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TITLE

Surface modification of non-viral nanocarriers for enhanced gene delivery

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[ABSTRACT]

Biomedical nanotechnology has given a new lease of life to gene therapy with the ever-developing and diversifying non-viral gene delivery nanocarriers. These are designed to pass a series of barriers in order to bring their nucleic acid cargo in the right subcellular location of the right cells. For a given application, each barrier has its dedicated strategy, which translates into a physico-chemical, biological, and temporal identity of the nanocarrier surface. Different strategies have thus been explored to implement adequate surface identities on nanocarriers over time for systemic delivery. In that context, this review will mainly focus on organic nanocarriers, for which these strategies will be described and discussed.

1 [INTRODUCTION]

The paradigm of non-viral nucleic acid delivery has been established for several years [1]. It is now widely acknowledged that viral and non-viral delivery systems are complementary to each other rather than competitors given the vast range of applications as well as yet to be met challenges [2]. Non-viral nucleic acid delivery systems are mostly based on nanocarriers, a majority of which are made from organic materials – polymers, lipids, peptides, and their derivatives. These agents have originally been designed to encapsulate and protect the nucleic acid cargo. Comprehensive design criteria have been well identified [3], and are the subject of steady encouraging ameliorations of non-viral nanocarriers. Besides their ability to encapsulate nucleic acids, a number of these criteria come down to the interactions between the nanocarrier and its surrounding biological environment – that is the nano-bio interface [4]. Figure 1 illustrates such interplay with the example of the systemic delivery of gene delivery nanocarriers for the

treatment of a solid tumor. Within seconds after injection, interactions of the nanocarrier surface with blood components occur. In particular, opsonization is thought to trigger the formation of a protein corona enveloping the nanocarriers. The resulting surface properties of the nanocarrier will greatly impact its fate in the biological milieu [5]. Furthermore, complex sequential hurdles along the intracellular trafficking of nanocarriers, as illustrated in Figure 2, highly depend on the presence of molecular cues on the surface of nanocarriers. That is why many efforts are being made to design and implement strategies to better control the surface properties of nanocarriers, without compromising their core properties, in order to enhance non-viral gene delivery.

2 THE NANOCARRIER SURFACE

2.1 DEFINITION

It is crucial to grasp the notion of nanoscale surface in order to perceive the stakes of modifying the surface of nanocarriers so as to enhance gene delivery. From the chemical outlook, the availability of functional moieties at the surface will often be a prerequisite, yet physical aspects such as the overhanging of polymer chains or the molecular flip-flop of lipids may in fact govern the whole process. Thus, far from a mere geometrical feature, the nanocarrier surface can be pictured as a dynamic interface between a solid or semi-solid core containing the nucleic acid payload and an aqueous environment (see Figure 3). That interface has a certain thickness: it is anchored to the nanocarrier core, and it displays biological cues – such as surface charges or specific biomolecules – outwards.

2.2 MODIFICATION STEPS: BEFORE OR AFTER SURFACE FORMATION?

In the field of biomaterials, surface modification is very common; yet in many cases macroscale surfaces have to be dealt with. When it comes down to nanocarriers, another aspect arises: the formation of the surface itself may be the critical step of the modification process.

In general, the surface of a nanocarrier forms as a result of the encapsulation of the nucleic acid cargo inside the assembly. A strategy to modify that surface can thus be implemented at three separate time frames: before surface formation, by chemically modifying the condensing agent; during the surface formation, by adding other agents in the formulation; or in a subsequent step involving additional agents. When nanocarriers have a limited stability and cannot be stored, surface modifications are preferably carried out before or during the formation of the surface. That is the case for many nanocarriers made of polymers, lipids and polypeptides, which are used within minutes to hours from their initial formation [6, 7]. In this review, we will first discuss the range of modifications carried out before surface formation. We will then focus on actual surface modifications of nanocarriers that are designed to enhance gene delivery.

Carrying out modifications before the formation of the surface offers the possibility to include extensive purification steps. Purification would otherwise be hardly scalable on semi-solid nanocarriers in suspension without compromising their integrity [7]. