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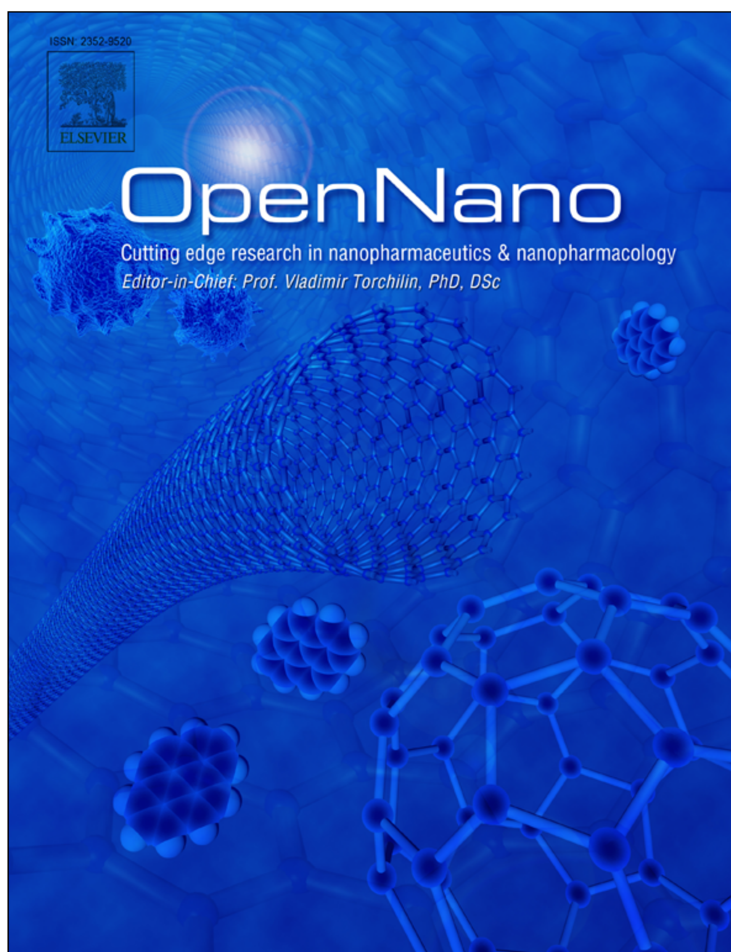

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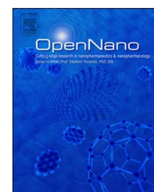

Lipid-based core-shell nanoparticles: Evolution and potentialities in drug delivery

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ABSTRACT

Over the last decade, impressive progress in the field of nanomedicine has led to the development of novel biomaterials and nanotechnology platforms. However, lipid-based nanovectors, i.e. liposomes, combining safety, versatility and delivery efficiency, remain the most "popular" approach. Lipids, especially charged lipids, have been used to design nanoparticles characterized by a core-shell structure. In these nanoparticles a lipid shell interacts with a core based on different biomaterials. Drugs characterized by a net charge can be condensed in the core, which is then covered by the lipid shell. This approach has been investigated in relation to the delivery of different active molecules, among them macromolecular drug, e.g. nucleic acids, and small molecules, e.g. bisphosphonates. This review reports the progress that has been made in the development of this technology and its potential applications in drug delivery.

1. Introduction

In the last ten years the number of published scientific studies in the field of nanomedicine has sharply increased leading to the development of different "families" of nanovectors tailored to meet specific requirements in drug delivery and targeting. Different biomaterials have been proposed depending on the drug and on the biomedical target. Moreover, the combination of different biomaterials, as well as the structure of the nanovectors, represents a further level of complexity.

Despite the number of nanotechnology-based platforms for drug delivery currently available, the number of clinical studies focused on these formulations is still very limited [1,2] with few products on the market [2,3]. The majority of studies do not go beyond the preclinical stage [1,4], remaining limited to laboratory scale. In many cases, the design of the nanotechnology based formulations has produced promising results in vivo, but is not suitable for clinical and industrial development. Other issues in the

Abbreviations: NPs, nanoparticles; siRNA, small interfering RNA; miRNA, microRNA; DC-Chol, 3b(N-(N¢,N¢-dimethylaminoethane)carbarmoyl)-cholesterol; DOPE, dioleoylphosphatidylethnolamine; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; Chol, cholesterol; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000; NGR, aspargine–glycine–arginine pepetide; DOX, doxorubicin; DSGLA, N,N-distearyl-N-methyl-N-2[N′-(N2-guanidino-L-lysinyl)] aminoethylammonium chloride; PTX, paclitaxel; VEGF, vascular endothelial growth factor; VAP, vapreotide; BBB, blood brain barrier; Tf, Transferrin; BMVECs, murine brain microvascular endothelial cells; U87, human glioblastoma cells; LPC, cationic liposomes; EGFR, epidermal growth factor receptor; MCF-7, human breast cancer cells; PPD, PEG-peptide-DOPE ternary conjugate; MMP, matrix metalloproteinase; pDNA, plasmid DNA; DPPE, dipalmitoylphosphatidylethanolamine; PC, phosphatidylcholines; PS, phosphatidylethanolamine; RAW 264.7, mouse macrophage cell line; HA, hyaluronic acid; CaP, calcium/phosphate; ZOL, zoledronic acid; Pgp, P glycoprotein; MDR, multidrug resistant; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; PEI, polyethylenimine; MCF-7, Human breast adenocarcinoma cell lines; GBM, glioblastoma; rPAA-Chol polymer, poly (amidoamine); T7, HAIYPRH; mPEG-PLA, poly(ethylene glycol)-block-polylactide; BHEM-Chol, N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesterylox-ycarbonylaminoethyl) ammonium bromide; PLA, polylactide; PLGA, poly(D,L-lactide-co-glycolide; CTAB, hexadecyltrimethylammonium bromide; EPC, ethylphosphocholine; PBAE, poly(β-amino-ester); PEI, polyethilenimine; DODMA, 1,2-Dioleyloxy-N,N-dimethyl-3-aminopropane

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clinical and industrial development of nanovectors are the difficult scale-up and the poor chemical and physical stability of the formulation during storage (i.e. aggregation of the nanovectors, degradation of the biomaterials, leakage of the encapsulated drug).

The use synthetic or polymeric components to deliver active molecules remains controversial due to their obvious limitations in term of systemic toxicity and high cost of production [5]. In the field of nanomedicine, lipid and mainly phospholipid-based delivery systems are certainly the most investigated platform due to their biocompatibility, biodegradability, very low toxicity and versatility [5–9]. Thus, lipid vesicles have been proposed to deliver both lipophilic and hydrophilic drugs by different routes of administration [6,10]. Liposomes can be considered the first generation of lipid nanocarriers, with several formulations already in clinical practice. Marketed formulations based on liposomes have been successfully used for the delivery of anticancer, antimicrobial, anaesthetic agents and vaccines [11].

