39). Subsequently, due to the instability of the molecule, a more stable analogue of HA14-1, called SV30, was prepared (40) and evaluated against glioma cells (38). SV30 was incorporated into LNCs at a high encapsulation rate, and its stability was maintained during the formulation process. Of note, unlike the other studies mentioned in this section, LNC size was affected by the loading of the lipophilic drug, resulting in a 20% increase compared to unloaded LNCs. Thus, LNC size and, more discretely, ZP modifications post-encapsulation combined with the relatively fast release of SV30 suggested that the drug might also be present on the external shell of the LNCs (38). Empty LNCs had no effect on glioma cells, while the SV30-LNCs triggered a two-fold increase in cancer cell death compared to free SV30. In addition, the nanoformulation was stable for at least one month at 4°C. The potential of LNCs to carry two different molecules was exploited to load a fluorescent probe, but could also have been used to load another synergistic drug. The labeled LNCs were used to confirm the ability of the SV30-LNCs to reach the mitochondria, which represent a major intracellular target for cell death induction and Bcl-2-related inhibition. The LNCs were able to accumulate next to the mitochondria, and the SV30-LNCs were more potent than empty-LNCs to do so. Thus, interactions of Bcl-2 (or a Bcl-2 family member) with SV30 and SV30-LNCs may have triggered specific modifications of subcellular trafficking pathways resulting in LNC redistribution within cells (38).

Paclitaxel (PTX) is a major antineoplastic agent with a widespread antitumor activity, but presents administration issues. Lacoeuille et al. (41) demonstrated the increased circulating properties of LNC and the therapeutic

efficiency of PTX-LNC compared to controls in a hepatocellular carcinoma model. The PTX-LNCs had the advantage of avoiding the conventional use of Cremophor EL for the solubilization and formulation of PTX, which is associated with severe side effects. Therefore, PTX-LNC has been proposed as a new drug delivery system for PTX (41). The LNCs were evaluated as new pulmonary delivery strategies, and the incorporation of the drug into breathable droplets for pulmonary delivery by nebulization is a potential therapeutic strategy with reduced systemic side effects. In this context, Hureaux et al. (42) demonstrated that PTX-LNCs could be delivered by nebulization and the physical and chemical parameters were optimized for aerosol delivery and storage.

The formulations were non-pyrogenic and remained sterile for up to 4 months, showing that PTX-LNC dispersions exhibited the principal parameters required for aerosol delivery in humans. Osmolarity and pH can be a problem for bronchial and alveolar cells; therefore, following LNC fabrication, the pH was increased to reach tissue pH by the addition of sodium hydroxide and the osmolarity was also increased to near-human iso-osmolarity by adding sodium chloride. Ultimately, only one mesh nebulizer was able to produce aerosols of the PTX-LNC dispersions without damaging the LNCs and sustaining good characteristics for human use. Stability during storage is important in order to allow preclinical and clinical studies to take place. The freeze-storage protocol developed did not alter or damage the PTX-LNCs. The cytotoxic effect of the stored PTX-LNCs was identical to that of the fresh PTX-LNCs, and the chemical parameters were not modified. As the PTX-LNCs are sustained-release drug nanocarriers that can be nebulized, the traditional limitations for the blood infusion of PTX, such as a low concentration in the lungs, insufficient drug delivery to tumor cells, resistance, and nonspecific distribution associated with systemic toxicity could be overcome with aerosols of PTX–LNCs in lung cancer patients (42).

More recently, Basile et al. (43) evaluated the protective effect of a new series of amphiphilic PEG derivatives obtained by conjugating either carboxy- or amine-PEG to lipoamino acid residues (LAA) on PTX-LNCs. Coated NCs were prepared by post-insertion. The behavior of the PEG-LAA conjugate was also compared to commercial DSPE-mPEG used to produce sterically stabilized vesicles. They investigated the ability of the novel amphiphilic PEG conjugate to

stabilize colloidal drug carriers with respect to their permanence in the bloodstream, and found that it was possible to prepare sterically stable nanocarriers, although future studies will be needed to assess the potentiality of these nanocarriers.

Layer by layer nanocapsules

One of the goals of an LbL process is to improve the stability of the core particles (44) or the controlled release of an encapsulated substance (45). Typically, LbL particles are formed by consecutive deposition of interacting polymers onto particle templates, resulting in the formation of thin, multilayered polymer coatings on particles of different size, shape, composition, thickness, permeability, and function (46). Several templates can be used to deposit multiple layers, which are subsequently removed by chemical or thermal means to yield the polymer capsules. The nonporous templates include: inorganic molecular precursors that have been used to prepare inorganic capsules, liquid droplets, and gas bubbles. The porous templates include mesoporous silica (MS) and calcium carbonate particles, both of which can be decomposed under adequate conditions (47). Nonporous colloidal particles (48) are convenient templates for capsules with nanometer-thick walls, while with porous particle templates (49) polymer chains can infiltrate the core and, upon core dissolution, give rise to nanoporous polymer particles. Such nanoporous polymer particles can be used as high load carriers of a desired molecule.

Since this method allows broad control of capsule properties through the choice of the sacrificial colloids and the film components, significant advances in the development of LbL particles have enabled efficient cargo encapsulation, triggered release, and antibody-mediated targeting (46). The feasibility of the LbL method improves the production of functionalized nanoparticles using polymers with free reactive groups for the outer layer. The sonication-assisted LbL nanoassembly technique can be used for the nanoencapsulation of poorly soluble anticancer drugs, and was exploited by Vergara et al. (50) to efficiently produce PTX-NCs. It was also used in the nanoformulation of two drugs in one NC, locating PTX in the core and lapatinib on the shell surface. Encapsulation increased the *in vitro* antitumor efficacy of PTX, which, in combination with

lapatinib, significantly overcame multidrug resistance in ovarian cancer cell lines (50).

Son et al. (51) developed a new type of hollow NC for use in combining photodynamic therapy (PDT) with chemotherapy. PDT uses a combination of light and dyes or photosensitizers to induce damage in cancer cells and tissue (52). To produce the photosensitizer, a negatively charged dendritic porphyrin (DP) was synthesized and combined as a bilayer component with poly(allylamine hydrochloride) (PAH) to fabricate hollow NCs. While most NCs used in drug delivery system (DDS) are prepared from linear polyelectrolytes whose only function is as a drug container, this system employed DP not only as a polyelectrolyte component in the LbL assembly for the formation of NCs, but also as a photosensitizing agent for PDT. The amount of DP was controlled by changing the number of deposited layers. The (PAH/DP)n multilayer NCs were filled with the anticancer drug doxorubicin hydrochloride (DOX), producing NCs with both PDT and chemotherapeutic actions. DOX could diffuse easily through the multilayer shells to fill the hollow NCs, and the release profile resembled the loading profile. To control the release rate, the shells were cross-linked between PAH and DP, and sustained release could be achieved by controlling the degree of cross-linking. Cell viability studies showed that the combined treatment resulted in higher toxicity than either chemotherapy or PDT alone (51).

Core-shell nanocapsules

Since tumor growth depends on the oxygen and nutrients supplied by tumor blood vessels, neovasculature in the tumor microenvironment is necessary for tumor pathogenesis (53). Therefore, targeting drugs directly to the tumor vasculature can provide an alternative therapy approach. However, there is evidence that tumor angiogenesis results from multiple associated and compensatory mechanisms, which could limit the therapeutic effects of a single antiangiogenesis therapy. Therefore, the therapeutic combination of a cytotoxic agent and an antiangiogenetic drug would probably enhance treatment (54). Moreover, a DDS that allows the sequential release of the antiangiogenic agent

from the same carrier, followed by the cytotoxic drug, could lead to a more effective cancer treatment.

Wang and Ho (55) designed a tumor vasculature- targeted, combinatorial, double self-assembled core-shell NC, with the aim to sequentially disrupt tumor vasculatures with combretastatin A4 (CA4), followed by induction of tumor cell apoptosis with PTX. The sequential drug release was accomplished by loading CA4 onto the lipid layer shell and PTX into the polymeric core. In addition, the arginine-glycine-aspartic acid (RGD peptide was used as a vascular-targeting ligand, since the proliferating vascular endothelial cells of the tumor overexpress integrin receptors, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, on the cell membrane. Thus, NCs could deliver drugs in a sustained and temporal manner to disrupt tumor vasculature and ablate tumor cells with the assistance of the RGD-targeting ligand (56).

The lipid-assisted nanoprecipitation method used in this study consisted of an one-step self-assembly process, where fewer chemicals and steps are involved, which makes the NC preparation simple and robust for large-scale production. The self-assembled PTX-loaded poly(lactic-*co*-glycolic acid) (PLGA) core surrounded by a hydrophilic PEG surface was coated with a lipid layer as a shell, and CA4 was loaded onto this lipid layer by hydrophobic interactions. The lipid shell served as a depot for CA4, as well as a molecular fence to control the release of PTX from the polymeric core, inducing cell death in a sequential manner (56).

An alternative design for an NC for the sequential delivery of CA4 and PTX employed the nanoprecipitation method using methoxyl PEG-poly(lactic acid) (mPEG-PLA) as the building-up matrix. PTX was conjugated to PLA, and CA4 was encapsulated in the core when the mPEGPLA self-assembled in an aqueous environment. This resulted in the production of uniform and stable nanoparticles exhibiting effective drug loading and sequential release of drugs. The *in vitro* evaluation of drug release showed a distinctive profile for PTX and CA4, with an initial fast release of CA4 followed by a sustained release. PTX was released in a sustained pattern with a slight peak on the first day of incubation, and longer incubation times accelerated the release of PTX from the NC, which was controlled by the slow hydrolysis of the ester bond between PTX and PLA (55).

Both *in vitro* and *in vivo* experiments have demonstrated significant therapeutic effects of dual drug-loaded sequential-delivery NCs. The NCs accumulated in the tumor mass over time and no abnormalities were detected in the liver, kidney or heart of treated mice, and there were no apparent side effects. Cancer cell proliferation was reduced in the drug treatment group and there was no significant tumor cell metastasis, compared to the control groups (PBS and empty NCs). Although the exact therapeutic mechanism of these NCs is not clear, the induction of cellular apoptosis and antiangiogenesis activity was improved compared to single agent-loaded NCs. In addition, expression of vascular endothelial growth factor (VEGF), a major pro-angiogenesis factor in the tumor microenvironment, was inhibited (55).

The combination of physical imaging for cancer diagnosis and anticancer drug delivery into one system is of great interest. Multifunctional core/shell structured hollow mesoporous silica NCs with ellipsoidal morphology for both MRI and drug delivery have been evaluated (57). Monodispersed ellipsoidal Fe_2O_3 nanocrystals were chosen as a template to obtain magnetic mesoporous NCs, which were fabricated by hydrothermal synthesis using iron perchlorate as the Fe precursor. A thick layer of dense silica was deposited onto the surface of Fe_2O_3 to form $Fe_2O_3@SiO_2$ core/shell nanostructures. A thin layer of mesoporous silica shell was deposited onto the surface of the $Fe_2O_3@SiO_2$ to form a trilayered composite nanostructure (Fe2O3@SiO2@mSiO2). The middle silica layer, with a lower degree of condensation, was etched away under alkaline conditions to form large cavities between the shell and core ($Fe_2O_3@mSiO_2$). The outer mesoporous silica shell (higher condensation degree) was maintained. Finally, the Fe_2O_3 core of the NCs was reduced in a gas mixture of H_2 and N_2 to produce hollow $Fe_3O_4@mSiO_2$ core/shell NCs with well-defined mesopores in the silica shell and controllable cavity sizes in the NCs (57). The $Fe_3O_4@mSiO_2$ nanocapsules demonstrated excellent blood compatibility and no significant coagulation effect was observed, ensuring biosafety for intravenous administration. There was no apparent *in vitro* cytotoxicity of the empty NCs even at high concentrations, and DOX could be loaded into the NCs with high efficiency, maintaining the pharmaceutical activities of the loaded DOX and enhancing its cytotoxicity against cancer cells (57).

Polymeric and shell cross-linked nanocapsules

To improve the conventional LbL technique for the preparation of NCs, Wang et al. (49) used a solid silica core and mesoporous silica shell (SC/MS) template to prepare thick-walled, single-component polymer NCs. The process involved the infiltration of polyelectrolyte or polymer-drug conjugates into the mesoporous shells of the SC/MS particles, followed by cross-linking of the infiltrated polymer chains and subsequent removal of the SC/MS silica template, producing polyelectrolyte or drug-conjugated polymer NCs. PAH was employed as the polyelectrolyte, glutaraldehyde was used as the cross-linking agent, and the SC/MS template was removed via treatment with hydrofluoric acid to obtain PAH NCs. For drug delivery applications, the preparation with polymers poly(L-amino acids), which are structurally related to natural proteins and considered to be biocompatible and biodegradable, was evaluated. Amine-functionalized positively charged SC/MS particles were used to adsorb poly(L-glutamic acid) conjugated with DOX. The polymer-drug conjugate, PGA-Dox, was loaded into the mesoporous shells and cross-linked using 2,2'-diaminodiethyl disulfide dihydrochloride (cystamine) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. After removal of the SC/MS template, PGADox NCs were obtained, and induced a significant decrease in the number of viable tumor cells *in vitro*, achieving >85% cell death (49).

Polymeric NCs can be designed to encapsulate proteins that are protected against proteolysis and denaturation, and have increased efficiency in transport across the cell membrane. One strategy consisted of the development of protease-modulated reversible NCs, in which monomers and cross-linkers were deposited onto the protein surface via physical adsorption. Free-radical polymerization was performed *in situ* to form the protective shell. Upon proteolytic cleavage of the cross-linker, the polymeric shell disintegrated and the protein was released in functional form (58). This has proved to be effective by successful delivery of caspase-3 (CP3), a peptidase in the apoptotic signaling pathway, through degradation of the cross-linked polymeric shell from within, promoting inhibition of proliferation and extensive apoptotic DNA fragmentation in cancer cell lines. Cancer cells treated with native CP3 or NCs

synthesized under identical conditions but with a nondegradable cross linker did not show such characteristics (58).

