



**Faculdade de Ciências da Saúde da
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ELIANA MARIA BARBOSA SOUTO

SUMÁRIO DA LIÇÃO

TARGETED LIPID NANOCARRIERS FOR THERAGNOSTICS

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1. INTRODUCTION

During the last years we have been witnessing the rising interest in the development of nanotechnology and its applications in medicine – a field currently known as “Nanomedicine”. The European Science Foundation¹²⁶ (ESF) defines Nanomedicine as the use of nanotechnology to improve healthcare, more specifically “the science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain and of preserving and improving human health” [1].

One of the most promising areas within the nanoscale technologies is that focusing on the design and development of nanocarriers for site-specific drug delivery and cancer diagnosis. Several types of nanocarriers have demonstrated usefulness for these purposes, being liposomes, micelles and microemulsions, so far the most exploited [2].

The success of chemotherapy lies on increased localized concentration of the drug in the vicinity of the tumour cells; otherwise, the treatment will result in non-discriminate drug distribution and severe adverse site toxicity. Localized drug release can be accomplished when using site-specific delivery systems, such as nanocarriers that target the drug to the tumour site while limiting exposure to non-target tissues and organs. In addition, given the recent advances on molecular imaging techniques (e.g. PET¹²⁷, MRI¹²⁸, SPECT¹²⁹, and optical imaging) [3, 4], for tumour detection and image resolution, novel nanocarriers can be developed to detect the tumour tissue and simultaneously delivery the anti-cancer drug – an emerging strategy currently known as “Theranostics”, *i.e.* “*Thera*” (prefix from “Therapy” or *θεραπεία*¹³⁰) and “*gnostics*” (suffix from “Diagnostics” or *διάγνωσης*¹³¹). These nanocarriers may act as vectors for the drug when the matrix solely behaves as the carrier [1], whereas in other cases the matrix is itself simultaneously the carrier and the drug [5].

To design a successful Theragnostic tool, some special attributes should be critically addressed [5, 6], namely: (i) the “stealth” properties of the nanocarrier to evade the immune system, (ii) the low toxicity of the nanocarrier, (iii) possibility of being traceable within the body and after administration, (iv) display controlled/sustained pharmacokinetic profiles, (v) appropriate half-life time within the blood; (vi) biodegradability, and (vi) selectivity to the target neoplastic tissue. An increasing number of publications suggests that nanocarriers may have more than one useful function by combining simultaneously, e.g. long-term stability, target ability, stimuli-sensitivity, and contrast properties [7].

Among several nanocarriers, those composed of lipid materials have been receiving particular attention over others of e.g. polymeric nature, because of better biocompatibility, *in vivo* tolerability, biodegradability with minimum and acute/chronic toxicity [8, 9]. In addition, nanocarriers composed of lipids allow the loading of a variety of imaging agents, ranging from fluorescent molecules to chelated metals and nanocrystals [2].

Application of nanocarriers for molecular imaging of cancer is currently in the pipelines of scientific discussions. In recent years, a large number of lipid nanocarriers have been tested for anti-cancer delivery and diagnosis, e.g. lipoplexes [10, 11], solid lipid nanoparticles (SLN) [12, 13], nanostructured lipid carriers (NLC) [14, 15], lipid-drug conjugates (LDC) [16, 17], self-emulsified drug delivery systems (SEDDS) [18], and hybrid structures containing inorganic nanocrystals (e.g. quantum dots (QDs) [19, 20], gold nanoparticles (AuNP) [21], iron oxide cores [22, 23]). Many of these carriers may show multimodal imaging ability (*i.e.* facilities of being used with more than one imaging technique), differing in size, morphology, and specificity for biological markers. The possibility of tackling physiological events at the molecular level offers unique opportunities to evaluate the function and dysfunction of living organisms. The developments of a variety of nanocarriers loading molecular imaging agents have made it possible to non-invasively image the specific biological interactions between the carrier and the target, the

¹²⁶ <http://www.esf.org/home.html>

¹²⁷ PET, Positron Emission Tomography

¹²⁸ MRI, Molecular Resonance Imaging

¹²⁹ SPECT, Single Photon Emission Computed Tomography

¹³⁰ *θεραπεία*, from the ancient Greek meaning “*therapeia*” *i.e.* “service, medical treatment”

¹³¹ *διάγνωσης*, from the ancient Greek meaning “*diagnōsis*”, *i.e.* “apart and discern”

pharmacokinetic profile (*i.e.* drug release kinetics), biodistribution, and therapeutic efficacy of many chemically different drugs.