Other families of lipid nanovectors have been proposed for drug delivery. For instance, solid lipid nanoparticles (SLNs) are delivery systems composed of a solid lipid core, generally based on glycerides, and stabilized by a surfactant coating [6,10]. SLNs are characterized by a core that remains solid at 37 °C, assuring nanoparticle stability in vivo. The techniques required to prepare SLNs are solvent-free, although high energy forces are often necessary [6]. Moreover, SLN are often characterized by low drug encapsulation efficiency as well as by premature, fast drug release [6,10,12]. Solid lipid nanocapsules (LNCs) can be considered a "second generation" of SLN, and consist, at room and body temperature, of a liquid lipid phase surrounded by a solid lipid shell. Compared to SLNs, LNCs are generally characterized by a higher encapsulation efficiency and finer control of drug release [13,14]. LNC have been successfully used as a drug delivery system for various applications and by different administration routes [14]. Recently, growing attention has been paid to lipid-based self-emulsifying drug delivery systems (SEDDSs) composed of a mixture of lipids, surfactant and co-surfactant able to emulsify in aqueous medium without the use of high energy forces. The resulting low production costs makes SEDDSs particularly attractive. However, their application has been proposed almost exclusively for the oral route [15–18]. Moreover, SEDDs, as well as SLNs and LNCs, are characterized by a lipid core, and are suitable for the encapsulation of lipophilic drugs in particular.

From a general point of view, lipid nanocarriers have been proposed for the delivery of drugs as which differ in terms of hydrophilicity/lipophilicity and molecular weight. Liposomal nanocarriers remain, in this context, the system with the highest versatility, due to the possibility to encapsulate lipophilic, hydrophilic molecules, independently of molecular weight. A great deal of work has been carried out to use lipid nanocarriers for the delivery of nucleic acids, i.e. small interfering RNA (siRNA) and microRNA (miRNA). These oligonucleotides are potentially useful for the development of novel therapies for the treatment of various diseases, but are also characterized by biopharmaceutical issues, thus requiring the development of delivery strategies [19,20]. In the case of liposomes, anionic molecules such as siRNA and miRNA have been efficiently encapsulated by using cationic lipids. Indeed, cationic liposomes have been used extensively in transfecting nucleic acid into cells and are the basis for a number of marketed commercial agents designed for in vitro experiments [21]. Liposomes based on cationic lipids have been used for local delivery of nucleic acid in humans. Encouraging results were found following nasal administration of DNA complexed with cationic liposomes in patients with cystic fibrosis [22–24]. DC-Chol (3b(N-(N′,N'-dimethylaminoethane)carbarmoyl)-cholesterol)/ dioleoylphosphatidylethanolamine (DOPE) cationic liposomes were also used in patients with different cancer types by injection directly into a cutaneous nodule, generating a strong local immune response [25]. Phase II of the clinical study demonstrated the usefulness of direct intratumoral injection with Allovectin-7, (a plasmid DNA encoding the genes HLA-B7 and beta2-microglobulin) complexed with a cationic lipid mixture, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide) /DOPE [26]. Although cationic liposomes have also been used in clinical trials for chemotherapeutics, e.g. paclitaxel [27,28], lipoplexes are characterized by heterogeneous size distribution and their size is very sensitive to the experimental conditions used in preparation [29–31]. SLNs containing cationic lipid, e.g. DOTAP, or other cationic additives, e.g. cetyl trimethylammonium bromide (CTAB), have been proposed to complex miRNA to obtain efficient protection of the nucleic acid towards enzymatic degradation and enhanced RNA delivery [32,33]. Moreover, they aggregate in presence of serum, mainly following interaction with the serum components, especially albumin [34] with consequent aggregation of the complexes and rapid uptake by the Kupffer cells of the liver [35]. Following intravenous administration, nucleic acid/lipid nanoparticles complexes transiently accumulate into the capillaries of the lung, gradually redistributing to the liver, especially in the Kupffer cells [35,36]. The addition of PEGylated lipids to the complexes prevents the complex aggregation in serum, also providing stealth properties [37]. However, this approach has achieved only limited in vivo applications, likely due to the difficulty to control the size as well as the complex structure/morphology [38]. Higher in vivo stability has been achieved by replacing positively charged lipids with ionisable cationic lipids i.e. 1,2-dioleyl-3-dimethylammonium propane (DODAP), 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane (DODMA). These nanovectors are known as stable nucleic acid lipid particles (SNALPs) [39,40]. The use of an ionisable lipid leads to neutral vesicles at physiological pH with higher physical stability and longer circulation time compared to cationic liposomes. [39,41]. Nowadays, different formulations based on ionisable lipids are under clinical trial [41,42].

Despite the huge number of studies and the encouraging results achieved over the last decade, the use of lipid nanocarriers in clinics remains in its infancy. Moreover, the use of lipid nanocarriers for the delivery of nucleic acids in clinical practice is still far off. Novel strategies to enhance the delivery efficiency are required as is greater effort in the design of strategies to facilitate the translation of these technologies from the lab to the large scale. In this direction, the core-shell nanoparticles, which has recently emerged for the delivery of different molecules, must be considered an opportunity. In this review, the peculiarities and the advantages of core-shell nanoparticles, compared to other lipid nanocarriers, are described and discussed. Special attention will be paid to the delivery of siRNA and miRNA, still considered an open challenge today. However, when worthy of note, novel applications of lipid core-shell nanoparticles for the delivery of other classes of drugs, i.e. peptides or small molecules, are outlined.

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Table 1
Examples of core-shell NP encapsulating DNA.

DNA	Core	Shell	Targeting moiety	Application	Administration route	Reference
Plasmid DNA	Protamine sulfate	DOTAP		Gene therapy	Intravenous	[38]
Plasmid DNA	Protamine sulfate	DOTAP/DOPE/CHOL/ PEG-peptide-DOPE	Peptide	Cancer	Intravenous	[58]
Plasmid DNA	Protamine sulfate	DOPE/CHOL/PC or PS	Anionic charge of NPs	Immune response regulation	Macrophages	[66]

2. Core-shell nanoparticles

Charged lipids have been also used to design core/shell nanoparticles. This approach allows encapsulating ionic molecules especially, but not exclusively, polyanionic nucleic acids. Drug/molecule can be highly condensed with different cationic additives forming a compact “core”; this first brick of the vector can be then covered with lipids, forming a core-shell nanovector. This strategy overcome some of the issues found in the case of lipoplexes, such as the high physical instability following preparation. On the other hand, the lipid shell assures biocompatibility but also possibility to modulate the surface by introducing PEGylated lipids or/and targeting moieties. Lipids characterized by a net charge, generally cationic lipids, have been used for a spontaneous interaction with the core containing the anionic drug. In this review, the different approaches used to design core-shell nanoparticles (NPs) and the consequent evolution of a such delivery systems are described. Moreover, the most meaningful application for the delivery of nucleic acids peptides and small molecules are reported.