For PDT, an increased photosensitizer affinity for tumor cells in order to reduce side effects on normal cells, as well as protection from thermal and photodegradation of the photosensitizer, are desired (59). Pietkiewicz et al. (60) reported polymeric oil-cored poly(n-butyl cyanoacrylate) (PBCA) NCs fabricated by means of o/w microemulsion templates formed by polyoxyethyleneted nonionics as promising nanocarriers for the delivery of hydrophobic cyanine-type photosensitizers. There was a discrepancy in size between the much bigger NCs and the corresponding microemulsion droplets, which probably resulted from the dynamic character of the microemulsion systems, assuming that the polymerization process did not involve a single droplet but several droplets at the same time. The preparation of NCs from the o/w microemulsion templates improved the encapsulation efficiency of cyanine IR-768, which was associated with the core rather than adsorbed on the surface, as shown by the sustained diameter and ZP, circumventing the undesirable characteristics of free cyanine.

Although PBCA NCs demonstrated low or moderate levels of hemolytic activity on human erythrocytes, it was concluded that intravenous injection of the NCs would result in lower serum concentrations compared to the *in vitro* experiments. Therefore, the NCs appeared to be relatively safe carriers of cyanine IR-768 in the circulation. The NCs were able to deliver cyanine IR-768 to cancer cell lines, and there was an initial burst release followed by a slow and sustained phase. The *in vitro* IR-768 leakage was dependent on the monomer concentration and not on the type of microemulsion. The dye did not aggregate inside the cells and could therefore act as an effective photosensitizer. Noteworthy, free cyanine uptake caused significant changes in cells morphology while cells treated with IR-768-loaded NCs maintained a regular shape (60).

Shell cross-linked NCs were found to be efficient delivery vehicles for water-insoluble anti-cancer drugs such as PTX, and achieved effective cancer therapy by reducing the nonspecific side effects on normal tissue (61). The conventional PTX formulation induces acute side effects that are associated with the oil-based vehicle (62). PTX shows high affinity for, and strong binding

to human serum albumin (HSA), and Lee et al. (61) employed HSA and amine-reactive multi-arm PEG to fabricate shell cross-linked NCs that incorporated PTX. Oil was not used and PTX was efficiently encapsulated into the hydrophobic interior by HSA-PTX interactions that were stabilized by a cross-linked shell layer consisting of PEG and HSA. As HSA is a negatively charged protein at neutral pH, it is necessary to overcome the strong electrostatic repulsion hindering entry into cells through the negatively charged cell membrane. This was achieved by conjugation, using a flexible PEG linker, of the drug-loaded NCs with the cell-penetrating peptide Hph1. Such modification enhanced the therapeutic performance of the loaded PTX against several cancer cell lines, and this could be induced by the Hph1-facilitated translocation of the NCs into the cells.

The biocompatible HSA/PEG PTX-NCs were structurally stable and the cytotoxic effect was attributed to PTX-induced apoptosis. In a mouse tumor model, clearance of the HSA/PEG NCs by the macrophages in the liver was minimal, suggesting that the NCs were targeted to and stayed in the tumor tissue for an extended period of time, with no severe nonspecific uptake by normal tissues. Tumor growth was significantly suppressed in mice that received a single *iv* dose of the NCs compared to untreated control mice (61).

Small interfering RNA (siRNA), a short double-stranded RNA less than 30 nucleotides in length, has received significant attention in biomedical research owing to its therapeutic potential. siRNAs function as effective mediators of post-transcriptional specific gene silencing via a mechanism called RNA interference (RNAi) (63). However, there are challenges in the application of siRNAs for anti-cancer therapy (64), as they exhibit poor *in vivo* stability by enzymatic degradation in the bloodstream. Lee et al. (65) developed pluronic/polyethylenimine shell cross-linked NCs with embedded magnetite nanocrystals (PPMCs) for magnetically triggered intracellular delivery of siRNA. Pluronic is a biocompatible amphiphilic tri-block copolymer, and polyethylenimine (PEI) is a cationic polymer used in non-viral gene delivery systems both *in vitro* and *in vivo* (66). However, its use to transfect tumors after systemic injection still represents an issue (67).

The formulation was produced by a modified emulsification/solvent evaporation procedure based on crosslinking at emulsion interfaces resulting in

the formation of a pluronic/PEI composite shell layer encapsulating iron oxide nanocrystals in their hollow interior space. Since these NCs had a highly cationic shell layer they could be used as delivery vehicles for anionic therapeutic agents such as siRNA. The PPMCs were expected to interact electrostatically with the negatively charged siRNA-PEG conjugates to form nanosized polyelectrolyte complexes favorable for cell internalization. The application of an external magnetic field could direct the PPMC/siRNA-s-s-PEG polyelectrolyte complexes into target cells. This enhances their uptake via endocytosis plus magnetically enforced cellular transport processes. The PPMCs suppressed gene expression in cancer cells, suggesting that they could be used as powerful nontoxic delivery vehicles for various nucleic acid-based therapeutic agents. In addition, the magnetic targeting of PPMCs greatly enhanced the efficiency of intracellular delivery and the gene silencing effect of the encapsulated siRNA (65).

The direct protein delivery to the cytosol in order to restore functions of interest is an interesting approach, but this has been hampered by some of the inherent characteristics of proteins. Therefore, vehicles for suitable protein delivery must meet certain criteria: they should protect the protein cargo from denaturation and proteolysis, shield the negatively charged protein, release the protein cargo in native forms when the desired destination is reached, not require covalent modification that can disturb protein folding and impair biological activity, and not exhibit low delivery efficiency or encounter difficulties due to colloidal instability (68-70). Zhao et al. (71) reported a method to encapsulate single protein molecules in a polymeric NC using a redox-responsible, disulfide-containing cross linker.

NCs interconnected by the cross-linker through interfacial polymerization should maintain the integrity of the thin polymer shell under the oxidative conditions outside the cell, but must be able to undergo rapid degradation and protein cargo release under the reducing conditions encountered upon entry into the cytosol. The formulation was internalized by the cells and a significant portion of the internalized NCs and the cargo escaped from the endosomal compartment and reached the desired destination. A human cancer cell line treated with CP-3 encapsulated in the NCs underwent extensive apoptotic DNA fragmentation, while apoptosis was not observed in cells treated with the

negative control. This demonstrated that CP-3-NCs could be internalized by cancer cells, and that functional CP-3 was released into the cytosol where it induced apoptosis. The redox-responsive encapsulation strategy was a simple yet effective method of intracellular protein delivery (71).

Liposome-like nanocapsules

The concept of directly using drug molecules as components of NCs was demonstrated using the hydrophobic drug Camptothecin (CPT) (72). CPT was used to replace the fatty acid(s) in phospholipids, in a formulation that contained a short nonionic ethylene glycol chain as the water soluble part and a β-thioester bond as the linker. The formed liposome-like NCs acted as nanocarriers that released the CPT inside cells due to the presence of estearase. In addition, these liposome-like NCs could be efficiently loaded with DOX for combination therapy and showed high *in vitro* and *in vivo* antitumor activity.

The frequently used platinum-based drug cisplatin has problems related to cumulative toxicity. Liposomal formulations of cisplatin reduced toxicity but exhibited relatively low concentrations of platinum in tumor tissue (73). An alternative method using cisplatin NCs was evaluated against a panel of human ovarian carcinoma cell lines. The cisplatin NCs consisted of nanoprecipitates of cisplatin encapsulated in a phospholipid bilayer. The increased cytotoxicity caused by the cisplatin NCs was observed to be cell line dependent (74). The *in vitro* cytotoxicity resulted from the uptake of the NCs by caveolae-mediated endocytosis. This led to increased intracellular accumulation of cisplatin and increased levels of platinum-DNA-adducts. In the absence of caveolin-1, the cisplatin NCs were taken up via clathrin-mediated endocytosis and became trapped in an endocytic compartment, resulting in a modest increase in platinum-DNA-adduct formation and toxicity of the encapsulated versus the free drug. The observation that cytotoxicity depended on the route of cellular entry may have identified a new strategy for predicting the efficacy of nanoparticulate anticancer DDS against different types of tumors. The cisplatin NCs may be able to circumvent the blocking mechanisms used by platinum-resistant cells and destroy them (74).

Protein nanocapsules

Phosphorylation by protein kinases plays an important role in cell growth and functions through the activation of the target proteins via signal transduction pathways. Abnormal activation of certain protein kinases has been associated with many diseases (75). Casein kinase 2 (CK2) is a highly conserved and ubiquitous protein serine/threonine kinase with an important role in cancer. Besides being implicated in cell proliferation and differentiation, CK2 can also be a potent suppressor of apoptosis (76). Therefore, downregulation of CK2 is a potential cancer therapy strategy if it can be targeted to cancer cells. The inhibition of CK2 using NCs could enhance the tissue-specific targeting and therapeutic efficacy of this approach (77).

Protein kinase CK2 may interact with both NF-κB and TP53 pathways and thereby promote a malignant phenotype and its progression. NCs containing tenfibgen (the fibrinogen binding domain of tenascin) have been shown to enhance the intracellular delivery to tumor cells of the siRNA anti-CK2α/α′ oligodeoxynucleotide (ODN) that acts against both of the CK2α and CK2α′ subunits (78). The anti-CK2α/α′ ODN was encapsulated in sub-50-nm tenfibgen NCs and targeted CK2α/α′ in a head and neck squamous cell carcinoma xenograft model (77). Tumor growth was significantly suppressed and the expression of multiple proteins involved in NF-κB, TP53, and apoptotic pathways was altered. An additional therapeutic approach was developed to target protein kinase C isozyme (PKCα) that is overexpressed or hyperactivated in cancer cells (79). This regulation system was provided by a PKCα-specific substrate peptide that could distinguish between normal and tumor cells but was not tissue-specific.

BNCs are hollow nanoparticles that can be produced efficiently in recombinant yeast cells. They consist of hepatitis B virus (HBV) surface antigen molecules and a lipid bilayer. Intravenous injection of BNCs has allowed the efficient delivery of incorporated payloads to human hepatocytes by using the HBV infection machinery (80). The pre-S1 peptide displayed on the BNCs specifically recognizes human hepatocytes, and therefore provided, in combination with the tumor cell specific gene regulation, the desired selectivity for hepatoma cells.

Two conjugates were designed using N-methacryloyl-PEG (NPEG), the positive polymer NPEG(S) containing serine at the phosphorylation site of the peptide (peptide(S)), and the negative polymer NPEG(A) containing alanine (peptide(A)) that blocked phosphorylation by PKCα. The NPEG(S) polymer was phosphorylated by PKCα after being incorporated into the polymer backbone and combined with DNA. The NPEG(S)-DNA complex was broken up by phosphorylation of the peptide and resulted in the release of the DNA from the complex. The delivery of the NPEG-DNA into cells was promoted by the BNCs. After transfection of the NPEG(S)-DNA-BNC complex, phosphorylation by PKCα induced gene expression. This complex increased transfection efficiency and resulted in cell-specific gene expression in hepatoma cells and tissues. No gene expression was observed in normal human hepatocytes or human epidermoid tumor cells, demonstrating that the system had hepatoma-specific gene expression both *in vitro* and *in vivo* (81).

A novel concept of immune-based therapy for lung cancer involved a vault NC for the delivery of immune potentiating cytokines. Vault NCs are naturally occurring ubiquitous cytoplasmic ribonucleoprotein particles, which may serve as a flexible therapeutic delivery vehicle. The effect of the vault NC delivery of CCL21, a lymphoid chemokine, on the growth of Lewis Lung (3LL) tumors *in vivo* was evaluated (82). The aim was to promote the recruitment of T lymphocytes and dendritic cells into the tumor microenvironment in order to stimulate a robust antitumor activity response. A single dose of the CCL21-vault NCs induced potent antitumor activity and inhibited tumor growth. Neutralizing antibodies to CCL21 inhibited the chemotactic activity of the NCs, demonstrating that the antitumor activity was CCL21 dependent (82).

Final remarks

In the development of more effective and less harmful anticancer therapeutic agents, improved carrier systems are urgently needed. Carriers should be biocompatible, with the lowest possible cytotoxicity, and should not influence the therapeutic effect of the loaded bioactive substances. NCs, nanovesicular systems that can act as carriers of therapeutic molecules, have shown great potential in cancer research. They can enhance drug accumulation

at a specific tumor site, thereby reducing the side effects associated with most chemotherapeutic drugs, and they can deliver DNA and siRNA. Most anticancer drugs have poor water solubility, rapid blood clearance, low tumor selectivity, and severe side effects for healthy tissues. Nanoencapsulated systems offer the appropriate physicochemical characteristics and modify the biodistribution of the encapsulated therapeutic agents. NCs can deliver higher payloads, shield the active molecules from degradation, prolong drug circulation time and delay their release. In addition they can facilitate cellular uptake, improve drug targeting and solubility, and provide controlled release.

A further advantage is that more than one drug can be carried by the same nanoparticulate DDS, and they can be released in a sequential manner (56). Thus, the activity of a number of anticancer hydrophobic compounds can be improved (83). In addition, cellular uptake of the NCs by the endocytosis mechanism can be achieved (55). The self-degrading NC approach (58) may be useful in protein drugs or vaccines, and may be extended to applications in which external proteases trigger the disassembly of the NCs. Encapsulated radioactive components can allow imaging of the distribution of the targeted NCs for diagnostic and therapeutic uses (84). Antibodies can be attached to the NC surface, and antibody functionalized NCs can bind specifically to cancer cells expressing the complementary antigen (85).