The present lecture addresses the current state-of-art knowledge in the field of cancer diagnosis and treatment using targeted nanocarriers composed of lipid materials. The selected topic falls within the scope of the Nanotechnologies and Nanomedicines, an emerging area of new knowledge that should be included in the academic curricula of Health Sciences courses. This lecture will start by disclosing the main limitations of current cancer chemotherapy, highlighting the advantages and special features of using targeted nanocarriers to address this problem. Most relevant examples of these carriers will be discussed in terms of development and characterization. Given their suitability for a variety of biomedical applications, solid lipid nanoparticles (SLN)¹³² will receive particular emphasis, pointing out successful achievements of these carriers in the area of drug delivery.

2. TARGETING NANOCARRIERS IN CANCER

The effectiveness of cancer chemotherapy requires understanding not only the mechanisms behind neoplastic cell growth, but also the pharmacological mechanisms of drug action, its pharmacokinetic and biopharmaceutic profile, and the phenomenon of multi-drug resistance (MDR) [24]. MDR is many times responsible for cancer relapse and, in many cases, patients' death. It is referred as a state of resilience against structurally and functionally unrelated drugs, and it can be inherent or acquired whether is intrinsic to the patient or as a result of continuous anti-cancer treatment [25]. Finding a successful anti-cancer therapy will firstly be dependent on understanding this MDR evading mechanisms that contributes to the persistence of the disease, in spite of high dose and combined chemotherapy. In addition, a large number of potential targets for anti-cancer drugs have been recently identified opening new opportunities in anti-cancer therapy and diagnosis.

From a pharmacological point of view, anti-cancers have different mechanisms of action which make them appropriate to one type of cancer or to another. Some drugs induce cytotoxic effects during specific phases of the cell cycle, whereas others show non-specific cytotoxic activity. Differences in the mechanism of action are therefore clinically relevant in cancer chemotherapy. Cell cycle non-specific agents are anti-cancer drugs being active at all or any phases of the cell cycle, whereas cell cycle specific agents are drugs affecting only one part of the cell cycle or only when the cell is in a specific part of the cell cycle [26]. Cell cycle non-specific agents (*e.g.* alkylating agents and platinum derivatives) usually have linear dose-response curves; therefore, when increasing the dose, it will increase cytotoxicity. On the other hand, the concentration dependent effects of cell cycle specific agents (such as anti-metabolites (*e.g.* fluorouracil and methotrexate) which are more active against S-phase cells, and taxanes (*e.g.* paclitaxel, docetaxel) and vinca alkaloids (*e.g.* vincristine, vinblastine) which are more active against M-phase) usually depict a plateau since only a subset of proliferating cells remain sensitive to drug-induced cytotoxicity. As a result, the chemotherapy efficiency will not be improved by increasing the dose, but by prolonging the drug release and tissue exposure to the anti-cancer.

Lipid nanocarriers have been referred to improve the therapeutic profile and effectiveness of conventional anti-cancer drugs, by providing a controlled/sustained release over time. However, it should also be pointed out that anti-cancers that interfere with cell DNA¹³³ will not only be toxic to the neoplastic tissue, but will also affect non cancerous cells, leading to adverse side effects. New strategies to diagnose, target, deliver and release the drug in a controlled way are still needed. These include micelles, liposomes, lipoplexes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and lipid-drug conjugates (LDC).

Micelles and liposomes are spherical vesicles produced from phospholipids. They differ from each other since the former is based solely on one phospholipid monolayer, whereas liposomes are self-closed structures formed by

¹³² SLN, Solid lipid nanoparticles, the main research topic of the author during the last 9 years.

¹³³ DNA, Deoxyribonucleic acid

one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. Both are attractive carriers for molecular resonance imaging (MRI) contrast agents, and to load both hydrophilic and lipophilic drugs.

