Recent Advances on Nanotechnology Applications to Cancer Drug Therapy

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Abstract: One of the greatest challenges in cancer drug therapy is to maximize the effectiveness of the active ingredient while reducing its systemic adverse effects. Conventional (non-targeted) systemic drug therapy is characterized by unspecific distribution of the anticancer drugs: both healthy and affected tissues are thus exposed to the chemotherapeutic agent, giving raise to off-target side-effects. Besides, a number of widely-used chemoterapeutic agents present unfavorable physicochemical properties, such as low solubility or low stability issues, limiting their available routes of administration and therapeutic applications. Nano-delivery systems seem as promising solutions to these issues. They can be used for targeted-drug release, diagnostic imaging and therapy monitoring. Nanosystems allow the formulation of drug delivery systems with tailored properties (*e.g.* solubility, biodegradability, release kinetics and distribution) that provide means to improve cancer patients' quality of life by lowering the administered dose and, incidentally, the cost of clinical treatments. This article overviews the main features of different nanovehicles (linear and non-linear polymeric nanosystems, lipid-based systems, inorganic nanoparticles) and presents a selection of reports on applications of such systems to cancer therapy published between 2010 and 2013.

Keywords: Anticancer Drug Therapy, Dendrimers, Inorganic Nanoparticles, Liposomes, Nanocapsules, Nanogels, Nanospheres.

1. INTRODUCTION

Multiple dosing regimens are the most common drug-based therapeutic interventions. Thinking of systemic therapies, conventional drug delivery systems rely on establishing a dynamic equilibrium or, more precisely, a *pseudo-equilibrium* between the free drug plasmatic concentration and the free drug concentrations in all the other body tissues (as open systems, a true equilibrium is never truly achieved within living systems due to the permanent mass exchange with the environment; furthermore, multiple dosing delivers the drug in discrete units while, once absorbed, elimination from blood is a continuous process). After a number of doses are administered, a steady state is reached, during which plasmatic concentration will fluctuate between practically fixed maximal and minimal steady state concentrations, as long as the treatment goes on. Since only the free, unbound drug can interact with its molecular target, the free drug levels at the vicinity of the site of action (generally) determine the extent of the pharmacological response [1]. An implication of the previous discussion is that, to attain effective concentrations of an active ingredient in its biophase or site of action, the patient is subjected to systemic exposure to the drug, which often leads to off-target undesirable side effects. In other words, conventional drug delivery systems are characterized by non-specific distribution: to attain therapeutic levels in the biophase, otherwise unneeded levels are accepted in the rest of the body. Patients undergoing drug therapy are thus exposed to: a) large doses to compensate such ubiquitous distribution within the organism and attain effective concentrations in the vicinity of the molecular target and; b) off-target drug effects, which could be ameliorated or avoided if targeted drug delivery systems were used. Patients receiving anticancer treatment constitute a very illustrative example of the consequences of the previous setting: a majority of the well-recognized adverse reactions to chemotherapy emerge from interactions between the drug molecules and healthy cells.

Besides safety issues, there are also a diversity of biopharmaceutical problems related to chemotherapeutic agents. Many commonly used anticancer drugs such as the *Vinca* alkaloids,

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ISSN: 1929-2260 / E-ISSN: 1929-2279/13

anthracyclines, epipodophyllotoxins, taxanes and actinomycin D are substrates of ATP-Binding Cassette (ABC) transporters involved in multi-drug resistance issues, which may reduce their bioavailability at tumoral cells expressing high levels of such carriers [2-4]. Several antineoplastic agents such as 5-fluorouracil, camptothecins, gemcitabine and curcumin are very rapidly metabolized or inactivated in the physiological environment [5-8]. Drugs with short half-life present difficulties to build up and sustain effective levels at the biophase (limiting the duration of the pharmacological effect or requiring large doses just to compensate metabolism). The poor aqueous solubility of a diversity of antineoplastic drugs (*e.g.* taxanes) precludes their IV administration or demands the use of highly toxic solvents [9].

Encapsulating or conjugating active ingredients within nano-sized vehicles that hide the drug from clearance mechanisms (in a sort of Trojan horse approach), maintaining their integrity throughout the distribution process and selectively directing the drug to its molecular target seems like a very appropriate strategy. An ideal drug delivery device should: a) compensate unfavorable physicochemical properties of the active ingredient; b) efficiently encapsulate, entrap or adsorb drug molecules; c) conceal the drug from enzymatic and non-enzymatic cleavage, undesired biotransformation and recognition by efflux transporters; d) extravasate; e) direct the active ingredient to its therapeutic target; in the case of intracellular targets, promote cellular uptake and deliver the drug to its subcellular location; f) once in the vicinity of the target (and not before), release the drug load in a controlled manner; g) present no toxicity nor accumulation within the body and, preferentially, be biodegradable. Some years ago, a device which gathered such a wide range of features would have been inconceivable. Today, burgeoning advances on nanobiotechnology have brought us closer to our dreamt delivery system, reviving Ehrlich magic bullet concept. Nanosystems are currently produced from a profusion of materials (and, more interestingly, materials combinations), in a wide range of tailored morphologies and sizes, and in a broad spectrum of surface coatings and functionalizations.

The pathological anatomy and physiology of cancerous tissue allow the development of both passive and active targeting strategies. On the one hand, a large number of genes (including many cell surface and nuclear receptors) are amplified or overexpressed in cancer cells [10-15]. On the other, the vasculature around cancer cells is poorly formed, which leads to large gaps between adjacent endothelial cells and consequently to enhanced permeation of large macromolecular delivery systems in the range of 20–200 nm [16]. Also, due to the rapid growth of the tumor, lymphatic drainage is deficient. Altogether, these phenomena are known as the Enhanced Permeability and Retention (EPR) effect [17-19]. In contrast, healthy tissues are much less permeable to macromolecules and large colloid particles, as pore sizes in the endothelia of blood vessels in most healthy tissues are ~2 nm, while ~6 nm pores are found in postcapillary venules [20]. The EPR phenomenon in cancer provides an opportunity to target diseased cells simply by controlling the size of the delivery system (*i.e.* passive targeting) [18, 19].

A last general consideration pertaining to the disposition of nanocarriers is that, while free drug usually follows biotransformation and excretion through bile and urine, drugs encapsulated within nanovehicles are mainly extracted through the mononuclear phagocyte system (MPS), mostly by fixed macrophages in the lymph nodes, the liver and the spleen [21]. Once in the bloodstream, non-coated nanoparticles (NP) are rapidly opsonized and massively cleared by those fixed macrophages. Both the composition (type, hydrophobicity, biodegradation profile) of the NP and the associated drug (molecular weight, charge, localization in the NP: adsorbed, dissolved or encapsulated) have a great influence on the drug distribution pattern in the MPS organs [22]. This propensity of nanosystems to localize in the MPS represents an excellent opportunity for passive targeting of drugs to the liver and the spleen [23, 24], and has been employed for chemotherapy of the MPS localized tumors: hepatocarcinoma or hepatic metastasis arising from digestive tract, gynaecological cancers, bronchopulmonary tumors, myeloma and leukemia, among others [22, 25]. In contrast, this characteristic biodistribution becomes a major obstacle for drugs whose site of action is located in other tissues. A great deal of work has been devoted to developing so-called ‘Stealth™’ particles, which are ‘invisible’ to macrophages [22]. These Stealth™ NP have been shown to be characterized by a prolonged half-life in the blood compartment and are able to directly target most tumors located outside the MPS regions [26-28]. Steric stabilization of NP has been achieved by adsorbing hydrophilic surfactants on the NP surface or by using block/branched copolymers. Poly(ethylene oxide) (PEO) and poly(ethylene glycol) (PEG) are the most successful nonionic hydrophilic polymers used for this purpose [29-31], However, a number of limitations to the use of PEG have also been described [32], such as the production of anti-PEG antibodies or the impairment of cellular internalization by the stealth coating. Depending on the nature of the nanovehicle, different approaches have been explored to circumvent these limitations, *e.g.* stimuli-responsive PEG-derivatized nanocarriers [33-35]. The effect of particle size on *in vivo* distribution has also been studied [36], suggesting that particle size below 100 nm tends to increase circulation lifetime.

This review article wills overview recent advances (2010-2013) on the development of nanocarriers for anticancer therapeutics. We have organized the discussion on the basis of the materials from which the presented developments have been derived (namely linear polymers, branched polymers, lipids and inorganic materials), even though the reader will appreciate throughout the review that the general tendency in the development of targeted drug nanovehicles seems to be the combination or arrangement of different materials (or even carrier-types) in complex platforms with very specific properties. Since the reports on the reviewed subject are abundant, excluding the possibility of an exhaustive review, we have selected those developments that from our point of view represent innovative contributions to the field and/or reflect a current tendency.

2. LINEAR POLYMERIC NANOPARTICLES

NP have been among the most widely studied particulate delivery systems over the past three decades. They are defined as submicrometer-sized, insoluble, solid-colloidal polymeric particles with sizes ranging from 10 to 1000 nm in which the drug can be dissolved, entrapped, encapsulated, or adsorbed [37, 38]. In the present work, we used the expression linear polymeric NP to denote the nanosystems formed by initially linear polymers that may be cross-linked in the synthesis pathway, but not branched or star-shaped, as in the case of dendrimers, which will be covered in section 3.

Depending on the preparation process of NP, nanospheres (NS) or nanocapsules (NC) can be obtained. NS are matrix-like structures where the drug can either be firmly adsorbed on the surface of the particle or dispersed/dissolved in the matrix itself. NC, on the other hand, consist of a polymer shell and a core, where the drug can either be dissolved in the inner core or adsorbed onto the surface [39]. NP formulation of anticancer drugs has attracted intensive research interest in the past decades and has become an important area in cancer-oriented nanotechnology applications: in all cases, the effectiveness of the treatment is directly related to the treatment's ability to target and kill the cancer cells while affecting as few healthy cells as possible [40].

One general problem that may occur after NP administration is the premature, off-target burst release of drugs in the bloodstream, which redounds in low efficiency and toxicity to healthy tissues [41]. After the initial burst release, the drug release from the NP may become very slow. Cancer cells have many drug resistance mechanisms; therefore, if the drug influx into the cancer cell is lower than the capacity of drug removal by ABC transporters and other detoxication mechanisms, the drug cannot build up an effective concentration [2-4, 37, 42-44]. The initial burst release is determined by poorly entrapped drugs or drugs weakly adsorbed onto the particles’ surface. In this sense, the interactions between the drug molecules and the NS/NC should be strong enough to provide good encapsulation and not too fast or too slow release. Appropriate cross-linking can be used to modify the drug release kinetics, which is one of the main advantages of these vehicles [45-47].

The purpose of this section is to highlight the most recent advances related to the use of polymeric NS and NC that have been used in chemotherapeutic drug delivery. A comprehensive review of this area of research is beyond the scope of this section and hence the readers are referred to other sources for additional information [22, 25, 38, 39, 48-50].

2.1. Combining Strategies: Nanospheres for Targeted and Triggered Drug Delivery

The addition of targeting ligands onto the surface of NP aims to increase selective cellular binding and internalization through receptor-mediated endocytosis, which is known as *active targeting*. Without the incorporation of targeting ligands, NP rely on non-specific interactions with cell membranes, which can be especially low when covered with a layer of PEG polymers. As has been underlined in the general introduction, to differentiate healthy from cancerous cells, ligands having specificity for receptors that are over-expressed on cancerous cells, but are normally or minimally expressed on normal cells can be selected [40, 51-53].

The folic acid receptor alpha (FRα), a glycosylphosphatidylinositol-linked protein, is over-expressed on the surface of numerous human cancer cells (including the malignant tumor cells of ovary, brain, kidney, breast, lung and uterine cervix) [54, 55]. Consequently, several different polymeric NP have been synthetized with folic acid (FA) conjugated onto their surface, loaded with drugs like doxorubicin (Dox) [56], paclitaxel [57] and 17-AAG [58], among several other chemical compounds [59-62]. Other targeting moieties that have been used in the design of targeted NP include: antibodies or antibodies fragments [63-65], aptamers [66-69], proteins and peptides [70-74].

Some of the main applications of polymeric materials to form NP arise from their stimulus-responsive nature, that is, their ability to undergo reversible volume phase transitions in response to environmental stimuli such as pH [75-78], temperature [79-81], redox environment [82, 83], ionic strength [84, 85], and/or the action of an external electromagnetic field [86-89]. This behavior is governed by the balance between repulsive and attractive forces acting in the particles: swelling occurs when ionic repulsion and osmotic forces exceed attractive forces, such as hydrogen bonding, hydrophobic interactions and van der Waals interactions, among others [90-92].