LNCs can be coated by a post-insertion technique to improve their circulation times in order to give them the adequate features for *in vivo* injection and accumulation in tumors (26). Nevertheless, accessibility to ions decreases with PEG length, and since the conformation of PEG tends to be globular, the length of PEG should be adapted for each application (28). For a delivery vehicle to be effective, it is desirable that it should not allow degradation of its content in the blood stream; however, it should easily release its cargo after reaching its destination within tumor cells (47). The size of nanodevices is also an important feature: if they are too large they will be removed from the bloodstream by the macrophages in the liver and spleen, whereas if they are too small they will be filtered by the kidneys. The ideal diameter of nanoparticles should be less than 200 nm for their efficient capture by the tumor vasculature (86), and NCs will be able to reach the targeted site through gaps in the endothelium (87).

LNC formulations are prepared using FDA-approved constituents in a solvent-free and low-energy process (18). Other formulation approaches also use biocompatible and biodegradable materials, with minimal, if any, cytotoxic effects, to construct a self-assembled core-shell NC (56). Besides, inorganic NCs can provide excellent cell uptake and high delivery efficiency of encapsulated drugs. Functional inorganic nanocrystals in the core of the NCs could allow simultaneous imaging and drug delivery functionalities (57). LbL assembly has proved to be advantageous (17), but alternatives have been proposed without multiple polymers and/or multiple polymer adsorption steps, such as template synthesis (88), providing NCs with thick shells. The nanoprecipitation technique can be used to encapsulate hydrophobic drugs into an amphiphiphlic copolymer, which can be self-assembled into a micellar structure in an aqueous environment (89). Another alternative is the use of natural functional macromolecular assemblies and structures that can be manipulated for nanobiotechnology applications. Protein engineering coupled with synthetic chemical strategies can be used to redesign structure and function, yielding biodegradable particles with very narrow size distributions and multifunctional control afforded by recombinant synthesis strategies (90).

In conclusion, the capability to generate a wide range of materials with tunable properties that can interact in a unique fashion with biological systems is providing exciting new opportunities for designing advanced NCs for use in a range of therapeutic and diagnostic applications. While several challenges remain to be addressed, undoubtedly NCs represent an interesting alternative for the development of new tools that can inhibit the growth of cancer cells.

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Table 1. Recent strategies for cancer treatment using Nanocapsules

Strategies	Encapsulated molecule	Encapsulation rates	Particle size	Zeta Potencial	Study designs	Results	Reference
Anti-tumour effect	Ferrociphenol (FcdiOH)	6.5 mg/g, 2% dried weight	44–53 nm	From −3 to −5 mV	Orthotopic gliosarcoma model	Increased survival time of tumour-bearing rats	(27)
Deliver foreign genetic material to target cells	DNA		117-142 nm (different coatings)	from +30 mV to -41 mV	*In vitro* Macrophage uptake evaluation; Complement activation study; *In vivo* hepatotoxicity study after IV injection; Blood kinetic study	Inhibition of complement activation; increase of *in vivo* circulation time and tumor accumulation	(26)
Anti-angiogenesis and anticancer drugs sequentially	Paclitaxel (PTX) and combretastatin A4 (CA4)	27.2% and 20.7%	180 nm	—	*In vitro* Drug Release; apoptosis assay; immunofluorescence staining of tubulin Disruption; cellular uptake by human umbilical vein endothelial cells	Temporal ablation of endothelial cells and cancer cells	(54)
Anti-tumour effect	Camptothecin (CPT)and doxorubicin salt (DOX· HCl)	40 or 58 wt %	132 nm and 192 nm	—	*In vitro* cytotoxicity on SKOV-3 and MCF-7 cancer cell lines; *in vivo* antitumor activity on mice bearing intraperitoneal tumors	High *in vitro* and *in vivo* antitumor activity	(72)
Overcome multidrug resistance	Paclitaxel (PTX)	70% wt	150 ± 50 nm	—	*In vitro* cell assays	Enhanced cell growth inhibition In a multidrug-resistant ovarian cancer cell line	(50)
Anti-tumour effect	Paclitaxel (PTX)	9.58 wt-%	280 nm	—	*In vitro* inhibition of cell growth; confocal microscopy and cell cycle analysis; *in vivo* tumor accumulation, biodistribution, and anti-tumor effect	Preferential accumulation in the tumor site and suppression of tumor growth	(61)
Combinatorial sequential drug delivery system for antiangiogenesis and anticancer activities	Combretastatin A4 in a matrix made up of paclitaxel (PTX) conjugated	52%	68.3 ± 1.4 nm	—	Biodistribution experiments; *In vivo* artificial proangiogenesis and tumor xenograft assays; intrasplenic liver metastasis experiment.	Efficient cellular uptake; long circulation in body fluid and accumulation in tumor; therapeutic effect o on tumor vasculature disruption, tumor cell proliferation and apoptosis induction; liver metastatic prevention capacity.	(55)
Expression of CK2 subunits and CK2 effects on mediated signal	Anti-CK2α/α′	90%	Sub- 50 nm	—	Western blot; immunohistochemistry; siRNA; luciferase reporter assays; quantitative PCR; MTT; flow	Suppression of tumor growth; down-modulation of NF-κB and up-modulation of TP53; induction of	(77)

activation and gene expression.	oligodeoxynucleotide				cytometry; migration assays; *In vivo* xenograft models.	apoptosis; efficacy enhancement of cisplatin-based chemotherapy; downregulation of CK2 in HNSCC models *in vitro* and *in vivo*.	
Cytotoxic effects of MIAMI cells loaded with LNCs.	Fc-diOH	2.6 ± 0.1 mg/ml	76 ± 2 nm	−7± 2mV	*In vitro* uptake of LNCs by MIAMI cells; i*n vitro* toxicity on U87MG cells; *in vivo* toxicity after intratumoral injection in a heterotopic U87MG glioma model	MIAMI cells internalized Fc-diOH-LNCs with no induction of MIAMI cell death, and Fc-diOH-LNC-loaded MIAMI cells showed cytotoxic effect	(36)
Hepatoma-targeted gene delivery system	PKCα-specific substrate peptide	0.9 and 1.2 mol%	‹ 100 nm	—	Human tumors xenografted mice; gene expression in hepatoma cells and tissues and human hepatocytes or epidermoid tumor cells	Increased transfection efficiency; cell-specific gene expression in hepatoma cells and tissues.	(81)
Internal radiation with LNC188Re-SSS	Rhenium-188	approximately 97%	49.7 ± 2.7 nm	—	Chemically induced hepatocellular carcinoma rat model; biodistribution study following hepatic artery injection; survival evaluation.	Increase in the median survival in treated rats; decreased vascular endothelial growth factor expression	(37)
Mitochondrial targeting	SV30, an analogue of the pro-apoptotic molecule HA14-1	92 ± 20%	~57 nm	~ −10 mV	*In vitro* survival assays; Western blots; HPLC; flow cytometry; confocal microscopy; spectral imaging	Improved SV30 biological activity.	(38)
Photodynamic therapy	Cyanine IR-768	from 65.7% to 91.7%.	from 192 to 642nm	from ~ -23 to ~ -41mV	*In vitro* erythrocyte hemolysis; cell viability of breast cancer MCF-7 cells; Fluorescence microscopy	Efficient delivery to doxorubicin-sensitive and resistant cells; decreased cell viability by photoirradiation with entrapped photosensitizer.	(60)
Chemokine delivery	Lymphoid chemokine CCL21	20–30 molecules of the CCL21-INT/particle.	40 nm in width and 70 nm in length	—	Intratumoral administration of CCL21-vaults in mice bearing lung cancer	Inhibition of tumor growth and reduction of immune suppressive and T regulatory cells; induction of systemic antitumor responses.	(82)
Magnetically triggered siRNA delivery	siRNA		500 nm	—	*In vitro* cell assays	Efficient intracellular uptake and silencing effect of siRNA upon exposure to a magnet.	(65)
Intracellular protein delivery	Caspase 3		11.3 nm	3.6 ± 0.1mV	*In vitro* assays: cellular uptake, internalization and trafficking; cytotoxicity and apoptosis assays	Efficient internalization and releasing the active protein in reducing cytosol; induction of apoptosis.	(71)

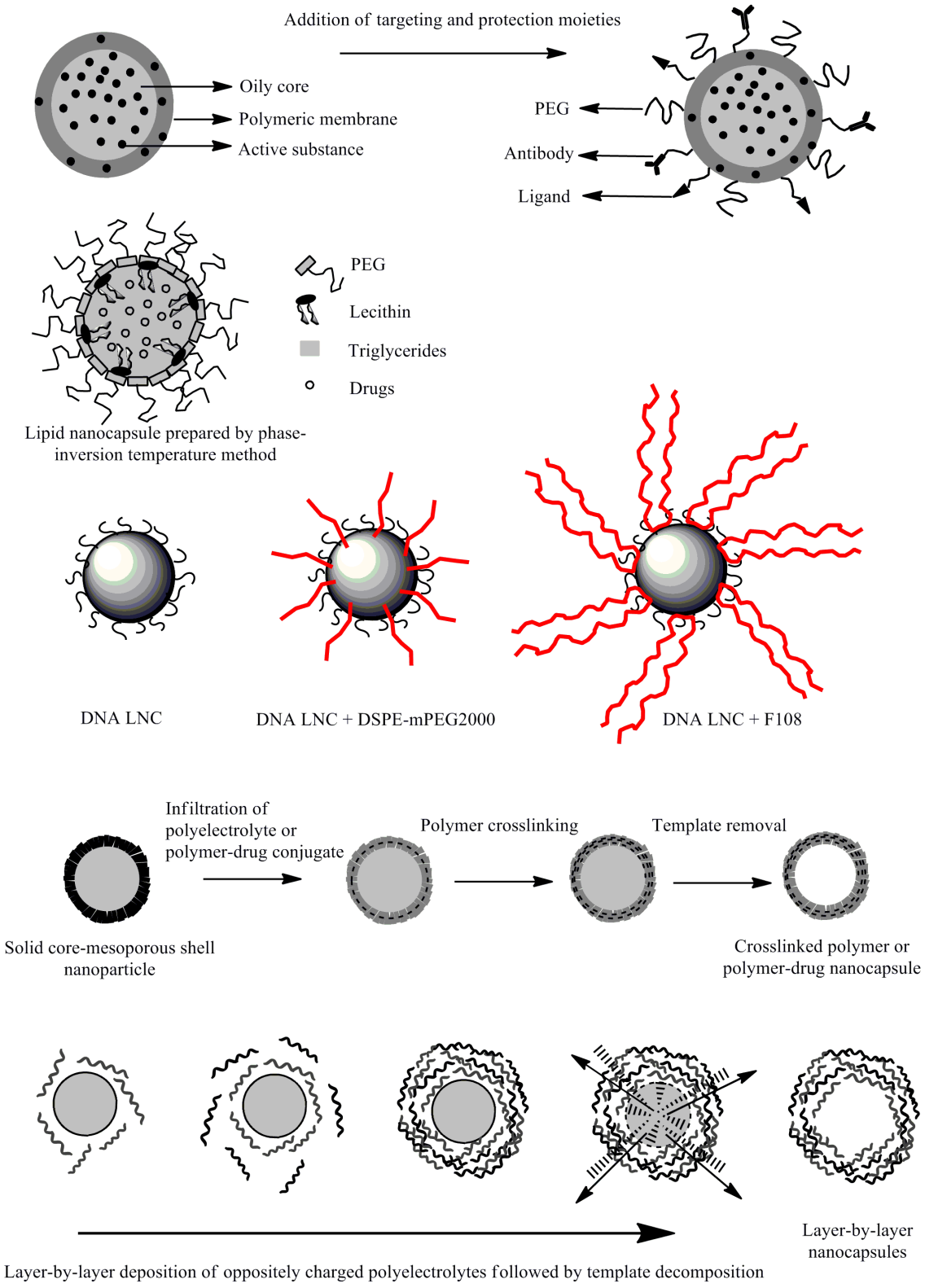

Figure 1. Schematic examples of nanocapsular structures.

4 ARTIGO 3

Novel Methotrexate derivative and its nanocapsules formulation as potential antitumor agents

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Este artigo refere-se ao estudo da ação *in vitro* do dietil éster de metotrexato e sua formulação nanoencapsulada em diferentes linhagens celulares submetido à revista Nanomedicine: Nanotechnology, Biology, and Medicine.

NOVEL METHOTREXATE DERIVATIVE AND ITS NANOCAPSULES FORMULATION AS POTENTIAL ANTITUMOR AGENTS

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Abstract

MTX is a widely used drug for treatment of many cancer forms. Considering drawbacks of its utilization, nanotechnology can allow therapeutic improvement. For increasing encapsulation of this drug in lipid core nanocapsule suspensions, the esterified derivative, diethyl ester of methotrexate ($MTX(OEt)_2$), was prepared. The efficacy of $MTX(OEt)_2$-loaded lipid core nanocapsules and of $MTX(OEt)_2$ solution were studied in three cancer cell lines: human bladder carcinoma 5637, human colorectal adenocarcinoma HT29 and human lung carcinoma A549. Cytotoxicity was also evaluated on the skin normal fibroblast cell line CCD-1059Sk. Cell viability and proliferation were determined by MTT assay, apoptosis was evaluated by flow cytometry, and expression of apoptotic related genes were investigated by qRT-PCR. We demonstrate a clear loss of cell viability after drug exposure in tested cancer cell lines, partly due to induction of apoptotic cell death indicated by flow cytometry and by overexpression of caspase-3 and caspase-8 genes, and also reduced expression of bcl-2 gene and increased bax/bcl ratio. No citotoxic effect was observed in the MTT assay for the tested normal cell line.