Micelles composed of PEGylated lipids¹³⁴ have been recognized of particular relevance to load poorly soluble drugs. For diagnostic purposes, Mulder *et al.* developed paramagnetic and fluorescent micelles surfaced with PEG and exposing maleimide moieties to enable functionalization [2]. These PEGylated micelles depicted a mean diameter of 15 nm being interesting for molecular imaging of extravascular targets, since they overcome the dysfunctional endothelial barrier, and reach the tumour.

Iron oxide cores can be loaded within PEGylated micelles following functionalization by subsequent conjugation with ligands. Fluorescent dyes may also be included in the structure. Mulder *et al.* has reported the usefulness of these nanocarriers for the detection of apoptotic cells by conjugating Annexin A5 [2]. This protein has high affinity for phosphatidyl serine, a phospholipid exposed in the outer leaflet of the plasma membrane of apoptotic cells [2]. Fluorescent dyes may be useful *in vivo* for detecting apoptotic cells and tumour treatment. Micelles can also be loaded with paramagnetic quantum dots and their PEG chains functionalized with certain ligands for site-specific targeting [27].

The first milestone however to confirm the clinical usefulness of lipid nanocarriers was published by Felgner *et al.* who described a DNA transfection protocol using small unilamellar liposomes composed of a cationic lipid [28]. For gene delivery, the bilayer of liposomes should have a lipid component (usually a cationic lipid and/or a fusogenic lipid) and cholesterol. Some liposomes may also be surfaced with PEG chains [29]. The aqueous compartment and the lipid bilayer have both been used to carry anti-cancer drugs. The most typical examples of anti-cancers loaded in liposomes include anthracyclines (Doxil®, Caelyx®, Myocet®) [30-32], taxanes (Taxol®, Taxotere®), anti-metabolites (e.g. methotrexate [33], 5-fluorouracil [34, 35]), alkylating agents (e.g. temozolomide [36], cytarabine [37]), Vinka alkaloids [38, 39], and topoisomerase inhibitors [40-43]. Liposomes have reported to increase the *in vivo* stability of the anti-cancers and protection from biodegradation [42, 43], to increase tumour uptake reducing the adverse side effects [44, 45], and improving the pharmacokinetic profile [46]. For MRI contrast agent, PEGylated liposomes were produced labelled with fluorophores to be tracked by fluorescence analysis [47]. The carrier was functionalized with maleimide to allow covalent linkage with thiol-exposing molecules via the sulfhydryl-maleimide-coupling method. When surfaced with monoclonal antibodies (*i.e.* H18/7¹³⁵, which has high affinity for E-selectin), the nanocarrier was preferentially linked to stimulated endothelial cells.

Functionalizing the nanocarriers may also allow manipulating the anti-cancer pharmacokinetic profile. Van Tilborg *et al.* produced liposomes cross-linked upon incubation with avidin [48]. Biotinylated liposomes were removed from circulation upon infusion of avidin by intravenous administration. The active avidin-induced clearance of the nanocarriers from the circulation allowed assessing the targeting kinetics, the specificity of signal changes by MRI, as well as vascular leakage and permeability.

For gene delivery and targeting there are two main types of self-assembled lipid nanocarriers, namely, (i) by mixing a positively charged liposome with the negatively charged DNA or siRNA¹³⁶ to form a lipoplex (extensively used for *in vitro* gene transfection or silencing); or (ii) by developing lipopolyplex nanoparticles such as the liposome-polycation-DNA (LPD) [49]. The first lipoplex mediated *in vivo* tumour siRNA was developed by Verma *et al.* [50], delivering via intraperitoneal injection the commercially available oligofectamine to a HCT116 colon cancer xenograft model. The authors reported the successful reduction of β -catenin expression and inhibition of HCT116 tumour growth. Systemic siRNA delivery was published by Sorensen *et al.* using DOTAP¹³⁷ liposomes [51]. Despite these relevant findings, lipoplexes are not physicochemically stable on the shelf, requiring fresh

¹³⁴ PEGylated, conjugates of lipids with polyethylene glycol (PEG) chains

¹³⁵ H18/7 F(ab')₂ E-selectin monoclonal antibody conjugated to cross-linked iron oxide nanoparticles

¹³⁶ siRNA, small interfering ribonucleic acid

¹³⁷ DOTAP, N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane

preparation before administration. Therefore, improved formulations are required to target siRNA to solid tumours through intravenous administration.

