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Recent studies on the delivery of hydrophilic drugs in nanoparticulate systems.

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Delivery of Hydrophilic Drugs in Nanoparticulate Systems: the Contribution Made by the Technology Group of Turin University

Silvia Arpicco, Luigi Battaglia, Paola Brusa, Roberta Cavalli, Daniela Chirio, Franco Dosio, Marina Gallarate, Paola Milla, Elena Peira, Flavio Rocco, Simona Sapino, Barbara Stella, Elena Ugazio, Maurizio Ceruti*

*To whom correspondence should be addressed at: Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, 10125 Torino, Italy.

Dedication: this review is dedicated to Maria Rosa Gasco (†) and Luigi Cattel

Abstract

This review discusses some approaches developed by the Turin University Pharmaceutical Technology group to enhance the entrapment efficiency of mainly hydrophilic molecules within nanoparticulate systems, and to optimize their delivery. Many attempts have been made to increase, either passively or actively, the delivery of hydrophilic drugs. One approach consists of their association to liposomes, in some cases by developing lipophilic prodrugs of anticancer hydrophilic molecules, and encapsulating them in liposomes; in some cases these are then further conjugated with suitable vectors, to increase targeting efficiency. The transformation of hydrophilic drugs into lipophilic prodrugs can also overcome problems of poor entrapment efficiency, and the frequently-observed rapid release from polymer nanocarriers, for example nanospheres or nanocapsules obtained from various PEGylated poly(alkylcyanoacrylate) copolymers. Strategies have also been developed to enhance hydrophilic drug entrapment in solid lipid nanoparticles (SLN). Of these, hydrophobic ion pairing (HIP) was designed to enable various antitumor drugs to be entrapped in SLN produced by the coacervation method. The w/o/w emulsion solvent dilution technique may also be employed to entrap peptide drugs, such as insulin, directly in the inner aqueous phase. Another strategy entails covalent linkage of antitumoral and antiviral drugs to a squalenoyl-derived chain; this produces bioconjugates that spontaneously self-assemble in an aqueous medium as stable nanoparticles. Another development comprises mesoporous silica nanoparticles with immobilized hydrophilic antioxidants, for topical applications. Ordered mesoporous silica (MCM-41) nanoparticles were complexed with hydrophilic antioxidants (Trolox[®] or rutin) employing different inclusion procedures, varying solvent and pretreatment of the silica matrix; on exposure to UV illumination, mesoporous silica significantly improved stability over time. Finally, polymer-shelled and perfluoropentane-cored nanobubbles have been designed as versatile multifunctional carriers for the delivery of gases, drugs and genes; the size range is below 500 nm, with shell thickness in the 30-50 nm range.

Introduction

This review presents the expertise of the Pharmaceutical Technology Group of Turin University (Italy). Over several years the Group has developed strategies for delivering hydrophilic molecules employed in the anticancer field. In the 1980s, two Pharmaceutical Technology research groups began exploring new routes in the field of delivering and targeting anticancer agents. One team, coordinated by Prof. M.R. Gasco, began by studying and characterizing lipophilic ion-pairs of several amine drugs. The research aimed to develop microemulsions for use as drug delivery systems (DDS): the novelty of this approach lay in the use of biocompatible surfactants as phospholipids. These microemulsions were proposed for the administration of hydrophilic and lipophilic drugs. M.R. Gasco patented a novel technique to produce solid lipid nanoparticles by preparing a hot microemulsion and dispersing it in cool water.

The other research team, coordinated by Prof. L. Cattel, took a medicinal chemistry approach. They began a novel drug-targeting strategy using monoclonal antibodies to delivery drugs, proteins

(lectins), or macromolecular conjugates to tumor tissues. Further, collaboration with several European research groups lead to the development of particulate delivery systems (liposomes, polymer nanoparticles) and, more recently, to the squalenoylation platform.

Colloidal systems, such as liposomes, nanoparticles, and microemulsions, have mainly been reported in the literature for use as carriers of hydrophobic drugs. Delivery of hydrophilic molecules is a challenging goal, which requires multidisciplinary approaches. This review essentially focused on the various strategies that may be employed to deliver hydrophilic active substances.

Many drugs are hydrophilic, and many of these are low-molecular weight molecules (less than 500 Da). According to the United States Pharmacopeia (USP), hydrophilic drugs are classified in the range very soluble to soluble in an aqueous medium, if their solubility is higher than 33 mg/ml. Hydrophilic drugs are often subject to low intracellular absorption, enzymatic degradation, rapid clearance, suboptimal distribution, development of resistance, poor pharmacokinetics, low therapeutic index and, in the case of antitumoral drugs, lack of accumulation and retention within the tumor. Drug entrapment in colloidal delivery systems can in many cases overcome these difficulties, since it may improve pharmacokinetics, protect the drug against *in vivo* degradation, sustain drug release, increase patient comfort by avoiding repetitive injections, and reduce side effects. In the treatment of cancer, nanoparticulate systems also possess the advantage of enhancing permeability and retention (the EPR effect), resulting in their higher accumulation in tumors [1,2]. The possibility of modifying the surface of nanoparticulate systems, for example by PEGylation, leads to stealth colloidal systems, which can escape rapid uptake by the mononuclear phagocyte system; the addition of specific ligands onto their surface can also provide more effective release at the target site.

This review will briefly examine the different approaches developed by our group, pointing out the originality of our contribution and the most relevant preclinical results obtained thus far. Some of the new technologies developed for the delivery of hydrophilic drugs are also applicable also to some very low water soluble molecules, which will thus also be mentioned in brief.

1) Liposomes

Liposomes are nanoconstructs, usually sized at the nanoscale, and consist of natural or synthetic phospholipids surrounding a water core. Since phospholipids are the major component of liposomes, they are little toxic, biodegradable, and biocompatible. Liposomes form spontaneously when phospholipids are dispersed in water. Liposomes composed of natural phospholipids are biologically inert and weakly immunogenic, and possess low intrinsic toxicity. Depending on the number of layers and the diameter, liposomes are classified as multilamellar vesicles (MLVs, diameter >200 nm), large unilamellar vesicles (LUVs, diameter 100–400 nm), and small unilamellar vesicles (SUVs, diameter <100 nm). By surface charge (zeta potential) they are classified as cationic, neutral, or anionic liposomes.

Thanks to their favorable characteristics, liposomes have been widely used as carriers for different kinds of drugs. In particular, different approaches and preparation methods have been reported for the encapsulation of either hydrophilic or hydrophobic antitumoral drugs [3].

A number of different anticancer drugs with different physico-chemical features have been encapsulated in liposomes by Turin University Group, with the goals of improving their cytotoxic activity, extending their plasma half-life, and reducing their side effects. The encapsulation of low-molecular-weight water-soluble drugs is, in most cases, characterised by poor trapping efficiency and rapid leakage *in vivo* [4], limiting both shelf life and clinical utility of these liposomes. To overcome these problems, lipophilic prodrugs of hydrophilic drugs were developed and encapsulated in liposomes. This approach was initially used to prepare liposomes and immunoliposomes containing 5-fluorouridine (5-FUR) lipophilic prodrugs [5]. The parent drug, 5-FUR, and the prodrugs 5'-succinyl-5-FUR and 5'-palmitoyl-5-FUR were encapsulated in liposomes, and encapsulation efficiency, drug leakage, stability, and size were evaluated. The results showed that 5'-palmitoyl-5-FUR was the most suitable for incorporation in liposomes, in terms of minimum leakage and high

encapsulation efficiency. Moreover, differential scanning calorimetry (DSC) analysis showed that the compound strongly interacted with the liposomal bilayer. To increase the drug delivery efficiency, these liposomes were then further conjugated with an AR-3 monoclonal antibody, targeted to human colon carcinoma. The immunoliposomes were prepared from phospholipidic vesicles incorporating a maleimido reactive derivative of phosphatidylethanolamine, able to react with the thiolated monoclonal antibody, as shown in Figure 1. The *in vitro* antitumor activity of liposomes and immunoliposomes was determined on the HT-29 human colon carcinoma cell line; the results showed that the targeted liposomes were more cytotoxic than their conventional counterparts. Both plain and targeted liposomes were then i.p. injected into athymic mice grafted with the HT-29 cell line; also in this case, the immunoliposomes were more effective than the plain variety as antitumor agents. Only 5% of residual tumor mass was present at the end of the therapy in mice treated with the immunoliposomes.

Gemcitabine (GEM) is an anticancer drug, indicated for pancreatic cancer, that is rapidly deaminated to an inactive metabolite; it must therefore be administered at a very high dose. A series of prodrugs of GEM was synthesized with the aim of improving its metabolic stability and facilitating its encapsulation in liposomes [6]. The GEM 4-amino group was derivatized with C5, C12 and C18 linear-chain acyl derivatives (Figure 2). Stability of the prodrugs at storage, in buffers, in plasma, and with the lysosomal intracellular enzyme cathepsins was evaluated. The results in buffers confirmed the high stability of the amide bond. Also in plasma, the prodrugs showed high stability and marked resistance to deamination. They were incubated with cathepsin B and D to evaluate the selective hydrolysis of the amide bond inside the cell by liposomal enzymes. After 24 h, 60% of the prodrug had undergone hydrolysis of the amide bond, without degradation of free GEM occurring. The characteristics of liposomes containing these prodrugs were also studied, and it emerged that GEM lipophilic derivatives containing C12 and C18 acyl chains showed the best results, in terms of incorporation into liposomes, and the corresponding drug-loaded carriers showed higher cytotoxic activity than the free drug, against both HT-29 colon and KB nasopharyngeal human carcinoma cell lines [6]. The pharmacokinetic behavior and the *in vivo* antitumor activity of the parent drug, and of the gemcitabine prodrug alone and encapsulated in liposomes, were evaluated. Liposomes containing the prodrug showed both longer plasma half life and greater tumor regression than either control or gemcitabine [7].

The GEM lipophilic prodrugs were studied in interaction with biomembranes through DSC, in order to evaluate the effect of the length of the acyl chain on the ability to interact with a model biomembrane in an aqueous compartment: the goal was to obtain biomimetic information on the prodrugs' lipophilic character and solubility [8]. As synthetic biomembrane models, liposomal MLV and LUV were used; these were composed of dimyristoylphosphatidylcholine (DMPC) and of distearoylphosphatidylcholine (DSPC). These studies involved the addition of GEM lipophilic prodrugs to the biomembrane models, the transfer of these compounds through an aqueous medium, and the migration to empty liposomes from liposomes containing a known quantity of GEM or of its 4-(*N*)-acyl derivatives. The results showed that the free drug alone does not interact with the biomembrane models used; conversely, the 4-(*N*)-acylgemcitabine derivatives interacted with the biomembrane models, this interaction being dependent on the acyl chain length of the phospholipids in the model biomembranes, and on the acyl chain length of the prodrugs.

The lipophilic GEM prodrugs were also studied to determine their interaction with biomembrane models, through the Langmuir-Blodgett (LB) technique [9]; this is one of the commonest ways to study the interaction between drugs and phospholipids. Monolayers are an excellent model to study two-dimensional ordering, with two thermodynamical variables, temperature and pressure, being readily controlled. Conclusions about the mixing process of two pure monolayers can be drawn by comparing surface pressure-area isotherms of mixed and pure films. Results provide indications on the compounds' capacity to dissolve in the phospholipid molecules used as model membrane. The

interactions enable hypotheses to be formulated concerning the intake and collocation of the compounds within the membrane.