Keywords: Methotrexate, Methotrexate diester derivative, Nanocapsules, *in vitro* citotoxicity, chemotherapy

Background

Methotrexate (MTX) is a folic acid structural analog extensively used as an antimetabolite drug in cancer chemotherapy. This molecule acts by inhibiting the metabolic enzyme dihydrofolate reductase (DHFR), a key enzyme in cell replication [1]. By affecting intracellular folate metabolism and blocking the synthesis of pyrimidines and purines, MTX impairs nucleotide production leading to disruption of DNA synthesis and consequent cessation of cell proliferation. This drives to reduction of tumor growth and induction of cell death through secondary genotoxic effects or apoptosis [2,3]. Induction of apoptotic cell death is recognized as the major cytotoxic mechanism of anticancer therapies [4], and MTX has been shown to promote apoptosis [5,6,7] .However, this effect may

vary among different cell lines, for instance, although it was revealed that MTX inhibits non small cell lung cancer A549 cell growth via induction of apoptosis [6], HT29 colon cancer cells showed resistance to develop an apoptotic response [8].

The MTX is transpoted into cells mostly by the reduced folate carrier (RFC), than the drug becomes polyglutamylated to prevent efflux, followed by its cytotoxic effect resulting in depletion of intracellular reduced folates. MTX also presents inhibition of thymidylate synthase (TS) and *de novo* purine synthesis [1]. It was demonstrated that methotrexate can also affect the actin organization [5]. Besides, a novel function for MTX has been proposed, suggesting that it is able to inhibit histone deacetylase (HDAC) activity. HDAC is usually found in tumors and inhibitors can reactivate tumor suppressor genes and serve as potential anti-cancer drugs [9]. Treatment with high dose MTX was also found to induce senescence-like growth arrest [10].

Since the drug blocks the biosynthetic pathway of nucleotides and proteins, MTX can be toxic for both cancer and normal cells. This gives rise to dose-limiting side effects [1]. In addition, the use of MTX can be also limited by its low solubility, rapid diffusion throughout the body, short half-life in the bloodstream, and drug resistance [11,12]. Cancer cells acquire resistance to MTX through mechanisms that include: impairment of drug transport into cells by decreased RFC mediated uptake, increased drug efflux from cells, reduced polyglutamylation of the drug, and overexpression of DHFR [1,13,14]. Methotrexate resistance by certain cancer cells significantly restricts the effectiveness of the drug, and this phenomenon is of much concern.

Nanotechnology is particularly suitable for providing new drug delivery systems [15,16,17] and increases the interest in binding MTX to nanoparticles. This strategy may alter its pharmacokinetic behavior in order to enhance anti-tumor activitity, reduce toxicity, and overcome drug-resistance mechanisms [18,19,20,21]. Among various nanoparticulate systems, polymeric nanocarriers are of particular interest since drug-loaded nanoparticles prepared using biodegradable polymers have potential to provide solution for problems encountered in chemotherapy [22].

Nanoparticles can carry a large payload of drug and protect it from degradation. Besides, the nanoparticles mediated delivery could have the potential to bypass multidrug resistance mechanisms that involve cell-surface

protein pumps, as they enter cells via endocytosis [15]. Higher efficiency could be achieved due to the higher intracellular drug concentrations as compared to conventional drug administrations. Also, nanoparticle-based drug delivery can present various advantages over conventional chemotherapy, including specificity and solubility [23].

Hence, new formulations may allow therapeutic improvement. Obtaining nanostructured formulations containing MTX can potentially decrease the side effects of the drug, maximizing the therapeutic effects. The polymeric nanoparticles can be classed as nanospheres or nanocapsules, when a matricial or a vesicular structure is formed [24]. In the past few years, lipid-core nanocapsules (LNC) have been developed and studied as new nanocapsules for drug delivery and drug targeting [24,25]. The main difference between the polymeric nanocapsules and the lipid-core nanocapsules is the composition of their cores. The former has a liquid core composed of oil and the latter has a dispersion of sorbitan monostearate in triacylglyceride, an organogel, giving a more rigid structure for those nanocapsules [26]. Despite the low water solubility of MTX, their carboxylic and amine groups provide a negative log D value (pH 4.8) allowing a drug distribution mechanism type II when formulated in lipid-core nanocapsules [27]. In this case, the drug is manly dissolved in the dispersant phase and poorly adsorbed at the polymeric wall of the nanocapsules. With the purpose of increasing the distribution of the drug in the core of the nanocapsules (mechanism type III), the MTX diester derivative ($MTX(OEt)_2$) was prepared and encapsulated [27].

In vitro cell culture studies are valuable instruments for screening chemotherapeutic agents and for provide preliminary data for *in vivo* studies. The efficacies of $MTX(OEt)_2$-loaded lipid-core nanocapsules and of the free drug in solution were studied *in vitro* using three cancer cell lines: human bladder carcinoma 5637, human colorectal adenocarcinoma HT29 and human lung carcinoma A549. To the best of our knowledge, this is the first report to determine the anticancer effects of $MTX(OEt)_2$ in vitro. The present study also evaluated the cytotoxicity on a skin normal fibroblast cell line (CCD-1059Sk). The *in vitro* experiments were designed to establish the anti-tumor efficacy of the drug-loaded LNC. In parallel, we also aimed to verify if the unloaded nanocapsules present any cytotoxic effect in the cell lines studied.

Methods

Materials

Poly(ε-caprolactone) (PCL) (MW=14,000 g mol^{-1}) was supplied by Sigma-Aldrich (Strasbourg, France). Caprylic/capric triglyceride (CCT) and polysorbate 80 were obtained from Delaware (Porto Alegre, Brazil). Span 60® (sorbitan monostearate), dicyclohexylcarbodiimide (DCC), 4-(*N*,*N*-dimethyl)aminopyridine (DMAP) were obtained from Sigma Aldrich (St. Louis, USA). Methotrexate was supplied by Pharma Nostra (Anapolis, Brazil). All other chemicals and solvents used were of analytical or pharmaceutical grades. All reagents were used as received.

Methotrexate diethyl ester

The MTX diethyl ester was prepared as previously reported [27]. The strategy was based on the classical condensation of carboxylic acids and alcohols using DMAP (6 mg, 0.4 mmol) as catalyst and DCC (416 mg, 2 mmol) as coupling agent. Briefly, a solution of methotrexate (MTX) (455 mg, 1 mmol) in ethanol (30 mL) was added of DMAP. After 10 min under argon and magnetic stirring, the coupling agent was added at 0 ^{o}C. After the addition, the reaction was carried out at 40 ^{o}C for 90 h. The solvent was evaporated under reduced pressure at 35 ^{o}C and the residue dispersed in dichloromethane (30 mL). The product was isolated by filtration. The residue was discarded (dicyclohexylurea) and the filtrate was extracted with $NaHCO_3$ saturated aqueous solution (3 x 30 mL), dried with anhydrous Na_2SO_4, and evaporated under reduced pressure. The raw product was purified by preparative column chromatography using silica gel 60 (70-230 mesh) and chloroform/methanol (99:1 v/v) with traces of ammonium hydroxide as the eluent. A yellow solid was obtained (70%). The chemical identity of diethyl (2*S*)-2-[(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzoyl)-amino]-pentanedioate was confirmed by ^{1}H-NMR (INOVA-300, Varian, USA).

Lipid-core nanocapsules

Lipid-core nanocapsules were prepared using the methodology of self-assembling as previously described [25,28]. Poly(Ɛ-caprolactone) (0.100 g), sorbitan monostearate (0.038 g), capric/caprylic triglyceride (160 µL) and $MTX(OEt)_2$ (0.005 g) were dissolved in acetone (27 mL) at 40 °C. A turbid solution was instantaneously obtained by injecting this organic phase into an aqueous phase containing polysorbate 80 (0.077 g) dissolved in water (53 mL) at 40 °C. After 10 min, the acetone was evaporated and the suspension concentrated under reduced pressure at 40 °C. The final volume was adjusted to 10 mL in a volumetric flask. This formulation was named $MTX(OEt)_2$-LNC.

A blank formulation of lipid-core nanocapsules (LNC) was also prepared as control as described above but omitting the $MTX(OEt)_2$.

Particle size and size distribution of lipid-core nanocapsule aqueous suspensions

The mean volume-weighted diameters ($D_{4,3}$) were determined using laser diffraction (Malvern Mastersizer® 2000, Malvern Instruments, UK). Lipid-core nanocapsules and $MTX(OET)_2$-loaded lipid-core nanocapsules were directly inserted in the wet unit (distilled water) at room temperature. Measurements were carried out in triplicate (n = 3).

Quantification of $MTX(OET)_2$ in the LNC formulation

$MTX(OET)_2$ was quantified by high performance liquid chromatography (HPLC). The HPLC system consisted of a Perkin Elmer S-200 with an S-200 injector, a UV-VIS detector, a guard-column and a column (Spherisorb® ODS2, 150 mm × 4 mm, 4 µm, Waters, USA). Methotrexate ethyl ester was detected at 303 nm using a mobile phase (1.0 mL min^{-1}) of methanol/water (80:20 v/v) adjusted to an apparent pH of 4.0 ± 0.5 with 10% (v/v) acetic acid. The method was previously validated [27] considering the linearity (*r*=0,998), precision and repeatability (relative standard deviation <4%), accuracy (100 ± 1%), limit of quantification (1 µg mL^{-1}) and recovery data (101 ± 1).

The total drug content was determined by HPLC after dissolving an aliquot of the formulation in acetonitrile using a volumetric flask (10 mL). The

solution was filtered (0.45 µm, Millipore® and injected (20 µL). This experiment was carried out in triplicate batches (n = 3). The MTX(OET)$_2$ retention time was 22.4 minutes.

The encapsulation efficiency (*EE*) was determined by ultrafiltration-centrifugation using a Microcon® centrifugal filter device (10 kDa, Millipore®). An aliquot of the formulation was directly placed in the filter device for centrifugation at 1844 ×*g* for 5 min (Sigma® 1-14, Germany). The ultrafiltrate was analyzed by HPLC without dilution. The encapsulation efficiency was calculated using equation (1).

$$\%EE{=}Ct{-}CfC\%EE = \frac{C_t - C_f}{C_t} \quad (1)$$

where C_t is the total drug content and C_f is the drug concentration in the ultrafiltrate.

Cell culture

Human bladder carcinoma 5637, human colorectal adenocarcinoma HT29, human lung carcinoma A549, and human skin fibroblast CCD-1059Sk cell lines were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil), and routinely cultured in our laboratory. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin, purchased respectively from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA). Cells were grown at 37°C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth and all experiments were performed in triplicate. The treatments tested consisted of diethyl ester of methotrexate (MTX(OEt)$_2$) and MTX(OEt)$_2$-LNC formulation at concentrations ranging from 20 µM to 1.125 µM of MTX(OEt)$_2$, and also LNC at the equivalent amounts used for the drug nanoformulation.

Morphologic observations

Cells were grown on 6-well plates (2 × 10^5 cells/well) and treated with MTX(OEt)$_2$ solution and MTX(OEt)$_2$-LNC at concentrations of 20 µM, and also

with LNC at the equivalent amount. The morphological characteristics were observed under a inverted microscope Olympus IX71 (Olympus Optical Co., Ltd. Tokyo, Japan). Images were stored as TIFF files using a digital camera (Nikon, Tokyo, Japan) attached to the microscope.

Determination of cytotoxicity

Cell viability and proliferation were determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide] to water insoluble formazan. Cells were seeded in 96-well plates at a density of 2×10^4 cell per well in a volume of 100 µL and allowed to grow at 37°C in a 5 % CO_2 atmosphere for 24 h before drug treatment for the cell viability assay. Cells were then exposed to free $MTX(OEt)_2$ in solution and $MTX(OEt)_2$-LNC at appropriate amounts to obtain the concentrations of 20 µM, 10 µM, 5 µM, 2.25 µM, and 1.125 µM of $MTX(OEt)_2$, and also to unloaded LNC in the equivalent amount used for drug-loaded LNC. The $MTX(OEt)_2$ powder was dissolved in dimethyl sulfoxide (DMSO). Equal volumes of pure DMSO were used in control experiments showing no cytotoxicity. At time of experiment, all formulations tested were diluted in culture medium to obtain the various concentrations, cells with no treatment were kept as control, and incubation was performed for 48 h at 37°C in a 5 % CO_2 atmosphere.

After that time, the medium was aspirated, cells were washed with phosphate-buffered saline (PBS; Gibco), and a 5mg MTT/mL solution (180 µL of medium and 20 µL of MTT per well) was added to each well. The plates were incubated for 3h at 37°C, then the solution was removed and the produced formazan was solubilized by adding 200 µl of DMSO to each well followed by 20 minutes on a shaker at 150 rpm. The absorbance of each well was read on a microplate reader at a test wavelength of 492 nm. The inhibition (%) of cell proliferation was determined as follows: inhibitory growth = (1- Abs492 treated cells/Abs492 control cells) x 100% [29]. All observations were validated by three independent experiments in triplicates for each experiment.

Flow cytometric analysis of apoptosis

Cells were seeded in 6-well plates at a density of 2×10^5 per well and grown at 37 °C in a humidified atmosphere of 5% CO_2, 95% air for 24 h. The cells were then incubated for 48h with $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC at concentrations of 20 µM and 5 µM, and also with LNC at the equivalent amounts. Apoptotic cell death was evaluated by flow cytometry using Guava Nexin® Reagent kit following manufacturer's instructions, and analyzed in a Guava EasyCyte plus flow cytometer. After the 48h treatment, culture medium was removed and placed in a centrifuge tube. Cells were washed with PBS, which was also placed in the centrifuge tube. Then, cells were harvested by trypsinization, transferred to the tube and centrifugated at 300*g* for 7 minutes. The medium was aspirated, carefully to not disturb the pellet, and cells were ressuspended in fresh serum-containing medium. Cells were counted to adjust cell concentration and stained as instructions.