LPD consist of a solid core inside a lipid bilayer [52]. Several methods have been described to develop these carriers where a high molecular weight calf thymus DNA to condense DNA or siRNA into a solid core may be used [53, 54], as well as poly-L-lysine to condense shRNA encoding plasmid DNA or siRNA into their octaarginine modified [55, 56].

Other lipid nanocarriers have been developed in the last 20 years to overcome the main problems of membrane stability and drug leakage associated with liposomes, lipoplexes, and related structures. These include SLN and NLC. These lipid nanocarriers are derived from o/w emulsions replacing the liquid lipid (oil) by a lipid being solid both at room and body temperature [57, 58]. The particle matrix needs to be in the solid state, and its melting temperature can be adjusted by the choice of the lipid. Depending on the administration purpose, it varies between 50°C (122°F) and up to almost 100°C (212°F).

In the mid-1980s, Speiser developed the first micro and nanoparticles, called “nanopellets”, composed of solid lipids for oral delivery [59], and since then lipids became attractive materials for drug delivery. SLN are characterized as being colloidal particles having sizes between 50 to 500 nm, depending on the method and materials employed for production. They were introduced in the early 1990s by Müller *et al.* [60], who produced them by a high pressure homogenization method and by Gasco, produced by the dilution of a warm microemulsion [61]. Advantages of SLN are the use of biodegradable physiological lipids, the bioavailability improvement of poorly water-soluble drugs, and protection of chemically labile agents from degradation. The carriers are produced in aqueous dispersions stabilized by surfactants, such as as polysorbates, poloxamers and tweens. With respect to drug loading, SLN show some limitations e.g. the risk of expulsion of the encapsulated drug during storage and relatively low payload. This expulsion phenomenon occurs over time due to the polymorphic changes the matrix suffers during the shelf-life, which changes the crystallization pattern of the lipid entrapping the drug. The step from an imperfect crystalline lattice of the lipid to a more perfect lipid crystalline structure leads to drug expulsion [62, 63]. Therefore, the loading capacity of drugs depends not only on the physicochemical properties of the drug, but also on the miscibility, lipid solubility of the drug, type of matrix material, the crystallinity degree and polymorphic form [63-65].

The limitations of drug loading with SLN can be minimized using NLC, which are characterized by the creation of a lipid particle matrix with many imperfections in their lattice to load a higher amount of drug. A blend of solid lipid and liquid lipid is used to produce NLC, remaining solid at temperatures up to 40°C. NLC show considerable crystal disorder, providing higher drug loading and less drug expulsion during storage [65]. However, controlled release properties of NLC may be compromised due to the decrease in diffusion length of the lipid matrix due to the presence of the liquid lipid. Modifying the ratio between solid and liquid lipids allows redefining the release profile and pharmacokinetics of the drug loaded in the matrix [65].

Due to their lipophilic nature, SLN and NLC present a great advantage for highly efficient incorporation of hydrophobic drugs, but with only a moderate loading capacity for hydrophilic drugs. To overcome this limitation, LDC were developed [66]. In this case, the hydrophilic drug is transformed into a more hydrophobic molecule by conjugation with a lipid molecule. LDC show advantages for oral and parenteral administration. After oral administration, the bioavailability will be enhanced in comparison to the administration of micronized conjugate powder. Upon intravenous delivery, LDC allow drug targeting and distribution according to the partition coefficient of the drug [67]. LDC can be prepared by salt formation or alternatively by covalent linkage. Most of the lipid conjugates melt between 50–100°C. These conjugates are melted and dispersed in a hot stabilizing agent solution. LDC may be recognised as a nanosuspension of a pro-drug.

3. ADMINISTRATION ROUTES FOR ANTI-CANCERS LOADED SLN

Anticancer formulations based on SLN may be administered orally, intravenously, or intraperitoneally, therefore their profile will depend on the selected route.