The interfacial behaviour of monolayers comprising DMPC, plus GEM or lipophilic prodrugs at increasing molar fractions, was studied at the air/water interface at temperatures below (10 °C) and above (37 °C) the lipid phase transition. The results show that the compounds interact with DMPC, producing mixed monolayers that are subject to an expansion effect, depending on the prodrug chain length. A comparison of the results shows that GEM-derivatives in mixed monolayers substantially exert an expansion effect, which is particularly strong in the case of GEM-C12. GEM-C5, although it possesses a much shorter hydrophobic tail than C-18, exerts a similar effect: GEM-C18, possessing a longer chain than DMPC, interacts with the phospholipid chains; the slight expansion effect might be due to the long chain tilting.

Liposomes containing the C12 acyl chain of GEM were further modified with a vector, to increase their targeting ability. Hyaluronic acid (HA) was chosen as vector, since its principal surface receptor, CD44, is overexpressed on a variety of tumors [10,11]. Moreover, HA is biodegradable, biocompatible, and can be chemically modified. Initially, conjugates between a phospholipid and HA of two different molecular weights (4800 and 12000 Da) were synthesized; they were purified and characterized, and then introduced into liposomes during preparation. Different liposomal formulations were prepared and their physico-chemical characteristics were evaluated. *In vitro* biological studies, including confocal microscopy, showed that HA facilitates recognition of liposomes by CD44+ cells, and that the uptake increased as the HA molecular weight increased. It was also observed that the cytotoxicity was higher in HA-liposomes than in plain liposomes [12]. The *in vivo* antitumoral activity of the liposomal formulations was further analysed in a mouse xenograft tumor model of human pancreatic adenocarcinoma, showing that liposomes obtained using high molecular weight HA were the most efficient.

2) Solid lipid nanoparticles (SLN)

Nanoparticles are making steady progress as drug carriers, because of their size-dependent properties. Owing to the biocompatibility and versatility of the lipid component, lipid nanoparticles have many advantages over polymer nanoparticles, and have been widely used for active drug delivery [13]. Solid lipid nanoparticles (SLN) are composed of a solid lipid matrix, for example glycerides, fatty acids or waxes, and are stabilized by physiologically-compatible emulsifiers, such as phospholipids, bile salts, polysorbates, polyoxyethylene ethers or polyvinyl alcohols. The lipids used for their production are solid at room temperature, and most of them have low toxicity, giving them approved status, for example GRAS (Generally Recognized as Safe). SLN are colloidal systems with approximate mean diameter between 50 and 1000 nm. They are produced with different methods [14]: in many cases these start from emulsions, such as the hot homogenization method [13], or from microemulsions [15], such as the microemulsion dilution method. These methods require high operating temperatures, which may be unsuitable for encapsulating thermosensitive drugs. SLN can also be produced by solvent-based methods (solvent injection [16], solvent evaporation method [17], solvent diffusion method [18]); one of the main advantages of these methods is the mild operating temperature, which can be useful for encapsulating thermosensitive drugs; however, toxicological issues concerning the solvent used are a limiting aspect.

The fatty acids coacervation method was recently developed to prepare SLN (Figure 3); this production method starts from alkaline salts of fatty acids, and enables drugs, including thermosensitive drugs, to be incorporated without using very complex equipment or dangerous solvents [19]. This method is based on a slow interaction between a micellar solution of a sodium salt of a fatty acid and an acid solution (coacervating solution) in the presence of an appropriate amphiphilic polymeric stabilizing agent. By lowering the pH, nanoparticles can be precipitated [20].

Drugs can be encapsulated in SLN in different ways, depending on the preparation method employed. However, in all preparation techniques, an interaction occurs between drug and lipid, which leads to the entrapment phenomenon. Drugs can be distributed in the lipid matrix in different ways: in a homogeneous matrix (the drug is molecularly dispersed or is present in amorphous clusters in the lipid matrix), in the nanoparticle shell (the drug is mainly in the outer shell that covers a lipid core) or as a lipid-coated core (the drug starts to precipitate before the lipid, and the lipid shell forms around this core).

Briefly, drugs can be divided into hydrophilic and hydrophobic, by their water solubility and partition coefficient. During SLN preparation, different strategies must be adopted to reduce partitioning of the drug into the outer water phase, and to enhance drug entrapment [21]. The most important ways of loading hydrophilic drugs into SLN are:

1. starting from a template that allows encapsulation of hydrophilic drugs: i.e. w/o/w emulsion with an organic solvent [17,18] or w/o/w microemulsion [22] or o/w emulsion with partially water miscible organic solvents [23];
2. using the hydrophobic ion pairing (HIP) technique: this is possible between certain charged hydrophilic drugs and opposite-charged surfactants. It decreases the drug's water solubility and increases the drug's apparent partition coefficient, enhancing drug encapsulation within the nanoparticles [24,25];
3. using polymer lipid hybrid nanoparticles: a complex between drug and ionic polymer is formed by neutralizing the charges of the drug with a polymer counterion, and the formed complex is entrapped into nanoparticles [26,27];
4. preparing lipid-drug conjugate nanoparticles [28];
5. using water free preparation methods [29];
6. using lipophilic prodrugs of the hydrophilic drug [30,31].

Insulin-loaded glyceryl monostearate nanoparticles have been prepared by the Turin University Group starting from w/o/w by simple water dilution [32]. SLN of spherical shape, with mean diameter in the 600–1200 nm range, and with up to 40% insulin entrapment efficiency (EE%) were obtained. Through preliminary solvent screening, the stability of the entrapped insulin was maintained, as was its biological activity after administration *in vivo*.

The HIP technique has been widely applied. Doxorubicin (DOX) was entrapped within behenic acid SLN, prepared by the coacervation method, by employing HIP with sodium dioctylsulfosuccinate (AOT), a negatively-charged surfactant used as counter-ion [33]. DOX is one of the leading anticancer drugs in clinical oncology, with a broad spectrum of activity against various solid and hematologic neoplastic diseases; it is commonly employed in numerous anticancer therapeutic regimens. DOX-SLN characterization indicated that DOX-AOT was loaded into SLN with good EE%, and that SLN-entrapped DOX maintained its cytotoxic activity against glioma cell lines; moreover, its permeation through hCMEC/D3 cell monolayer, taken as model of the blood-brain barrier, was increased when the drug was entrapped in SLN.

Stearic acid SLN loaded with different peptides were prepared using the coacervation method [25]. Leuprolide acetate and insulin were loaded into SLN, using AOT and sodium dodecylsulphate (SDS) respectively as counter ions. All peptide-loaded SLN had diameter in the 320-520 nm range; insulin EE% was about 90%, and leuprolide EE% depended on ion pair stoichiometry (from 48% to 86%). The mild heating necessary for the coacervation process did not affect the chemical stability of the peptides. The same team [24] also prepared stearic acid cisplatin-loaded SLN. Cisplatin, a hydrophilic anti-tumor agent, could be loaded thanks to the formation of a hydrophobic ion-pair with AOT. Particle size was in the 275-525 nm range; cisplatin EE% was up to 90%, depending on both stearic acid concentration and stabilizer type and concentration. *In vitro* cisplatin release showed a burst effect of about 10-20%, corresponding to the non-entrapped drug, and then complete drug release was reached after 24 h. The HIP technique was also used to prepare methotrexate (MTX)-loaded SLN by the coacervation method [34]. The ion pair between MTX and dequalinium

dichloride (DEQ), a cationic surfactant currently used as antimicrobial agent, markedly increased drug EE% within SLN. MTX-DEQ-loaded SLN also showed increased cytotoxicity against breast cancer cell lines compared with drug solution, while blank SLN showed good biocompatibility. Increased MTX plasmatic concentration and increased accumulation within breast neoplastic tissue were achieved when MTX was loaded into SLN as an ion pair, compared with drug solution.

Higher molecular weight drugs have also been entrapped in SLN, including bevacizumab (BVZ) [35]. BVZ is a recombinant humanized monoclonal antibody that inhibits angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A). It is used to treat various cancers, including colorectal, lung, breast, glioblastoma, kidney and ovarian. BVZ was entrapped in stearic acid SLN as AOT-BVZ ion pair with 30% EE%. When entrapped in SLN, BVZ increased its *in vitro* activity as VEGF-A inhibitor and increased by up to 70%, its *in vitro* capacity to permeate through the hCMEC/D3 cell monolayer, taken as model of the blood-brain barrier, compared to BVZ solution.

HIP have also been used to enhance the loading of highly water-soluble drugs [26,27], in studies that loaded cationic hydrophilic anticancer drugs, such as DOX-HCl, verapamil and quinidine, following the formation of ionic complex with various anionic polymers, such as dextran sulphate, sodium alginate, sodium stearate. The team prepared SLN by the microemulsion dilution technique, and obtained mean diameters in the 180-300 nm range, depending on the type and content of both drug and polymer. Ionic complexation with dextran sulphate generally improved (two- to fourfold) loading of the three cationic drugs tested.

The lipid drug conjugates preparation strategy has been used to incorporate hydrophilic drugs into SLN [28]. The antitrypanosomal drug diminazene, used as model drug, reacts with fatty acids to form water-insoluble salts, for example diminazene distearate and dioleate; these salts were transformed into nanoparticles using the high-pressure homogenization technique. Nanoparticle size was in the 360-440 nm range, and high drug loading (33%, w/w) was obtained for the highly-water-soluble drug diminazene diacetate.

Another strategy that can be utilised to load hydrophilic drugs into SLN is the free water preparation method. Insulin-loaded SLN may be prepared by the electrospray technique [29]. An electric field is applied to a lipid-drug solution in a syringe connected to a pump. The applied high-voltage electric field forces the polymer solution out of the syringe in jet form; along the jet's trajectory, the solvent gradually evaporates and the particles are deposited on the collecting plate. The apparatus is contained in a closed transparent case, at 20 or 40 °C. The great advantage offered by electrospraying over other commonly-used methods is that it is a one-step process. Insulin was entrapped into the particles with high encapsulation efficiency, by formation of an ion-pair with SDS. Far-ultraviolet circular dichroism spectroscopy indicated that electrospraying did not modify the secondary structure of insulin. *In vitro* prolonged release over 24 hours was observed after an initial burst effect.

3'-5'-dioctanoyl-5-fluoro-2'-deoxyuridine (DO-FUdR, a lipophilic derivative of the hydrophilic drug 5-fluoro-2'-deoxyuridine, FUdR), has been entrapped in SLN using a thin-layer ultrasonication technique [30]. The mean particle size of DO-FUdR SLN was 76 nm and EE% was 96.6%. It was found that SLN improved the ability of the drug to penetrate through the blood-brain barrier: after intravenous administration of DO-FUdR SLN, the brain concentration of FUdR was more of 10 fold that achieved with plain FUdR.

In another study [31] lipophilic prodrugs were used to entrap hydrophilic molecules in SLN. A myristic ester of RU 58841, a potent hydrophilic nonsteroidal antiandrogen used in the treatment of acne and androgenetic alopecia, was synthesised to improve entrapment of the drug in SLN. It was found that the lipophilicity of the drug and that of the carrier improved drug uptake by the hair follicles, and that SLN can act as a slow release system, even achieving low systemic drug levels.