Early apoptotic cells can be identified by annexin V-PE binding, as annexin V has a high affinity toward phosphatidyl serine (PS) residues which are externalized from inner to outer surface of the cell membrane during early stages of apoptosis. On the other hand, the cell impermeant dye 7-AAD identifies late apoptotic and necrotic cells having damaged plasma membrane [30].

Quantitative Real-Time PCR (qRT-PCR)

The gene expression profiles of apoptotic related genes were investigated by qRT-PCR. Cells were seeded in 6-well flat bottom plates at a density of 2×10^5 per well and grown at 37°C in a humidified atmosphere of 5 % CO_2 for 24 h. The cells were then incubated for 48 h with $MTX(OEt)_2$ solution and $MTX(OEt)_2$- LNC at concentrations of 20 µM and 5 µM, and LNC in the amount equivalent to the higher drug-loaded nanocapsules concentration tested. After this period the cells were washed with PBS and RNA extraction was performed. Total RNA extraction, cDNA synthesis and qRT-PCR were conducted as previously [29,31]. Briefly, RNA was isolated using TRIzol Reagent (Invitrogen) and samples were DNase-treated with a DNA-free kit (Ambion, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 1 µg of RNA using the High Capacity cDNA Reverse

Transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. Realtime PCR reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK) and the primers described in Table 1, also used by Nedel *et al.* [31]. The housekeeping gene GAPDH was used as a control to normalize the levels of the other genes.

Data analysis

Data sets were analyzed using one-way ANOVA followed by a Tukey test for multiple comparisons. Significance was considered at $P<0.05$ in all analyses. Data were expressed as mean±SEM.

Results

$MTX(OEt)_2$-loaded lipid-core nanocapsules

The MTX diethyl ester derivative was obtained in 70% of yield. The NMR spectrum showed the characteristic signals attesting its chemical identity. The $MTX(OEt)_2$-LNC formulation was obtained as a liquid opaque solution with $D_{[4,3]}$ of 190 ± 33 nm and SPAN of 1.6 ± 0.1. The drug content and the encapsulation efficiency were 0.50 ± 0.06 mg mL^{-1} and 99± 1%. The control formulation (LNC) prepared without drug had $D_{[4,3]}$ of 206 ± 8 nm and SPAN of 1.8 ± 0.1.

Morphological changes

After 48 h incubation with $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC at a drug concentration of 20 µM, or with LNC at an equivalent volume, cells were observed under an inverted microscope and compared in a 200x increase (data not shown). Significant reduction on cell number per field caused by the drug treatments can be noticed. Distinct morphological changes can be detected in treated cells, what seems to be more severe for $MTX(OEt)_2$-LNC treatment. Cells treated with LNC demonstrated no significant reduction on cell number per field and no drastic morphological alterations.

Inhibition of proliferation

Human bladder carcinoma (5637), human colorectal adenocarcinoma (HT29) and human lung carcinoma (A549) cell lines were incubated with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-LNC or LNC for 48 h. As demonstrated in Figure 1, $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC showed *in vitro* cytotoxic activity in all three cell lines. $MTX(OEt)_2$-LNC inhibited more than 50% of tumor 5637 cells growth at concentrations of 20 µM (58.7%), 10 µM (57.8%), 5 µM (55.2%) and 2,5 µM (53.4%); and 20 µM (69.2%), 10 µM (67.1%) and 5 µM (60.1%) for HT-29 cells. For A549 cells $MTX(OEt)_2$-LNC treatment exceeded 50% growth inhibition only at 20 µM (58.8%), so this treatment appears to be more effective for 5637 and HT-29 cells than for A549 cells. For HT-29 cell line, drug loaded nanocapsules were significantly more effective than the free drug ($p<0.05$). Although LNC showed some inhibition of proliferation on HT-29 and A549 cells, such inhibition was significantly lower than inhibition promoted by loaded nanocapsules for HT-29 cell line, and was not at all observed for 5637 cell line. The volumes of DMSO used to solubilize $MTX(OEt)_2$ presented no cytotoxicity.

Cell proliferation was also evaluated on a non cancerous cell line to investigate selectivity of the treatments. Skin normal fibroblast CCD-1059Sk cell line was incubated with the treatments at the same conditions as tumor cells and there was no cell proliferation inhibition, as demonstrated in Figure 2.

Induction of apoptosis

The effect of $MTX(OEt)_2$ solution, $MTX(OEt)_2$-LNC and LNC on apoptosis in 5637, HT-29 and A549 cells was assessed by flow cytometry with annexin V-PE/7-AAD staining. The results showed in Figure 3 indicate that $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC significantly increased ($p<0.05$) the early apoptotic ratio in 5637 and HT-29 treated cells compared with untreated cells and cells incubated with LNC. Moreover, $MTX(OEt)_2$-LNC were significantly ($p<0.05$) more efficient in initiating apoptosis than the solution of the free drug. For A549 treated cells, only $MTX(OEt)_2$-LNC significantly increased early apoptosis percentage compared with the other groups ($p<0.05$). In regard of 5637 and HT-29 late apoptosis and dead cells, drug treatments were significantly different from untreated ($p<0.05$) cells but with less distinguishable differences between

groups. For A549 late apoptosis and dead cells rates, only $MTX(OEt)_2$-LNC treatment was significantly different from other groups ($p<0.05$). Concentration of 20µM was more efficient in initiating apoptosis than 5µM for $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC treatments in 5637 and HT-29 cells, and only for $MTX(OEt)_2$-LNC treatment in A549 cells.

Expression changes

The expression level of anti-apoptotic genes bcl-2 and survivin, pro-apoptotic gene bax, apoptosis-inducing factor (AIF) and Endonuclease G (Endo G) genes, and also caspase-3, caspase-8 and caspase-9 genes were evaluated by qRT-PCR. No significant differences in survivin, AIF and Endo G expressions were observed for all three cancer cell lines (data not shown). Whereas, for Bcl-2, mRNA expression levels in HT-29 cells treated with $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC significantly decreased compared to control cells (Figure 4a). Also, for A549 cell line, the Bcl-2 expression levels for cells exposed to $MTX(OEt)_2$-LNC were significantly lower than control (Figure 4a). Significant increases in bax expression were noted only for free $MTX(OEt)_2$ treatment in A549 cells (data not shown), nevertheless the bax/bcl ratio incresed substantialy for HT-29 and A549, compared to that observed for control cells (Figure 4b). Regarding caspases expression, initiator caspase-8 and effector caspase-3 were significantly upregulated in 5637 treated cells compared to control cells and cells exposed to unloaded nanocapsules (figure 4c). No significant caspase upregulations were observed for the other cell lines.

Discussion

MTX is an important antitumoral agent and improvement of its efficacy and reduction of undesirable effects through nanotechnology is of major interest. In the present study we evaluated the effect of a novel MTX derivative and the influence of its encapsulation in lipid-core nanocapsule aqueous suspension on different cellular models. Our results indicated selectivity and effectiveness of the drug and the $MTX(OEt)_2$-LNC against tumor cell lines. The

mechanism by which cytotoxicity is achieved seems to be in part by apopsosis induction.

Biological effects of nanoscale materials can vary greatly depending on the physicochemical properties and the cell type used as model in vitro systems [32]. The $MTX(OEt)_2$-LNC formulation demonstrated some improvement in effectiveness compared to the free drug. Regarding MTX, previous efforts to design nanoformulations demonstrated conflicting results. An attempt to bind MTX to maghemite biodegradable nanoparticles was successful in preserving the efficacy of the drug against cancer cells, however the drug loaded nanoparticles showed similar *in vitro* efficacy to that of free MTX without any significant improvement [33]. Other nanoparticles have been successfully formulated as delivery systems for the anticancer drug MTX and displayed cytotoxic effects against a cancer cell line [20,21].

Our microscopical observations made clear the inhibition of proliferation verified by the reduced number of cells per field, and also indicate substantial morphologic alterations induced by drug treatments. Treatment with MTX causes changes in the cellular metabolism that are manifested by increase in cell granularity and cell shape flattening [34]. The microscopical observations are in accordance with the results obtained when examining cell viability by MTT assay, where both drug treatments significantly inhibit proliferation in 5637 cell line, and drug loaded nanocapsules treatment significantly inhibit proliferation in HT-29 cell line. The effectiveness of treatments on viability test was reduced for A549 cell line for concentrations under 20µM. It has been stated that A549 cells lack of folic acid receptor [35], what compromises the mechanism of entrance in cells by MTX and may explain the lower cytotoxic effect, although uptake through an unknown endocytic pathway can take place but significantly different from the receptor mediated entry [35].

The inhibition observed for LNC on MTT assay in not in accordance with microscopical observations, where it is clear that there is not a reduction on cell number and no meaningful morphological alterations can be noted as compared to drug treated cells. Measurement of mitochondrial metabolic rate using MTT to indirectly reflect viable cell numbers has been widely applied [6,20,34]. However, metabolic activity may be changed by different conditions or chemical

treatments which can cause considerable variation in results reported from these assays [36].

For chemotherapeutic drugs, poor selectivity between neoplasic cells and normal cells, often leading to adverse toxicity and narrower therapeutic window, has been a huge problem [37]. It is remarkable the absence of cytotoxic effect by $MTX(OEt)_2$ treatments observed in the MTT assay for the normal cell line tested. This important selectivity towards cancer cells as compared to normal cells was also reported for MTX in the study conducted by Mazur et al. [5] which was attributed to a different organization and distribution of the tested cells actin cytoskeleton. Selectivity was also reported for MTX-mesoporous silica nanoparticles [19].

Apoptosis is considered a highly regulated cell defense mechanism that allows a cell to self-degrade in order to eliminate an unwanted or dysfunctional cell, and plays an important role in preventing tumor development [38]. In this study we demonstrate by flow cytometry that treated cells exhibited significantly higher rates of apoptosis than control cells (untreated and treated with unloaded nanocapsules). We observed more consistent results for early apoptosis, which is a good indicative of apoptosis since the kit used is not able to distinguish between late apoptotic cells and necrotic cells. Perhaps longer treatment incubation times could enhance and differentiate late apoptosis rates among treatments. Both $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC were able to increase early apoptosis rates in 5637 and HT-29 cells, but the drug-loaded LNC were significantly more efficient than the solution of the free drug. Since HT-29 colon cancer cells showed resistance to develop an apoptotic response by MTX [8] it is noteworthy that the $MTX(OEt)_2$-LNC formulation somehow assists in overcoming this resistance and induces better initial apopototic response than the $MTX(OEt)_2$ in solution. Although MTX have been shown to inhibit non small cell lung cancer A549 cell growth via induction of apoptosis [6], our results for A549 cells showed that only $MTX(OEt)_2$- LNC increased both initial and late apoptosis, what can be related to a facilitation on cell entry given by the nanoformulation [39]. In a previous study the level of MTX-nanoparticle induced apoptosis was higher than that observed with an equal concentration of free MTX [19], supporting the fact that nanotechnology can improve the effectiveness of therapies.

Although significantly different from control cells, the observed apoptotic rates were not very high, indicating that maybe another response is involved in the proliferation inhibition. This is in accordance with previous data stating that the MTX induced decrease in cell survival might be attributed either to a decrease in cell proliferation or to an increase in apoptotic cell death [40]. Also, drug treatment was proved to be capable of inducing cessation of proliferation leading to a senescence-like phenotype of cancer cells, and accelerated senescence was identified as the outcome of cells exposure to high doses MTX, which is considered an important factor contributing to the outcome of cancer therapy [41]. Most cells are capable of both responses to the damage, apoptosis and senescence, depending on the nature of stress signal [42]. It was suggested that senescence might be a response particularly important for the antiproliferative effect of differentiating agents [43].

Tumour suppression frequently involves the modulation of signal transduction pathways, leading to alterations in gene expression [44]. Many conventional anti-cancer therapies work primarily inducing apoptosis through regulating apoptosis-associated signaling. The intrinsic apoptotic pathway is mediated by the mitochondria and is mainly controlled by the balance and interactions between pro and anti-apoptotic members of the bcl-2 family proteins, which regulate the permeability of the mitochondrial membrane [45]. Anti-apoptotic protein Bcl-2 is located in the mitochondrial outer membrane where it inhibits pro-apoptotic molecule Bax to maintain the integrity of the mitochondrial outer membrane and prevent the release of Cyt-c and other apoptotic factors [46].

The expression of anti-apoptotic gene Bcl-2 was significatively downregulated by drug treatments in HT-29 cells and especially by drug loaded nanocapsules in A549 cells. It has been proposed that the ratio between bcl-2 and bax genes is more important in the regulation of apoptosis than the level of each bcl-2 family protein alone [47]. Our data indicated that the bax/bcl-2 ratio in HT-29 and A549 cell lines increased post-treatments with free $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC, manly for 20μM concentration, suggesting that bax and bcl-2 are involved in the apoptotic events associated with the cytotoxic effects of $MTX(OEt)_2$ treatment.

The proteases termed caspases play a central role in apoptosis. Caspase-8 is activated by the extrinsic apoptosis pathway and leads to caspase-3 activation which is a late signal that accomplishes apoptosis [48]. Caspase-3, member of the family of cysteinyl aspartate proteases, activation constitutes a crucial event for the entering the execution phase of apoptosis. However, there are reports demonstrating the existence of caspase-independent forms of cells death [49]. When activated, the caspases cleave a series of substrates, activate DNases and orchestrate cell death and its removal by scavenger macrophages [45]. A sharp increase in caspase-3 activity might be sufficient to trigger the induction of an irreversible death program [50]. The activation of Caspase-8 and Caspase-3 were substantially upregulated in 5637 drug treated cells. The results indicate the involvement of the caspase signaling pathway in the apoptotic cell death of 5637 cells treated with $MTX(OEt)_2$.