Therefore, the combination of these tools may result in a synergistic effect: ligand-functionalized surfaces and stimulus-responsive materials are two different strategies aimed to achieve a common goal: to minimize the side effects of systemic exposure to cytotoxic chemotherapeutic agents. Fox example, a 2013 work by Shen *et al.* [70] proposes the synthesis of NS presenting RGD peptide-mediated tumor targeting, embedded with drug-loaded magnetic NC. RGD peptides target integrin expressing tumor vasculature [93, 94] while the superparamagnetic properties facilitate biomedical applications such as imaging, hyperthermia therapy, and magnetic response in an external magnetic field [95].

Two drugs, Dox and verapamil (Ver) were adhered by hydrogen bonds and adsorption to the chitosan shell of the coating of magnetic nanoparticles (MNP), which were later entrapped into the poly(lactic-co-glycolic acid) NP (PLGA-NP) by the double emulsion (W/O/W) solvent evaporation method, in order to prepare a dual-drug delivery system against both cancer and Dox-induced cardiotoxicity. Further modification was conducted by conjugating the tumor-targeting ligand, cyclo(Arg-Gly-Asp-D-Phe-Lys) (c(RGDfK)) peptide onto the end carboxyl groups on the PLGA-NP. The size of the resulting cRGD-Dox/Ver-MNP-PLGA NP was approximately 144 nm under simulated physiological environment. Figure **1** shows a representation of the NP synthesis and the drugs release mechanism.

In vitro cytotoxicity evaluation on HepG2 and S-180 murine sarcoma cells suggested that, due to the cRGD-mediated targeting strategy, the developed NP

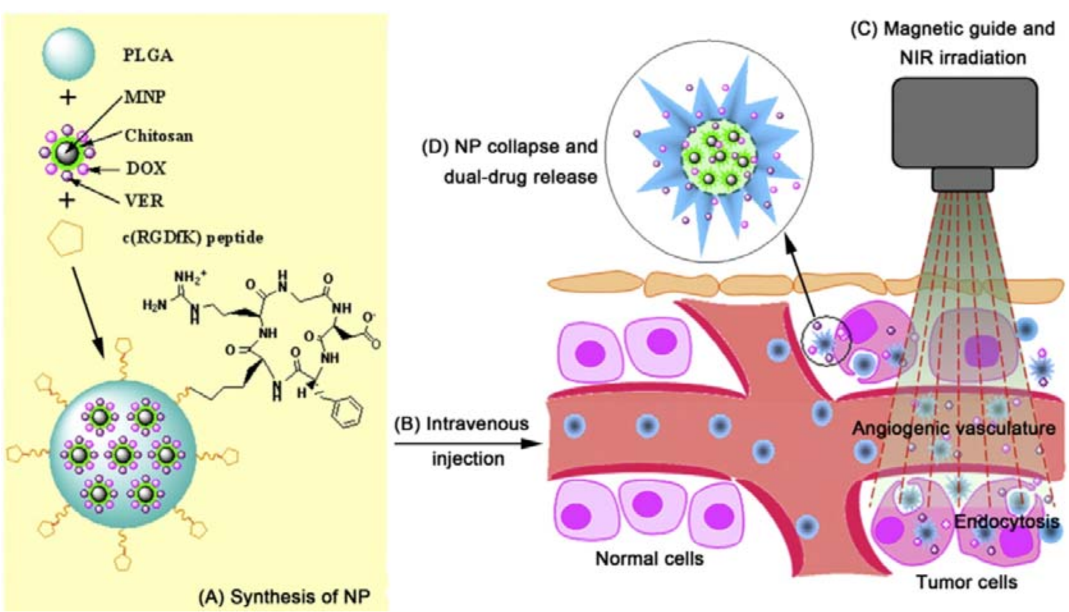

Figure 1: Schematic representation of the cRGD-Dox/Ver-MNP-PLGA NP synthesis and the following Dox and Ver release. *Reprinted with permission from Shen et al.* cRGD-functionalized polymeric magnetic nanoparticles as a dual-drug delivery system for safe targeted cancer therapy. Pharmacological Research 2013; 70(1): 102-115, Copyright 2013 Elsevier.

possessed higher growth inhibition properties on cancer cells than free drug or cRGD-unconjugated NP. Biodistribution studies and whole-mouse optical imaging on S-180 sarcoma-bearing Balb/c mice demonstrated consistently preferential accumulation capability of the cRGD-Dox/Ver-MNP-PLGA NP in the tumoral tissue under magnetic guidance.

Another example of synergistic combinations is presented in the work of Khoee *et al*. [96], who developed a pH-sensitive polymeric NP targeted with folate groups and loaded with the anti-cancer drug quercetin. The formulation was produced by radical polymerization of three monomers: methacrylated poly(lactic-co-glycolic acid) (mPLGA) as a lipophilic domain, acrylated methoxy poly(ethylene glycol) (aMPEG) as hydrophilic part and *N*-2-[(tert-butoxycarbonyl)amino] ethyl methacrylamide (Boc-AEMA) as pH-responsive segment, followed by the removal of the protecting amine group (Boc) and the conjugation of the resulting copolymer with activated FA. Finally, the drug -which is poor water soluble- was loaded into the NP by a nanoprecipitation method. *In vitro* release experiments showed that quercetin release from the NP was pH-dependent, and much faster at pH 5.8 than at pH 7.4. The results indicated a conformational change in AEMA chains from a compacted shape to an expanded one with a decrease in the pH values. In expanded conformation, drug can diffuse out from the NP more easily than in a compact form. Even though the authors did not present results of the affinity nor the drug release in the presence of cancer cells, the folate group is expected to increase the specificity in the delivery of the encapsulated by the pH-sensitive NP.

Similarly, recent publications presenting new combinations of well-known systems can be found in the field of stimulus-responsive targeted NP for cancer drug therapy: magnetic NP targeted with folate [97], biotin-conjugated pH-responsive polymeric micelles [98], pH and redox dual responsive NP functionalized with cRGD peptide [99] and pH-sensitive chitosan-silica NS conjugated with an antibody molecule to ErbB 2 [100], among others.

A 2013 work by Zhao *et al.* [101] presents a polymeric NC for the delivery of recombinant apoptin fused with maltose binding protein (MBP—APO), in which the protein complex is non-covalently protected in a water soluble polymer shell (Figure **2**). Apoptin is a protein encoded by chicken anemia virus which induces apoptosis in a variety of cells. The formulation of the NC is produced by addition of acrylamide (AAm) monomer in the protein solution; followed by addition of a second monomer, N-(3-aminopropyl)methacrylamide (APMAAm). Different crosslinkers (N,N'-methylene bisacrylamide and N,N'-bis(acryloyl)cystamine) were added after the addition of APMAAm. Finally, the polymerization was produced by the addition of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine.

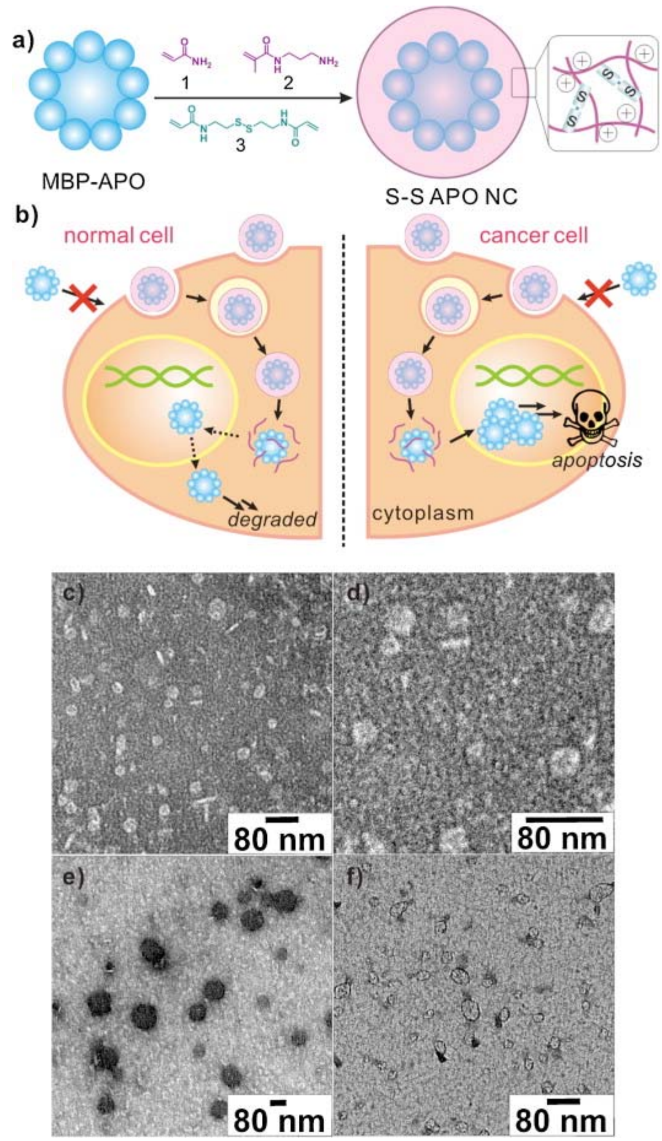

Figure 2: Degradable NC for apoptin delivery. (**a** and **b**) Schematic diagram of synthesis of degradable apoptin nanocapsules (S-S APO NC) and delivery into tumor cells to induce apoptosis; TEM images of (**c**) native MBP–APO; (**d**) enlarged image of MBP–APO; (**e**) S-S APO NC; and (**f**) degraded S-S APO NC after treatment with 2 mM GSH for 6 h at 37 °C. *Reprinted with permission from Zhao et al.* Degradable polymeric nanocapsule for efficient intracellular delivery of a high molecular weight tumor-selective protein complex. Nano Today 2013; 8: 11-20, Copyright 2013 Elsevier.

The slightly positively charged shell shields the MBP—APO from serum proteases and surrounding environment, while enabling cellular uptake of the polymer—protein complex through endocytosis [102]. The polymeric layer is weaved together by the redox-responsive cross-linkers containing disulfide bonds that are degraded once the NC are exposed to the reducing environment in cytoplasm [103]. No covalent bonds are formed between the protein cargo and the polymer shell, which ensures complete disassembly of the capsule layer and release of native MBP—APO inside the cell. A xenograft study verified that the degradable NC effectively delivered MBP—APO proteins to tumor cells *in vivo*, which was highly effective in limiting tumor progression. Upon further optimization of the pharmacokinetics of the APO NC, including surface derivatization with active targeting ligands, these particles may be IV administered as an anticancer therapy.

2.2. Layer-by-Layer Nanoshells

Layer-by-Layer (LbL) NP are an emerging class of therapeutic carriers that afford precise control over key design parameters, facilitating improved, controlled drug release features, and enhanced molecular-targeting capabilities. LbL technique, first introduced at the beginning of the 1990s by Decher, was first used to assemble multilayered films through successive ionic later deposition [104], that is, alternate deposition of polycations, such as PAH (poly (allylamine hydrochloride)) and PRM (protamine dextran) and polyanions, such as PSS (poly (styrene sulfonate)) and DXS (dextran sulfate) [105]. Because the assembly mechanism is so simple (though tedious), there is a minimum requirement of apparatus necessary [106], and this methodology rapidly spread within various research communities especially for the preparation of regular assemblies of various materials including polymers [107], biomaterials [108, 109] and inorganic substances [110]. The technique takes advantage of attractive electrostatic forces between charged polymers and oppositely charged surfaces, and film growth is achieved stepwise by the repetitive exposure of substrates to dilute polycation and polyanion solutions. For example, positively charged substrates are immersed into the solution of polyanion (negatively charged polymer, for example, PSS) for several minutes. As a result, a thin layer (thickness 1–2 nm) of the polymer is adsorbed on the surface. Charge overcompensation leads to a negative surface re-charging. Then, the substrate is washed (a washing step is obligatory to remove excess reagents and thus precisely control the growing layer thickness) and placed into the solution with polycation (positively charged polymer, for example, PAH). The polymer is attached electrostatically to the charged surface. The process can be repeated several times to reach a defined multilayer thickness controlled by layer coating cycling. [111-114]. A milestone innovation in the LbL technique was achieved through its application to assemblies onto a colloidal particle core [115], which allowed overcoming the limitation of the flat supports and obtaining micro and nanocapsules by the LbL technique. Often, an inorganic porous sacrificial template is used (usually, silica and calcium carbonate), which after dissolution leads to a hollow capsule (empty shell) [116, 117].