In conclusion, SLN may be considered promising DDS, owing to their biocompatibility and versatility; through easy formulative strategies, it is possible to entrap hydrophilic molecules with good EE%.

3) Polymer nanoparticles

Thanks to the wide range of polymers and nanoparticle preparation methods, hydrophilic molecules can also be encapsulated in polymer-based nanoparticles, both nanospheres and nanocapsules. Nanospheres are matrix systems in which the drug is dispersed within the polymer, throughout the particle. Nanocapsules are vesicular systems in which the drug is located in an inner cavity surrounded by a thin polymer layer.

To overcome the poor entrapment efficiency of hydrophilic drugs in polymer nanocarriers, and the frequently observed rapid release, several strategies have been developed [36]. Generally, hydrophilic molecules can be loaded onto polymer nanospheres, either by entrapping the drug within the polymer matrix (by adding the drug during nanoparticle formation) or by adsorption onto preformed nanocarriers. If the drug is encapsulated during the nanoparticle fabrication process, several parameters are involved. As an example, in the emulsion polymerization of alkylcyanoacrylate monomers, the monomer type, pH, and drug concentration play crucial roles in the polymerization rate, which, for instance, is faster for monomers with shorter chain lengths, facilitating mechanical trapping of the drug [37]. When nanospheres are prepared from a preformed polymer by nanoprecipitation, generally low entrapment efficiency levels are obtained, because of the hydrophobic nature of polymers, for instance, occurs with polyesters [38]. Nevertheless, it is possible to increase the loading of an ionizable drug during the nanoprecipitation process by adjusting the pH of the outer media [39]; this modification affects the ionization of the drug and, hence, the solubility: an ionic substance tends to stay in the water phase, while the molecular form is more likely to be attached to the hydrophobic polymer phase and more efficiently encapsulated.

In the light of these findings, the Turin University Group prepared DOX-loaded polymer nanospheres and nanocapsules *via* solvent displacement in confined impinging jets reactors (CIJR) [40,41]. CIJR are small passive mixers in which very fast turbulent mixing of the solvent (i.e., acetone) and of the antisolvent (i.e., water) solutions occurs under controlled conditions. These devices can be operated continuously, and can easily be scaled up. Two different types of DOX were considered: the commercially-available DOX hydrochloride (DOX-HCl), which has minimal solubility in organic solvents, and DOX free base (DOX-FB), which, conversely, is soluble in organic solvents but has very low solubility in water [41]. A PEGylated amphiphilic biodegradable copolymer, poly(methoxypolyethyleneglycol cyanoacrylate-*co*-hexadecyl cyanoacrylate) (poly(MePEGCA-*co*-HDCA)), was used to prepare DOX-loaded nanoparticles, and the effect of the operating parameters was investigated. For the nanoprecipitation process, DOX-FB was dissolved in the organic solvent (acetone or tetrahydrofuran), while DOX-HCl was added to the water phase. The results showed that DOX-FB-loaded nanoparticles were smaller than DOX-HCl-loaded nanoparticles, and the zeta potential was less sensitive to operating conditions. These results suggested that DOX-FB molecules could be properly incorporated inside the polymeric matrix, whereas DOX-HCl could only be partially incorporated inside the particles, with a fraction interacting with the surface and altering its properties [41].

Other preparation methods afford greater drug loading into polymer nanoparticles, such as the double w/o/w emulsion technique [42] and the use of reverse micelles-containing polylactic acid (PLA) nanoparticles [43]. Moreover, the addition of a polyanion can be a useful strategy to compensate a polymer positive charge, such as that of chitosan, a naturally positively-charged polymer. Although chitosan nanoparticles are able to imbibe large quantities of water, thus facilitating the encapsulation of hydrophilic molecules, the positive charge limits cationic drug encapsulation. The addition of a negatively-charged molecule, such as polyaspartic acid, can promote drug entrapment [44]. Conversely, negatively-charged nucleic acids are efficiently loaded in pure chitosan nanoparticles [45]. Alginate, a natural water-soluble polymer, also forms hydrogel nanoparticles suitable for the encapsulation of hydrophilic compounds [46].

To efficiently adsorb a drug onto the polymer nanoparticle surface, the physico-chemical characteristics of both drug and polymer must be taken into account, in order to achieve a good

interaction between drug and nanoparticle surface [37]. The association may arise from ionic interactions between the polymer and a charged drug [47]. Moreover, drug absorption is dependent on the concentration of the drug in the incubation medium [48] and can be increased by modifying the nanoparticle surface [49].

Another strategy to entrap hydrophilic compounds in nanocarriers consists of preparing polymer nanocapsules with an aqueous inner core. In these carriers, the hydrophilic drug is directly solubilized in the internal water. The aqueous-core nanocapsules are generally produced by a two-step method consisting first of generating aqueous droplets in a continuous organic phase, then reinforcing the polymeric interface (for a review see [36]). The advantages of aqueous-core nanocapsules are high drug encapsulation efficiency and low polymer content compared to polymer nanospheres.

To make possible their encapsulation in the lipophilic matrix of nanoparticles, hydrophilic drugs can also be modified chemically, by transforming them into prodrugs with higher lipophilicity. This approach requires a linkage strategy that modifies the drug without causing a loss of activity. The Turin group encapsulated GEM and a series of its lipophilic prodrugs into nanospheres and oily-core nanocapsules (Figure 2) [50]. The nanoparticles were prepared by nanoprecipitation of an amphiphilic biodegradable PEGylated poly(alkylcyanoacrylate) copolymer, the poly(aminopolyethyleneglycol cyanoacrylate-*co*-hexadecyl cyanoacrylate) (poly(H₂NPEGCA-*co*-HDCA)) [51]. GEM normally suffers from rapid plasmatic metabolization. Its transformation into lipophilic derivatives (in which an acyl chain of increasing length is covalently coupled to its 4-amino group), together with encapsulation in nanospheres or nanocapsules, protected GEM both chemically and physically. The results showed that the increased lipophilicity enabled GEM to be efficiently encapsulated in poly(H₂NPEGCA-*co*-HDCA) nanocarriers, while the hydrophilic parent drug was not entrapped. Moreover, GEM lipophilic derivatives were found to release the free drug once inside the cell.

4) Squalene-derived nanoparticles

In 2004, some researchers of the Turin University team, together with Prof P. Couvreur's group (University of Paris-Sud, France), discovered that the covalent linkage of various hydrophilic nucleoside drugs, such as GEM, affords bioconjugates that spontaneously self-assemble in an aqueous medium to form stable nanoparticles, having diameters in the 100-200 nm range [52,53]. This strategy can be exploited to give impressively high drug loading, together with high therapeutic efficiency and, in most cases, the possibility to overcome tumour's resistance to currently-available treatments and/or to reduce the drug's side effects. This therefore opens the way to a new and original approach for delivering therapeutic compounds. In particular, the first squalenoyl derivative, 4-(*N*)-squalenoyl-gemcitabine (Sq-GEM), administered by the intravenous route, has been shown to improve the drug therapeutic index, at the same time increasing its *in vitro* toxicity against tumor cells about six-fold, while decreasing its toxicity against healthy cells [54]. The Sq-GEM nanoassemblies also possessed much stronger antitumoral activity than free GEM when administered by the oral route to rats in which leukaemia had been induced by RNK-16 LGL cells [55]. The possibility of administration by several routes, the resistance to deamination, the increased bioavailability, the improved pharmacokinetics and tissue distribution, the decreased drug resistance and toxicity against healthy cells, together with enhanced accumulation and retention within tumor cells, are the key advantages of nanoassembled Sq-GEM versus GEM as such. A comprehensive review on this topic was recently published [56].

In general, the squalenoylation of antitumoral and antiviral drugs was achieved starting from 1,1',2-tris-nor-squalene acid. This intermediate was obtained from squalene, following a general procedure that was developed many years ago: it is the sinthon for obtaining many inhibitors of oxidosqualene cyclase and squalene epoxidase [57,58]. By a multistep reaction, starting from squalene treated with *N*-bromosuccinimide, closure to 2,3-epoxide, and subsequent cleavage to 1,1',2-tris-nor-squalene

aldehyde, 1,1',2-tris-nor-squalene acid was finally obtained by oxidation. Sq-GEM was obtained by reacting GEM with 1,1',2-tris-nor-squalene acid, previously activated with ethylchloroformate (Figure 4) [52,53].

Sq-GEM was also studied in regard to its interaction with biomembranes through DSC [59]. As synthetic biomembrane model, liposomal DMPC MLV were used. These studies entailed:

- 1) DSC analysis of DMPC MLV containing increasing amounts of GEM, squalene, 1,1',2-tris-nor-squalene acid, or Sq-GEM;
- 2) transfer of these compounds through an aqueous medium;
- 3) migration of these compounds from liposomes containing a known quantity of GEM or Sq-GEM to empty liposomes.

The results showed that neither GEM nor squalene alone interact with the biomembrane model used. Conversely, 1,1',2-tris-nor-squalene acid and Sq-GEM interacted strongly with the biomembrane model. The interaction of Sq-GEM with a biomembrane model was also studied, through the LB technique: the molecular area/surface pressure isotherms of GEM/lipid, Sq-GEM/lipid, and pure compound monolayers, taken together with the calorimetric results, indicate that Sq-GEM interacts with the phospholipid monolayer with the squalene moiety in contact with the phospholipid chains, while the GEM moiety protrudes into the aqueous medium.

1,1',2-tris-nor-squalenoyl-citarabine (Sq-ARA-C) was synthesised and formulated as nanoparticles (Figure 4) [60]. The antitumor activity was studied on murine leukaemia cell lines (L1210, responsive to ARA-C, and L1210R, resistant), a human leukaemia cell line (K562) and a human breast tumor cell line (MCF-7). Sq-ARA-C reduced the IC₅₀ value by 2.5 times, for the L1210R cells, and by 4 times, for the K562 and MCF-7 cells, versus ARA-C. The activity of Sq-ARA-C on the murine leukaemia model L1210R was also studied, and the results showed a marked reduction in disease development. The biodistribution study of tritiated Sq-ARA-C in DBA/2 mice after 1 h showed the nanoparticles to be mainly localized in the spleen, liver and lungs, and to a much lesser extent in other organs, whereas a large quantity was still in the blood. After 24 h, the quantity present in the blood was greatly reduced, with a higher concentration in the RES organs. Sq-ARA-C showed a plasma half-life about 6 times that of ARA-C. The interaction of ARA-C and Sq-ARA-C with liposomal biomembranes (MLV) studied by DSC confirmed the results: the new compound interacted with the liposome system in different ways, since ARA-C and squalene had no effect on the transition temperature (T_m), while both Sq-ARA-C and 1,1',2-tris-nor-squalene acid caused a decrease in the T_m, destabilising the membrane by inserting compounds between the phospholipid molecules [61].