2.1. Protamine-based core-shell NPs

In 1996, the research team of prof. Huang demonstrated that the introduction of polypeptides, such as protamine, in the lipoplexes reduced particle size and increased the stability of the complexed nucleic acid toward nucleases [43]. Thus, protamine sulfate was proposed to condense plasmid DNA, followed by the addition of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) cationic liposomes (Table 1). Protamine sulfate (USP quality) is nontoxic and only weakly immunogenic. The highest transfection efficiency was ascribed to the higher stability of DNA in presence of nucleases, but also to the more favourable structure of the highly condensed DNA [28]. The same authors reported that the developed NPs were more stable upon storage and were less toxic than DNA-cationic liposome complexes [38]. In addition, a higher +/- charge ratio improved DNA protection and its delivery by core-shell NPs. This should be probably ascribed to a better neutralizing effects of the cationic charges of DOTAP against serum components that could biodistribution profile of core-shell NPs, improving the amount of intact DNA into the lung and the in vivo gene expression [38]. The same core/shell lipid-based nanoparticles have been adapted to deliver DNA or RNA oligonucleotides. SiRNA was mixed with calf thymus DNA (1:1 wt ratio) and condensed by mixing with protamine sulfate. High molecular weight DNA was used to improve compaction of the NP core; although calf thymus DNA was preferred to plasmid DNA due to the limited contents of immunostimulating CpG motifs [44], (Table 2). Compared to plasmid DNA, the higher molecular weight of calf-thymus DNA led to a more condensed core of the NPs with a reduction of the particles size up to 30% and a consequent improvement of siRNA delivery [45]. The resulting RNA-containing core was covered by a lipid shell consisting on a mix of DOTAP/chol. NPs were then mixed with DSPE-PEG by post-insertion method to decrease the particle size and to prevent the NP aggregation in serum [44]. Steric hindrance introduced with PEGylation reduced about 80% of their delivery efficiency; however, the conjugation of anisamide, a small molecule to target human lung cancer cells, to the DSPE-PEG restored the delivery efficacy of these NPs. In the same study, a significant improvement of siRNA localization in lung cancer was observed by using anisamide-targeted core-shell NPs [44]. Core-sell NPs modified with the NGR (aspargine–glycine–arginine) peptide, a targeting aminopeptidase N (CD13) overexpressed in different tumor cells or tumor vascular endothelium were also developed [46]. Pharmacokinetics of siRNA-containing core-shell NPs was investigated in an animal model of lung cancer. An increased circulation time of siRNA encapsulated into the NPs was observed, compared to free siRNA, while any significant difference was found when comparing targeted and untargeted NPs [47]. It is worthy of note that NPs were removed from the circulation slower in healthy than tumor-bearing mice. In the same study, a prolonged siRNA half-life, a higher area under the curve and a reduced clearance were observed when using these core-shell NPs [47]. The use of anisamide as targeting agent did not influence biodistribution of siRNA, which was found into the tumor for about 80–70% of the total injected siRNA [44,47]. However, once into the tumor, the use of anisamide-targeted NPs resulted in an enhanced siRNA cytosolic delivery, compared to untargeted NPs. Protamine-based core-shell NPs co-encapsulating a siRNA and doxorubicin (DOX) were also developed [46], (Table 3). In particular, this approach was developed to overcome chemoresistance in cancer cells overexpressing c-myc gene. Thus, siRNA against c-myc was used to sensitize HT-1080 cells to DOX. Co-encapsulation of siRNA and DOX was possible due to the ability of DOX to intercalate into double stranded DNA. However, when mixing the protamine-siRNA-DOX complex with cationic liposomes, only 20% of the DOX initially added in the preparation was effectively retained into the NPs. This was probably due to the competition between the positively charged DOX and the cationic lipid, with consequent dislodgement of DOX from the DNA. The replacement of DOTAP with a novel non-glycerol based cationic lipid, termed DSGLA (N,N-distearyl-N-methyl-N-2[N′-(N2-guanidino-L-lysinyl)] aminoethylammonium chloride), containing both a guanidinium and a lysine residue as the cationic headgroup showed an enhanced antitumor effect in vitro and in vivo [48]. Interestingly the authors demonstrated that the addition of DSPE-PEG

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Table 2
Examples of core-shell NP encapsulating RNA.

RNA	Core	Shell	Targeting moiety	Application	Administration route	Reference
siRNA	Calf thymus DNA/ protamine sulfate	DOTAP/CHOL/DSPE-PEG	Anisamide	Lung cancer	Intravenous	[44]
siRNA	Calf thymus DNA/ protamine sulfate	DOTAP/CHOL/DSPE-PEG	Aspargine– glycine–arginine peptide	Solid tumors	Intravenous injection	[46]
siRNA	Calf thymus DNA/ protamine sulfate	DOTAP or DSGLA/chol/ DSPE-PEG	Anisamide	Lung cancer	Intravenous	[48,49]
siRNA	chondroitin sulfate/ protamine	DOTAP/DOPE/CHOL/ DSPE-PEG	Vapreotide	Breast cancer	Intravenous	[50]
siRNA	Chondroitin sulfate/ protamine	DOTAP/DOPE/CHOL/ DSPE-PEG	T7 peptide	Breast cancer	Intravenous	[53]
			transferrin	glioma		[54]
siRNA	Protamine sulfate	PC/Chol/DPPE/ hyaluronan	Integrin β7	Inflammation	Intravenous	[62]
siRNA	Protamine sulfate/ hyaluronic acid	DOTAP/CHOL/ DSPE-PEG	Anisamide	Metastatic tumors	Intravenous	[73]
siRNA and miRNA			GC4- scFv	Lung metastasis of melanoma	Intravenous	[74]
siRNA	Calcium/phosphate	DOTAP/CHOL/ DSPE-PEG	Anisamide	Xenograft tumor model	Intravenous injection	[80]
Pooled siRNAs	Calcium/phosphate	DOTAP/DOPE/CHOL/ DSPE-PEG	Anisamide	Lung metastasis	Intravenous	[81]
siRNA	Calcium/phosphate	DOTAP or DOPC/CHOL/DSPE-PEG	Anisamide	Lung cancer	Subcutaneous injected	[93]
siRNA	Calcium/phosphate	PEI-CHOL/DOPE	–	Breast cancer	Intratumor injection	[94]
siRNA	calcium/phosphate	DOTAP/CHOL	–	melanoma	Peritumoral injection	[95]
siRNA	rPAA-Chol polymer	DOTAP/DOPE/CHOL/DSPE-PEG	T7 pepetide	Breast cancer	Intravenous	[103]
siRNA	mPEG-PLA/PLA	BHEM-Chol	–	Breast cancer	Intravenous	[104]
siRNA	EPC/PLGA	Lecithin/DSPE-PEG	–	Xenograft tumors	Intratumoral injection	[108]
miRNA vaccine	PBAE	DOTAP/ DOPC/DSPE-PEG	–	Vaccine	Intranasal	[111]
siRNA	PEI	DODMA/ PC/Chol/DSPE-PEG	–	Down-regulation of VEGF protein	Tumor cells	[112]

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Table 3
Examples of core-shell NP encapsulating chemotherapeutics, peptides and small molecules.

Encapsulated drug	Core	Shell	Targeting moiety	Application	Administration route	Reference
Doxorubicin	Calf thymus DNA/ protamine sulfate	DOTAP/CHOL/DSPE-PEG	Aspargine–glycine–arginine peptide	Solid tumors	Intravenous injection	[46]
Paclitaxel	Chondroitin sulfate/ protamine	DOTAP/DOPE/ CHOL/ DSPE-PEG	Vapreotide	Breast cancer	Intravenous	[50]
F1-V vaccine	HA	DOTAP/DOPE/thiol-PEG	–	Pneumonic plague	Intranasal vaccination	[75]
Insulin	Chitosan	DSPC/DPPS/CHOL	–	Diabet	Oral	[79]
Zoledronic acid	Calcium/phosphate	DOTAP/CHOL/DSPE-PEG	– Transferrin	Prostate cancer Glioblastoma	Intravenous	[87,88] [97,100]
Gemcitabine	Calcium/phosphate	DOTAP/DOPE/ DSPE-PEG	Anisamide	Pancreatic cancer	Intravenous	[91]
Zoledronic acid	Calcium/phosphate	DOTAP/CHOL	–	Melanoma	Peritumoral injection	[95]
Daunorubicin Lornoxicam	PLGA/lecithin	CTAB	–	Lung cancer Inflammation	Intravenous oral	[105]
Amphotericin B	PLGA	Stearylamine	Stearylamine	Leishmaniasis	Intravenous	[106]

by post-insertion lead to stripping of a large percentage of lipids from the NPs, reducing the lipid content to only the 32%; similarly, only 20% of the DSPE-PEG remained associated to the core-shell NPs, while a larger amount formed smaller particles, presumably micelles [49]. Moreover, the use of a multivalent cationic lipid, such as DSGLA, compared to the monovalent cationic lipid DOTAP, resulted in an enhanced stability of the core-shell NPs in presence of high concentrations of DSPE-PEG, suggesting that stronger charge–charge interaction between shell and the core contribute to a higher stability [49]. In the same study, the authors speculate that polyethylene glycol (PEG) on the surface of NPs arranged in the brush mode that should provide the full protection of the NPs from opsonization [35].

Self-assembled nanoparticles based on protamine with a core/shell structure were developed to co-deliver siRNA and the chemotherapeutic drug paclitaxel (PTX) to treat breast cancer [50]. The siRNA used in this study was involved in VEGF (vascular endothelial growth factor) regulation responsible of tumor progression. In this case, core-shell NPs with a negative protamine/ chondroitin-siRNA core surrounded by a DOTAP/ DOPE/CHOL (1:1:1 mol ratio) phospholipids coating encapsulating PTX were designed. Chondroitin sulfate is a glycosaminoglycan with a polymerized disaccharide base linked to a sulfate group able to condense siRNA and therefore electrostatically interact with protamine [50,51]. The lipid shell offered the possibility to simultaneously deliver not only the nucleic acid but also a hydrophobic drugs as PTX. In addition, DSPE-PEG and/or DSPE-PEG-VAP (vapreotide) were also added in order to obtain PEGhilated and/or targeted nanoparticles to target somatostatin receptors on tumor cells [52]. The introduction of a PEG moiety in the percentage of 5% did not lead to a reduction of a positive surface charge and allowed the obtainment of a reduced loss of siRNA. Although the use of high PEG concentration should favor the achievement of a more efficient delivery systems, this study demonstrated that a concentration of PEG higher of 5% had an effect of surfactant detergent on the liposome membrane leading to a drastic siRNA lost [50]. Furthermore, in vitro studies showed that all the VAP-siRNA-PTX-NPs efficiently downregulated VEGF expression with a more efficient uptake of siRNA into the MCF-7 cells also compared to the targeted nanoparticles without PLX (VAP-siRNA-NPs). This difference in siRNA delivery should be probably related to the highest positive surface charge of VAP-siRNA-PTX-NPs due to the presence of PTX. The authors also hypothesized that the presence of the helper lipid DOPE that promote the interaction of the lipid bilayer with the cells membrane and the proton sponge effects of the cationic lipid DOTAP as well as of protamine could justify the siRNA endosomal escape and its consequent major concentration in the cytoplasm after only 4 h [50,53]. The in vivo studies carried out on BALB/c mice bearing in situ MCF-7 tumor model showed an accumulation of PEGylated NPs into the liver due to their sequestration by the RES. Moreover, while the PEG-siRNA-NPs showed a major liver distribution of siRNA, VAP-siRNA-NPs allowed an highest siRNA distribution at the tumor site with a consequent tumor and neovascularization inhibition suggesting an efficient receptor mediate targeting delivery of siRNA LPD also in breast cancer [50].

Targeted core-shell NPs composed of protamine/chondroitin/siRNA-DOTAP/DOPE/CHOL/DSPE-PEG liposomes were also successfully used to the treatment of other different type of cancers [53,54]. Wei et al. developed targeted-self assembled core/shell NPs able to deliver siRNA across the blood brain barrier (BBB) for the treatment of glioma, one of the more aggressive brain tumors. Transferrin (Tf) was used to target glioma cells being transferrin receptor overexpressed on the cells surface of a multitude of tumors like glioma [55–57]. The in vitro experiments on murine brain microvascular endothelial cells (BMVECs) and human glioblastoma cells (U87 cells) evidenced an higher siRNA uptake and downregulation effect on EGFR (epidermal growth factor receptor) expression with Tf-targeted siRNA encapsulating NPs (Tf-siRNA-NPs) compared to the untargeted PEGylated formulation or naked siRNA. Moreover, in a co-culture model of BMVECs/U87 cells Tf-siRNA-NPs showed a better capacity to go across the BBB (Tf-siRNA-NPs > PEG siRNA-NPs- siRNA-NPs). It is important to underline that the absence of a PEG moiety in the lipid bilayer enhanced the toxicity of the delivery system inducing cell death, probably due the highest positive charge of the NP surface that led to aspecific interactions with untarget cells [54]. The in vivo results on nude mice bearing U87 glioma tumor confirmed the in vitro results; Tf-siRNA-NPs inhibited EGFR expression and the tumor growth after intravenous administration prolonging the mice survival up to 37 days.

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The same research group adapted the previous core/shell lipid nanoparticles to deliver siRNA in a model of breast cancer [53]. Nanoparticles offered siRNA protection since they remained intact after even 36 h of incubation in fetal bovine serum compared to free siRNA that was degraded only after 3 h. Moreover, the experimental results on MCF-7 human breast cancer cells and on Female Balb/c nude mice confirmed that also in this case targeted nanoparticles improved EGFR downregulation and the tumor growth inhibition [53].

To address the dilemma existing between use of PEG on the core-shell NPs, e.g. stability in the systemic circulation versus a reduced cellular uptake, a PEG-peptide-DOPE (PPD) ternary conjugate was used [58]. The conjugate was designed to induce the removal of PEG via cleavage by a matrix metalloproteinase (MMP) specifically expressed in tumor tissues. Core-shell NPs were prepared with a core of plasmid DNA condensed with protamine, then covered with a lipid shell of DOTAP, DOPE and cholesterol together with PEGylated lipid or PPD. *In vitro*, pDNA expression activity was found to be dependent on the MMP expression level in the cells. PPD-modified core-shell NPs were stable in the blood and successfully accumulated into the tumor, although with a lower extend compared to core-shell NPs prepared with conventional PEGylated lipids. This effect was attributed to binding to blood cells of the newly developed delivery system [58].