The main advantages of these capsules are their multifunctionality, modularity and structural control. Owing to the electrostatic driving force for multilayer build-up, a wide variety of constituents can be chosen, such as synthetic polyelectrolytes, enzymes, lipids, NP and others [118]. Mechanical and physicochemical properties of the capsules can be tailored by varying these constituents or by varying the capsule thickness. Furthermore, encapsulation within polyelectrolyte capsules can easily be achieved under mild conditions avoiding the use of organic solvents or mechanical stress, which is often applied during the synthesis of 'traditional' drug delivery particles such as, for example, liposomes. There are many approaches to attain drug loading. Direct coating of the drug substance itself, leading to drug particles cover by a polyelectrolyte membrane is one of the possibilities [119]. Alternatively, a post-loading procedure might be applied, in which the capsule permeability is reversibly altered by changes environmental factors (*e.g.* pH, light or solvent polarity) allowing drug inward diffusion [120-123] (naturally, the same principle can be used to provide controlled, triggered drug release). Another approach involves the use of mesoporous inorganic templates that are preloaded with the drug substances before being coated with polyelectrolytes. Although in the first decade of LbL study, most research was devoted to the assembly of synthetic polyelectrolytes, one current trend is to use natural biodegradable polyelectrolytes, such as chitosan, gelatins and dextran sulfate [104, 124]. Following this tendency, Zhou *et al.* recently used the LbL assembly technique for preparing LbL alginate/chitosan coatings on the top of biocompatible PLGA NP. FA or FA-grafted PEG (PEG-FA) was covalently bonded to the polyelectrolyte multilayer *via* carbodiimide chemistry. Cellular uptake experiments were carried out by co-culture of HepG2 cells in the presence of NP. Flow cytometry and confocal laser scanning microscopy (CLSM) were used to investigate the influence of the surface chemistry of the NP on uptake. A significantly lower uptake of PLGA NP coated with chitosan/alginate was observed compared with bare NP, but the uptake increased after the attachment of FA molecules [125].

Other advances that deserve mentioning include stimuli-responsive LbL systems. For example, Ochs *et al.* reported the modular assembly of polymer-drug conjugates into covalently stabilized, pH-responsive, biodegradable films and capsules [126]. To that end, Dox was conjugated to alkine functionalized poly(L-glutamic acid) (PGA_{Alk}). PGA_{Alk} was assembled with poly(N-vinyl pyrrolidone) (PVP) on planar and colloidal silica by using a combination of click chemistry and LbL assembly. PVP and the silica template were later removed, to achieve single-component PGA_{Alk} capsules. The polymer-drug conjugate could be incorporated at defined positions of the multilayer with controlled dose. The PGA_{Alk} capsules were stable in the pH range between 2 and 11 and exhibited reversible swelling/shrinking behavior. The drug-loaded capsules could be degraded enzymatically, resulting in the sustained release of active Dox for around 2 h.

3. DENDRIMERS

Dendrimers are regularly branched polymeric macromolecules with unique structural and topological features [127, 128]. The term dendrimer comes from the Greek *dendron*, which means tree. They differ from traditional (linear) polymers in that they have a multi-branched, 3D architecture with very low polydispersity and high functionality. A typical dendrimer comprises three different topological parts, which are: a) a focal core; b) building blocks with several interior layers composed of repeating units and; c) multiple peripheral functional groups. The focal core and the interior layers, composed of repeating units, can provide a flexible space created within the voids of dendritic building blocks, which may encapsulate various small guest molecules. The multivalent surface can accommodate a large number of functionalities that can interact with the external environment [129].

The dendrimeric structure is characterized by layers called generations (branching cycles). The number of generations corresponds to the number of branching points. A fifth generation dendrimer presents 5 focal points between the core and the surface. The core is often called generation zero (G0). For example, in propylene imine (PPI) dendrimers, the core molecule is 1,4-diaminebutane; in polyamidoamine (PAMAM) dendrimers, the initiator core is either ammonia or 1,2-etilendiamine. Dendrimers design can be based on a great diversity of functional groups, such as polyamines as in the case of PPI [130], a combination of amines and polyamides as in the case of PAMAM [127] or more hydrophobic poly(aryl ether) dendrimers [131]. Depending on the peripheral functional groups, dendrimers can be either neutral or charged. Their physical properties vary in a regular way depending on the number of generations. The diameter of dendritic molecules increases linearly with the number of generations [132]; the number of terminal groups duplicates with each generation [133].

Besides monodispersity, there are a number of other advantages associated with dendrimers that can be exploited in the drug delivery field. Biodegradable dendrimers might be obtained if cleavable functions (*e.g.* ester groups) are included in the polymer backbone. What is more, degradation kinetics might be controlled by adjusting the nature of the chemical bond connecting the monomer units, the hydrophobicity of the monomer units (hydrophilic monomers result in faster degradation), the size of the dendrimer (larger dendrimers determine slower degradation due to tight packing of their surface) and the cleavage susceptibility of the peripheral and internal dendrimer structure [134]. The large number of dendrimers' surface groups and the versatility in their chemical structures allow the conjugation of different drugs, imaging agents, and/or targeting moeities [134]. Asymmetric dendrimers might be prepared by coupling dendrons of different generations to a linear core, leading to "bow-tie" dendrimers; the asymmetry allows for tunable structures and molecular weights, control on the number of functional groups and improved versatility in relation to attachment of diverse drugs, imaging agents and targeting moieties. Finally, the presence of many polar termini redounds in high solubility; through entrapment of guest molecules in dendrimer's voids increased solubility of poorly soluble drugs may be achieved [135, 136]. The high specific surface and the spherical geometry confer dendrimers low intrinsic viscosity (compared to linear polymers) and high reactivity [133]. Among the limitations of these systems, we might highlight high production costs owing to multi-step synthesis [133], and difficulties to achieve controlled, sustained release in physiological conditions [134].

3.1. Dendrimers in Drug Delivery of Anticancer Drugs

As in the other reviewed nanosystems, recent advances in the application of dendrimers as drug delivery systems of anticancer agents seem to explore the use of complex multifunctional nanoplatforms.

Taratula *et al.* [137] developed a complex tumor-targeted drug delivery system for simultaneous delivery of siRNA and MRI contrast agents (superparamagnetic iron oxide NP). 5 nm superparamagnetic NP were complexed with G6 PPI dendrimers and siRNA targeted to B-cell lymphoma 2 (BCL2) mRNA. The resulting NP were modified with heterobifunctional PEG by coupling the polymer to amino groups in the surface of the complexes (which are introduced by the dendrimers). The distal end of PEG was coupled with a synthetic analog of the luteinizing-hormone-releasing hormone (LHRH) peptide as targeting moiety. *In vitro* testing of the targeted nanoplatform on A549 cancer cells which overexpress LHRH and LHRH negative SKOV-3 cells proved that the nanocomplex was preferentially incorporated by cells overexpressing the targeted receptor. The authors also tested the co-delivery of siRNA and cisplatin, finding that the cytotoxicity of cisplatin against multidrug resistant human cancer cells was enhanced in the presence of both non-targeted or LHRH targeted siRNA delivery vectors. Similar results were obtained *in vivo* in a xenograft model of human cancer, which showed that the combinatorial treatment (cisplatin plus siRNA) decreased tumor volume on 67.5% (compared to 36.2% for free cisplatin alone), while the LHRH targeted vector improved the results achieving a 75.5% decrease on tumor volume. A similar platform for the delivery of Dox was developed and tested by Chang *et al.* [138], who synthesized superparamagnetic iron oxide NP stabilized with PAMAM dendrimers conjugated to Dox. The chosen linker between the dendrimers and the drug was a hydrazone bond, which is acid-cleavable and can be used as pH-responsive release system.

Kirkpatrick *et al*. [139] complexed aqua cisplatin with half-generation PAMAM dendrimers in an attempt to design dendrimer-based cancer therapeutics. The amount of drug bound was found to proportionally increase with dendrimer generation. *In vivo* activity was examined using an A2780 tumor xenograft. The G6.5 cisplatin–dendrimer complex was administered in two doses (6 and 8 mg/kg equivalent of cisplatin) both of which were well tolerated by the mice. The lower dose displayed comparable activity to free cisplatin with a tumor volume reduction of 32%, but the higher dose was significantly more active than free cisplatin with a tumor reduction of 45%.

A dual-targeting drug carrier based on PEGylated G4 PAMAM dendrimers with transferrin (Tf) and wheat germ agglutinin (WGA) on the periphery and Dox loaded in the inner space was synthesized and its blood brain barrier (BBB) penetration and tumor targeting properties were explored by the group of He *et al.* [140]. The nanosystem reduced the cytotoxicity of Dox to normal cells, while efficiently inhibited the growth of C6 glioma cells. Transport assays across the BBB showed that PAMAM-PEG-WGA-Tf delivered 13.5% of Dox in a period of 2 h, demonstrating an enhanced transport compared to single-targeted dendrimers (8% for PAMAM-PEG-WGA, 7% for PAMAM-PEG-Tf) and free Dox (5%).

4. HYDROGEL-BASED NANOSYSTEMS

Hydrogels are hydrophilic polymeric networks composed of either homopolymers or copolymers, which can entrap large amounts of water or biological fluids [38]. The hydrophilic polymer components are cross-linked into a network that provides dimensional stability to maintain the network structure of the hydrogels and to prevent dissolution of the hydrophilic chains [141], while the high solvent content gives rise to the fluid-like transport properties. Therefore, they can be defined as highly water-absorbing materials that remain insoluble in aqueous solutions owing to the internal chemical or physical cross-linking of their macromolecular chains, which vary in size and structure. They are considered nanogels when the particle size is less than 200 nm [142].

In general, hydrogels can be classified according to their composition, route of administration, method of preparation, physical structure, responsiveness to physiologic environment stimuli, type of material being delivered or release kinetics [143-145]. The compositions can be divided into natural polymer hydrogels, synthetic polymer hydrogels and combinations of the two classes. Natural and synthetic polymers are the most frequently used in the pharmaceutical and biomedical fields [146]. Valuable articles reviewing different aspects of hydrogel polymeric materials, their classification and applications are available in the literature [16, 147-149].

One of the main areas of application of hydrogel-based nanosystems is drug delivery. They share a common feature of self-assembly with polymeric micelles [49], but with a major advantage: while polymeric micelles possess only one hydrophobic internal core with a hydrophilic shell [150-153], the interior of hydrogel nanosystems consists of dispersed multiple hydrophobic island domains in a hydrophilic sea domain due to the random association of hydrophobic moieties conjugated to soluble macromolecules. This characteristic makes them suitable carriers for poorly soluble drugs like chemotherapeutic agents. Furthermore, the possibility to control the distribution of drugs in the body to reach specific tumor cells by controlling the particle size or by attaching receptor-specific molecules to the hydrogel NP (HNP) surface enhance their attractiveness as drug delivery system [154, 155]. Other special functions such as crossing the BBB, stimulus responsive nature and more sophisticated controlled release patterns may also be achieved [16].

4.1. Hydrogels-Based Nanosystems for Passive and Active Drug Targeting

Passive targeting may be achieved by taking advantage of the already commented EPR effect (see Introduction) [16, 18, 19]. Nanogels are drug delivery systems with ideal characteristic to exploit the EPR effect, which is exemplified by several works that have manage to synthesis small, monodispersed nanogels with great control over particle size and the ability to synthesize a wide range of diameters [156-159]. For example, Table **1** shows the parameters for poly(N-isopropylacrylamide) (pNIPAm) core particles synthesized by free-radical precipitation polymerization [156].

It is important to note that by gradually increasing the surfactant and initiator concentrations the particle size could be modified, in this case to approach the target 50 nm radius, and for all the syntheses, the batch to batch size variation was within 10% [156].

There are many studies related to nanogel-based drug delivery systems that exploit the EPR effect to passively deliver a chemotherapeutic agent. Hydrogel-based nanosystems have been developed that passively deliver poorly soluble chemotherapeutic agents as Dox [160], curcumin [161] and nucleoside analogues [162, 163]. A very recent study by Saboktakin *et al*. [164] present the synthesis and characterization of carboxymethyl starch and dextran sulfate hydrogels with a porphyrin-based photosensitizer (PS) agent incorporated. Photodynamic Therapy (PDT) is a light-activated treatment for cancer tumors and other diseases based on the fact that some PS can be accumulated to a higher concentration in tumor cells than in healthy cells upon systemic administration. By matching the wavelength of the therapeutic light to the absorption peak of the sensitizers, the light is absorbed by the PS, and the excited PS molecules can then transfer their energy to surrounding oxygen molecules, which are normally in their triplet ground state, to generate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) or free radicals, which in turn are responsible for oxidizing various cellular compartments, resulting in irreversible damage to tumor cells [165].