The squalenoylation technology was also extended to develop a new ester prodrug with antiviral activity, squalenoyl-acyclovir (Sq-ACV). The interaction of Sq-ACV with biomembrane models made of DMPC MLV was studied by means of DSC and LB techniques [62,63]. The DSC study concerned the effects of ACV and of Sq-ACV towards the liposomal biomembranes (MLV) [62]. DSC was also run for squalene and 1,1',2-tris-nor-squalene acid. ACV and squalene had no effect on the T_m, while both Sq-ACV and 1,1',2-tris-nor-squalene acid produced a decrease in the T_m, causing destabilisation of the membrane, due to insertion of the compounds between the phospholipids; this shows that Sq-ACV is easily distributed in the phospholipid bilayers. In additional research, Sq-ACV nanoassemblies were obtained and the pharmacokinetics after ocular administration in rabbits was studied [64]. The lipophilic character of Sq-ACV optimizes permeation through the cornea, while the squalenoylation nanotechnology provides prolonged release of ACV at the absorption site. The ocular administration of Sq-ACV increased the bioavailability of ACV in both the tear fluid and the aqueous humor, to as much as four and six times the reference values, respectively. The intact prodrug was found in the lachrymal fluid, while it was not detected in the aqueous humor, thus ruling out passive transcorneal diffusion of the conjugate. The lipophilic character of Sq-ACV, associated with its high affinity for the ocular structure, might thus improve the availability of ACV on the corneal surface.

In the field of the squalenylation of anticancer drugs, the most interesting results to date have been those achieved with DOX. The squalenylation approach involved the synthesis of several derivatives having different characteristics: DOX-squalenoyl hydrazone (DOX-Hz-Sq), DOX-squalenoyl ester hydrochloride (DOX-E-Sq) and DOX-squalenoyl amide (DOX-A-Sq). DOX-Hz-Sq contains an acid-sensitive hydrazone linker that allows DOX to be released either extracellularly, in the slightly acidic environment often present in tumor tissue, or intracellularly, in acidic endosomal or lysosomal compartments after cellular uptake by the tumor cell, as reported for DOX [65]. DOX-E-Sq can also release the free drug under ester hydrolysis, while DOX-A-Sq appears to be very stable in *in vitro* studies. The synthesis of Sq-DOX derivatives is depicted in Figure 5. All DOX derivatives, with increasing hydrophobicity from DOX-E-Sq to DOX-A-Sq, were nanoprecipitated by simple solvent displacement in nanoassemblies of mean size 130-200 nm. It is significant that drug loading was impressively high (i.e. 57%). Preliminary *in vitro* data revealed DOX-E-Sq as the most promising compound of the series: *in vivo* toxicity studies, after intravenous administration, showed that the MTD of DOX-E-Sq nanoassemblies was up to five-fold that of the free drug and, more importantly, the squalene derivative did not cause any myocardial lesions, such as those observed after DOX treatment [66]. Concerning pharmacological activity, DOX-E-Sq nanoassemblies showed higher antitumor efficacy on both human pancreatic carcinoma and mouse lung tumor xenografts, compared to free DOX. Remarkably, tumor growth inhibition was marked also in DOX-resistant mouse lung tumor. Furthermore, DOX-E-Sq nanoassemblies also displayed lower toxicity and enhanced anticancer activity compared to the DOX liposomal formulations currently on the market [66].

The capacity of squalene-derived chains to form nanoassemblies when linked with hydrophilic drugs was thought to be due to the amphiphilic character of the bioconjugate: the squalene-derived part providing the lipophilic moiety, and the drug the hydrophilic part of the molecule. It was thus expected that this approach could only be applied to hydrophilic compounds. Unexpectedly, two studies showed that the squalenylation technique may also be appropriate to design nanomedicines loaded with hydrophobic compounds having very low water solubility, like paclitaxel (PTX) [67], podophyllotoxin, camptothecin and epothilone [68]. It thus appeared that squalenylation might also be a relevant technology for poorly water soluble compounds, which are difficult to administer intravenously. This approach was further developed in Prof. P. Couvreur's laboratories, by modulating the linker between PTX and the squalene-derived chain [69].

The new technology has been extended to other therapeutic areas, such as antibiotics, for example penicillin G, using either a pH-sensitive or a pH-insensitive linker [70]. Formation of nanoassemblies was confirmed, and a significant proportion of *S. aureus*-infected macrophage cell lines were rapidly killed [71]. Application of the squalenylation approach was also extended to statins (e.g. pravastatin), obtaining novel nanoassemblies with improved performance [72], and to antidiabetic agents, such as insulin [73]. Choosing different reactive conditions, several populations of squalenoylated insulin (one terminal amino term and two lysines) were fully identified and separated. Squalenoyl-insulin nanoassemblies were easily obtained by dialysis. The relevant patent also describes the preparation of derivatives and related nanoassemblies of 15-deoxyspergualine (Gusperimus), a hydrophilic drug useful in hyperactive inflammatory diseases, such as autoimmune diseases [74], that has also been used in an experimental approach to cystic fibrosis [75]. Other fields of application of squalenylation are the topical delivery of vitamin C [76] as a potential anti-aging cosmetic.

The squalenylation platform was further employed for diagnostic agents. The contrast agent gadolinium Gd (III+) coupled to a squalene-derived chain was also synthesised, and preliminary *in vivo* data examined [77]. In our laboratory, a novel luminescent ruthenium (II) complex was linked to a squalene-derived chain. The resulting nanoassemblies exhibit long-lived and intense luminescence, and are rapidly transported across cell membrane. This cell-imaging tool was further easily co-nanoassembled with Sq-GEM and Sq-PTX [78]. The combination of therapeutic and

diagnostic agents in the same structure is a powerful approach for nanomedicine delivery systems. Using the squalene-based platform, the combination by one-step nanoprecipitation of a squalenoyl bioconjugate solution containing magnetite nanocrystals (USPIO) was also achieved [79]; it can be guided by an external magnetic field towards the tumor tissue, where it can be tracked by magnetic resonance imaging. This nanotechnology approach offers drug delivery combining diagnostic and therapeutic activities. For this purpose, various squalene-drug derivatives were tested (Sq-E-DOX, Sq-cisplatin, Sq-GEM) and formulated by one-step nanoprecipitation with USPIO. The USPIO/Sq-GEM nanocomposites, injected in mice bearing the L1210 wt subcutaneous tumor model, was successfully guided by an external magnetic field towards the tumor tissue and tracked by magnetic resonance imaging.

The most recent approach in squalenoylation nanotechnology is active targeting of squalene-derived nanoparticles. One strategy involves the co-nanoprecipitation of Sq-GEM with a squalene derivative of the CKAANK peptide, which had been identified by phage display as an efficient homing device within the pancreatic pathological microenvironment. *In vivo* tests in RIP-Tag2 mice have showed that the CKAANK functionalization enabled actively-targeted Sq-GEM nanoparticles: (i) to specifically interact with both tumor cells and angiogenic vessels, and (ii) to simultaneously promote pericyte coverage, thus leading to normalization of the vasculature, and likely improving tumor accessibility for therapy [80].

5) Mesoporous silica nanoparticles as a drug delivery system for topical applications

Due to its siting, the skin is exposed to a large number of environmental threats; these include free radicals, which are implicated in a number of diseases and disorders. Antioxidants and free radical scavengers have thus been proposed as protective or therapeutic agents against these injuries. Oral treatment with antioxidants has been reported to provide skin protection against the deleterious effects of UV radiation and other oxidative stresses. Topical delivery of antioxidants has increasingly attracted interest, and recent developments include improved targeting to the upper skin layer. However, the cutaneous application of antioxidants is challenging, since this type of molecule is, in general, susceptible to degradation. The search for delivery systems that simultaneously preserve the stability of the active ingredient, and enhance skin deposition, constitute a new research field in drug delivery design [81].

Over several decades, the Turin Research Group has devoted much effort to identifying new vehicles meeting these two goals, in both the dermatological and the cosmetic fields. The formulations studied include microemulsions [82], multiple emulsions [83], and vesicular systems (like liposomes) [84]. However, the advent of new functional substances, and the expectations of users, have lead to the introduction of more complex vehicles, which not only stabilize and release the active molecule at the target site, but also give the preparation an acceptable and pleasant feel, thus combining efficacy and aesthetics. Examples of this approach involve solid lipid nanoparticles [85,86], nanosponges [87], cyclodextrins [88-90], and more recently mesoporous silica nanoparticles [91].

Ordered mesoporous silica is a new development in nanotechnology: this material is now being produced on an industrial scale in a growing number of commercial products, its fabrication being simple, cost-effective, and controllable. The first mesoporous material was introduced in 1992 at *Mobil Corporation laboratories* and named *Mobil Crystalline Materials* (MCM-41), but it was not investigated in biomedical studies until 2001, when it was demonstrated to act as a drug delivery system [92]. Two research groups [93,94] proposed synthesis methods for mesoporous silica, reporting particles small enough to be considered for use as carrier for active compounds. Since these initial reports, extensive studies have been performed involving improved matrix synthesis, introduction of multifunctionality, and *in vitro/in vivo* experiments. The considerable interest in applying MCM-41 as a drug delivery system arises from a series of unique features: high surface area (about 1000 m²/g), large pore volume (≥ 1 cm³/g), well defined and tunable pore size (2-10 nm),

uniform porous structure, thermal stability, water insolubility, and a silanol-containing surface that can easily be functionalized. Moreover, it has notable *in vivo* biocompatibility and biodegradability [95,96] and its porous interiors can be used as reservoirs for loading both hydrophilic and hydrophobic substances [92,97,98]. In addition, MCM-41 is reported to be able to increase the stability of included molecules and improve their bioavailability [99].

The procedure for producing MCM-41 is simple, and the parameters can easily be controlled; the typical synthesis mixture contains four major components: inorganic precursors, organic template molecules, a solvent, and an acid or base catalyst [96,100]. The ordered mesoporous material is obtained by a supramolecular assembly of silica around surfactant micelles. The formation of the resulting material occurs by means of a “templating” mechanism in which the silicates form walls of inorganic material around the ordered micelles of surfactant.

The immobilization of active molecules in this inorganic matrix for topical delivery has been reported. Particularly, in a recent paper [101], in order to reduce the sunscreen release from the formulation and to prevent contact between the UV absorbent and the skin, MCM-41 was proposed as a carrier for sunscreens; thus oxybenzone was included in its nanopores. The same group suggested MCM-41 as a matrix to host octylmethoxycinnamate, a well-known UV chemical filter: they reported a broader photoprotection range, enhanced photostability and reduced release of the included sunscreen [102].

Another potential advantage of using MCM-41 as a topical carrier is its water sorbent property, which is due to the abundant presence of free silanol groups on its surface structure. Therefore, by forming hydrogen bonds, a large number of water molecules can be adsorbed; this can contribute to reducing the moisture present in the skin, responsible for maceration and the growth of microorganisms [103].

In the light of these interesting developments, we studied the potential of MCM-41 as a carrier for Trolox[®] [104] and rutin [105], two hydrophilic derivatives of natural antioxidants, vitamin E and quercetin (Figure 6). The complexes between Trolox[®] and MCM-41 nanoparticles were prepared by employing different inclusion procedures, varying solvent and pretreatment of the silica matrix. The objectives of this study were to determine Trolox[®] loading, analyze its integrity and availability after immobilization on mesoporous silica, evaluate the influence of MCM-41 on its photostability, and establish whether the preparation method significantly influences complex properties. The physico-chemical characterization, carried out by several techniques (X-ray diffraction, thermogravimetric analysis, DSC, and infrared spectroscopy), confirmed the host-guest interaction and Trolox[®] structure preservation. Gas-volumetric (BET) analysis showed a marked decrease in surface area and pore volume with respect to bare MCM-41, indicating that the antioxidant was present in the mesopores, and not simply located on the external surface. *In vitro* diffusion tests showed a slower release of Trolox[®] after immobilization in the inorganic matrix; at the same time, UV irradiation studies revealed increased photostability of the complex, particularly when dispersed in an emulsion system. Moreover, the radical scavenging activity of this hydrophilic derivative was maintained even after immobilization.

For rutin, a complex was prepared using aminopropyl functionalized nanoparticles (NH₂-MCM-41) to improve interaction with the guest molecule; the formulation was compared to that obtained with the parent MCM-41. Physico-chemical characterization, particularly BET analysis, evidenced the high rate of inclusion of rutin in the pores of NH₂-MCM-41. Infrared spectroscopy demonstrated the formation of hydrogen-bonded adducts in both silica matrices, irrespective of surface functionalization, but in the case of functionalized silica, spectroscopy suggested that the aminopropyl groups play an important role in interaction with the quercetin aglycone, while on bare silica the interaction takes place through the rutinose disaccharide. When exposed to UV illumination, mesoporous silica significantly improved rutin's stability over time. Significantly, the protection appears to be correlated with both the presence of aminopropyl groups and the nature of the dispersion medium (*i.e.* solution or emulsion system). The antioxidant activity of immobilized

rutin was demonstrated by both 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH[•]) assay and the ferrous ion chelating test. *Ex vivo* experiments on Franz diffusion cells demonstrated that complexation with NH₂-MCM-41 enhanced accumulation in the upper layer of the pigskin model. Considering these findings, MCM-41 may rationally be proposed as an innovative carrier for the topical delivery of unstable active molecules, although further investigation into MCM-41/skin surface interactions is necessary to assess the potential of this vehicle.

6) Nanobubbles as carriers of hydrophilic molecules

Recently, microbubbles and nanobubbles, spherical gas-filled core-shell structures, have gained increasing attention for drug and gene delivery. Their shell can be composed mainly of lipids or polymers, whereas the core can be filled with various gases, such as perfluorocarbons, sulphur hexafluoride, air, or carbon dioxide [106,107]. Nanobubbles have mean diameter in the nanometer order of magnitude, while microbubbles have mean diameter in the 1-6 micrometers range. The latter are currently on the market as contrast agents for ultrasound (US) imaging, because they undergo volumetric oscillations or acoustic cavitation when insonified by US and, particularly, they resonate at diagnostic frequencies [108,109]. More recently, nano-scaled bubbles have shown promising results for application as innovative nanocarriers, with improved stability and drug loading compared to microbubbles, as well as possessing extravasation capability. Thanks to their small size, extravasation from the blood vessels into the surrounding tissues is possible. Moreover, they might accumulate within tumor tissues *via* either the EPR effect [1,2] or through active targeting, i.e. by binding antibodies onto the bubble surface [110]. Ultrasound-triggered site-specific release of the incorporated drug, with minimal invasiveness, can then be achieved. Moreover, nanobubbles can act as a US-mediated nanocargo for the intracellular uptake of drugs [111]. All these features have been exploited for developing targeted delivery systems combined with ultrasonography. In addition, it is of note that a bubble-targeted strategy could make it possible to visualize the delivery and release of the loaded compound, through real-time echography imaging.

Nanobubbles are mainly prepared by sonication, high shear emulsification, coacervation or coalescence; these techniques are also used in microbubble preparation [112].

Nanobubbles can be loaded with either hydrophilic or lipophilic molecules, and different technological approaches have been proposed to associate molecules within the bubble structure [113]. Drugs might be electrostatically bound to the bubble surface, or might otherwise be incorporated within or just beneath the nanobubble shell. Alternatively, they can be previously loaded into a nanosystem, which can then be linked to the nanobubble surface.

Considering hydrophilic drugs, a number of purpose-built formulations have been designed. Particularly, hydrophilic molecules can be directly attached to the outer nanobubble surface, exploiting electrostatic interactions; they can also be included in the nanobubble inner core using technological approaches.

The US-mediated therapeutic strategy associated with hydrophilic drugs was firstly proposed for cancer chemotherapy [114,115], but this nanotechnology showed potential for other applications. A polymeric nanobubble system has been produced to increase the sensitivity of cancer cells to Cox associated to US [116]. The developed systems comprise perfluorocarbon nanodroplets stabilized by a biodegradable block copolymer wall. Upon heating to physiological temperatures, the nanodroplets convert into nano/microbubbles. Under the action of tumor-directed ultrasound, microbubbles cavitate and collapse, releasing the encapsulated drug. Due to the ultrasound-enhanced intracellular drug uptake, DOX-loaded nanobubbles proved effective in *in vivo* tumor chemotherapy.

More recently, nanobubbles have been developed by encapsulating DOX-HCl into poly(lactic-co-glycolic acid) (PLGA) shells for drug delivery into cancer cells; pH-triggered release profile of DOX from nanobubbles was achieved. DOX-loaded nanobubbles were internalized in HeLa cells in both a concentration- and a time-dependent manner, and has a stronger cell-growth inhibition effect *in vitro* than free DOX [117].

Nanobubbles have also been developed with the aim of obtaining more efficient gene delivery systems [118]. For this purpose, bubble liposomes have been produced with sizes between 150-200 nm. After injection into mice, they successfully showed that gene uptake was limited to the area exposed to US, indicating that the system could be used to increase DNA transduction [119]. In the context of gene delivery, US-sensitive nanobubbles bearing siRNA for tumor therapy have been synthesized by a hetero assembling strategy, in order to target the anti-apoptosis gene sirtuin 2. The application of US enhanced the gene silencing effect of siRNA-nanobubbles both *in vitro* and *in vivo*; this led to a high rate of cancer cell apoptosis. Consequently, a significantly improved therapeutic effect was achieved in a nude mouse glioma model. The US-sensitive siRNA nanobubbles may be an ideal delivery vector to mediate highly effective RNA interference for tumor treatment [120].

Nanobubbles carrying androgen receptor (AR) siRNA have been prepared using poly-L-lysine and electrostatic adsorption methods. Treatment with siRNA-nanobubbles combined to ultrasonic irradiation significantly inhibited cell growth and resulted in the suppression of AR mRNA and protein expression. Based on these results, nanobubbles carrying AR siRNA might potentially be used as gene vectors in combination with ultrasonic irradiation for the treatment of androgen-independent prostate cancer [121].

Lipid-based nanobubbles containing perfluoropentane were prepared for the delivery of apomorphine hydrochloride, a chemically-unstable dopamine receptor agonist with low bioavailability. Its encapsulation within a delivery system might overcome these drawbacks. The *in vitro* drug release was sustained over time and it was enhanced by insonation, demonstrating a possible drug-targeting effect [122].

In work done by the Turin Group, polymer-shelled and perfluoropentane-cored nanobubbles were designed as versatile multifunctional carriers for the delivery of gases, drugs and genes (Figure 7). These nanobubbles were of size below 500 nm, with shell thickness in the 30-50 nm range. For the nanobubble core, perfluoropentane was chosen, because it is a perfluorocarbon that is liquid at room temperature (boiling point 29 °C); it converts into vapor in the presence of US, through a mechanism known as acoustic droplet vaporization [123]. Consequently, it is possible to prepare stable perfluoropentane nanoemulsions with a simple preparation set-up (compared with the use of gases), which then convert into bubbles after the liquid-to-gas transition. These perfluoropentane-cored nanobubbles were found to be echogenic and stable without morphological changes or shrinkage, after insonation for 5 minutes at 2.5 MHz. They did not affect the viability of several different cell lines. Confocal microscopy showed the nanobubble capacity to be internalized in cells.

Based on the premise that large amounts of oxygen can be dissolved in perfluoropentane, oxygen-filled nanobubbles were obtained using either chitosan or dextran for the shell [124,125]. They were able to store oxygen and then slowly release it in hypoxic environments, after exposure to US [126]. To increase the oxygen loading, alternative US-responsive nanodroplets, consisting of decafluoropentane and chitosan, were developed and formulated in a hydrogel preparation, to treat hypoxic diseases of the dermal and subcutaneous tissues [127].

A peculiar perfluoropentane-cored nanobubble formulation, comprising a diethylaminoethyl-dextran (DEAE)-based shell with positive surface charge, was designed to complex DNA. DEAE nanobubbles were able to protect their cargo from the action of proteases, and to transfect plasmid DNA without any resulting cytotoxic effects [128]. Subsequently, chitosan-based shelled formulations were proposed for DNA delivery [129]. Chitosan was selected for the nanobubble shell because it is a polycationic polymer with low toxicity, low immunogenicity, and excellent biocompatibility. DNA-loaded chitosan nanobubbles were formed with mean diameter below 300 nm and positive surface charge. *In vitro* transfection experiments were performed on COS7 cells. In the absence of US, nanobubbles failed to trigger transfection at any of the concentrations tested; in contrast, 30 seconds of US (2.5 MHz) promoted a moderate degree of transfection without affecting cell viability [129].

In addition, dextran-shelled nanobubbles were also exploited to deliver vancomycin, a hydrophilic antibiotic used in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. This drug has low oral bioavailability and its systemic administration can cause severe adverse effects. These drawbacks could be overcome by the topical administration of vancomycin loaded in nanobubbles. Vancomycin-loaded nanobubbles were about 300 nm in size with a negative surface charge, an EE% of 86% and a loading capacity of 29%. *In vitro* studies demonstrated prolonged release kinetics and absence of toxicity on keratinocytes. The antibacterial activity against MRSA of vancomycin-loaded nanobubbles was confirmed *in vitro*. The application of US in combination with nanobubbles enhanced both *in vitro* release and drug permeation through a pigskin model. Vancomycin properly formulated in US-responsive nanobubbles might be a promising strategy for the topical treatment of MRSA infections [130].

Conclusion

This review has discussed different DDS developed in Turin University. Although hydrophobic drugs also benefit from loading into nanostructures (e.g., increasing their aqueous solubility), the present article has mainly focused on strategies to improve the performance of hydrophilic drugs. The new DDS that have been developed possess improved properties compared to the free drug or parent substance, namely:

- regulated drug release from the DDS, reducing healthy tissue damage;
- protection of the encapsulated molecule from premature degradation, meaning lower doses are required;
- different pharmacokinetics, often similar to that of the DDS, lowering the volume of distribution, and avoiding rapid clearance;
- in the case of antitumor drugs, increased concentration in diseased tissues caused by the EPR effect, and in some cases by ligand-mediated targeting, further improving drug specificity.

Some of these delivery systems are made of lipidic compounds: natural or synthetic phospholipids for liposomes, fatty acids for SLN, or squalene-derived chains for squalenoyl nanoparticles. Other systems are mainly composed of natural or synthetic polymers, such as drug-loaded nanobubbles, or totally synthetic polymers, such as polyalkylcyanoacrylate carriers, both nanospheres and nanocapsules. Inorganic materials, such as mesoporous silica particles, are receiving increasing attention as modern drug delivery systems essentially for their high biocompatibility and considerable versatility.