To reduce the systemic toxicity associated to the presence of positive charges on the NP surface [59–61], a lipid shell based on neutral phospholipids, e.g. DPPE (dipalmitoylphosphatidylethanolamine), was proposed by the research group of Prof. Peer. NPs with a protamine core condensed with siRNA and a lipid bilayer composed of PC (phosphatidylcholines): Chol:DPPE (60:20:19.9 M ratio) were developed [62]. In this case, liposomes bearing glycosaminoglycan hyaluronan on their surface were prepared and freeze-dried. Then, the dry lipid layer was hydrated with siRNA/protamine complexes forming the final core-shell NPs. In this work, a siRNA directed against the over expressed Cyclin D1, to regulate intestinal inflammation, was used. Moreover, integrin β7 was covalently attached to DPPE to achieve nanovector targeting leukocytes. The presence of glycosaminoglycan hyaluronan on the NP surface allowed to preserve the liposome integrity after the lyophilization process, increase the stability in vivo and prolong the circulation time [62,63]. When integrin-targeted core-shell NPs were systemically administrated in a animal model of colitis, selective cellular targeting, siRNA uptake and gene silencing was reported, resulting in a suppressing the aberrant proliferation of mucosal leukocyte in colon, the restoration of the damaged tissue and of the hematocrit values [62]. The same NPs formulation was successfully used to efficiently deliver siRNA for a significant reduction of multidrug resistance extrusion pump, p-glycoprotein (P-gp) in A549 cancer cells [64].

Protamine-based anionic core-shell NPs were also developed to delivery pDNA to macrophages. The authors proposed to replace cationic lipids with dioleoyl phosphatidyl ethanolamine (DOPE), thus achieving pH sensitive liposome [65,66]. The use of DOPE was used to prevent the toxicity on macrophage cells as well as the aspecific interactions and promoted the endosomal escape [67–70]. Thus, the lipid mix DOPE: CHOL:PS (molar ratio of 8:2:2 and 8:2:4) was used, which showed pH sensitive propriety with a release of calcein dependent to the acidic pH conditions. These observations were in contrast with previous study in which DOPE/PS liposomes did not show pH sensitivity [71]. This different behavior was attributed to different lipid molar ratio and to different condition used in the two studies. Thus, the use of a higher amount of CHOL reduced the pH-sensitivity of the NPs, by stabilizing the bilayer. *In vitro* studies on RAW 264.7 confirmed that anionic core-shell NPs had a transfection efficiency higher compared to Lipofectamine 2000 [66].

2.2. Hyaluronic acid-based core-shell NPs

A issue of the above described core-shell NPs based in protamine was the potential toxicity and immune response expected when using them in humans [72]. Thus, an evolution of this system consisted on the replacement of calf thymus DNA with a less immunostimulating hyaluronic acid (HA) into the core of the NPs [73]. These NPs were PEGylated adding DSPE-PEG by the post-insertion method; once more, DSPE-PEG-anisamide was used for targeting cancer cells (e.g. melanoma cells). These NPs, tested in a model of metastatic melanoma, showed little immunotoxicity compared to liposome-protamine-DNA nanoparticle previously proposed by the same research group [47]. Simultaneously deliver siRNA and miRNA (miR34a) was also successfully used for a combined antitumor therapy in an experimental model of lung metastasis of melanoma [74].

HA-based hybrid NPs were proposed as delivery system for antigen intended for intranasal vaccination [75]. NPs were prepared by ionic complexation of negatively charge thiolate HA and cationic DOTAP/DOPE liposomes. Optimal ionic complexation between HA and DOTAP liposomes was obtained at the w/w ratio between polymer and total lipids of 1:10. Thus, the authors found that higher concentration of HA limited the polymer-liposomal fusion and increased NPs size. As suggested by the decrease of the surface charge value, this could be ascribed to the distribution of the HA molecules on the liposomal surface that impede the +/- charge interaction between DOTAP and HA causing also nanoparticles aggregation. NPs showed higher stability up to 3 days of incubation at 37 °C in PBS and stability in serum with a release of antigen of about 40% in 21 days compared to cationic liposomes that aggregates. NPs strongly reduce (20 fold) the cytotoxicity associated to cationic liposomes and enhanced the humoral immune responses against subunit protein antigens compared to the classic vaccine solution when administrated in vivo [75].

2.3. Chitosan-based core-shell NPs

Chitosan (CHI) lipid nanoparticles were also developed especially for mucosal delivery of actives by Prof. Alonso's group [76]. CHI is a polysaccharide largely investigated for drug delivery and characterized by bioadhesive and penetration enhancer properties [76,77]. The approach was to combine the advantages of CHI nanoparticles, e.g. adhesion to the ocular mucosa and efficient penetration through cornea and conjunctiva, together with those of liposomes for topical/mucosal administration of actives [78,79]. In

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particular the authors aim to achieve a highest protection of the encapsulated drug as well as a slower drug release. Thus, CHI lipid NPs were obtained mixing suspensions of CHI NPs and liposomes (with different lipid compositions) at the ration 1:2 w/w after a freeze drying process, exploiting the electrostatic interactions between the positive CHI NPs and the negative DPPS lipid [78,79]. The positive zeta potential of CHI lipid NPs facilitated the interaction with the eye surface. Compared to CHI NPs, CHI lipid NPs showed a higher stability in the simulated lacrimal fluid; finally, in vitro and in vivo experiments showed that all CHI lipid NPs penetrate the conjunctiva without altering cells viability. These hybrid CHI lipid NPs were also used for the delivery of insulin by oral route [79]. CHI lipid NPs encapsulating insulin the hydration method (hydration of thin lipid film composed of CHI NPs suspension) and lyophilization method (as previously described) were used. In this case, the saturated lipids DSPC and DPPS with high phase transition temperatures, allowed to obtain delivery systems stable both in gastric and intestinal fluid [79]. Release studies carried out in artificial gastric juice showed a controlled release of insulin due to the external lipid coating compared to CHI NPs formulations. Thus, in the case of nanoparticles prepared with the hydration method, nanoparticles composed of DSPC: DPPS:Chol showed a better release of insulin with higher reduction of glucose plasma level (about 50% after 24 h) due to the precence of the negative lipids that improved the shell coating of CHI NPs with the liposomes [79]. Nanoparticles prepared by lyophilization showed different characteristics in term of charge surface. Moreover, the same formulation prepared with a different method showed a completely different surface charge (positive) with a consequent more rapid insulin release. This was probabily due to the lower time of interaction between the positively charges of CHI and the negatives of lipids that impaired the formation of an homogeneous lipid shell around the CHI nanoparticle [79]. Finally, in the case of the others two formulations (neutral zeta potential) the reduction of glucose levels was significant and stable for about 24 h [79].