One potential challenge of PDT is that many PS agents are lipophilic, making parenteral administration problematic [166, 167]. In addition, systemic administration of a PS leads to generalized photosensitivity and the temporary need to avoid light exposure. Various strategies to overcome these limitations have been investigated, including conjugation of PS agent to water soluble polymers and

Table 1: Parameters for Poly(N-isopropylacrylamide) (pNIPAm) Core Particle Synthesis. Bis: *N, N'*-methylenebis(acrylamide); AAc: acrylic acid; AFA: 4-acrylamidofluorescein (fluorescent monomer); SDS: sodium dodecyl sulfate; APS: ammonium persulfate. Extracted from Blackburn *et al.* [156]

	Monomer	Cross-linker	Co-monomer	[SDS, Surfactant] mM	[APS, Initiator] mM	[Total Monomer], mM	*Rz*, nm
1	pNIPAm- 96%	BIS- 2%	AAc- 2%	2	2	70	86
2	pNIPAm- 96%	BIS- 2%	AAc- 2%	3	3	70	73
3	pNIPAm- 96%	BIS- 2%	AAc- 2%	4	4	70	53
4	pNIPAm- 98%	BIS- 2%	AFA- 0.1 mM	4	4	70	57
5	pNIPAm- 95%	BIS- 5%	AFA- 0.1 mM	4	4	40	44

colloidal administration, as well as encapsulation in different nanocarriers [168]. On the other hand, the advantage of porphyrin-based PS compounds include their ability to efficiently absorb a wide range of light spectra, especially red light ($\lambda \geq 600$ nm) (a region of the spectrum to which tissues are more transparent) [165], as well as high quantum yield of $^1O_2$ [169]. The nanogels developed by Satoktakin *et al.* present several combined advantages: the ability to solubilize the porphyrin-based hydrophobic agents, the uniform size of hydrogels, and the potential for passive targeting of solid tumors *via* the EPR effect, hence decreasing systemic photosensitization [164].

A 2013 work by Murphy *et al.* [170] describes the preparation of lipid-coated nanogels, which enable versatile and stable loading of drug cargoes and imaging agents within a crosslinked core. The authors used a previously described *in vivo* optimized bilayer composition [171] containing cholesterol, dioleoyl-phosphatidylethanolamine (DOPE), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine-PEG2000 (DSPE-PEG2000), and DSPE-$(PEO)_4$-cRGDfK as a template, in the molar ratio cholesterol:DOPE:DSPC:DSPE-$(PEO)_4$-cRGDfK: DSPE-mPEG2000 (6:6:6:1:1). The synthesis of the cyclic peptide cRGDfK (f denotes D-phenylalanine) using standard Fmoc solid phase chemistry and its coupling to DSPE is also described [171]. This peptide is a ligand for integrin αvβ3, which is widely accepted as a targeting moiety for the tumor neovasculature [172]. Once the lipid film was dried, it was rehydrated with a solution containing the desired monomers (hydroxyethylmethacrylated-PEG, albumin, α1-acid glycoprotein), the drug and a photoinitiator (Irgacure 2959 [173]) in phosphate buffer, and sonicated to form multilamellar vesicles. Extrusion, purification, and photo-crosslinking of the encapsulated monomers create a targeted lipid-coated nanogel, which enables stable loading of a wide array of chemotypes. The lipid bilayer acts as a template for the core and can be extruded to a defined size (100 nm hydrodynamic diameter) before photo-crosslinking to form the nanogel, which enables precise control of nanogel formation. Photo-crosslinking of the nanogel core enhances drug retention, thereby improving a major limitation associated with liposomes, micelles, and co-block polymeric systems.

The resulting nanogels were loaded with paclitaxel, docetaxel (taxanes), bortezomib (peptide mimetic), 17-AAG (antitumor antibiotic that targets Hsp90), sorafenib, sunitinib, bosutinib, or dasatinib (kinase inhibitors), and tested in cell viability assays with M21 human melanoma cells, which express integrin αvβ3. In addition to the versatile drug loading capability, the assayed formulations demonstrated enhanced potency when compared to free drug. Furthermore, the authors proved that the docetaxel- and paclitaxel-loaded nanogels improve the *in vitro* efficacy beyond a clinically approved nanoparticle formulation (Abraxane™) in breast and pancreatic cancer cell lines [170].

Other examples of actively targeted nanogels include, among many other, a chitosan/alginate hydrogel-based NP funcionalized with antibodies toward death receptor 5 (DR5) that efficiently encapsulate photodynamic agents to treat colorectal tumours [174]; targeted delivery of Dox by chitosan NP surface functionalized with Trastuzumab (Herceptin™), a humanized monoclonal antibody directed against the Her2 receptor for the treatment of advanced breast cancer [175] and; hydrophilic nanosized particles of PEG-polyethylene imine (PEG-PEI) cross-linked cationic polymer network that actively target the triphosphate form of cytotoxic nucleoside analogues with folate as targeting agent [176].

In a 2010 work, Galmarini *et al.* present nanogels conjugated with multiple molecules of tumor lymphatic-specific peptide (LyP1) that enhanced the binding efficacy of nanocarriers to lymphogenic cancer cells. The authors assessed the performance of the targeted nanoformulation loaded with gemcitabine when injected in subcutaneous gemcitabine-resistant RL7/G xenograft tumor model, which demonstrated a 2-fold more efficient tumor growth inhibition than gemcitabine at a higher dose, with no systemic toxicity during the treatment, hence extending the versatility of nucleoside analogs in the treatment of drug-resistant lymphogenic tumors [177].

4.2. Stimulus-Responsive Hydrogels

The stimulus-responsive nature is a characteristic that these nanosystems have in common with polymer-based nanosystems (see section 2.1). Therefore, the capability of responsive nanogels to adapt to surrounding environments has been extensively exploited to develop drug delivery systems with increased specificity and targeting abilities to cancer cells and tissues [178-180].

A near infrared (NIR) triggered remote control drug delivery platform based on chemically reduced graphene oxide (CRGO) modified by conjugation with chitosan (chitosan modified CRGO) and incorporated into a thermosensitive nanogel (CG-TSN) was recently introduced by Wang *et al* [181]. CRGO was chosen as photosensitizer since it has high absorption in the NIR region. Furthermore, the two-dimensional graphene structure with high specific surface area and functional groups enables facile biological/chemical functionalization. The thermo-responsive carrier employed was pNIPAm, which undergoes a reversible discontinuous phase transition in water, changing from hydrophilic (swelling) to hydrophobic (shrinking) in response to temperature changes. At last, Dox was incorporated into CG-TSN (Dox-CG-TSN) to demonstrate the NIR-light-triggered release of this widely used anticancer agent. Figure **3** shows a scheme of the synthesis and releasing mechanism of Dox-CG-TSN.

Confocal fluorescence images of TRC1 cells (mouse prostate cancer cells) demonstrated that the Dox-CG-TSN is taken up by cells *via* the endocytic pathway and transported into the endosome. Cytotoxicity assays in TRC1 cells showed that Dox-CG-TSN toxicity was less than free Dox at 37 °C but comparable to free Dox at 42 °C. Upon irradiation with NIR light (808 nm), a rapid Dox release from the Dox-CG-TSN was observed *in vitro*. When cancer cells (TRC1 and Lewis lung cancer cells, LLC1) were incubated with Dox-CG-TSN and irradiated with NIR light, the irradiated system displayed significantly greater cytotoxicity than without irradiation, owing to NIR-triggered increase in temperature leading to nuclear Dox release [181].

The group of Matyjaszewski *et al.* possesses vast experience in the preparation of functional gel materials by atom transfer radical polymerization (ATRP), formed from a dual cross-linked polymeric network including a fraction of stable cross-links and a second fraction of cleavable cross-links [83, 157, 182-184]. The interesting characteristic about the double-crosslinking system is that it can predictably open and close the polymeric network upon exposure to different redox environments. The authors prepared stable biodegradable nanogels cross-linked solely with disulfide linkages, which possess a uniformly cross-linked network that can improve control over the release of encapsulated agents. They are biodegraded into water-soluble polymers in the presence of a biocompatible glutathione tripeptide commonly found within cells. This biodegradation process triggers the release of encapsulated molecules, exemplified by rhodamine 6G, a fluorescent dye, and Dox. The results obtained from cytotoxicity assays in HeLa cancer cells suggested that the released Dox molecules could penetrate cell membranes to suppress the growth of cancer cells. Further experiments with glutathione in

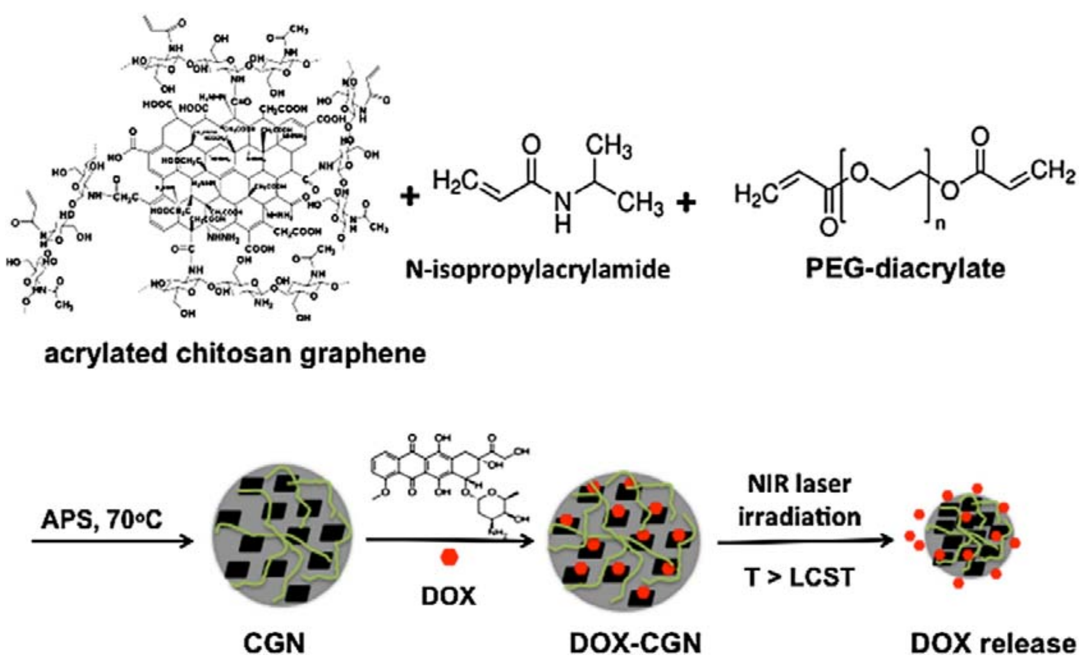

Figure 3: A schematic representation of Dox-CG-TSN synthesis and Dox release. The acrylated chitosan-CRGO monomers were copolymerized with NIPAm and PEG-diacrylate crosslinker to form a nanogel. *Reprinted with permission from Wang et al.* A chitosan modified graphene nanogel for noninvasive controlled drug release*.* Nanomedicine: Nanotechnology, Biology and Medicine IN PRESS (http://dx.doi.org/10.1016/j.nano.2013.01.003), Copyright 2013 Elsevier.

the reaction media showed that the Dox-loaded nanogels are essentially non-toxic before addition of the reducing agent, but after the reducing agent is added, the drug is released, and the cell growth is significantly inhibited due to the contact with the drug [83]. The presented nanosystem may also be targeted by its conjugation with biotin. The data presented show that each nanogel particle may bond 142,000 biotin molecules, which are promising results, since previous works have shown that the biotin-conjugated NP could improve the selective delivery of drugs into cancer cells *via* interactions with over expressed biotin receptors on the cells' surfaces [185].

Stimulus-responsive hydrogels also allow the preparation of drug delivery systems able to respond to a combination of stimulus. Many examples can be found of these dual-responsive materials [91, 186-188], like the work of Zhou *et al*. [189], who designed a chitosan-based nanogels through the physical interpenetration of chitosan chains into a nonlinear PEG chain network. The resultant PEG-chitosan nanogels not only respond to the changes in environmental pH over the physiologically important range of 5.0–7.4 but, more importantly, also allow the remotely modulation of the pH-response by external cooling/heating.

5. LIPID BASED NANOSYSTEMS

Achieving nanocarriers with low or no toxicity either *in vivo* or to the environment (as a byproduct) is one of the biggest challenges in designing drug delivery nanosystems. Ideally, the drug carrier should be removed from the body after drug release. But, unless the nanocarrier is biodegradable, it will remain in the body and be dealt as a foreign body, stimulating innate elements of the immune system (inflammation, foreign body reaction) [190, 191]. Another concern in relation to the accumulation of non-degradable materials inside the body is the potential induction of malignancy resulting from frustrated phagocytosis and prolonged inflammation [190, 192]. Lipid-based nanoparticles (LN) are probably the least toxic for clinical applications [193], especially when developed from natural lipids. The hydrophobic constituents of lipid-based systems also provide a suitable environment for entrapment of hydrophobic drugs (*e.g.* many anticancer agents), which represent about 40% of newly developed drugs [194]. LN have been used for enhancing lipophilic drug absorption, bioavailability and, of course, their clinical efficacy [39]. In the light of these advantages, three main types of LN have been extensively explored for anticancer drug delivery: liposomes (LP), solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

LP are, by far, the most studied LN. They can be defined as artificial vesicles composed of one or more closed, concentric lipid (in general, phospholipid) bilayer membranes surrounding an aqueous core [195-199]. Conventional LP are formed spontaneously by dispersion of amphiphilic lipids (and usually cholesterol) in aqueous media, which, upon hydration, self-assemble to form bilayers surrounding an aqueous interior [200-202]. Because of its characteristic biphasic structure, LP can entrap both lipophilic and hydrophilic drugs [196, 199, 202-204]. The size of LP vary widely [195, 196], but to be considered as a nano-liposome, it must be below 1000 nm. These nanocarriers can be classified in terms of their size, the number of concentric bilayers (lamellae), and the composition and physical properties of the lipids used in their formulation [199, 205, 206]. LP are generally composed from naturally occurring phospholipids, cholesterol, sphingolipids and long chain fatty acids among others, so they are readily biodegradable [201, 207]. Besides, a wide variety of phospholipids (synthetic or natural) can be used, and it is possible to change the LP size, charge, and surface properties by adding new ingredients to the lipid mixture [195]. This allows designing LP which provide control over certain important properties for drug delivery such as elimination half-lives, permeability, biodistribution and targeting specificity [200, 208]. For example, the stability of the membrane bilayer as well as the retention of encapsulated drugs depend on the lipid composition and cholesterol content of the liposomal membranes [203].

LP have been used to encapsulate and deliver chemotherapeutics drugs for more than three decades now, and currently they are extensively researched as potential vectors for gene therapy. The first nanoscale delivery system that received, in 1995, clinical approval for the treatment of acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma was a Dox hydrochloride (Dox-HCl) liposomal injection (Caelyx in Europe, Doxil in the USA) [209, 210]. Since then, other liposomal formulations have entered the market such as DaunoXome™ (daunorubicin citrate in LP from Diatos, France) for advanced AIDS-related Kaposi's sarcoma and AmBisome™ (amphotericin B in LP from Gilead Sciences, USA) for fungal infections [197].

Currently, most traditional anticancer drugs have been encapsulated into LP using different technologies and many of them have entered clinical trials, indicating that this is a fast developing field [200].

Despite the great advantages offered by the LP, there are still some important drawbacks related to the organic solvents used in their manufacturing process, instability in biological fluids and in aqueous solutions, poor batch-to-batch reproducibility and difficulties in their sterilization [211, 212]. In the search for other LN, NLC and SLN have been developed and used as parenteral drug delivery systems, mainly in cancer chemotherapy [213, 214].

The SLN were developed in the middle of the 1990s, by replacing the oil of an oil-in-water nanoemulsion by a solid lipid or a blend of solid lipids [215]. The use of solid lipids instead of oils follows the idea of achieving more control over drug release, since the drug molecule mobility in a solid matrix should be intuitively lower compared with an oily phase [194]. The diameter of SLN ranges between 50 and 1000 nm [216], large-scale manufacturing is possible (while other systems such as polymeric NP have faced scaling-up issues) and solvent use can be avoided using high-pressure homogenization with extant machinery [193, 194, 217]. The drawbacks to the use of SLN come from the formation of a highly ordered, perfect lipid crystal matrix, which limits their loading capacity [218]. After preparation, at least some of the particles crystallize in higher energy modifications that, during storage, evolve to a low energy modification that leads to drug expulsion [193].

In order to solve these limitations, a second generation of LN, NLC, has been developed by blending the solid lipids in SLN with oils, which provides a less-ordered (and even amorphous) solid lipid matrix, so that the active ingredient load can be increased and the expulsion of the drugs during storage avoided [193, 194, 216, 219, 220]. Different types of NLC can be obtained depending on the method of production and the composition of the lipids blend: imperfect, amorphous and multiple type. In the imperfect type, lipid crystallization is altered by small amounts of oils. In the amorphous type, the lipid matrix is solid but not crystalline (amorphous state): this can be achieved by mixing specific lipids, for example, hydroxyoctacosanyl hydroxystearate and isopropyl myristate. In the multiple type, the solid lipid matrix contains tiny oil compartments: they are obtained by mixing a solid lipid with a higher amount of oil [219].

5.1. Triggerable LN

As said before, an important requirement for effective drug delivery is the precise spatial and temporal release of therapeutic agents at the target site. For this purpose, stimuli-responsive (or stimuli-triggered or smart) LP have been conceived. Various triggering mechanisms have been described in the literature, including those that rely on changes in local microenvironment such as decreased pH [221, 222] and the presence of specific enzymes [223], as well as the use of externally applied triggers such as light [224], ultrasound [225] and heat [226, 227]. Electromagnetic radiation-sensitive LP present a promising system and rely on strategically designed phospholipid molecules to initiate light-induced release [224]. The principles of phototriggering include photopolymerization of lipids, photosensitization by membrane anchored hydrophobic probes, or photoisomerization of photoreactive lipids [228, 229].

A novel class of photo-triggerable LP prepared from dipalmitoyl phosphatidylcholine (DPPC) and a photopolymerizable phospholipid, (1,2 bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$), which efficiently release entrapped calcein (a water soluble fluorescent dye) upon UV (254 nm) treatment has been recently developed [230]. The photopolymerizable diacetylene phospholipid $DC_{8,9}PC$ is present in lower organisms [231] and, due to the highly reactive diacetylene groups uniquely assembled in the lipid bilayer, photopolymerization by UV treatment results in chains of covalently linked lipid molecules within the bilayer [232]. To date, various photo-triggerable liposome formulations are described in literature as candidates for localized drug delivery, however, none of these studies have demonstrated light-triggered delivery of cytotoxic agents to improve cell killing. The work of Yavlovich *et al.* [233] demonstrates that the light-triggered release of an anticancer agent (Dox) from light-sensitive LP (containing DPPC:$DC_{8,9}$PC:DSPE-PEG2000 at 86:10:04 molar ratio) promotes Raji (a B-lymphocyte cell line) and MCF-7 cell killing compared to untreated samples. However, these LP are only targeted by the EPR effect, so an improvement can be envisioned by integrating them with active targeting strategies as already commented in section 2.1.

Yuba *et al.* [234] developed highly pH-sensitive LP for the delivery of antigenic molecules (ovalbumin, OVA) into cytosol. Remarkably, the authors rely on a combination of materials, in this case, polymer-modified LP. pH-sensitive fusogenic polymers which confer fusion ability under weakly acidic conditions, were developed by carboxylation of poly(glycidol)s, which are known to be highly biocompatible [235], with acid anhydrides of various kinds [236-238]. The surface modification of stable egg yolk phosphatidylcholine (EYPC)/DOPE LP with 3-methylglutarylated poly(glycidol) of linear (MGlu-LPG) or hyperbranched (MGlu-HPG) structure [237] can provide pH-sensitive destabilizing properties. These polymer-modified LP are stable at neutral pH, but they become strongly destabilized below pH 6, which corresponds to the pH of endosomes. The polymer-modified LP loaded with OVA were taken up by murine dendritic cells (DCs) more efficiently than the unmodified LP. Administration of these LP loaded with OVA into mice bearing E.G7-OVA tumor (which is a chicken egg OVA gene-transfected clone of EL4, a C57BL/6 mice-derived T lymphoma, and which presents OVA with MHC class I molecules) induced strong OVA-specific cellular immune responses sufficient to entirely reject the engraftment of OVA-expressing tumor cells, and even established tumor burdens of the mice were completely eliminated by immunization with the polymer-modified LP. These highly pH-sensitive polymer-modified LP are extremely effective for the induction of antigen-specific immunity. Therefore, they are promising for use as antigen delivery systems for efficient cancer immunotherapy.

Cationic LP have the capability of facilitating the tumor cellular uptake by electrostatic absorptive endocytosis [239, 240], since many cancer cell membranes possess an overall negative charge resulting from the presence of sialic acid and proteoglycans. Besides, cationic LP tend to fuse with the endosomal/lysosomal membrane under the presence of specific lipids for membrane fusion, thus releasing their contents into the cytoplasm [241-243], or producing a proton sponge effect similar to PEI, which in turns leads to the swelling and disruption of the endosomes/lysosomes for cytoplasmic liberation of the intact LP [244, 245].

Despite these advantages, positively charged NP cause severe cytotoxicity, serum inhibition and a rapid clearance from the reticulo-endothelial system (RES) as a result of aggregation with plasma proteins [246]. Introduction of a PEG-modified lipid (PEG-lipid) into the cationic liposomal membrane is a common approach for application of cationic LP *in vivo*, which partially diminishes the net surface charge and the interaction with opsonin, thus increasing the circulation time. LP functionalized with a PEG deshielding mechanism introduced by a degradable pH-sensitive bond between PEG and lipid (such as hydrazone bonds) have been studied [247-249], as anticipated in the introduction. Obata *et al*. developed a pH-responsive LP containing synthetic glutamic acid-based zwitterionic lipids, which can quickly change surface charge from negative to positive at endosomal/lysosomal pH (pHi, pH 4.0-6.0), producing efficient release of drugs into the cytoplasm [250]. However, these LP still present negative charge at tumor microenvironmental pH (pHe, pH 6.0-7.0) and had no effect on subcellular targeting.

In order to integrate the merits of anionic LP for lower hematotoxicity, PEGylated LP for longer circulation in the blood, and cationic LP for enhanced uptake at the tumor site and efficient intracellular delivery in the tumor cells, Mo *et al*. have studied pH-sensitive zwitterionic oligopeptide LP [221, 251]. In their last work, the pH-sensitive LP developed were based on two synthetic amino acid-based lipids, 1,5-dioctadecyl-L-glutamyl 2-histidyl- hexahydrobenzoic acid (HHG2C18) and 1,5-distearyl N-(N-α-(4-mPEG2000) butanedione)-histidyl-L-glutamate (PEGHG2C18), which have a multistage pH-response to pHe and pHi successively [221]. Therefore, two types of pH-sensitive LP were developed and evaluated for effective intracellular delivery and enhanced antitumor activity: HHG2C18-L, which contains only HHG2C18, and PEGHG2C18-L, which includes both HHG2C18 and PEGHG2C18. Both of them have the capability of charge conversion in response to the surrounding pH, achieving increased tumor cellular uptake at pHe, and the effect on endosomal/lysosomal escape and mitochondrial targeting for enhanced antiproliferation and apoptosis (Figure **4**). In particular, both LP loaded with temsirolimus (CCI-779) had significantly higher antiproliferative and apoptosis-inducing effect on the human renal carcinoma (A498) cells. *In vivo*, CCI-779/PEGHG2C18-L displayed higher blood persistence and antitumor efficacy against xenograft renal cancer (Renca) tumor models in comparison with CCI-779/HHG2C18-L.

Cell-penetrating peptides (CPPs) that facilitate the cellular uptake of various cargos without causing any cellular injury have been widely investigated in the fields of gene and drug delivery for cancer therapy [252-254]. Specially, pH-responsive CPPs [255-257] have been developed based on the pH gradient between the tumor milieu and physiological environment [258]. Unfortunately, recent studies have

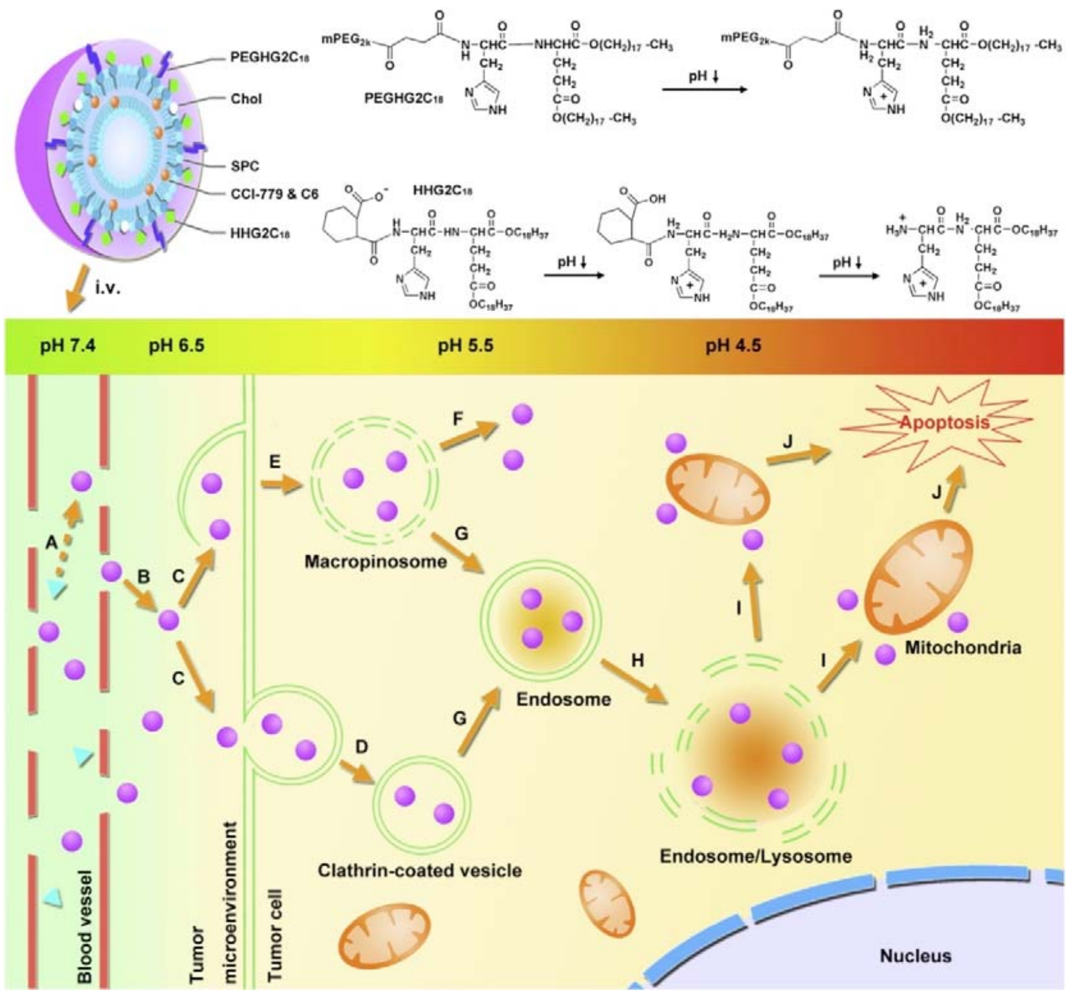

Figure 4: Scheme of the mechanism of action proposed for the PEGylated zwitterionic oligopeptide LP (PEGHG2C18-L), composed of soy phosphatidycholine (SPC), cholesterol (Chol) and two synthesized amino acid-based zwitterionic lipids (PEGHG2C18 and HHG2C18). PEGHG2C18 and HHG2C18 can respond to tumor extracellular and intracellular pH to endure PEGHG2C18-L with efficient intracellular delivery and enhanced antitumor efficacy. (**A**) The PEG outer corona and negatively charged surface provide a good protection for LP (spheres) away from the attack of plasma proteins (triangles) in the blood. (**B**) Targeting of LP through EPR effect. (**C**) Charge conversion from negative to positive for enhanced cellular uptake at tumor pH. (**D**) Clathrin-mediated endocytosis. (**E**) Macropinocytosis. (**F**) Leakage of LP from the porous macropinosomes. (**G**) Delivery to endosomes. (**H**) Endosomal/Lysosomal escape as a result of the proton sponge effect. (**I**) Cytoplasmic liberation and subsequent mitochondrial targeting. (**J**) Promotion of cell death *via* mitochondrial apoptotic pathway. *Reprinted with permission from Mo et al.* Intracellular delivery and antitumor effects of pH-sensitive liposomes based on zwitterionic oligopeptide lipids. Biomaterials 2013; 34: 2773-86, Copyright 2013 Elsevier.

suggested that CPPs on the surface of LP and micelles are susceptible to enzymatic cleavage by enzymes present in human plasma [259]. To address this dilemma, surface hyaluronic acid (HA) coating of CPP-modified LP (HA-CPP-LP) was used since HA with negative charge in neutral pH condition can be easily adsorbed on the surface of cationic LP [260-262]. HA is generally regarded as non-toxic and biodegradable, and overexpression of HA-binding receptors, such as CD44 and RHAMM, has been found on the cell surface of several malignant tumors [263-265], which explains the broad applications of HA-based polymers in active tumor targeting for anticancer drugs [266-268]. More importantly, hyaluronidase (HAase) is widely distributed in the acidic tumor extracellular matrix, playing a significant role in tumor growth, invasion and metastasis [269, 270]. HA is susceptible to hydrolysis by HAase, allowing exposure of CPPs in HA-CPP-LP to facilitate effective admission of LP into the tumor cells.

In order to combine the advantages of pH-responsive CPPs for efficient intracellular delivery and HA for both improved blood persistence and tumor targeting, Jiang *et al.* [271] developed dual-functional LP with pH-responsive CPPs and active targeting HA. In fact, after HAase treatment, paclitaxel-loaded HA-CPP-LP presented a remarkably stronger cytotoxicity toward the hepatic cancer HepG2 cells at pH 6.4 relative to the cytotoxicity at pH 7.4. The LP showed efficient intracellular trafficking including endosomal/lysosomal escape and cytoplasmic liberation and stronger antitumor efficacy against murine hepatic carcinoma (Heps) tumor xenograft models *in vivo*.

Drug-loaded microbubbles (MB) in combination with therapeutic ultrasound (US) have also become a promising therapeutic approach for drug delivery to treat malignant tumors [272]. Drugs can either be incorporated into the MB shell by hydrophobic or electrostatic interactions [273, 274] and they are released by insonation with high-intensity focused US. Currently, the limited drug-loading capacity of MB with lipid monolayer shells presents a major drawback to this therapeutic strategy [275]. One solution may be the recent introduction of US-triggered liposome-microbubble complexes (LMC). There have been several reports of efficient *in vitro* controlled drug delivery by US-triggered LMC [276]. Yan *et al.* [277] developed paclitaxel-LMC (PLMC) as possible US-triggered targeted chemotherapy against breast cancer. PLMC were conjugated to the MB surface through biotin–avidin linkage, increasing the drug-loading efficiency of MB. Significantly increased release of payloads from LMC was achieved upon US exposure, and significant increase of drug concentration in tumors was observed in comparison to treatment with non-conjugated paclitaxel-LP or PLMC without US. In fact, fluorescent quantum dots (QDs) were used as a model drug to show that released QDs were taken up by 4T1 breast cancer cells treated with QD-LMC and US. Uptake was dependent on the exposure time and intensity of insonication. PLMC possessed significantly greater tumor growth inhibition effect both *in vitro* and *in vivo.*

Limitations of adenoviral (Ad) vectors for cancer gene therapy could be overcome by their combination with pharmaceutical technologies. Anionic LP (AL) significantly boosted the transduction efficiency of Ad in numerous cells such as LLC, B16, A549 and MDCK, and exhibited low cytotoxicity compared with cationic LP. Moreover, the AL-Ad vectors (AL-Ad) have demonstrated to confer some protection from neutralizing antibodies. However, the system lacked selectivity and specificity, and it could be completely inactivated in the presence of high titers of anti-adenovirus antibody *in vitro*. Therefore, the previously reported AL-Ad formulations were administered to tumor bearing mice by intratumor injections rather than intravenous injections [239, 240, 278]. Recently, matrix metalloproteases (MMPs) have attracted much attention owing to their ability to degrade the extracellular matrix (ECM), which is involved in the angiogenesis, invasion and metastasis of malignant tumors. In particular, type IV collagenases (MMP-2 and MMP-9) have been reported to play vital roles in this process [244], and the expression levels of MMPs were found to be relatively high in tumor cells compared to non-malignant cells. On the basis of these findings, Wan *et al.* [279] designed an enzymatically cleavable PEG-lipid material that was composed of a PEG/matrix metalloproteinase (MMP)-substrate peptide/cholesterol (PEG-peptide-chol, PPC) and is specifically cleaved by MMPs in the extracellular space in tumor tissues. The obtained MMP-cleavable lipids were inserted into the AL-Ad by the post-insertion method. The results of *in vitro* infection assays indicated that the enzymatically cleavable formulation (PPC-AL-Ad) displayed a much higher gene expression than both the naked Ad and a non-cleavable one. More importantly, PPC-AL-Ad induces a lower production of neutralizing antibodies and lower innate immune response; it also showed reduced liver toxicity *in vivo* on specific pathogen-free C57BL/6N mice. These findings suggest that PPC-AL-Ad is a promising system for gene delivery in tumor therapy.

Heat is another trigger actively investigated for controlled drug release. Thermosensitive LP (TLP) have become a very attractive vehicle for drug delivery applications [280-282]. Cargo drugs are released when the environmental temperature is above the membrane melting temperature (Tm) of TLP, which is tunable [281, 283]. The stability and release efficiency are among the main concerns for developing new TLP. Fe_3O_4 magnetic NP are also widely applied for molecular imaging, drug/gene delivery and drug removal due to magnetic and/or magnetocaloric effects [284-286]. Ding *et al.* [287] reported a combination of these two stimuli-responsive approaches by developing PEGylated thermosensitive magnetic LP (TMLP) entrapping oleic acid-coated Fe_3O_4 magnetic NP. TMLP were stable and impermeable at body temperature and the tested drugs (5-(and-6)-carboxylfluorescein and Dox) were released within 1 h at 42 °C.

5.2. LN to Overcome the Multi-Drug Resistance Profile of Cancer Cells

Multi-drug resistance (MDR) is a serious obstacle in cancer treatment. In recent years, nanocarriers have been explored for the potential to overcome tumor resistance and LP, in particular, are one of the most extensively studied systems. Various gene-silencing tools that specifically inhibit the expression of target genes have been developed, such as antisense oligonucleotides (ASOs) and short interfering RNA. Several ASOs targeting genes involved in neoplastic progression have been evaluated as potential therapeutic agents [288, 289]. Once the cancer cells have developed two major mechanisms of resistance to the chemotherapeutic treatment, namely pump and nonpump resistances, a rational antisense strategy involves targeting more than one oncogene or resistance-related gene simultaneously. P-glycoprotein (P-gp), Multidrug Resistance Protein MRP1, and MRP2 are three major ABC transporter proteins responsible for pump resistance, whereas BCL-2 and BCL-xL are well characterized as mediators of nonpump resistance for a wide variety of apoptotic stimuli [290-293]. Moreover, bispecific ASOs targeting a region of high homology shared by BCL-2 and BCL-xL as a suppressor of nonpump resistance induce apoptotic cell death in various tumor cells [294, 295]. In this sense, Lo *et al.* [296] developed PEGylated cationic LP loaded with epirubicin (Epi), and/or ASOs targeting MDR-associated protein (MDR1), MRP1, MRP2, and BCL-2/BCL-xL. Pegylated positively charged LP remarkably enhance the cytotoxicity of Epi on mouse colon adenocarcinoma CT26 cells. The liposomal Epi and ASOs (Epi-ASOs-LP) significantly increase the accumulation of Epi in CT26 murine colon carcinoma cells, indicating that pump resistance is effectively reversed. Epi-ASOs-LP have also demonstrated substantial improvements in tumor growth inhibition and survival percentage in CT26-bearing Balb/c mice *in vivo*. This pioneering study demonstrates that Epi-ASOs-LP targeting both pump and nonpump resistances increase antitumor efficacy *in vivo* through the simultaneous inhibition of MDR transporters and apoptosis induction.

5.3. Subcellular Targeting

Besides targeting specific cells or tissues, recent research on targeted nanosystems also explores subcellular targeting. It is believed that mitochondrial targeting may enhance efficiency and specificity of anticancer drugs for cancer treatment [297], since mitochondria, the powerhouses of the cells, are also implicated in the regulation of cellular differentiation and growth as well as programmed cell death, especially in tumor cells [298].

Zhou *et al.* synthesized a D-α-tocopheryl polyethylene glycol 1000 succinate-triphenylphosphine conjugate (TPGS1000-TPP) as a mitochondrial targeting molecule, which was incorporated onto the surface of paclitaxel LP to treat drug-resistant lung cancer [299]. Triphenylphosphine (TPP) is a delocalized lipophilic cation with the ability to transport across mitochondrial membranes [300]. It has been reported that TPP can accumulate into highly negatively charged mitochondria of cells, including the ones in cancer cells [301, 302]. The system was tested on human lung cancer A549 cells, drug-resistant lung cancer A549/cDDP cells, and on the drug-resistant lung cancer A549/cDDP cells xenografted nude mice. In comparison with Taxol^{TM} and regular paclitaxel LP, the targeting paclitaxel LP exhibited the strongest anticancer efficacy *in vitro* as well as in the drug-resistant A549/cDDP xenografted tumor model. The targeting paclitaxel LP were selectively accumulated into the mitochondria, and enhanced apoptosis by acting on the mitochondrial signaling pathways.