In conclusion, studies on the mechanism of the cytotoxic effect of MTX derivatives and novel nanoformulations on different cellular models are very important. In our experiments we used three different cancer cell lines to evaluate the effect of $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC on cell survival. We demonstrate for all tested cancer cell lines a clear loss of cell viability after drug exposure partly due to the induction of apoptotic cell death. $MTX(OEt)_2$-LNC formulation exhibited greater efficiency. However, further studies are needed to elucidate the mechanism by which $MTX(OEt)_2$-LNC modulates pathways that are crucial for cancer cell survival.

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Table 1. Primers used for qRT-PCR

Gene	Sequence 5′–3′
Bcl-2	For-GTGTGGAGAGCGTCAACC Rev-CTTCAGAGACAGCCAGGAG
Survivin	For-CTGTGGGCCCCTTAGCAAT Rev-TAAGCCCGGGAATCAAAACA
Bax	For-ATGCGTCCACCAAGAAGC Rev-ACGGCGGCAATCATCCTC
Caspase-3	For-CAGTGGAGGCCGACTTCTTG Rev-TGGCACAAAGCGACTGGAT
Caspase-8	For-GGATGGCCACTGTGAATAACTG Rev-TCGAGGACATCGCTCTCTCA
Caspase-9	For-CCAGAGATTCGCAAACCAGAGG Rev-GAGCACCGACATCACCAAATCC
Endo G	For-GTACCAGGTCATCGGCAAGAA Rev-CGTAGGTGCGGAGCTCAATT
AIF	For-GGGAGGACTACGGCAAAGGT Rev-CTTCCTTGCTATTGGCATTCG
GAPDH	For-GGATTTGGTCGTATTGGG Rev-TCGCTCCTGGAAGATGG

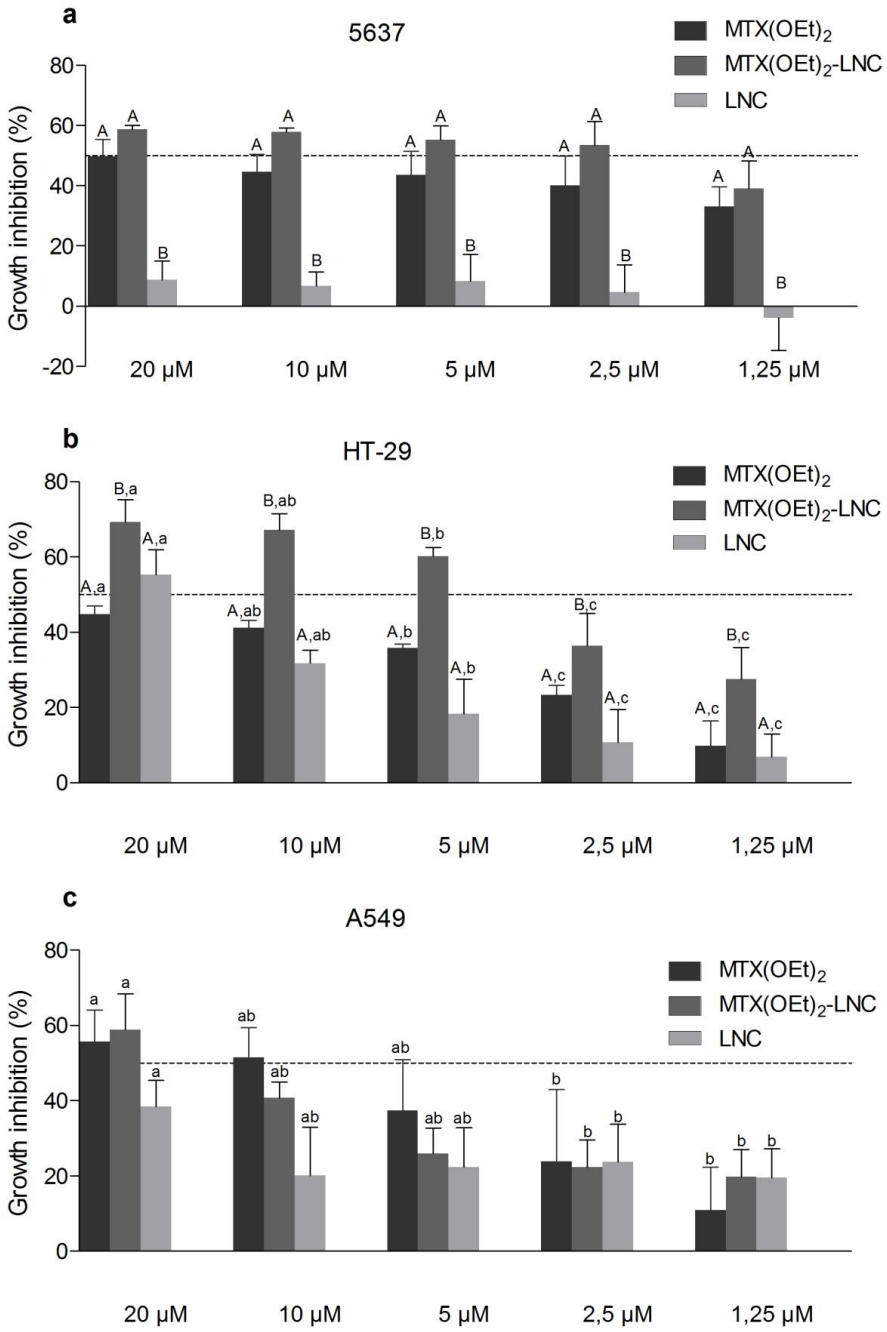

Figure 1. Inhibition of 5637 human bladder carcinoma (a), HT-29 human colorectal adenocarcinoma (b) and A549 human lung carcinoma (c) cell proliferantion after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Cell proliferation was measured by MTT assay. Data are expressed as means ± SEM from three independent experiments, each performed in triplicate. Distinct capital letters indicates significant differences between treatments, and lowercase letters represent differences between concentrations. Differences were considered significant at $P< 0.05$. Dashed line denotes 50% growth inhibition.

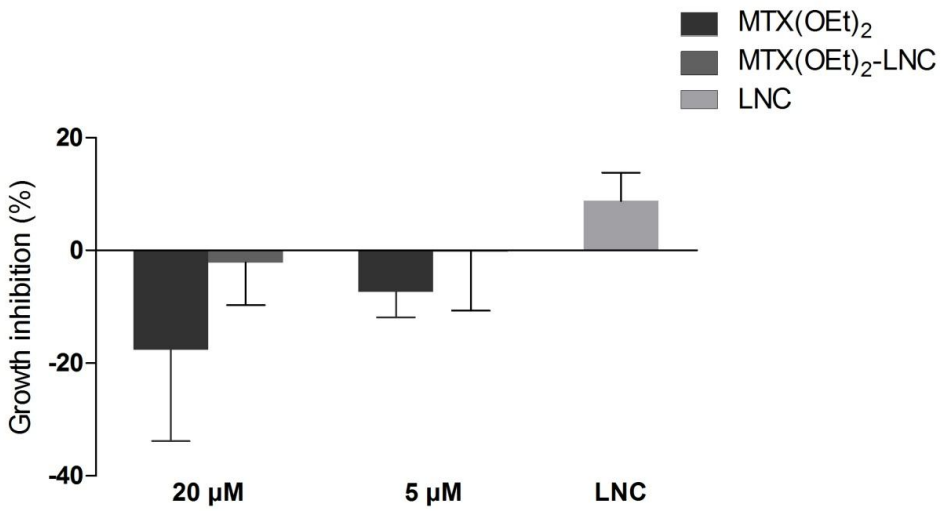

Figure 2. Inhibition of cell proliferation measured by MTT assay on CCD-1059Sk skin normal fibroblast cells after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Data are expressed as means ± SEM from three independent experiments, each performed in triplicate. There were no significant differences between groups (P >0.05).

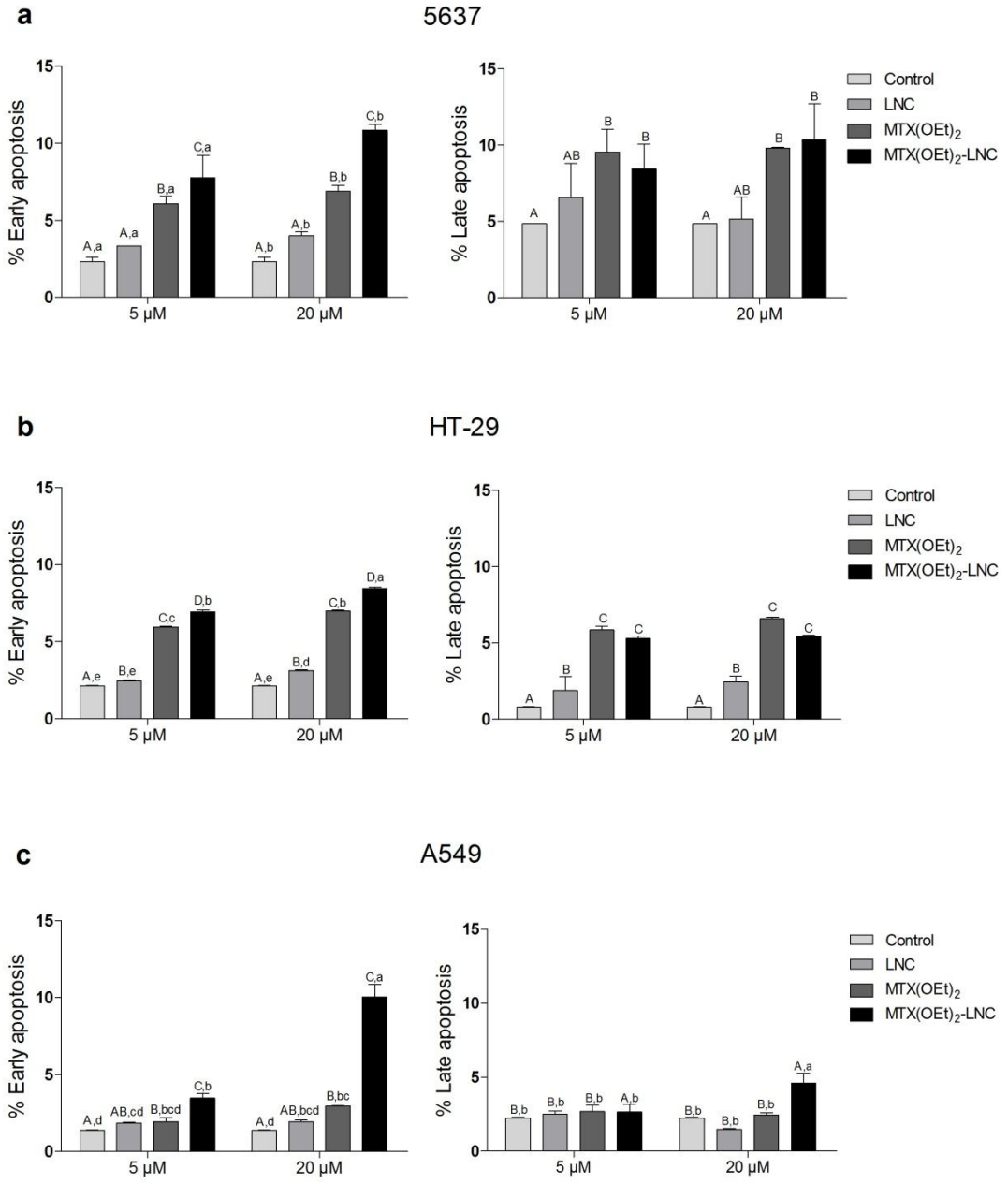

Figure 3. Early and late apoptosis percentages of 5637 human bladder carcinoma (**a**), HT-29 human colorectal adenocarcinoma (**b**) and A549 human lung carcinoma (**c**) cells after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Untreated cells were kept as control. Apoptosis was evaluated by Flow citometry with annexin V-PE/7-AAD staining. Data are expressed as means ± SEM from three independent experiments. Distinct capital letters indicates significant differences between treatments, and lowercase letters represent differences between concentrations. Differences were considered significant at $P< 0.05$.