2.4. *Calcium/phosphate-based core-shell NPs*

A further evolution of the lipid coated core-shell NPs can be considered the replacement of the HA core with calcium/phosphate (CaP) core, proposed by prof. Huang [80]. The aim of this strategy was to use a materials able not only to condense the nucleic acid into the core of the NPs, but also increase the intracellular delivery. Indeed, once entered into cells by endocytosis, the lower pH of the lysosomes should disassemble the CaP core, thus increasing the endosome internal pressure and swelling, with consequent release of the encapsulated molecule (e.g. siRNA) into the cytosol [80]. The core of the NP was prepared by mixing three aqueous solutions containing Ca^{2+}, HPO_4^{2-} and a siRNA, respectively; the resulting aqueous dispersion was mixed with an organic solution to form a microemulsion. Nanoprecipitation of CaP/siRNA NPs was carried out by addition of a citrate solution followed by purification. The CaP/siRNA nanoprecipitates were then mixed with DOTAP/cholesterol liposome and then extruded [80]. By this approach three different siRNA, directed against three different targets, respectively, have been successfully delivered for an efficient anticancer activity in vitro and in vivo in an animal model of lung metastasis [81]. The new formulation allowed to achieve 3–4 folds increase of the in vitro silencing effect compared to the previous core-shell nanoparticles; this effect was associated to a negligible immunotoxicity [80]. It is worthy of note that also with the formulation based on CaP, an efficient delivery was observed only in the case of anisamide-targeted NPs, while a very low silencing effect was reported in the case of untargeted NPs.

A different protocol was designed to prepare CaP-based core-shell NPs encapsulating bisphosphonates, and in particular zoledronic acid (ZOL). This is a powerful aminobisphosphonate used in the clinical practice to prevent skeletal related events in the case of bone metastasis, osteoporosis and Paget's disease [82]. Despite the growing interest toward possible direct anticancer activity of ZOL largely demonstrated in vitro [83], its anticancer effects in extraskeletal tissue cannot be observed in vivo due to the rapid accumulation of ZOL into the bone [83–86]. From here, the interest to encapsulate ZOL in nanovector to change its biodistribution and to increase the accumulation in extraskeletal tumor. CaP-based core-shell NP encapsulating ZOL have been developed. These NPs have been prepared starting from a simple protocol to prepare in the first step a core of CaP/ZOL, then covered by a lipid shell [87]. In particular, two solutions of Ca^{2+} and HPO_4^{2-} were mixed at pH 9.5 and the resulting dispersion filtered on 0.22 µm was mixed with the aqueous solution of ZOL and then with DOTAP/cholesterol liposomes. Nanoparticles characterized narrow size distribution and high ZOL encapsulation efficiency were obtained [87]. Two different protocols have been investigated to obtain PEGylated NPs. in the first approach, DSPE-PEG was added to the preformed core-shell NPs by post-insertion method; in alternative, DOTAP/cholesterol/DSPE-PEG liposomes were directly mixed with the CaP/ZOL nanoaggregates. The latter approach resulted in the lowest mean diameter (about 140 nm) and narrowest size distribution together with a higher drug loading into the NPs [87]. The use of these core-shell NPs led to a significant increase of ZOL cytotoxicity in several cancer cell lines [87]. Moreover, in an animal model of prostate cancer, the treatment with core-shell NPs encapsulating ZOL resulted in strong inhibition of the tumor growth, with a negligible effect observed in animals treated with free ZOL [87]. Interestingly, core-shell NPs were compared with "conventional" stealth liposomes showing superior delivery efficiency in vitro and in vivo [88]. It is worthy of note that in a model of prostate cancer the use of core-shell NPs resulted in the complete disappearance of the tumor mass in a significant number of animals [88]. The same formulation was found to be effective also to reduce the migration of mesenchymal stem cells as well as their secretion of chemokines and angiogenic factors, thus blocking the cross-talk between mesenchymal stem cells and tumor cells involved in tumor progression and occurrence of metastasis [89]. Once more, these effects were negligible when using free ZOL. Core-shell NPs encapsulating ZOL have also been investigated in the multidrug resistant (MDR) cancer cells [90]. It has been found that ZOL reduced the activity and the expression of P glycoprotein (Pgp) in MDR cells. Indeed, the ZOL-encapsulating core-shell NPs were found to revert the sensitivity of MDR cancer cells (lung cancer) to chemotherapeutics such as DOX or carboplatin [90], while any effect was found when associating chemotherapy with free ZOL.

Another phosphate-containing drug, gemcitabine triphosphate, has been encapsulated into core-shell NPs based on a core of CaP NPs and a DOTAP/DOPE-PEG shell. This system was proposed to overcome the occurrence of resistance against gemcitabine [91].

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Different mechanism have been identified in the cancer cells that hamper the conversion of prodrug gemcitabine in the active metabolite gemcitabine triphosphate [92]. Thus, CaP-based core-shell NPs encapsulating the bioactive form of gemcitabine were developed to overcome the above-mentioned issues. The formulation was tested in a model of pancreatic cancer, showing a inhibition of the tumor growth higher than that observed in the gemcitabine treated group [91]. As in previous works of the same research group, untargeted and anisamide-targeted NPs were used, with a higher antitumor activity found in the case of the targeted formulation. However, it is worthy that untargeted NPs showed, in this study, an effective delivery, although lower than targeted-NPs; these findings were different than that observed in the case of NPs encapsulating nucleic acids in which only targeted NPs were effective [91]. It is difficult to hypothesize that the different protocols used to prepare the NPs could influence the delivery efficiency, while it is reasonable hypothesized that differences could be ascribed to the different encapsulated active molecule. Basically, RNA oligonucleotides require a higher degree of complexity in the delivery, requiring not only an efficient delivery into the target cells but also a protection against nucleases. Moreover, the high molecular weight of siRNAs (about 14,000 g/mol) limits the number of molecules that can be encapsulated and efficiently released into the cytoplasm. On the other hand, gemcitabine triphosphate is a single triphosphate nucleotide (molecular weight 503.14 g/mol) that can be loaded and released at a higher drug dose. This hypothesis is in line with the results obtained with the CaP-based core-shell NPs encapsulating ZOL (molecular weight about 280 g/mol) that resulted in very efficient drug delivery without any strategy for active targeting [87–89].

An approach to enhance the delivery efficiency of RNA oligonucleotides could be optimization of the formulation, in terms of lipid composition. This preparation protocol was optimized by pre-coating the CaP NPs encapsulating the siRNA with the anionic phospholipid dioleoylphosphatidic acid (DOPA) [93]. By this approach, the aggregation prevented the CaP core during the centrifugation step and make the CaP NPs soluble in $CHCl_3$ in which the neutral (dioleoylphosphatidylcholine, DOPC) and the cationic (DOTAP) lipid of the outer leaflet, together with the PEGylated lipid, were dissolved. The authors speculate that DOPA could easily interact with the Ca^{2+} ions arranging themselves on the core surface; in addition, DOPA could mediate stronger bindings between the core and the external lipid coating leading to a more stable delivery system, leading to an improved silence activity compared to the previous developed CaP-based core-shell NPs [93]. Very recently, core-shell NPs encapsulating siRNA were designed with a core of CaP complexed with siRNA and a shell obtained by PEI (polyethylenimine)-cholesterol/DOPE liposomes [94]. The authors clearly showed the encapsulation of CaP NPs into the lipid bilayer by TEM analysis. The formulation was very effective in reducing VEGF expressiong in MCF-7 cells and inhibiting tumor growth in a MCF-7 xenograft [94]. Compared to previous works, these core-shell NPs encapsulating siRNA seem to have superior delivery efficiency that could be ascribed to the replacement of DOTAP with PEI-cholesterol.