Oral delivery is the easiest and the most convenient route for drug administration; however, several drugs are not administered orally because they are not absorbed or are degraded in the gastrointestinal tract. SLN have been proposed to overcome these problems. For oral administration, SLN have shown ability to enhance the oral bioavailability of a variety of drugs by physicochemical (e.g. drug solubility enhancement) or by biochemical (e.g. efflux transporters inhibition) mechanisms [68, 69].

Several factors are responsible for controlling the intestinal absorption, e.g. nanocarrier' size and structure, nature of the lipid material, and the employed surfactants [70]. Particle size reduction is a key factor for improving the peroral performance of SLN of poorly soluble drugs, resulting in increased surface area and saturation solubility, and allowing to reach high concentrations of SLN in the gastrointestinal tract. Due to their small particle size, SLN also exhibit bio/mucoadhesive properties to the wall or enter the intervillar spaces, thus increasing their residence time in the gastrointestinal tract. This adhesion enhancement will result in improved bioavailability.

The physicochemical mechanism by which SLN increase oral bioavailability of drugs relies on the simultaneous administration of lipid and drug, where the lipids are degraded by the enzymes in the gut forming surface active mono and diacylglycerols, which can improve the solubility of poorly soluble drugs [69]. Interaction with bile salts takes place, leading to the formation of mixed micelles, which will promote drug absorption. The lipids and the drug are simultaneously taken up by the gut wall, following a mechanism of absorption promotion effect. For a maximum bioavailability enhancing effect, the drug should be closely associated with the lipid. When the lipid is degraded to surface active molecules (*i.e.* molecules with tensioactive properties), the drug should be simultaneously present to be solubilized. The drug should be preferentially dissolved (molecularly dispersed) in the lipid to be digested. Movements of the gut wall in combination with the presence of surface active molecules, such as bile salts, transfer oils and fat to a coarse emulsion [71]. The emulsion droplets are then degraded. Degradation and subsequent solubilization of drugs is faster if the droplets are in the nanometer size range. Close association of the drug with the lipid and ultrafine dispersion in the nanometer range are obtained with SLN.

Nanoparticles in general possess adhesive properties; therefore, the absorption enhancing effect of orally administered SLN are also expected. Drug should be released exactly at the place of its absorption, leading to a higher concentration gradient between gut wall and blood. In addition, SLN are degraded by the lipases. Degradation is relatively fast due to the large surface area of SLN. Lipase/co-lipase complex degrades SLN in a similar mechanism as the liquid lipids [72-74]. In case of drug being molecularly dispersed in the solid lipid matrix (solid solution), during the *in situ* formation of the surface active mono- and diacylglycerols the drug being present is solubilized in the acylglycerols micelles. Subsequently, the acylglycerols micelles can directly lead to absorption of lipid and drug. Alternatively, they can interact with bile salts leading to mixed micelles prior to absorption.

Transport through endothelial tight junctions, endocytosis by the epithelial membranes, and M-cell mediated transport has also been proposed to explain the mechanism of drug absorption by SLN in the gut. The main SLN uptake pathway in rats is the M-cells transport overlaying the Peyer's patches [75, 76]. The particle size is a critical factor, where larger particles (> 300 nm) may be retained for longer periods in Peyer's patches, while smaller particles are transported to the thoracic duct. The composition of SLN is also relevant since a wide variety of solid lipids, such as highly purified triglycerides, complex glyceride mixtures, phospholipids or even waxes are used in SLN production [9]. Surfactants are used to stabilize SLN. Liquid lipid vehicles composed of unsaturated fatty acids and triglycerides can favour intestinal lymphatic absorption of orally administered drugs. Transport of such drugs or carriers through the intestinal lymphatics, via the thoracic lymph duct to the systemic circulation joining at the junction of the jugular and left subclavian vein, avoids pre-systemic hepatic metabolism, and as a

result, enhances the amount of orally administered drugs reaching into systemic circulation. Aji Alex recently reported the SLN enhanced lymphatic uptake duodenal administration to Male Wistar rats [77]. Surfactants (e.g. poloxamers, tweens) also contribute to an increase in the permeability of the intestinal membrane or improved the affinity between SLN and intestinal membrane [78]. Lipid nanocapsules loading anti-cancers have also led to an improvement of oral exposure compared to free drug solutions. The inhibition of P-glycoprotein by the surfactants surfacing the lipid nanocapsules could explain the improvement of oral paclitaxel exposure when it was administered in lipid nanocapsules to male Sprague rats [79].