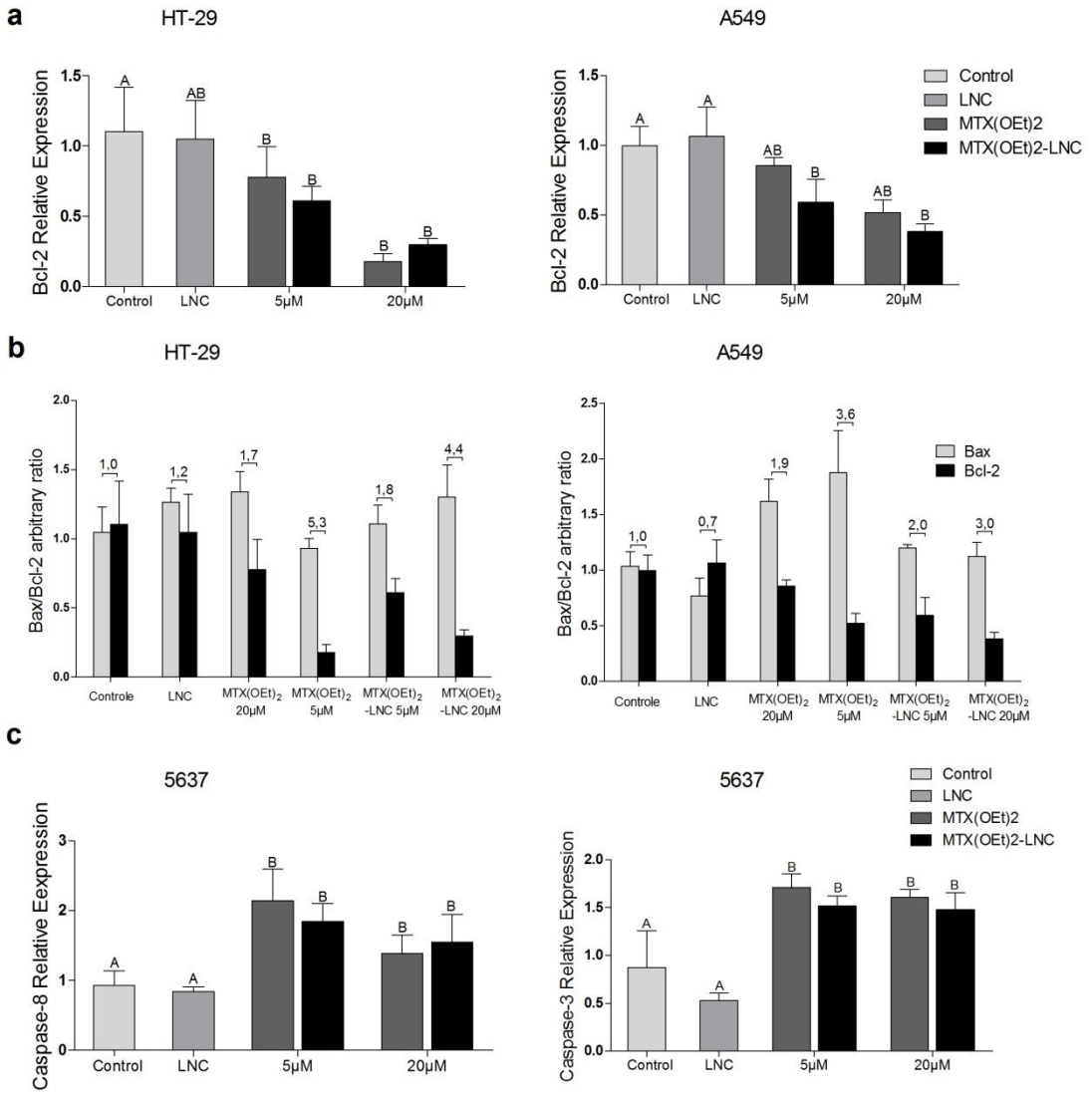

Figure 4. Gene expression profile determined by qRT-PCR after 48 h treatment with unloaded nanocapsules (LNC), $MTX(OEt)_2$ solution and $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC). Untreated cells were kept as control. **a** anti-apoptotic gene bcl-2 expression in HT-29 and A549 cell lines. **b** bax/bcl 2 arbitray ratio for HT-29 and A549 cell lines. **c** caspase-3 and caspase-8 expression levels for 5637 cell line. Data are expressed as means ± SEM from three independent experiments. Distinct letters indicates significant differences between treatments. Differences were considered significant at $P< 0.05$.

5 ARTIGO 4

Methotrexate derivative and its nanocapsules formulation against breast adenocarcinoma cell lines

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Este artigo refere-se ao estudo da ação *in vitro* do dietil éster de metotrexato e sua formulação nanoencapsulada em duas linhagens celulares de adenocarcinoma de mama e será submetido em formato de *short communication* à revista European Journal of Medicinal Chemistry.

METHOTREXATE DERIVATIVE AND ITS NANOCAPSULES FORMULATION AGAINST BREAST ADENOCARCINOMA CELL LINES

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Abstract

Breast cancer is the most frequent cancer affecting women. Methotrexate (MTX) is an antimetabolic drug that remains important in the treatment of breast cancer. Its efficacy is often compromised by resistance in cancer cells that occurs through a variety of mechanisms. This study evaluated apoptotic cell death and cell cycle arrest induced by a MTX diester derivative ($MTX(OEt)_2$) and $MTX(OEt)_2$-loaded lipid-core nanocapsules in two resistant breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231. The treatment responses were evaluated through flow citometry. We demonstrated significant differences in apoptotic response between treatments, and S-phase cell cycle arrest induced by $MTX(OEt)_2$ solution.

Keywords

Methotrexate derivative; breast cancer; resistance; apoptosis

Introduction

Breast cancer is the most frequent cancer affecting women. Most of the deaths associated with breast cancer are result of metastasis and its physiologic effects on morbidity and mortality [1]. Methotrexate (MTX) is an antimetabolic drug that remains important in the treatment of a variety of malignancies, such breast cancer [2]. MTX presents high structural homology to folic acid is able to bind folic acid receptor [3]. The mechanism of action consists of transportation into cells mostly by the reduced folate carrier (RFC), than the drug becomes polyglutamylated to prevent efflux, what effectively traps the molecule inside the cells, followed by its cytotoxic effect. MTX exerts its toxicity by competitively inhibiting the enzyme dihydrofolate reductase (DHFR). This enzyme is a key factor in DNA synthesis and cell division, due to its involvement in the production of reduced folate needed for the synthesis of thymydilate and purines [2, 3].

Unfortunately, efficacy is often compromised by resistance in cancer cells, which constitutes a major barrier in the effectiveness of anticancer therapy with MTX [4]. The resistance to MTX occurs through a variety of mechanisms that

include: reduction of MTX uptake by reduced folate carrier (RFC), increase of MTX efflux, a decrease of MTX polyglutamation, the over-expression of DHFR, among others [4, 5]. Transport of MTX is an essential determinant for generating a sufficient MTX intracellular concentration [6].

The breast cancer cell lines MCF-7 and MDA-MB- 231 both demonstrated resistance to MTX, but by distinct mechanisms. MCF-7 cells displayed an increased *dhfr* gene copy number [7], and overexpression of *dhfr* gene is an important mechanism of MTX resistance. The mRNA upregulation can be explained either by gene amplification of the *dhfr* locus or by an increase of *dhfr* transcription rate. The metastatic MDA-MB-231 cells lack expression of RFC [8] and therefore are highly resistant to MTX. Heavy promoter methylation was the underlying mechanism for the complete lack of RFC expression in MDA-MB-231 breast cancer cell line [9].

Aiming to bypass drug resistance, strategies may consist of chemical modification of methotrexate [10, 11] and of the design of new drug delivery systems. Nanocarriers have recently emerged as potential drug carriers in cancer therapy due to increased drug efficacy, low toxicity, and ability to minimize multi-drug-resistance mechanisms [12].

In vitro cell culture systems provide a rapid and effective mean to assess parameters and responses. This study evaluated apoptotic cell death and cell cycle arrest induced by a MTX diester derivative ($MTX(OEt)_2$) and $MTX(OEt)_2$-loaded lipid-core nanocapsules in two breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231.

Materials and Methods

Methotrexate diethyl ester

The MTX diethyl ester was prepared as previously reported [13]. Basically, the strategy was based on the classical condensation of carboxylic acids and alcohols using DMAP (6 mg, 0.4 mmol) as catalyst and DCC (416 mg, 2 mmol) as coupling agent. The chemical identity of diethyl (2*S*)-2-[(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzoyl)-amino]-pentanedioate was confirmed by ^{1}H-NMR (INOVA-300, Varian, USA).

Lipid-core nanocapsules

Lipid-core nanocapsules were prepared using the methodology of self-assembly as previously described [14, 15]. Briefly, Poly(Ɛ-caprolactone) (0.100 g), sorbitan monostearate (0.038 g), capric/caprylic triglyceride (160 µL) and $MTX(OEt)_2$ (0.005 g) were dissolved in acetone (27 mL) at 40 °C. The organic phase was injected into an aqueous phase containing polysorbate 80 (0.077 g) dissolved in water (53 mL) at 40 °C. A turbid solution was obtained as result. After 10 min, the acetone was evaporated and the suspension concentrated under reduced pressure at 40 °C. The final volume was adjusted to 10 mL. This formulation was named $MTX(OEt)_2$-LNC. A blank control formulation of lipid-core nanocapsules (LNC) was prepared as described above but omitting the $MTX(OEt)_2$.

The mean volume-weighted diameters ($D_{4,3}$) were determined using laser diffraction (Malvern Mastersizer® 2000, Malvern Instruments, UK). Lipid-core nanocapsules and $MTX(OET)_2$-loaded lipid-core nanocapsules were directly inserted in the wet unit (distilled water) at room temperature. Measurements were carried out in triplicate (n = 3).

Quantification of $MTX(OET)_2$ in the LNC formulation

$MTX(OET)_2$ was quantified by high performance liquid chromatography (HPLC). The HPLC system consisted of a Perkin Elmer S-200 with an S-200 injector, a UV-VIS detector, a guard-column and a column (Spherisorb® ODS2, 150 mm × 4 mm, 4 µm, Waters, USA). Methotrexate ethyl ester was detected at 303 nm using a mobile phase (1.0 mL min^{-1}) of methanol/water (80:20 v/v) adjusted to an apparent pH of 4.0 ± 0.5 with 10% (v/v) acetic acid. The method was previously validated [13] considering the linearity (r=0,998), precision and repeatability (relative standard deviation <4%), accuracy (100 ± 1%), limit of quantification (1 µg mL^{-1}) and recovery data (101 ± 1).

The total drug content was determined by HPLC after dissolving an aliquot of the formulation in acetonitrile using a volumetric flask (10 mL). The solution was filtered (0.45 µm, Millipore® and injected (20 µL). This experiment

was carried out in triplicate batches (n = 3). The $MTX(OET)_2$ retention time was 22.4 minutes.

The encapsulation efficiency (*EE*) was determined by ultrafiltration-centrifugation using a Microcon® centrifugal filter device (10 kDa, Millipore®). An aliquot of the formulation was directly placed in the filter device for centrifugation at 1844 ×*g* for 5 min (Sigma® 1-14, Germany). The ultrafiltrate was analyzed by HPLC without dilution. The encapsulation efficiency was calculated using equation (1).

$$\%EE=Ct-CfC\%EE = \frac{C_t - C_f}{C_t} \quad (1)$$

where C_t is the total drug content and C_f is the drug concentration in the ultrafiltrate.

Cell culture

Human breast adenocarcinoma MCF-7 and MDA-MB-231 cell lines were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil), and routinely cultured in our laboratory. MCF-7 cells were cultured in RPMI 1640 medium, purchased from Vitrocell Embriolife (Campinas, Brazil), supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. MDA-MB-231 cells were cultured in LEIBOVITZ L-15 medium, purchased from Cultilab, supplemented with 10% FBS, 1% L-glutamine and 1% penicillin and 0,2 mg/mL sodium bicarbonate. FBS was purchased from Gibco (Grand Island, NY, USA). Cells were grown at 37°C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth. Apoptosis experiments were performed in triplicate, and cell cycle experiments were performed in duplicate. The treatments tested consisted of diethyl ester of methotrexate ($MTX(OEt)_2$) and $MTX(OEt)_2$-LNC formulation at 20 µM and 5 µM concentrations and also LNC at the equivalent amounts used for the drug nanoformulation.

Flow cytometric analysis of apoptosis

Cells were seeded in 6-well plates at a density of 2×10^5 per well and grown at 37 °C in a humidified atmosphere of 5% CO_2, 95% air for 24 h. The cells were then incubated for 48h with $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC at concentrations of 20 µM and 5 µM, and also with LNC at the equivalent amounts. Untreated cells were kept as control. Apoptotic cell death was evaluated by flow cytometry using Guava Nexin® Reagent kit following manufacturer's instructions, and analyzed in a Guava EasyCyte plus flow cytometer. After the 48h treatment, culture medium was removed and placed in a centrifuge tube. Cells were washed with PBS, which was also placed in the centrifuge tube. Then, cells were harvested by trypsinization, transferred to the tube and centrifugated at 300*g* for 7 minutes. The medium was aspirated, carefully to not disturb the pellet, and cells were ressuspended in fresh serum-containing medium. Cells were counted to adjust cell concentration and stained as instructions.

Early apoptotic cells can be identified by annexin V-PE binding, as annexin V has a high affinity toward phosphatidyl serine (PS) residues which are externalized from inner to outer surface of the cell membrane during early stages of apoptosis. On the other hand, the cell impermeant dye 7-AAD identifies late apoptotic and necrotic cells having damaged plasma membrane [16].

Flow cytometric analysis of late apoptosis

Cells were treated with $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC at concentrations of 20 µM and 5 µM, and also with LNC at the equivalent amounts for 48 h. Untreated cells were kept as control. The Guava® TUNEL assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, cells were subjected to cells fixation procedure with 50 µL of 4% (w/v) paraformaldehyde in PBS for 60 min at 4°C and then with 200 µL of ice-cold 70% (v/v) ethanol at -20° C for at least 18 hours. For staining procedure, $1.5x10^4$ to $1.0x10^5$ of fixed cells was washed twice and was added to 25 µL of DNA Labeling Mix for 60 minutes at 37°C. At the end of the incubation time, cells were centrifugated and resuspended in 50 µL of the Anti-BrdU Staining Mix. Cells were incubated in the dark at room temperature for 30

min and samples were acquired on the flow cytometry (Guava® Flow Cytometry easyCyte™ System; Millipore Corporation™). In this assay, terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of BrdU residues into the fragmenting nuclear DNA of apoptotic cells at the 3'-hydroxyl ends by nicked-end labeling. TRITC-conjugated anti-BrdU antibody binds to the incorporated BrdU residues, labeling the mid- to late-stage apoptotic cells.

Cell Cycle analysis

Cell populations in the different phases of cell cycle (subG_1, G_0 /G_1, S, and G_2/M) was analyzed by flow cytometry in cells maintained in the presence or absence of MTX$(OEt)_2$ solution and MTX$(OEt)_2$-LNC at concentrations of 20 µM and 5 µM, and also of LNC at the equivalent amounts for 48h. Before treatment, cells were sincronized by removal of FBS suplementation of culture medium. Cell cycle evaluation was performed by propidium iodide (PI) sataining using Guava®Cell Cycle reagent kit, and analyzed in a Guava EasyCyte plus flow cytometer. After 48 h treatment, cells were detached, fixed and stained acordinding to manufactors prococol.

Data analysis

Data sets were analyzed using one-way ANOVA followed by a Tukey test for multiple comparisons. Significance was considered at $P<0.05$ in all analyses. Data were expressed as mean±SEM.

Results

MTX$(OEt)_2$-loaded lipid-core nanocapsules

The MTX diethyl ester derivative was obtained in 70% of yield. The NMR spectrum showed the characteristic signals attesting its chemical identity. The MTX$(OEt)_2$-LNC formulation was obtained as a liquid opaque solution with $D_{[4,3]}$ of 190 ± 33 nm and SPAN of 1.6 ± 0.1. The drug content and the encapsulation

efficiency were 0.50 ± 0.06 mg mL^{-1} and 99± 1%. The control formulation (LNC) prepared without drug had $D_{[4,3]}$ of 206 ± 8 nm and SPAN of 1.8 ± 0.1.

Induction of apoptosis

The effect of $MTX(OEt)_2$ solution, $MTX(OEt)_2$-LNC and LNC on apoptosis in MCF-7 and MDA-MB-231 cells was assessed by flow cytometry with annexin V-PE/7-AAD staining. The results showed in Figure 1 indicate that $MTX(OEt)_2$-LNC significantly increased (P<0.05) the apoptotic ratio in both cells compared with untreated cells. For MCF-7 cells, $MTX(OEt)_2$ solution and $MTX(OEt)_2$-LNC presented significantly higher (P<0.05) apoptotic rates than both untreated cells and cells incubated with LNC. For MDA-MB-231 cells, $MTX(OEt)_2$-LNC were significantly (P<0.05) more efficient in inducing apoptosis than the solution of the free drug. LNC treatment induced apoptosis in MDA-MB-231 cells, but this induction was significantly different than $MTX(OEt)_2$-LNC treatment group. Concentration of 20 µM was more efficient in inducing apoptosis than 5 µM.

Late apoptosis rates

The rates of late apoptosis induced by $MTX(OEt)_2$ solution, $MTX(OEt)_2$-LNC and LNC in MCF-7 and MDA-MB-231 cells was assessed by flow cytometry with Tunel staining. The results are show in Figure 2. There was no significant difference between groups for MCF-7 cells (P>0.05). For MDA-MB-231 cells, $MTX(OEt)_2$-LNC was significantly (P<0.05) more efficient than the solution of the free drug, but LNC treatment also demonstrated induction of apoptosis.

Cell Cycle analysis

The number of cells at the diferent phases of cell cycle (G_0 /G_1, S, and G_2/M) after treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-LNC and LNC, and also of untreated cells, was analyzed by flow cytometry with PI staining. The results shown in figure 3 demonstrate that only $MTX(OEt)_2$ solution, at both concentrations, significantly (P<0.05) altered the cell cycle, causing S-phase arrest.

Discussion

Recently, nanoparticulate drug delivery systems containing anticancer agents have received much attention [17, 18]. Nanotechnology can be suitable for providing advantages over conventional chemotherapy, since more efficient drug delivery can be achieved with higher intracellular drug concentrations. This approach can serve to minimize the development of MTX resistance by cancer cells, which remains a primary cause of therapy failure in cancer treatment.

We aimed to examine whether induction of apoptosis or alterations in the normal cell cycle were the possible molecular mechanisms involved in the antitumor activity of $MTX(OEt)_2$ and $MTX(OEt)_2$-LNC. Association of a MTX derivative in a lipid-core nanoformulation may increase cellular uptake and cytotoxicity. Nanocapsules could reduce the multidrug resistance that characterizes many anticancer drugs by a mechanism of cell internalization by endocytosis and by lowering drug efflux from the cells [19]. When cell internalization of the drug increases, the pharmacological action of the compound can be optimized [11].

The two breast cancer cell lines used in this study demontrated that diferent resistance mechaninsms can be exploited for design diferent aproaches. MCF-7 cell line presents MTX resistance by overexpression of DHFR [7]. In that sense, facilitation of drug transportation into cells by a nanoformulation does not seem to be the aproach that will overcome the resistance, although pharmacological action can be improved . Accordly, we did not observed more efficiency of $MTX(OEt)_2$-LNC compared with the solution of the free drug in MCF-7 cells.

On the other hand, MDA-MB-231 cell line presents MTX resistance by lack of transport receptors [8, 9]. For that cell line, $MTX(OEt)_2$-LNC treatment was much more effective than $MTX(OEt)_2$ demonstrating greater antitumor effects *in vitro*. Also, the results suggests that the molecular mechanisms associated with apoptosis within the highly tumorigenic MDA-MB-231 cell line is more sensitive to the nanoformulation elvaluated in this study. In this cell line we observed apoptosis induction by LNC without the presence of the drug. Another study showed that unloaded nanoparticles reduced cell viability and they attribuated this effect, most likely, to the combined effect of the free amines of the polymer

at the nanoparticle surface and the cytotoxic potential of the surfactant by itself [20].

In normal cells, the cell cycle is controlled by a complex series of signaling pathways by which a cell grows, replicates its DNA and divides [21]. It was demonstrated that a MTX nanoformulation arrested the cell cycle in the S-phase [20]. In contrast, we were not able detect any significant alteration in cell cycle caused by our $MTX(OEt)_2$ -LNC tratment. However, $MTX(OEt)_2$ solution caused cell cycle arrest in S-phase.

It is relevant to search for new effective drug delivery systems. This study aimed to investigate whether the association of $MTX(OEt)_2$ and a lipid-core nanocapsules formulation could increase antineoplastic effects in resistant cell lines. Relying on the mechanism of resistance, improvement can be achieved. Further study models are required to understand the pharmacokinetic and toxicological profiles of the formulation as well as to investigate its therapeutic efficacy.

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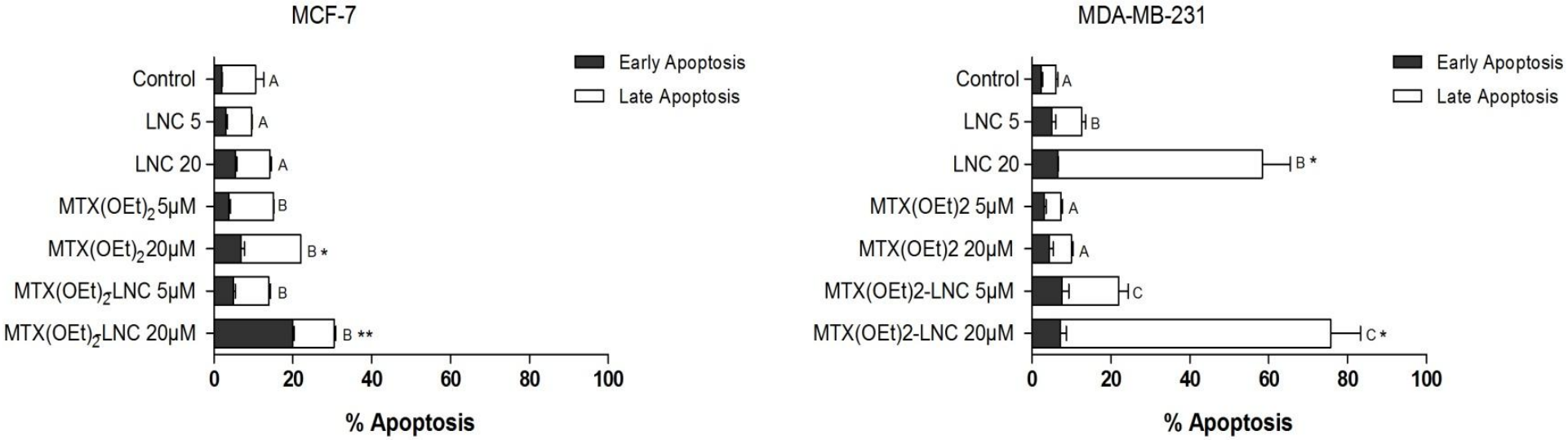

Figure 1. Early and late apoptosis percentages of MCF-7 human breast adenocarcinoma and MDA-MB-231 human breast adenocarcinoma cells after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Untreated cells were kept as control. Apoptosis was evaluated by flow cytometry with annexin V-PE/7-AAD staining. Data are expressed as means ± SEM from three independent experiments. Distinct capital letters indicates significant differences between treatments groups, and * indicates significant differences considering treatment and concentration. Differences were considered significant at $P< 0.05$.

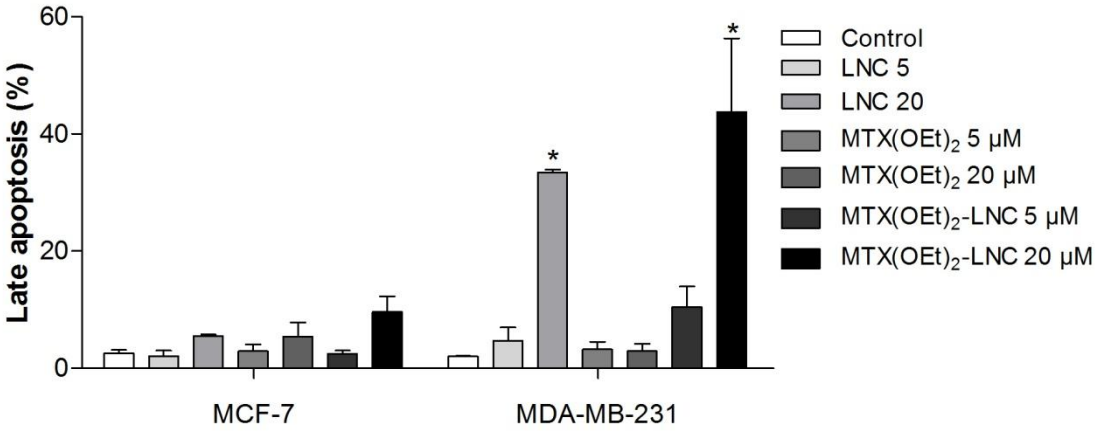

Figure 2. Late apoptosis percentages of MCF-7 human breast adenocarcinoma and MDA-MB-231 human breast adenocarcinoma cells after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Untreated cells were kept as control. Late apoptosis was evaluated by flow cytometry with Tunel staining. Data are expressed as means ± SEM from three independent experiments. * indicates significant differences considering treatment and concentration. Differences were considered significant at $P< 0.05$.

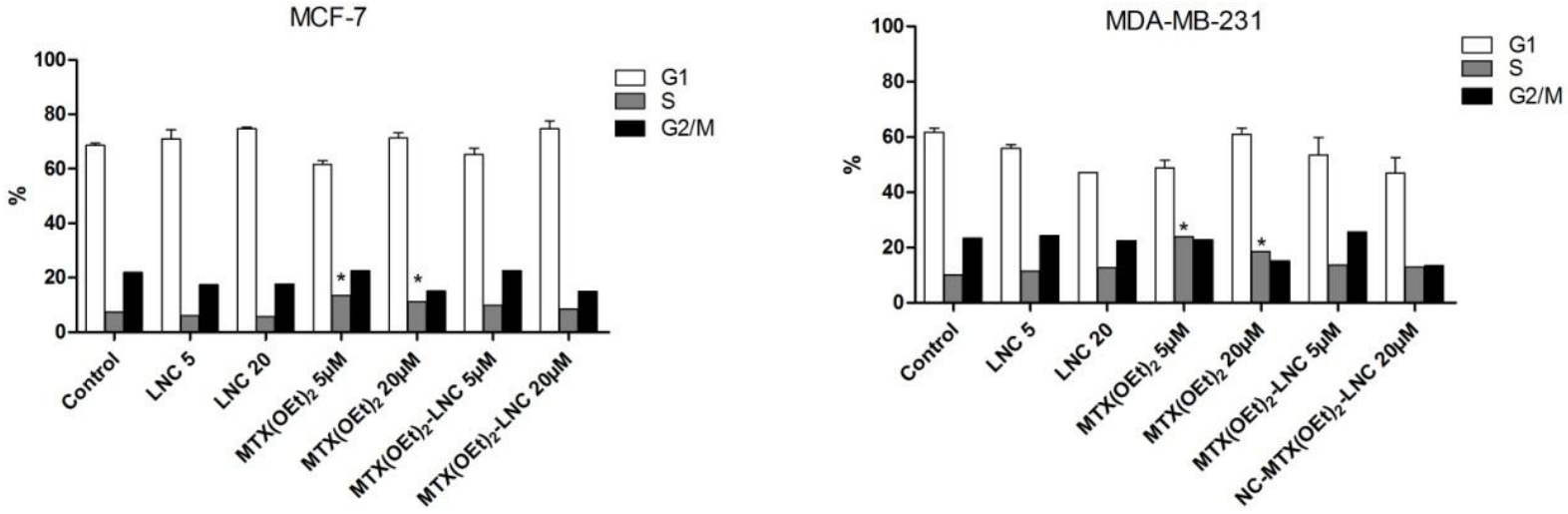

Figure 3. Number of MCF-7 human breast adenocarcinoma and MDA-MB-231 human breast adenocarcinoma cells in each phase of the cell cycle (G1, S, G2/M) after 48 h treatment with $MTX(OEt)_2$ solution, $MTX(OEt)_2$-loaded nanocapsules ($MTX(OEt)_2$-LNC) and unloaded nanocapsules (LNC). Untreated cells were kept as control. Cell cycle was evaluated by flow cytometry with propidium iodide staining. Data are expressed as means ± SEM from two independent experiments. * indicates significant differences between treatment groups within the phase. There were no significant differences between groups in G1 and G2/M phases. Differences were considered significant at $P< 0.05$.

6 CONCLUSÃO

1. A aplicação da nanotecnologia na área da saúde, especialmente na terapia do câncer, é um campo extremamente promissor.

2. A incorporação do éster dietílico de metotrexato na suspensão de nanocapsulas de núcleo lipídico apresentou habilidade em melhorar o efeito antitumoral *in vitro* do composto.

3. A interação da nanoformulação com a membrana das células ainda deve ser melhor avaliada.

4. Os testes utilizados complementaram-se e mostraram-se eficazes em determinar as respostas aos tratamentos.

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