CaP-based core-shell NPs have also been proposed for the co-encapsulation and co-delivery of polyanionic molecules, to be used in combination therapies. A synthetic RNA poly (I:C) and ZOL were encapsulated in CaP-based core-shell NPs and tested in B16BL6 melanoma-bearing mice [95]. It has been demonstrated that poly (I:C) could interfere with the gene responsible of melanoma cells differentiation [96]. The authors found that a RNA: ZOL weight ratio of 3:3 was needed to achieve a stable core and high ZOL encapsulation (about 94%). Interestingly, cationic liposomal shell around the CaP core prolonged the colloidal stability of the NP preventing CaP aggregfvation until 30 days. Finally, results showed that the co-delivery of ZOL and poly (I:C) RNA with core-shell NPs was effective to inhibit melanoma tumor growth [95].

Also in the case of core-shell NPs showing efficient delivery efficiency, further modification of the NPs can be taken into account to adapt the formulation to specific applications. For example, CaP-based core-shell NPs encapsulating ZOL were modified to target brain tumors. In particular, Tf-targeted core-shell NPs encapsulating ZOL were developed for the treatment of glioblastoma (GBM) [97]. Tf was used to target cells overexpressing Tf receptors (e.g. GBM cells) and to facilitate crossing of blood brain barrier (BBB) [98,99]. Moreover, a self-assembling protocol was maintained in order to avoid stability issues and favor a further scale-up process. Indeed, the NPs were prepared starting from 4 components to be mixed and without any following purification step. Moreover, Tf was added to NPs without any covalent binding. *In vitro*, Tf-targeted NPs, did not show higher inhibition activity on different GBM cells, compared with un-targeted NPs. On the contrary, in vivo experiments showed superior delivery efficiency in both a heterotopic and an orthotopic model of GBM. In detail, untargeted self-assembling NPs showed a significant inhibition of the tumor growth; on the other hand, the Tf-targeted NPs encapsulating ZOL allowed achieving regression of the tumor mass in all the animals and, in all a significant number of animals, complete disappearance of the tumor mass [100]. The discrepancy between in vitro an in vivo results has been justified with the high quote of free Tf associated to the targeted NPs [97]. Indeed, free Tf could compete with bound Tf for the Tf receptor overexpressed on GBM cells; it is expected that this competition should not arrive in vivo due to the different distribution of free Tf and Tf-targeted NPs. Interestingly, treatment with Tf-targeted NPs also showed superior antitumor activity in vivo, compared to the temozolamide that is considered the gold standard for the treatment of GBM [97]. Due to these results, the above mentioned product received the Orphan Drug Designation by the European Medicine Agency (EU/3/16/1735 granted on 8/11/2016) and Food and Drug Administration (granted on 11/29/2016) [101,102].

2.5. Core-shell NPs based on synthetic polymers

Other studies proposed alternative materials for the NP core, then covered by cationic lipids. Synthetic polymers represent certainly an attractive alternative due to the previous materials previously described, especially for the possibility to design novel advanced functional biomaterials with tailored properties to optimize delivery and biocompatibility.

Thus, a bioreducible cholesterol-grafted poly(amidoamine) (named as rPAA-Chol polymer) was used to complex siRNA anti EGFR, then covered with a lipid shell of DOTAP/DOPE/Cholesterol (25:43:25, mol/mol) lipid mix, then followed by PEGylation (DSPE-PEG) by post-insertion method [103]. A peptide (HAIYPRH, named as T7) modified DSPE-PEG-T7 was also used to prepare targeted NPs.

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Both PEGylated and targeted NPs efficiently delivered an anti-EGFR siRNA with efficient downregulation of EGFR protein expression level in vitro and inhibition of tumor growth in vivo. The authors also demonstrated the superiority of the "core-shell approach" compared to the naked siRNA lipoplexes.

A number of studies investigate the potential to use polyesters PLA and PLGA, or their PEGylated derivates as core materials of NPs, then covered with a lipid shell, generally based on cationic lipids. NPs composed of a polymeric core of poly(ethylene glycol)-block-polylactide (mPEG-PLA) or mPEG-PLA and PLA able to electrostatically interact with siRNA and decorated by a cationic shell with the lipid N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonylaminoethyl) ammonium bromide. BHEM-Chol were developed to delivery siRNA in breast cancer [104]. NP composition was optimized to obtain nanoparticles with a mean diameter of lower than 100 nm. Moreover, the use of PEG (at the molar ratio of 1:1 PEG/PLA) prevented NP aggregation. The lipid/polymer ratio was fixed to 1:10 w/w, while a higher lipid concentration led to the simultaneous formation of liposomes. Finally, an optimized BHEM-Chol/siRNA ratio (5:1 molar ratio) assured a high encapsulation efficiency, e.g. 95%. The use of mPEG-PLA, compared to PLA, resulted in lower toxicity on breast cancer cell line BT474 but also to a lower siRNA uptake. These observations suggest that, in this formulation, PLA-PEG is not only located into the core, but also at the NPs surface. Finally, the systemic administration of siRNA/mPEG-PLA/PLA in mice with BT474 xenograf murin model of tumor allowed siRNA downregulation of poli-like kinase 1 expression and tumor growth inhibition [104]. In another study, hybrid lecithin poly(D,L-lactide-co-glycolide (PLGA)) NPs named Lecithemer® covered by different cationic lipids to deliver anticancer drugs were developed [105]. Daunorubicin and lornoxicam were encapsulated into the NPs, intended for systemic and oral administration, respectively. Hexadecyltrimethylammonium bromide (CTAB) was used as cationic lipid. The polymer/lecithin ratio was fixed at 3:1 mol/mol since highest amount of PLGA led to an increase of the NPs diameter. Indeed, lecithin acted as a surfactant and it stabilized NPs, while an high concentration of PLGA led to a more viscous core with a consequent increased of particles size. An additional reduction of NPs size was obtained after NPs coating with the cationic lipids. This phenomena was probably due to a surfactant effects of lipids but also to their cationic charge. Moreover, the encapsulation efficiency of both anticancer drugs was high (about 90%) although in the case of lornoxicam the decrease of lecithin led to a reduction of the encapsulation efficiency probably caused by the lower affinity of lornoxicam and PLGA matrix [105]. When comparing these hybrid PLGA/lipid NPs with PLGA NPs, the coating of PLGA core resulted in a slower sustained release of daunorubicin and lornoxicam, and in more efficient delivery of the encapsulated drugs [87]. Hybrid NPs with an inner region of PLGA were also successfully proposed to deliver Amphotericin B for the treatment of leishmaniasis [106]. In this case, the cationic shell of stearylamine was also used as targeting ligand for macrophages cells. The antiprotozoan activity of stearylamine has been previously reported [107]. These core-shell NPs were stable in storage condition and serum, and had a showed an high encapsulation efficiency as well as slow release of Amphotericin B. The in vivo studies confirmed the sustained release of NPs and showed a higher distribution of targeted NPs in liver and spleen with about 90% of parasites inhibition and a reduction of Amphotericin B in kidney with consequent low nephrotoxicity [106].

PLGA-based core-shell NPs with a higher complexity degree, described as a "sandwich structure", was proposed by Shi *et al.* [108]. These NPs were prepared combining the double emulsion and self-assembling methods. The core of the NPs was constituted by the cationic lipid ethylphosphocholine (EPC) able to strongly interact with the negative charges of siRNA and previously used to promote its delivery in the cytosol [109,110]. Thus, in the first emulsion EPC rearranged and self-assembled at the water/oil interface leading to siRNA encapsulation in the aqueous core and to the formation of the external poly(lactic-co-glycolic acid) PLGA layer. Finally, in the second emulsion, PLGA was coated with lecithin and DSPE-PEG molecules that self-assembled on polymer surface forming the external neutral layer to confer stealth proprieties to the NPs. The obtained NPs showed a size of about 200 nm and a zeta potential near to the neutrality that limited nanoparticles interactions with serum components and improved their stability in culture medium. Moreover, NPs were stable in PBS and siRNA encapsulation was significantly high (80%) specially if compared to conventional PLGA NPs (max 7%). The introduction of a EPC inner region allowed a better siRNA condensation in the inner region. The multi layered structure, and in particular the polymer coating, resulted in a siRNA sustained release with a release of siRNA molecules of about 50% after 12/20 h. The efficacy and the safety of NPs were also confirmed in vitro and in vivo. Finally, core-shell multilayer NPs allow to achieve a siRNA-mediate gene silencing activity higher than siRNA lipoplexes prepared with marketed cationic liposomes, e.g. Lipofectamine 2000 [108]. Hybrid core-shell NPs based on a polymeric core of poly(β-amino-ester) (PBAE) were also used to deliver a miRNA vaccine in dendritic cells after non-invasive intranasal administration [111]. The polymeric core of PBAE was coated with a lipid shell of DOPC: DOTAP (70:20 mol) and DSPE-PEG. The pH-responsive PBAE was used to improve NPs endosomal escape. The inclusion of the lipid shell significantly reduced the toxicity of naked PBAE NPs. Moreover, the endosomal escape experiments demonstrated the ability to PBAE NPs to promote endosomal escape compared to hybrid PLGA-based core-shell and non-pH sensitive NPs. Finally, in in vitro and in vivo tests, NPs showed a very safe profile and an efficient miRNA transfection in dendritic cells after intranasal administration in mice [111]. Recently, Li et al. proposed to combine a pH sensitive polymer with a pH-sensitive lipid into the core of core-shell NPs intended for systemic siRNA delivery [112]. Thus, the cationic polyethilenimine (PEI) 800 was chosen to condense the nucleic acid in the inner region of the NPs and also for its safer profile compared to other form of PEI [113]. In addition, the pH sensitive 1,2-Dioleyloxy-*N*,*N*-dimethyl-3-aminopropane (DODMA) was added in the core of the NPs, finally covered with PC: Chol: (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-amino(polyethylene glycol)-2000) DSPE-mPEG (40/19/35/1/5 mol/mol). In this study, the selected hybrid formulation was very stable in serum until 12 h showing a very high siRNA affinity for the core as well as efficient protection toward enzymatic degradation. Moreover, these core-shell NPs allowed to achieve high siRNA transfection and VEGF downregulation in different cell lines [112].

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3. Conclusions

Progress in nanomedicine over the last decade has provided novel technological platforms for improved delivery of drugs. In this context, core-shell NPs are certainly one of the most attractive strategies. From literature, several peculiarities emerge. Firstly, the possibility to combine materials overcomes the limits of the individual materials. In detail, the use of protamine, salts (e.g. CaP), or synthetic polymers results in a high encapsulation of anionic molecules by electrostatic interactions. However, these complexes are suitable for local or systemic administration because they are not stable enough and are often characterized by high toxicity. The use of a lipid shell hides the characteristic of the core, enabling the administration in vivo of these formulations. In many studies cited in this review, cationic lipids have been used to achieve more efficient interaction with the core, but also to promote the interaction with cell and the uptake into the cells. Moreover, some authors suggest using negatively charged lipids to increase core-shell NPs stability in biological fluids.

Core-shell technology has been proposed mainly for the delivery of nucleic acids, e.g. siRNA and miRNA. However, it seems to be very promising for the delivery of other anionic drugs, such as bisphosphonates. For this last category of drugs, core-shell NPs not only improve the intracellular delivery, but also change the biodistribution thus opening novel therapeutic opportunities. Finally, some studies also reported the use lipid core-shell NPs for the delivery of chemotherapeutics, combining a polymeric core with a lipid shell.

On the basis of these studies, we conclude that core-shell NPs for drug delivery are worthy of further investigation, not only to demonstrate their performance as delivery systems, but also to verify strategies for the transition from bench to bed-side. In this sense, the self-assembling approach used to prepare the formulation facilitates scale-up and the consequential development of such technology. In turn, this enlarges the number of nanomedicines in an advanced stage of development which, when compared to the number of ongoing research studies, is still very limited.

4. Future perspectives

Despite the promising results described in this review, the studies focusing on core-shell NPs generally lack of a deep physical-chemical characterization. The core-shell structure of these NPs is often only hypothesized and not really demonstrated. The measure of zeta potential of NPs can only indirectly provide information on the external shell of the particles. Thus, future studies should be carried out to provide evidence of the core-shell architecture. To this aim, scattering techniques, such as small-angle neutron scattering and small-angle X-ray scattering would be useful to provide information on the reorganization of the different components used in the preparation of core-shell NPs. Moreover, cryo-trasmission electron microscopy can allow the visualization of nanoparticle architecture.

Moreover, a limited number and combination of lipids (cationic, anionic, neutral, PEGylated) have been tested to prepared core-shell NPs. Thus, in order to move towards the clinical use and industrialization of these NPs, a large spectrum of lipids and lipid combinations should be tested to obtain higher delivery efficiency, lower toxicity and higher stability after preparation. Indeed, the physical stability of the different formulations described in this review is poorly addressed. It is worthy of note that core-shell NPs are often prepared by mixing different components of the formulation, forming NPs by self-assembling process. This characteristic preparation strategy could partially overcome the poor physical stability of NPs. Indeed, NP preparation by self-assembling process could be used to design a kit for the preparation of the formulation in the hospital pharmacy before administration. This approach is already in use for products based on nanocarrier-containing formulations already on the market (e.g. Myocet®) or under clinical evaluation (e.g. CALAA-01). On the other hand, when designing a formulation based on such a strategy, the complexity of the preparation procedure to be performed in hospital must be carefully evaluated. Otherwise, in the case of poor stability of the formulations post-preparation, freeze-drying of the pre-formed core-shell formulations should be evaluated.

All these issues should be addressed in future studies on core-shell NPs in order to seriously evaluate their potential to move from bed to bed-side.

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