For intravenous administration, the particle size of SLN must be below 5 μm to avoid blocking of fine capillaries leading to embolism. More relevant than the mean particle size, is the polydispersity index, which is requested to be as lowest as possible, preferentially below 0.24. Because of their minimum size below 400 nm, SLN formulations can be used for systemic body distribution with a minimized risk of blood clotting and aggregation. A three to five-fold enhancement of intravenous plasma peaks of loaded drugs, as well as controlled release and prolonged residence time are generally observed upon intravenous administration of SLN. Uptake of SLN by the cells of the reticuloendothelial system (RES) or mononuclear phagocyte system (MPS), differ depending on the size and composition of the particles. Uptake of SLN can be avoided by PEGylation leading to long circulating particles, so-called stealth SLN. However, it has been reported that stealth SLN showed similar circulating time and pharmacokinetic behaviour as non-stealth SLN [80]. The similar low surface hydrophobicity of both stealth and non-stealth systems could explain the results obtained. Minimizing opsonin protein binding is the key point for developing a long circulation nanocarrier formulation. The nature of the lipid material and the surfactant employed in the SLN preparation might play an important role to avoid adsorption of blood proteins that mediates liver uptake [81]. The loaded drug is gradually released by lipid matrix erosion (e.g. degradation by enzymes) or by diffusion from SLN. The release rate may be controlled by the nature of the lipid material, particle size, and choice of surfactant and also by inner structure of SLN.

When developing cationic SLN for site-specific targeting of siRNA, endocytosis by the cells may be relatively easy to accomplish. The problem however, relies on the release of siRNA out of the endosome. Xu and Szoka described the mechanism through which lipid nanocarriers can trigger endosome release [82]. In the endosome, the cationic lipid of SLN may form ion-pairs with the anionic endosomal membrane, and by excluding the interfacial water molecules, the ion-pairs destabilize the endosomal membrane and release siRNA. Cullis *et al.* further proposed that binding of cationic lipid with anionic lipids an inverted hexagonal H_{II} phase can be formed [83], leading to membrane fusion and drug release. However, despite some successful reports in xenograft mouse models, the endosome escape is still inefficient. Most of the siRNA delivered to tumour cells are still trapped inside the endosome compartment. Reasons include the presence of PEG molecules surrounding the carriers, lack of ion-pair formation, not small enough particle size, and insufficient de-assembly of the carriers.

As previously mentioned, PEGylation is the most commonly used method to protect SLN in the blood circulation (*i.e.* stealth SLN). However, as PEG chains prevent the attachment of opsonins, they also limit the contact between the carrier and the target cells. Inside the endosome, PEG may also limit the interaction between the cationic lipids and the anionic endosomal membrane. To overcome this limitation, suggestions include using cleavable PEG-lipid linkers or acid labile PEG molecules [84].

Besides endosome escape, de-assembly of cationic SLN is also essential for efficient siRNA release. If the structure of the carrier is so stable that they will not release the siRNA inside, the siRNA will not be bioavailable. De-assembly may take place either in the endosome or in the cytoplasm after the endosome escapes. It should take place in the endosome with the release of endosome disrupting cationic materials, because, even though the endosome is disrupted or burst, the opening of the endosomal membrane may not be enough to allow intact carrier to come out. If carriers are smaller than e.g. 100 nm, not only they could escape from the endosome more efficiently, but the required siRNA dose for tumour killing would also decrease. In addition, they may reach those tumours with less leaky vasculatures [85].

For topical use, SLN and NLC are considered to be the latest generation of lipid nanocarriers. Compared with other vehicles, such as creams, ointments or emulsions, and other delivery systems e.g. liposomes and

microparticles, they combine advantages as local tolerance, good adhesive properties, the protection of the encapsulated drugs against chemical degradation, and the possibility of modulating the drug release [86, 87]. The bioavailability of drugs in SLN and NLC may be increased by the close contact between the small particle size of nanoparticles and stratum corneum. However, the limited voids in the lipid bilayers of stratum corneum make the diffusion of intact particles into the skin impossible. SLN and NLC provide enhanced occlusiveness than other pharmaceutical formulations, increasing drug permeation through the skin. SLN and NLC form adhesive films of densely packed spheres on the surface of the skin, increasing skin hydration [57, 64, 87, 88]. The enhanced skin permeation of SLN and NLC may also be attributed to the surfactants, which may act as permeation enhancers. SLN have been used as skin penetration enhancers for several types of drugs [89-92].