To conclude, this review has described the wide range of new drug delivery technologies developed by the Turin research group to encapsulate hydrophilic molecules, also examining some issues concerning physical, chemical, technological, pharmacological, and biological aspects of the DDS.

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References

1. Maeda H. Emergence of EPR effect theory and development of clinical applications for cancer therapy. *Therapeutic Delivery* 2014; 5:627-630.
2. Fang J, Nakamura H, Maeda H. The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev.* 2011; 63:136-151.
3. Mallick S, Choi JS. Liposomes: versatile and biocompatible nanovesicles for efficient biomolecules delivery. *J Nanosci Nanotechnol.* 2014; 14:755-765.
4. Drbohlavova J. Nanocarriers for anticancer drugs - New Trends in Nanomedicine *Current Drug Metab.* 2013; 14:547-564.
5. Crosasso P, Brusa P, Dosio F, Arpicco S, Pacchioni D, Schuber F, Cattel L. Antitumoral activity of liposomes and immunoliposomes containing 5-fluorouridine prodrugs. *J Pharm Sci.* 1997; 86:832-839.
6. Immordino ML, Brusa P, Rocco F, Arpicco S, Ceruti M, Cattel L. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. *J Control Release.* 2004; 100:331-346.
7. Brusa P, Immordino ML, Rocco F, Cattel L. Antitumor activity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. *Anticancer Res.* 2007; 1:195-199.
8. Castelli F, Sarpietro MG, Ceruti M, Rocco F, Cattel L. Characterization of lipophilic gemcitabine prodrug-liposomal membrane interaction by differential scanning calorimetry. *Mol Pharmaceut.* 2006; 3:737-744.
9. Castelli F, Sarpietro MG, Rocco F, Ceruti M, Cattel L. Interaction of lipophilic gemcitabine prodrugs with biomembrane models studied by Langmuir-Blodgett technique. *J Colloid Interf Sci.* 2007; 313:363-368.
10. Arpicco S, Lerda C, Dalla Pozza E, Costanzo C, Tsapis N, Stella B, Donadelli M, Dando I, Fattal E, Cattel L, Palmieri M. Hyaluronic acid-coated liposomes for active targeting of gemcitabine. *Eur J Pharm Biopharm.* 2013; 85:373-380.
11. Arpicco S, Milla P, Stella B, Dosio F. Hyaluronic acid conjugates as vectors for the active targeting of drugs, genes and nanocomposites in cancer treatment. *Molecules* 2014;19:3193-3230.
12. Dalla Pozza E, Lerda C, Costanzo C, Donadelli M, Dando I, Zoratti E, Scupoli MT, Beghelli S, Scarpa A, Fattal E, Arpicco S, Palmieri M. Targeting gemcitabine containing liposomes to CD44 expressing pancreatic adenocarcinoma cells causes an increase in the antitumoral activity. *BBA Biomembranes* 2013; 1828:1396-1404.
13. Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. *Eur J Pharm Biopharm.* 2000; 50:161-177.
14. Battaglia L, Gallarate M. Lipid nanoparticles: state of the art, new preparation methods and challenges in drug delivery. *Expert Opin Drug Del.* 2012; 9:497-508.
15. Gasco MR. Method for producing solid lipid microspheres having a narrow size distribution. US5250236 patent; 1993.
16. Schubert MA, Mueller-Goymann CC. Solvent injection as a new approach for manufacturing lipid nanoparticles – evaluation of the method and process parameters. *Eur J Pharm Biopharm.* 2003; 55:125-131.
17. Siekmann B, Westesen K. Investigation on solid lipid nanoparticles prepared by precipitation in o/w emulsion. *Eur J Pharm Biopharm.* 1996; 43:104-109.
18. Trotta M, Debernardi F, Caputo O. Preparation of solid lipid nanoparticles by a solvent emulsification-diffusion technique. *Int J Pharm.* 2003; 257:153-160.

19. Hao J, Wang F, Wang X, Zhang D, Bi Y, Gao Y, Zhao X, Zhang Q. Development and optimization of baicalin-loaded solid lipid nanoparticles prepared by coacervation method using central composite design. *Eur J Pharm Sci.* 2012; 47:497-505.
20. Battaglia L, Gallarate M, Cavalli R, Trotta M. Solid lipid nanoparticles produced through a coacervation method. *J Microencapsul.* 2010; 27:78–85.
21. Attama AA. SLN, NLC, LDC: State of the art in drug and active delivery. *Recent Pat Drug Deliv Formul.* 2011; 5:178-187.
22. Morel S, Terreno E, Ugazio E, Aime S, Gasco MR. NMR Relaxometric investigations of solid lipid nanoparticles (SLN) containing gadolinium (III) complexes. *Eur J Pharm Biopharm.* 1998; 45:157-163.
23. Battaglia L, Trotta M, Gallarate M, Carlotti ME, Zara GP, Bargoni A. Solid lipid nanoparticles formed by solvent-in-water emulsion-diffusion technique: development and influence on insulin stability. *J Microencapsul.* 2007; 24:672-684.
24. Gallarate M, Trotta M, Battaglia L, Chirio D. Cisplatin-loaded SLN produced by coacervation technique. *J Drug Deliv Sci Tech.* 2010; 20:343-347.
25. Gallarate M, Battaglia L, Peira E., Trotta M. Peptide-loaded solid lipid nanoparticles prepared through coacervation technique. *Int J Chem Engin.* 2011, doi:10.1155/2011/132435.
26. Wong HL, Bendayan R, Rauth M, Wu XY. Development of solid lipid nanoparticles containing ionically complexed chemotherapeutic drugs and chemosensitizers. *J Pharm Sci.* 2004; 8:1993-2008.
27. Wong HL, Rauth AM, Bendayan R, Manias JL, Ramaswamy M, Liu Z, Erhan S.Z, Wu XY, A new polymer-lipid hydrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug resistant human breast cancer cells. *Pharm Res.* 2004; 23:1574–1585.
28. Olbrich C, Gessner A, Kayser O, Müller RH. Lipid-drug-conjugate (LDC) nanoparticles as novel carrier system for the hydrophilic antitrypanosomal drug diminazenediaceturate. *J Drug Target.* 2002; 10:387–396.
29. Bussano R, Chirio D, Costa L, Turci F, Trotta M. Preparation and characterization of insulin loaded lipid-based microspheres generated by electrospray. *J Disper Sci Technol.* 2011; 32:1524-1530.
30. Wang JX, Sun X, Zhang ZR. Enhanced brain targeting by synthesis of 3',5'-dioctanoyl-5-fluoro-2'-deoxyuridine and incorporation into solid lipid nanoparticles. *Eur J Pharm Biopharm.* 2002; 54:285-290.
31. Münster U, Nakamura C, Haberland A, Jores K, Mehnert W, Rummel S, Schaller M, Korting HC, Zouboulis Ch C, Blume-Peytavi U, Schäfer-Korting M. RU 58841-myristate - prodrug development for topical treatment of acne and androgenetic alopecia. *Pharmazie.* 2005; 60:8-12.
32. Gallarate M, Trotta M, Battaglia L, Chirio D. Preparation of solid lipid nanoparticles from W/O/W emulsions: Preliminary studies on insulin encapsulation. *J Microencapsul.* 2009; 26:394-402.
33. Battaglia L, Gallarate M, Peira E, Chirio D, Muntoni E, Biasibetti E, Capucchio MT, Valazza A, Panciani PP, Lanotte M, Schiffer D, Annovazzi L, Caldera V, Mellai M, Riganti C. Solid lipid nanoparticles for potential doxorubicin delivery in glioblastoma treatment: preliminary in vitro studies. *J Pharm Sci.* 2014; 103:2157-2165.
34. Battaglia L, Serpe L, Muntoni E, Zara GP, Trotta M, Gallarate M. Methotrexate-loaded SLN prepared by coacervation technique: in vitro cytotoxicity and in vivo pharmacokinetics and biodistribution. *Nanomedicine.* 2011; 6:1561-1573.
35. Solazzi I, Battaglia L, Gallarate M, Peira E, Chirio D, Giordano S, Dolo V, Dianzani C. Oral communication at 54° Simposio AFI, Rimini 11-13 Giugno 2014.

36. Vrignaud S, Benoit J-P, Saulnier P. Strategies for the nanoencapsulation of hydrophilic molecules in polymer-based nanoparticles. *Biomaterials* 2011; 32:8593-8604.
37. Arias JL, Ruiz MA, López-Viata M, Delgado AV. Poly(alkylcyanoacrylate) colloidal particles as vehicles for antitumour drug delivery: a comparative study. *Colloids Surfaces B* 2008; 62:64-70.
38. Barichello JM, Morishita M, Takayama K, Nagai T. Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method *Drug Dev Ind Pharm.* 1999; 25:471-476.
39. Peltonen L, Aitta J, Hyvönen S, Karjalainen M, Hirvonen J. Improved entrapment efficiency of hydrophilic drug substance during nanoprecipitation of poly(l)lactide nanoparticles *AAPS PharmSciTech*, 2004; 5:1-6.
40. Lince F, Bolognesi S, Marchisio DL, Stella B, Dosio F, Barresi AA, Cattel L. Preparation of poly(MePEGCA-co-HDCA) nanoparticles with confined impinging jets reactor: experimental and modeling study. *J Pharm Sci.* 2011; 100:2391-2405.
41. Lince F, Bolognesi S, Stella B, Marchisio DL, Dosio F. Preparation of polymer nanoparticles loaded with doxorubicin for controlled drug delivery. *Chem Eng Res Des.* 2011; 89:2410-2419.
42. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(*d,l*-lactide-co-glycolide) and its derivatives. *J Control Release.* 2008; 125:193-209.
43. Gao H, Jones M, Chen J, Liang Y, Prud'homme R, Leroux JC. Hydrophilic nanoreservoirs embedded into polymeric micro/nanoparticles: an approach to compatibilize polar molecules with hydrophobic matrixes. *Chem Mater.* 2008; 20:4191-4193.
44. Zheng Y, Yang W, Wang C, Hu J, Fu S, Dong L, Wu L, Shen X. Nanoparticles based on the complex of chitosan and polyaspartic acid sodium salt: Preparation, characterization and the use for 5-fluorouracil delivery. *Eur J Pharm Biopharm*, 2007; 67:621-631.
45. Rudzinski WE, Aminabhavi TM. Chitosan as a carrier for targeted delivery of small interfering RNA. *Int J Pharm.* 2010; 399:1-11.
46. Chavanpatil MD, Khdair A, Patil Y, Handa H, Mao G, Panyam J. Polymer-surfactant nanoparticles for sustained release of water-soluble drugs *J Pharm Sci.* 2007; 96:3379-3389.
47. Yang SC, Ge HX, Hu Y, Jiang QX, Yang CZ. Doxorubicin-loaded poly(butylcyanoacrylate) nanoparticles produced by emulsifier-free emulsion polymerization. *J Appl Polym Sci.* 2000; 78:517-526.
48. Arias JL, Reddy LH, Couvreur P. Polymeric nanoparticulate system augmented the anticancer therapeutic efficacy of gemcitabine. *J Drug Target.* 2009; 17:586-598.
49. Fattal E, Vauthier C, Aynie I, Nakada Y, Lambert G, Malvy C, Couvreur P. Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of oligonucleotides. *J Control Release.* 1998; 53:137-143.
50. Stella B, Arpicco S, Rocco F, Marsaud V, Renoir J.-M, Cattel L, Couvreur P. Encapsulation of gemcitabine lipophilic derivatives into polycyanoacrylate nanospheres and nanocapsules. *Int. J Pharm.* 2007; 344:71-77.
51. Stella B, Arpicco S, Peracchia MT, Desmaële D, Hoebeke J, Renoir M, D'Angelo J, Cattel L, Couvreur P. Design of folic acid-conjugated nanoparticles for drug targeting. *J Pharm Sci.* 2000; 89:1452-1464.
52. Stella B, Rocco F, Rosilio V, Renoir J.-M, Cattel L, Couvreur P. Nanoparticles of Gemcitabine derivatives. French patent deposited 6/6/2004, n° 04 51365; European Patent PCT/FR2005/050488 deposited 23/06/2005.

53. Couvreur P, Stella B, Reddy H, Hillaireau H, Dubernet C, Desmaële D, Lepetre-Mouelhi S, Rocco F, Dereuddre-Bosquet L, Clayette P, Rosilio V, Marsaud V, Renoir J-M, Cattel L. Squalenoyl nanomedicines as potential therapeutics. *Nano Lett.* 2006; 6:2544-2548.
54. Reddy LH, Renoir J-M, Marsaud V, Lepetre-Mouelhi S, Desmaële D, Couvreur P. Anticancer efficacy of squalenoyl gemcitabine nanomedicine on 60 human tumor cell panel and on experimental tumor. *Mol Pharmaceut.* 2009; 6:1526-1535.
55. Reddy LH, Ferreira H, Dubernet C, Mouelhi S, Desmaële D, Rousseau B, Couvreur P. Squalenoyl nanomedicines of Gemcitabine is more potent after oral administration in leukemia-bearing rats: study of mechanisms. *Anti-Cancer Drugs.* 2008; 19:999-1006.
56. Desmaële D, Gref R, Couvreur P. Squalenoylation: a generic platform for nanoparticulate drug delivery. *J Control Release.* 2012; 161:609-618.
57. Ceruti M, Balliano G, Viola F, Cattel L, Gerst N, Schuber F. Synthesis and biological activity of azasqualenes, bis-azasqualenes and derivatives. *Eur J Med Chem.* 1987; 22:199-208.
58. Viola F, Brusa P, Balliano G, Ceruti M, Boutaud O, Schuber F, Cattel L. Inhibition of 2,3-oxidosqualene cyclase and sterol biosynthesis by 10 and 19-azasqualene derivatives. *Biochem. Pharmacol.* 1995; 50:787-796.
59. Castelli F, Sarpietro MG, Micieli D, Stella B, Rocco F, Cattel L. Enhancement of gemcitabine affinity for biomembranes by conjugation with squalene: differential scanning calorimetry and Langmuir-Blodgett studies using biomembrane models. *J Colloid Interf Sci.* 2007; 316:43-52.
60. Cosco D, Rocco F, Ceruti M, Vono M, Fresta M, Paolino D. Self-assembled squalenoyl-cytarabine nanostructures as a potent nanomedicine for treatment of leukemic diseases. *Int J Nanomed.* 2012; 7: 2535-2546.
61. Sarpietro MG, Ottimo S, Giuffrida MC, Rocco F, Ceruti M, Castelli F. Synthesis of N-squalenoyl-cytarabine and evaluation of its affinity with phospholipid bilayers and monolayers. *Int J Pharm.* 2011; 406:69-77.
62. Sarpietro MG, Micieli D, Rocco F, Ceruti M, Castelli F. Conjugation of squalene to acyclovir improves the interaction with biomembrane models. *Int J Pharm.* 2009; 382:73-79.
63. Sarpietro MG, Rocco F, Micieli D, Ottimo S, Ceruti M, Castelli F. Interaction of acyclovir and its squalenoyl-acyclovir prodrug with DMPC in monolayers at the air/water interface. *Int J Pharm.* 2010; 395:167-173.
64. Stella B, Arpicco S, Rocco F, Burgalassi S, Nicosia N, Tampucci S, Chetoni P, Cattel L. Nonpolymeric nanoassemblies for ocular administration of acyclovir: Pharmacokinetic evaluation in rabbits. *Eur J Pharm Biopharm.* 2012; 80:39-45.
65. Kratz F, Fichtner I, Graeser R. Combination therapy with the albumin-binding prodrug of doxorubicin (INNO-206) and doxorubicin achieves complete remissions and improves tolerability in an ovarian A2780 xenograft model. *Invest. New Drugs.* 2012; 30:1743-1749.
66. Maksimenko A, Dosio F, Mougin J, Ferrero A, Wack S, Reddy LH, Weyn AA, Lepeltier E, Bourgaux C, Stella B, Cattel L, Couvreur P. A unique squalenoylated and nonpegylated doxorubicin nanomedicine with systemic long-circulating properties and anticancer activity. *Proc Natl Acad Sci USA.* 2014; 111:E217-E226.
67. Dosio F, Reddy LH, Ferrero A, Stella B, Cattel L, Couvreur P. Novel nanoassemblies composed of squalenoyl-paclitaxel derivatives: Synthesis, characterization, and biological evaluation. *Bioconj Chem.* 2010; 21:1349-1361.
68. Borrelli S, Christodoulou MS, Ficarra I, Silvani A, Cappelletti G, Cartelli D, Damia G, Ricci F, Zucchetti M, Dosio F, Passarella D. New class of squalene-based releasable

- nanoassemblies of paclitaxel, podophyllotoxin, camptothecin and epothilone A. *Eur J Med Chem.* 2014; 85:179-190.
69. Caron J, Maksimenko A, Wack SE, Bourgaux C, Morvan E, Leblanc K, Couvreur P, Desmaële D. Improving the antitumor activity of squalenoyl-paclitaxel conjugate nanoassemblies by manipulating the linker between paclitaxel and squalene. *Advanced Healthcare Materials.* 2013; 2:172-185.
 70. Semiramoth N, Di Meo C, Zouhiri F, Said-Hassane F, Valetti S, Gorges R, Nicolas V, Poupaert JH, Chollet-Martin S, Desmaële D, Gref R, Couvreur P. Self-assembled squalenoylated penicillin bioconjugates: an original approach for the treatment of intracellular infections. *ACS Nano.* 2012; 6:3820-3831.
 71. Couvreur P, Desmaële D, Zouhiri F. Synthesis of nanoparticles of beta-lactam-squalene derivatives as antibacterial agents. Patent FR2937549A1, 2010.
 72. Couvreur P, Desmaële D, Zouhiri F, Lakkireddy HR. Preparation of squalene-statin nanoparticles for pharmaceutical use. Patent WO2010049900A1, 2010.
 73. Couvreur P, Lakkireddy HR, Dosio F, Stella B, Cattel L. Preparation of nanoparticles of squalenoyl therapeutically active agents having low water solubility such as insulin, paclitaxel, epirubicin and deoxyspergualin. Patent WO2009071850A2, 2009.
 74. Lorenz HM, Schmitt WH, Tesar V, Mauller-Ladner U, Tarner I, Hauser IA, Hiepe F, Alexander T, Woehling H, Nemoto K, Heinzel PA. Treatment of active lupus nephritis with the novel immunosuppressant 15-deoxyspergualin: an open-label dose escalation study. *Arthritis Research and Therapy.* 2011; 13:R36
 75. Norez C, Pasetto M, Dehecchi MC, Barison E, Anselmi C, Tamanini A, Quiri F, Cattel L, Rizzotti P, Dosio F, Cabrini G, Colombatti M. Chemical conjugation of $\hat{P}^{\text{F508}}$ -CFTR corrector deoxyspergualin to transporter human serum albumin enhances its ability to rescue Cl⁻ channel functions. *Am J Physiol. - Lung Cellular and Molecular Physiology.* 2008; 295:L336-L347.
 76. Couvreur P, Zouhiri F, Gref R, Desmaële D. Preparation of nanoparticle squalenyl vitamin C complexes as skin antiaging cosmetics. Patent FR2988092A1, 2013.
 77. Othman M, Desmaele D, Couvreur P, Vander Elst L, Laurent S, Muller RN, Bourgaux C, Morvan E, Pouget T, Lepetre-Mouelhi S, Durand P, Gref R. Synthesis and physicochemical characterization of new squalenoyl amphiphilic gadolinium complexes as nanoparticle contrast agents. *Org Biomolec Chem.* 2011, 9, 4367-4386.
 78. Dosio F, Stella B, Ferrero A, Garino C, Zonari D, Arpicco S, Cattel L, Giordano S, Gobetto R. Ruthenium polypyridyl squalene derivative: A novel self-assembling lipophilic probe for cellular imaging. *Int J Pharm.* 2013; 440:221-228.
 79. Arias JL, Reddy LH, Othman M, Gillet B, Desmaële D, Zouhiri F, Dosio F, Gref R, Couvreur P. Squalene based nanocomposites: A new platform for the design of multifunctional pharmaceutical theragnostics. *ACS Nano.* 2011; 5:1513-1521.
 80. Valetti S, Maione F, Mura S, Stella B, Desmaële D, Noiray M, Vergnaud J, Vauthier C, Cattel L, Giraudo E, Couvreur P. Peptide-functionalized nanoparticles for selective targeting of pancreatic tumor. *J Control Release.* 2014; 192:29-39.
 81. Ascenso A, Ribeiro HM, Marques HC, Simoes S. Topical delivery of antioxidants. *Curr Drug Deliv.* 2011; 8:640-660.
 82. Carlotti ME, Sapino S, Gallarate M, Peira, Ugazio E. O/W microemulsions with vanillin as vehicles for antiacne actives: preparation, characterization and stability. *J Disp Sci Technol.* 2008; 29:991-998.
 83. Carlotti ME, Ugazio E, Sapino S, Peira E, Battaglia L, Cavalli R. Photodegradation of caffeic acid in W/O/W emulsions in the absence and in the presence of TiO₂. *J Disp Sci Technol.* 2008; 29:1435-1444.

84. Peira E, Ugazio E, Chirio D, Trotta M. Elastic positively charged liposomes for topical administration of acyclovir. APGI Skin and Formulation 2nd Symposium, Versailles, 9-10 october 2006.
85. Carlotti ME, Sapino S, Vione D, Pelizzetti E, Ugazio E, Morel S. Study on the photostability of octyl-p-methoxy cinnamate in SLN. *J Disp Sci Technol*. 2005; 26:809-816.
86. Carlotti ME, Sapino S, Ugazio E, Gallarate M, Morel S. Resveratrol in solid lipid nanoparticles. *J Disp Sci Technol*. 2012; 33:465-471.
87. Sapino S, Carlotti ME, Cavalli R, Ugazio E, Berlier G, Gastaldi L, Morel S. Photochemical and antioxidant properties of gamma-oryzanol in beta-cyclodextrin-based nanosponges. *J Incl Phenom Macrocycl Chem*. 2013; 75:69-76.
88. Carlotti ME, Sapino S, Marino S, Ugazio E, Trotta F, Vione D, Chirio D, Cavalli R. Influence of hydroxypropyl-beta-cyclodextrin on the photostability and antiradical activity of Trolox. *J Incl Phenom Macrocycl Chem*. 2008; 61:279-287.
89. Sapino S, Trotta M, Ermondi G, Caron G, Cavalli R, Carlotti ME. On the complexation of Trolox with methyl-beta-cyclodextrin: characterization, molecular modelling and photostabilizing properties. *J Incl Phenom Macrocycl Chem*. 2008; 62:179-186.
90. Carlotti ME, Sapino S, Ugazio E, Caron G. On the complexation of quercetin with methyl- β -cyclodextrin: photostability and antioxidant studies. *J Incl Phenom Macrocycl Chem*. 2011; 70:81-90.
91. Berlier G, Gastaldi L, Ugazio E, Miletto I, Iliade P, Sapino S. Stabilization of quercetin flavonoid in MCM-41 mesoporous silica: positive effect of surface functionalization. *J Colloid Interf Sci*. 2013 393:109-118.
92. Vallet-Regi M, Rámila A, del Real RP, Pérez-Pariente J. A new property of MCM-41: drug delivery system. *Chem Mater*. 2001; 13:308-311.
93. Fowler CE, Khushalani D, Lebeau B, Mann S. Nanoscale materials with mesostructured interiors. *Adv Mater*. 2001; 13:649-652.
94. Cai Q, Luo S, Pang WQ, Fan YW, Chen X. Dilute solution routes to various controllable morphologies of MCM-41 silica with a basic medium. *Chem Mater*. 2001; 13:258-263.
95. Hu YC, Zhao Q, Wu C, Zhao P, Jiang H, Jiang T, Wang S. 3D cubic mesoporous silica microsphere as a carrier for poorly soluble drug carvedilol. *Micropor Mesopor Mat*. 2012; 147:94-101.
96. Tang F, Li L, Chen D. Mesoporous silica nanoparticle: synthesis, biocompatibility and drug delivery. *Adv Mater*. 2012; 24:1504-1534.
97. Song B, Wu C, Chang J. Controllable delivery of hydrophilic and hydrophobic drugs from electrospun poly(lactic-co-glycolic acid)/mesoporous silica nanoparticles composite mats. *J Biomed Mater Res Part B*. 2012; 100B:2178-2186.
98. Fuertes AB, Valle-Vigon P, Sevilla M. Synthesis of colloidal silica nanoparticles of a tunable mesopore size and their application to the adsorption of biomolecules. *J Colloid Interf Sci*. 2010; 349:173-180.
99. Zhang Y, Zhi Z, Jiang T, Zhang J, Wang Z, Wang S. Spherical mesoporous silica nanoparticles for loading and release of the poorly water-soluble drug telmisartan. *J Control Release*. 2010; 145:257-263.
100. Vallet-Regi M, Balas F, Arcos D. Mesoporous materials for drug delivery. *Angew Chem Int Ed*. 2007; 46:7548-7558.
101. Ambrogi V, Latterini L, Marmottini F, Tiralti MC, Ricci M. Oxybenzone entrapped in mesoporous silicate MCM-41. *J Pharm Innov*. 2013; 8:212-217.
102. Ambrogi V, Latterini L, Marmottini F, Pagano C, Ricci M. Mesoporous silicate MCM-41 as a particulate carrier for octylmethoxycinnamate: sunscreen release and photostability. *J Pharm Sci*. 2013; 102:1468-1475.

103. Ambrogi V, Perioli L, Pagano C, Marmottini F, Moretti M, Mizzi F, Rossi C. Econazole nitrate-loaded MCM-41 for an antifungal topical powder formulation. *J Pharm Sci.* 2010; 99:4738-4745.
104. Gastaldi L, Ugazio E, Sapino S, Iliade P, Miletto I, Berlier G. Mesoporous silica as a carrier for topical application: the Trolox case study. *Phys Chem Chem Phys.* 2012; 14:11318-11326.
105. Berlier G, Gastaldi L, Sapino S, Miletto I, Bottinelli E, Chirio D, Ugazio E. MCM-41 as a useful vector for rutin topical formulations: synthesis, characterization and testing. *Int J Pharm.* 2013; 457:177-186.
106. Sirsi S, Borden M. Microbubble compositions, properties and biomedical applications. *Bubble Sci Eng Technol.* 2009; 1:3-17.
107. Hernot S, Klibanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Deliv Rev.* 2008; 60:1153-1166.
108. Dijkmans PA, Juffermans LJM, Musters RJP, van Wamel A, ten Cate FJ, van Gilst W, Visser CA, de Jong N, Kamp O. Microbubbles and ultrasound: from diagnosis to therapy. *Eur J Echocardiogr.* 2004; 5:245-256.
109. Klibanov AL. Preparation of targeted microbubbles: ultrasound contrast agents for molecular imaging. *Med Biol Eng Comput.* 2009; 47:875-882.
110. Fokong S, Theek B, Wu Z, Koczera P, Appold L, Jorge S, Resch-Genger U, VanZandvoort M, Storm G, Kiessling F, Lammers T. Image-guided, targeted and triggered drug delivery to tumors using polymer-based microbubbles. *J Control Release* 2012; 163:75-81.
111. Pitt WG, Hussein GA. Ultrasound in drug and gene delivery. *Adv Drug Deliv Rev.* 2008; 60:1095-1096.
112. Stride E, Edirisinghe M. Novel microbubble preparation technologies. *Soft Matter* 2008; 4, 2350-2359.
113. Tinkov S, Bekeredjian R, Winter G, Coester C. Microbubbles as ultrasound drug carriers. *J Pharm Sci.* 2009; 90:1035-1061.
114. Rapoport N, Kennedy A, Gao Z, Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *J Natl Cancer Inst.* 2007; 99:1-12.
115. Wang Y, Li X, Zhou Y, Huang P, Xu Y. Preparation of nanobubbles for ultrasound imaging and intracellular drug delivery. *Int J Pharm.* 2010; 384, 148-153.
116. Gao Z, Kennedy AM, Christensen DA, Rapoport N. Drug-loaded nano/microbubbles for combining ultrasonography and targeted chemotherapy. *Ultrasonics* 2008; 48:260-270.
117. Deng L, Li L, Yang H, Li L, Zhao F, Wu C, Liu Y. Development and optimization of doxorubicin loaded poly(lactic-co-glycolic acid) nanobubbles for drug delivery into HeLa cells. *J Nanosci Nanotechnol.* 2014; 14:2947-2954.
118. Cavalli R, Bisazza A, Lembo D. Micro- and nanobubbles: a versatile non-viral platform for gene delivery. *Int J Pharm.* 2013; 456:437-445.
119. Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Sawamura K, Tanaka K, Namai E, Oda Y, Matsumura Y, Maruyama K. Tumor specific ultrasound enhanced gene transfer in vivo with novel liposomal bubbles. *J Control Release.* 2008; 125:137-144.
120. Yin T, Wang P, Li J, Zheng R, Zheng B, Cheng D, Li R, Lai J, Shuai X. Ultrasound-sensitive siRNA-loaded nanobubbles formed by heteroassembly of polymeric micelles and liposomes and their therapeutic effect in gliomas. *Biomaterials* 2013; 34:4532-4543.
121. Wang L, Zhang M, Tan K, Guo Y, Tong H. Preparation of nanobubbles carrying androgen receptor siRNA and their inhibitory effects on androgen-independent prostate cancer when combined with ultrasonic irradiation. 2014; *PLoS ONE* 9: e96586. doi:10.1371/journal.pone.0096586.

122. Hwang T, Lin Y, Chi C, Huang T, Fang J. Development and evaluation of perfluorocarbon nanobubbles for apomorphine delivery. *J Pharm Sci.* 2009; 98:3735-3747.
123. Kripfgans OD, Fabilli ML, Carson PL, Fowlkes JB. On the acoustic vaporization of micrometer-sized droplets. *J Acoust Soc Am.* 2004; 116, 272-281.
124. Cavalli R, Bisazza A, Rolfo A, Balbis S, Madonnaripa D, Caniggia I, Guiot C. Ultrasound-mediated oxygen delivery from chitosan nanobubbles. *Int. J. Pharm.* 2009; 378:215-217.
125. Cavalli R, Bisazza A, Giustetto P, Civra A, Lembo D, Trotta G, Guiot C, Trotta M. Preparation and characterization of dextran nanobubbles for oxygen delivery. *Int J Pharm* 2009; 381:160-165.
126. Bisazza A, Giustetto P, Rolfo A, Caniggia IA, Balbis S, Guiot C, Cavalli R. Microbubble-mediated oxygen delivery to hypoxic tissues as a new therapeutic device. *Conf. Proc. IEEE Eng Med Biol Soc.* 2008; 2067-2070.
127. Magnetto C, Prato M, Khadjavi A, Giribaldi G, Fenoglio I, Jose J, Gulino GR, Cavallo F, Quaglino E, Benintende E, Varetto G, Troia A, Cavalli R, Guiot C. Ultrasound-activated decafluoropentane-cored and chitosan-shelled nanodroplets for oxygen delivery to hypoxic cutaneous tissues. *RSC Adv.* 2014; 4:38433-38444.
128. Bisazza A, Civra A, Donalisio M, Lembo D, Cavalli R. The in vitro characterization of dextran-based nanobubbles as possible DNA transfection agents. *Soft Matter.* 2011; 207:10590-10593.
129. Cavalli R, Bisazza A, Trotta M, Argenziano M, Civra A, Donalisio M, Lembo D. New chitosan nanobubbles for ultrasound-mediated gene delivery: preparation and in vitro characterization. *Int J Nanomed.* 2012; 7:3309-3318.
130. Cavalli R, Argenziano M, Banche G, Prato M, Khadjavi A, Mandras N, Allizond V, Scalas D, Roana J, Giribaldi G, Tullio V, Guiot C, Cuffini AM. Nanotechnology and infection: vancomycin-loaded dextran-shelled/perfluoropentane-containing nanobubbles as topical skin-friendly antibacterial vector. *Second Conference on Nanotechnology for Biological and Biomedical Applications, Trieste, 14-18 October 2013.*

Figure 1. Preparation of the immunoliposomes containing 5-fluorouridine prodrugs.

R=H	gemcitabine
R=CO-(CH ₂) ₃ -CH ₃	4-(N)-valeroylgemcitabine
R=CO-(CH ₂) ₁₀ -CH ₃	4-(N)-lauroylgemcitabine
R=CO-(CH ₂) ₁₆ -CH ₃	4-(N)-stearoylgemcitabine

Figure 2. Chemical structures of gemcitabine and 4-(*N*)-acylgemcitabine derivatives.

Figure 3. Scheme of the coacervation method.

Figure 4. Synthesis of 4-(*N*)-squalenoyl-gemcitabine (Sq-GEM) (**3**) and of 1,1',2-tris-nor-squalenoyl-citarabine (Sq-ARA-C) (**4**).

A

B

C

Figure 5. Synthesis of doxorubicin squalenoyl derivatives: A) DOX-Hz-Sq; B) DOX-E-Sq; C) DOX A-Sq

Figure 6. Immobilization of the hydrophilic antioxidant (Trolox® or rutin) in mesoporous silica nanoparticles.

Figure 7. Schematic representation of polymer-shelled nanobubble structure and different loading strategies.