Malhi *et al*. developed a similar strategy, based on the delivery of redox cycler-Dox to the mitochondria of cancer cells, where it acts as a source of exogenous ROS production [303]. Cancer cells present higher levels of ROS in comparison to the normal cells [304, 305], thus being more vulnerable to further oxidative stress induced by exogenous ROS-generating agents [306] known as 'Redox Cyclers' [307]. Dox is an example of redox cycler agent [308]. Under this idea, the purpose of the authors was to perform surface modifications of LP with dual ligands, FA for cancer cell targeting and TPP cations for mitochondria targeting, resulting on a FA-TPP-LP. The cytotoxicity, ROS production and cellular uptake of Dox loaded LP were evaluated in FR (+) KB cells and found to be considerably increased with FA-LP. As confirmed by confocal microscopy, the TPP-LP delivered Dox to mitochondria of cancer cells and also showed higher ROS production and cytotoxicity in comparison to FA-LP and non-targeted LP. Most importantly, FA-TPP-LP showed superior activity over mitochondria targeted LP, which confirms the synergistic effect imparted by the presence of dual ligands on the enhancement of cellular and mitochondrial delivery of Dox in KB cells.

5.4. Brain Targeting

Brain delivery of drugs is frequently impaired by the presence of the BBB, a physical and biochemical barrier that regulates drug transference between the blood and the central nervous system. A number of recent reports present SLN successfully targeting the brain, and thus potentially useful in the delivery of chemotherapeutic agents to brain tumors.

The work of Agarwal *et al*. [309] introduces SLN surface engineered with cationic bovine serum albumin (CBSA) as vectors to bypass the BBB and provide improved therapeutic efficacy of encapsulated anti-cancer drug methotrexate (MTX). CBSA shows good accumulation in the brain and preferential distribution in brain tissue compared to other organs like liver, heart and lung [310-312]. This ligand promotes transport of fluorescent probes across the BBB, apparently undergoing transcytosis-mediated absorption [313, 314]. Hemolytic studies on the CBSA-SLN from Agarwal *et al*. suggested that the formulation is biocompatible. A transendothelial transport study on brain capillary endothelial cells (BCs) confirmed that CBSA-SLN are up-taken through transcytosis. The CBSA-SLN increases the uptake of MTX by human neuroglial culture HNGC1 tumor cells compared to unconjugated SLN and free MTX, exhibiting a more potent cytotoxic effect than free MTX.

Venishetty *et al*. [315] presented surface modified SLN for combined therapy with docetaxel and ketoconazole. Even though docetaxel inhibitory effect on cancer cells is potentiated when administered with ketoconazole, potential drug-drug interactions between these two drugs may result from the fact that both agents are hepatically metabolized by the cytochrome P-450 system [316, 317], while ketoconazole can also inhibit P-gp efflux of docetaxel at the BBB [318]. Plasma and brain pharmacokinetics have shown increased brain uptake of docetaxel with surface-modified dual drug-loaded SLN. Brain permeation coefficient of folate-grafted docetaxel and ketoconazole loaded SLN was higher than that of Taxotere^{TM}.

Kuo and Liang have developed innovative catanionic SLN (CASLNs) carrying carmustine (BCNU) and grafted with anti-epithelial growth factor receptor (anti-EGFR) antibody [319]. The catanionic microemulsion is a recently developed colloidal system containing a mixture of oppositely charged surfactants to form vesicles or micelles [320]. Anti-EGFR/BCNU-CASLN demonstrated an effective delivery of BCNU to human brain malignant glioblastoma U87MG cells and antiproliferative efficacy against the growth of human GBM U87MG cells. Moreover, the same authors tested the anti-EGFR-CASLN for encapsulation of Dox with good results [321].

5.5. Multifunctional LN

LP were the first nanopharmaceuticals introduced to market; thus they have reached a high level of development and optimization, which led to more than 2000 papers and 200 reviews published only in 2011 and many commercialized liposomal drugs for cancer therapy [322, 323]. Multifunctional liposomal nanocarriers (MLN, Figure **5**) are at the top of the art and they have been intensively explored in the last years in order to combine in a single nanodevice a

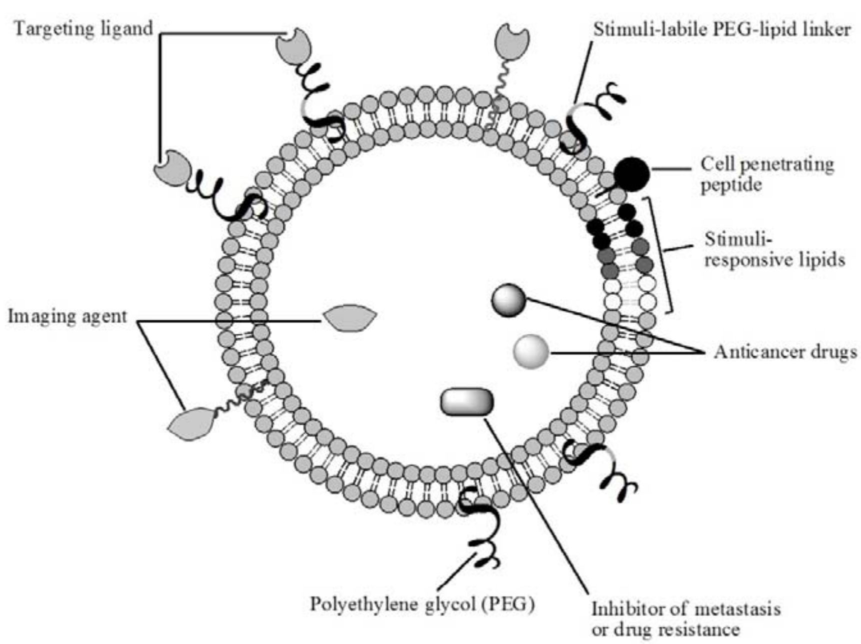

Figure 5: Scheme of a generic multifunctional liposomal nanocarrier (MLN).

number of desired features such as long blood circulation, high stability *in vivo*, high drug and/or imaging agent loading, selective distribution to the neoplasm lesion relative to healthy tissues, remote-controlled or stimuli-sensitive extravasation, surface modifications to enhance cell internalization, intracellular payload release and even intracellular targeting to a specific organelle of the tumor cell [324].

Some of the works discussed in the previous subsections showed a diversity of examples of MLN, such as the pH-sensitive zwitterionic oligopeptide LP which combine higher blood persistence, capability of charge conversion in response to the surrounding pH and efficient intracellular delivery of the loaded drug(s) within the tumor cell; the dual-functional LP with pH-responsive CPPs and active HA-targeting which allow efficient intracellular delivery and enhanced tumor targeting; the PEGylated thermosensitive magnetic LP entrapping oleic acid-coated Fe_3O_4 magnetic NP and; the Epi and ASOs loaded PEGylated cationic LP which can target both pump and nonpump resistances increasing antitumor efficacy *in vivo* through the simultaneous inhibition of MDR transporters and apoptosis induction (see 5.1 and 5.2 subsections). In the following paragraphs we will briefly describe some other outstanding works in the field of MLN.

To provide a novel early diagnostic method and targeted therapy for pancreatic cancer, a multifunctional nanoimmunoliposome with high loading of ultrasmall superparamagnetic iron oxides (USPIOs) and Dox, conjugated with anti-mesothelin monoclonal antibody to target anti-mesothelin-overexpressed pancreatic cancer cells was developed by Deng *et al.* [325]. The *in vitro* and *in vivo* properties of this anti-mesothelin antibody-conjugated PEGlyated liposomal Dox and USPIOs (M-PLDU) and PEGlyated nanoimmunoliposomes without antibody conjugation (PLDU) were evaluated both in human pancreatic cancer cell line Panc-1 and in a pancreatic cancer xenograft animal model. The *in vitro* study demonstrated that the M-PLDU possessed good MRI capability and significant inhibitory effect. The *in vivo* antitumor study demonstrated that compared with free Dox and PLDU, M-PLDU possessed higher inhibitory effect on tumor growth. The tissue distribution assay further proved that M-PLDUs could selectively accumulate in the tumor xenograft. These results indicated that M-PLDU not only retained the inherent MRI capability of USPIOs, but significantly improved the targeting distribution of USPIOs and therapeutic agents to pancreatic tumor tissues.

It has been pointed out that nitric oxide (NO) may function as a double-edged sword in cancer therapy, with relatively low concentrations promoting tumor growth and proliferation, and high concentrations of NO and other related reactive nitrogen species mediating cell apoptosis and inhibiting cancer cells growth [326-333]. NO also acts as a sensitizer leading to enhanced cell death when irradiating with γ-radiation [334-336]. For therapeutic NO delivery, it is then critical to be able to control the concentration of NO released in the cell. With the intention of designing stimuli responsive NO releasing complexes for therapeutic applications, Ostrowski *et al.* developed the NO precursor trans-$Cr(L)(ONO)2^+$ (L = cyclam = 1,4,8,11-tetraazacyclotetradecane, CrONO, or L = mac = 5,7-dimethyl-6-anthracenylcyclam, mac-CrONO) and related compounds that release NO after irradiation with UV or visible light [337-341]. Photochemical triggering of such a “caged” bioactive agent provides the ability to control the timing, dosage, and location of the NO release by controlling the timing, intensity, and location of light irradiation, respectively [342, 343]. The most recent work of the group describes the development of CrONO complexes encapsulated in phosphatidylcholine LP [344]. The LP provide a mean to maintain a localized high concentration of NO releasing complexes and are easily modified for *in vivo* targeting. Encapsulated mac-CrONO showed NO release after photolysis with low-intensity blue light. Furthermore, the fluorescence of mac-CrONO allows for development of theranostic NO delivery vessels where tracking and imaging can occur simultaneously with therapeutic NO release.

The search on the MLN field includes the development of novel materials which allow improving the features of the MLN. For example, novel hydrazine functionalized PEG-phosphatidylethanolamine-based amphiphilic polymer have been developed by Biswas *et al.*, which can conjugate the LP to a variety of ligands *via* a reversible pH-cleavable bond [345]. The work by Zhu *et al.* describes two novel functional polymers: TATp-PEG2000-1,2-dioctadecanoyl-*sn*-glycero-3-phosphoethanolamine (TATp-PEG2000-DSPE) and maleimide-PEG3400-MMP-2 cleavable peptide-1,2-dioleoyl-*sn*-glycero-3- phosphoethanolamine (MALPEG3400-peptide-DOPE) for surface modification of pharmaceutical nanocarriers [346]. By using these polymers, a novel multifunctional stimulus sensitive liposome was developed, which includes several functions: (i) passive tumor targeting by the EPR effect due to longevity imparted by long PEG chains; (ii) prevention of the nonspecific intracellular uptake on the way to the tumor by steric shielding of surface-attached cell-penetrating function (TATp) moieties with the long-chain PEG; (iii) the active tumor targeting by a surface-attached cancer specific monoclonal antibody (mAb 2C5); (iv) the detachment of the long-chain PEG in the tumor due to the cleavage of the MMP-2-cleavable linker between the long chain PEG and the nanocarrier, resulting in the exposure of the cell-penetrating TATp; and (v) the enhanced cellular internalization by TATp-mediated endocytosis. Novel lipids to be applied in the development and optimization of multifunctional drug-carriers in order to modulate drug release and track lipid-based drug-carriers have been developed [347].

Other reports that deserve mentioning include the multifunctional HaT (Hyperthermia-activated-cytoToxic) thermosensitive liposome formulation consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and Brij78 surfactant, that co-encapsulates Gd-DTPA (Gd-diethylene triamine pentaacetic acid, an MRI probe) and Dox for enhanced drug targeting to locally heated tumors and real-time monitoring of clinical response [348]; and a hybrid nanosystem consisting in cisplatin and quantum-dots loaded LP (QDLs) developed by Zhang *et al*. [349]. These cisplatin QDLs include CdSe or CdSe/ZnS QDs for both drug delivery and bioimaging and have demonstrated effective internalization, significant fluorescence and higher cytotoxic activity in melanoma cells compared to an equal dose of free cisplatin. As can be appreciated, the multifunctional systems have become the goal to pursue in the field of nanocarriers, with numerous attempts to combine the best features of different systems, materials and functionalities.

6. INORGANIC NANOSYSTEMS

Inorganic nanosystems are extensively investigated for imaging and therapeutic applications owing to their unique properties (*e.g.* optical and magnetic properties), which made them particularly suitable for diagnosing and monitoring applications, and for the design of stimuli-responsive nanovehicles. Some materials (such as gold) allow preparing highly monodisperse NP in a wide range of arrangements. The inert nature of materials such as silica or gold constitutes both bless and curse for their bioapplications. In the light of the safety considerations briefly exposed at the beginning of section 5, the reader can imagine the important health concerns posed by a chemically and physically inert nanovehicle whose size exceeds the renal filtration threshold. Among all nanosystems, inorganic NP are thus the ones that raise more safety concerns. Noteworthy, a study on the *in vivo* toxicity of gold NP showed that administration of 8 mg/kg/week of 8 to 37 nm particles produced, from day 14, severe side-effects such as camel-like back and crooked spine in mice [350]. Histological examination revealed various degrees of abnormality in the liver, lung and spleen of gold nanoparticle-treated mice. The median survival time was also significantly reduced. These important results underline the fact that *in vitro* assessment on a single cellular line may often not be representative of the behavior of the nanosystem in a whole organism, claiming for the development of complex cellular models and the unavoidable use of animal models. The previous study clearly states the cautions that should be taken regarding the clinical use of nanosystems as drug delivery devices, particularly if they are non-biodegradable and in long-term treatment settings, and the need to develop standardized procedures to perform nanotoxicology studies [351, 352]. We will present a brief overview on three kind of inorganic nanosystems that have attracted increasing attention for the treatment of cancer: gold nanocarriers, magnetic NP and mesoporous silica NP, as well as the combination of these three systems (and the ones described in previous sections) to give nanocomposites that merge the properties of the separate materials and arrangements.

Gold NP join fascinating optical properties with their chemical inert nature, which creates a great number of potential applications in diagnostics and therapeutics. Metals can be represented as confined plasma of positive ions and mobile conduction electrons. An interacting electromagnetic field (*e.g.* light) can induce a coherent oscillation against the restoring force of positive nuclei. The collective oscillations of conduction electrons upon excitation with electromagnetic radiations are known as plasmons, which give rise to a strong absorption band attributed to resonance (surface plasmon resonance) between the oscillating electrons and the incident radiation. When conductive NP whose size is smaller than the wavelength of light are irradiated, a local surface plasmon resonance phenomenon that lies in the UV-vis range is observed [353-356]. Equally important, the frequency and intensity of the surface plasmon absorption bands are highly sensitive to NP composition, sizes, size-distribution, geometry, morphology, surface coating, environment and separation distance. In other words, optical properties of metallic NP are tunable. Through manipulation of those variables a band shift to even the infrared region can be achieved, which is interesting since biological tissues are transparent to this range of the electromagnetic spectrum [357]. The unique and customized optical properties make these systems ideal candidates for phototermal therapies (light-induced plasmonic heating), photo-triggered controlled drug release and imaging purposes, as well as integrative diagnostics and therapeutics (theranostics). On the other hand, unlike fluorescent materials, gold experiences no photobleaching, while thiol-gold association allows for ready functionalization [353]. The aqueous synthesis of gold NP is well established (they can be easily prepared by reducing their salts in aqueous solutions) [354] and they can be easily adjusted to a desirable size between 0.8 and 200 nm [358].

Superparamagnetic NP are obtained by scaling ferromagnetic and ferrigmagnetic materials to nano-sizes. In such small systems, the magnetic moment of the NP is free to fluctuate in response to thermal energy, while the individual atomic moments maintain their ordered state [359, 360]. In the absence of an external magnetic field, their magnetization appears to be in average zero. Under a magnetic field, however, they exhibit a magnetic signal far exceeding that of biomolecules and cells. What is more, under an alternating magnetic field, the magnetization of the superparamagnetic NP can be switched back and forth turning the NP into local heaters (magnetic fluid hyperthermia), which seems auspicious for cancer treatment. Evidently, targeted delivery through application of an external, high-gradient magnetic field has also been proposed [358].

Mesoporous silica NP have attracted a lot of attention for controlled delivery applications. This can be explained by considering their particular properties [354, 361, 362]: they are chemically and physically stable (they are very stable against coagulation) and present a rigid framework; they show very uniform, tunable pore size (2-6 nm), which allows loading different drugs and studying the release kinetics with high precision; they have an enormous specific surface (up to more than 1000 m^2/g) and large pore volume (more than 0.9 cm^3/g), allowing high payloads; they can be readily functionalized *via* silylation and; they present a unique porous structure with no inter-connectivity between them that minimizes the chance of premature drug release even in the case of non-perfect capping (in other systems such as dendrimers pore encapsulated guest molecules can leak through the interconnected porous matrix when some of the pores are not capped).

6.1. Nanocomposites of Different Inorganic Or Organic/Inorganic Materials

As observed in other materials, recent progress on application of inorganic NP to cancer treatment emerges from juxtaposition of carriers and materials that were studied in a separate manner in the past years, allowing multifunctionality.

For example, Liu *et al.* have recently developed dendrimer stabilized gold-silver alloy NP [363]. The amine terminated G5 PAMAM dendrimers that provide colloidal stability to the metallic particles and augment their aqueous solubility were modified with FA to achieve active targeting.

Yang *et al*. reported a novel multifunctional nanocomposite where superparamagnetic Fe_3O_4 NP were fixed between a pH-sensitive polymer conjugated with FA, and a mesoporous silica NP core [364]. The silica core provides high drug loading capacity, while the pH-responsive polymer allows controlled release in the mildly acidic environment of cancer cells. The targeting process may be traced through the magnetic signal. Zhang *et al.* developed multifunctional silica NP capped with cleavable disulfide bonds bridged to amino-β-cyclodextrin [365]. The β-cyclodextrin strongly complexes PEG polymers functionalized with adamantine and FA. The cleavable cyclodextrin capping retains the tested drug (Dox) inside the mesopores, preventing premature release. Once inside the cell, the acidic environment and the reduction of the disulfide bonds by high glutathione concentrations trigger the liberation of the active ingredient.

Ma *et al.* synthesized a multifunctional nanoplatform based on a Fe_3O_4 core surrounded by a mesoporous silica shell capped by gold nanorods (Figure **6**) [366]. Such system integrates chemotherapy, magnetic resonance and IR imaging and photothermal therapy. Anticancer drugs can be efficiently loaded into the mesopores of the silica shell (an encapsulation of up to 30 wt% Dox was achieved). The system is practically inert at blood pH, but releases Dox in the acidic environment of cancer cells, in a pH-responsive way. *In vitro* and *in vivo* experiments confirmed the feasibility of using this platform in photothermal therapy under near IR irradiation, while *in vitro* experiments showed a synergistic effect of the combined chemo- and photothermotherapy between 39-42 ºC, suggesting

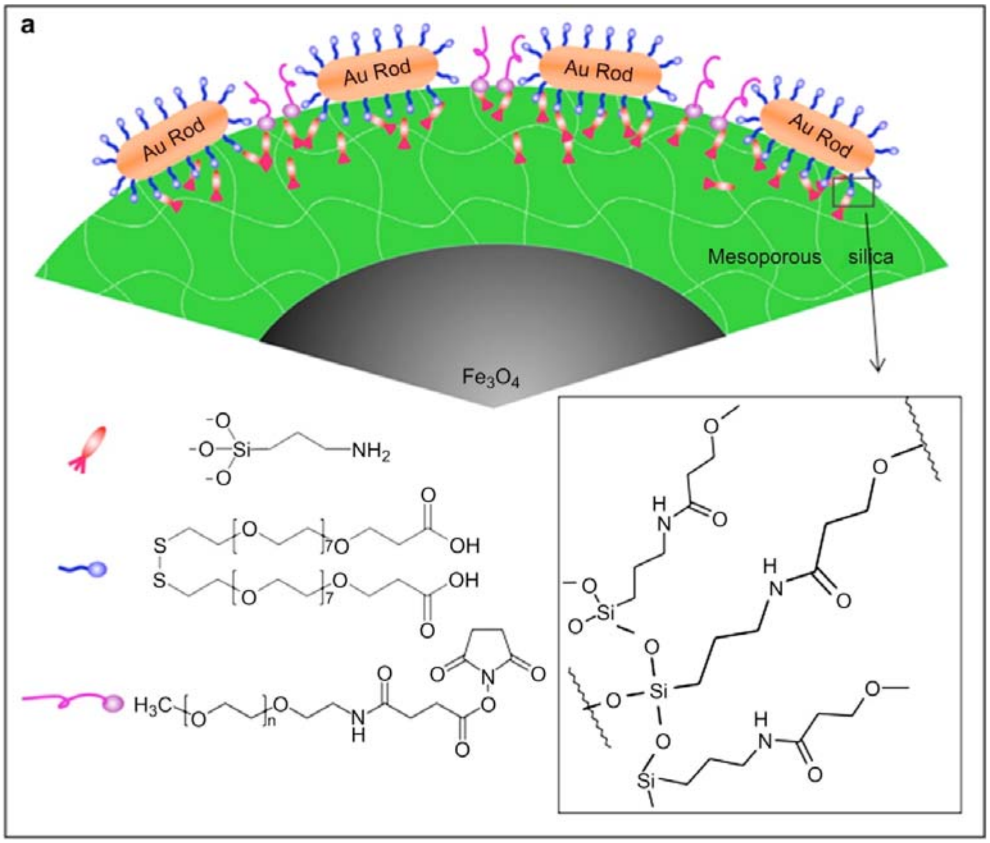

Figure 6: Scheme of the nanoplatform introduced by Ma *et al.* Covalently gold linked nanorods cap the mesopores of the silica shell, which coats a superparamagnetic iron oxide core. *Reprinted with permission from Ma et al.* Au capped magnetic core/mesoporous silica shell NP for combined photothermo-/chemo-therapy and multimodal imaging. Biomaterials 2012; 33(3): 989-998, Copyright 2012 Elsevier.

tumoral cells may be damaged without affecting surrounding healthy tissue. Confocal fluorescence microscopic images confirmed that Dox was preferentially delivered to the nuclei of MCF-7 cells.

Shi *et al.* recently reported the design of a multi-functional magnetic and plasmonic nanocomposite [367], based on graphene oxide decorated with both iron oxide and gold, modified later with lipoid acid-modified PEG and FA modified PEG for molecular targeting. The effectivity of this platform for photothermal therapy was demonstrated both *in vitro* (KB and 4T1 cells, FA receptor+ and FA receptor-, respectively) and *in vivo* (BALB/c mice bearing 4T1 tumors).

The previously discussed examples clearly illustrate the possibilities of multifunctional systems, and the current tendency to integrate different nanoplatforms into more complex ones.

CONCLUSIONS

In this review we have discussed prominent nanosystems (overviewing their limitations and advantages) and we have also presented a selection of what we believe are representative advances in the field of cancer nanotherapies.

The delivery of anticancer agents (both conventional, small molecule-based, and next-generation, biomolecule-based therapies) through nanocarriers has shown significant advances in the last few years. Cancer diagnosis and treatment is, without hesitation, the field of medicine that has attracted more attention from the nanobiotechnology sector. This seems natural if one bears in mind that a critical point to successful chemotherapy is to maximize the exposure of the cancerous or tumoral cells to the active ingredient, while minimizing the exposure of healthy tissue. Furthermore, many anticancer agents present limitations related to either stability or solubility issues, which could be ameliorated by encapsulating the drugs in adequate vehicles.

The current tendency in the field of cancer nanotherapeutics seems to be the combination of strategies that have been tested or applied in a separate manner in the past. The first combination that we have envisaged while reviewing recent literature in this field was the combination of different targeting strategies, *e.g.* different active targeting moieties have been introduced on a single nanocarrier or smart, stimuli-responsive systems have been improved through molecular targeting. The second combination relates to the development of multifunctional, complex nanoplatforms merging different separate nanosystems. These multifunctional systems join the advantages of the separate platforms, *e.g.* plasmonic behavior of metallic NP, MRI imaging of superparamagnetic NP, high loading capacity of mesoporous silica NP, or high and easy surface functionalization of dendrimers.

An issue that should be taken into consideration is the potential toxicity of those non-biodegradable nanosystems with sizes above the renal filtration threshold, whose final fate in the body has not been fully studied yet.

AKNOWLEDGEMENTS

We thank Elsevier for the permission to reproduce Figures **1-4** and **6**. The authors would like to thanks UNLP and CONICET. C. L. Bellera, M. E. Gantner and M. E. Ruiz are fellowship holders from the National Council of Scientific and Technical Research (CONICET). A. Talevi is a member of the Scientific Research Career at CONICET. Alan Talevi and María E. Ruiz thank Incentivos UNLP.

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Received on 15-04-2013 Accepted on 07-05-2013 Published on 01-07-2013

DOI: http://dx.doi.org/10.6000/1929-2279.2013.02.03.3