For subcutaneous injection, administration of etoposide loaded in SLN to tumour bearing BALB/c mice showed that biodistribution of the drug was slow from the injection site [93]. The increased concentration of etoposide in tissues at 6 h and 24 h post-injection allowed inferring the longer residence time of the drug at the injection site.

With respect to intra-tumour administration, few studies have been carried out using SLN. Mitoxantrone is an example with which after subcutaneous injection it induced more serious local toxicity than intravenous injection or intraperitoneal injection. After multiple subcutaneous administration of mitoxantrone loaded SLN every four days, up to 28 days, mitoxantrone loaded in SLN showed a strong affinity to the tumour tissue, contributing the enhanced therapeutic effect, in comparison to the administration of mitoxantrone solution through the same route [94].

4. SURFACE MODIFIED NANOCARRIERS FOR ACTIVE TARGETING

For targeting nanocarriers to solid tumours, there are several important barriers that have to be overcome. The pharmacokinetic and pharmacodynamic profiles of these systems are entirely distinct from conventional small chemical drugs or some protein drugs that are usually eliminated or metabolized by the kidneys, liver, or lungs [95]. Nanocarriers are cleared from the blood circulation primarily by the RES, especially the liver Kupffer cells [96] and by the spleen macrophages. Upon intravenous administration, opsonins (e.g. IgM, IgG, fibronectins, or complement C3) will adsorb to their surface. Phagocytic cells will recognize the opsonins and will rapidly and effectively take up the opsonized nanocarriers. Their uptake by the tumour is a slower and less efficient process. Thus, the RES uptake represents a major kinetic barrier for drug delivery to the tumour, but there are other physical barriers that need to be taken into account.

Due to the leakiness of the vasculature in the solid tumour, macromolecules and nanocarriers that are too big to penetrate normal blood vessels could penetrate the leaky vasculature and accumulate at the tumour site. This is so called Enhanced Permeability and Retention (EPR) effect [97]. Lacking lymphatic drainage might also contribute to the enhanced retention effect [97-99]. To take advantage of the EPR effect, nanocarriers must be within an optimal size range. The optimum diameter should be around 100 nm [100]. However, it is dependent on the leakiness of the tumour vasculature.

Nanocarriers need to remain in the blood circulation long enough to overcome the kinetic barrier for extravasating the leaky tumour vasculature. The primary elimination mechanism from the blood is the uptake by phagocytic cells after opsonization. Modifying their surface with PEG chains is a common strategy for protecting and shielding the surface charge.

To trigger receptor mediated endocytosis, targeting ligands are required, such as monoclonal antibodies, peptides, proteins, aptamers and small molecular weight ligands.

Antibodies have been extensively used because of their high binding affinity to specific epitopes. Examples of antibodies approved by FDA include the anti-Her2/neu monoclonal antibody Herceptin® for breast cancer and the anti-VEGF monoclonal antibody Avastin® for metastatic colorectal cancer.

Transferrin has also been used as ligand for targeting nanocarriers to tumours [101, 102] and for brain targeting [103]. It is an iron transporting protein that can specifically react with its receptor that is expressed in various

tissues, being over-expressed in different types of cancers. The protein has already been tested in SLN to target curcumin to MCF-7 breast cancer cells, increasing its photostability, and enhancing the anticancer activity [104]. Jain *et al.* administered 5-fluorouracil, SLN and ferritin-coated SLN to MDA-MB-468 tumour bearing mice [105]. The administration of ferritin-coated SLN also resulted in effective reduction of tumour growth as compared with free 5-fluorouracil and non-loaded SLN. Beduneau *et al.* conjugated a whole monoclonal antibody to carriers directed against transferrin receptors, enhancing the uptake of these particles by tissues over-expressing such receptor [106].

Small molecule ligands that have good binding affinity and specificity, such as anisamide, haloperidol, and folic acid, may also be suitable for tumour targeting. The advantages of using small molecule ligands compared to small peptides, proteins, or antibodies are: (i) relatively easy production, (ii) better tolerance to chemical modification/conjugation, (iii) low immunogenicity, and (iv) long-term stability. Anisamide [107] and haloperidol [108, 109] are small molecule ligands for cancer cells over-expressing the sigma receptor (e.g. melanoma, non-small cell lung carcinoma, breast tumours of neural origin, and prostate cancers [108, 110-112]). Folic acid is the high affinity natural ligand for the folate receptor which is over-expressed in a wide range of human cancers, including ovary, lung, breast, endometrium, kidney, and brain cancers. Monostearin composed SLN were produced by Yuan *et al.* containing folic acid-stearic acid (FA-SA) to target paclitaxel to A549 lung cancer cells with improved endocytosis mediated by folate receptor [113]. In another study, paclitaxel was loaded in SLN modified with folate and PEG chains (folate-PEG-SLLN¹³⁸) and the pharmacokinetic properties were evaluated in male Sprague-Dawley rats [114]. Treatment efficiency was investigated with the mouse with sarcoma 180 ascites tumour, revealing that these folate-PEG-SLLN exhibited higher tumour inhibition rate, in comparison to animals administered with conventional paclitaxel injection. Stella *et al.* exploited folic acid as a targeting agent to actively target ovarian cancer [115]. Stevens *et al.* used the same drug to treat mice bearing lung carcinoma M109 tumours with folate receptor-targeted SLN, resulting in significantly greater tumour growth inhibition and animal survival, compared to treatment with non-targeted SLN or free paclitaxel formulation [116].

Other ligands include aptamers, which are nucleic acid based ligands ranging in size from 20 to 80 bases (6 to 26 kDa). Because of their specific nucleotide sequences, aptamers fold into unique 3D structures and are able to recognize, with high affinity, various molecules including proteins, sugars, phospholipids, or even small chemicals. Binding motifs between ligands and receptors usually involves only several amino acids. This has driven researchers to set up phage display libraries allowing selection of special amino acid sequences that show strong binding affinities to specific tissues, cells, or organs [117]. Increasing numbers of peptide ligands have been identified with high affinities against neo-vasculature, various kinds of cancer cells, proteins, receptors, organs and even lymphatic vessels [118]. The RGD (Arg-Gly-Asp) peptide has been used for targeting nanocarriers to tumour neo-vasculature or to cancer cells that express integrin $\alpha_v\beta_3$ cell surface receptor [119-121]. The same has also been reported for NGR (Asn-Gly-Arg) peptide targeting aminopeptidase N (APN, CD13) [122]. This knowledge will certainly allow developing new strategies for targeted nanocarriers to specific tumours.

5. CONCLUSIONS

Cancer is characterized by abnormal cell growth in an uncontrolled way and the current anti-cancer drugs lack of selectivity for tumour tissue, resulting in severe side effects and low cure rates. Recent advances in the field of molecular oncology have led to the identification of large numbers of potential targets for new anti-cancers. In addition, lipid nanocarriers have been known for their high biological compatibilities. Administration of anti-cancer drugs loaded in SLN provides distinct pharmacokinetic profiles from those obtained with free drug solutions. Depending on the administration route, when loading drugs in SLN, the molecules may be protected from biochemical degradation, bypass the effect of extrusion membrane proteins, reduce the drug exposure to hepatic

¹³⁸ Folate-PEG-SLLN, folate and poly(ethylene glycol) solid-to-liquid lipid nanoparticles

metabolizing enzyme activity and avoid the renal clearance, prolong the residence time in the circulation, increased skin penetration. Reduced liver metabolism and renal clearance often results in prolonged blood circulation, with an increased chance of accumulation in the target tissue. SLN have also been developed for cancer diagnosis [93, 123], however much still need to be done so that a successful formulation can reach the market.

This field shows a solid ground for further development of advanced lipid nanocarriers taking advantages of the last developments in imaging analysis, combined with phage display libraries to develop a versatile multifunctional Theragnostic. Theragnostic tools will be a new generation of Nanomedicines.

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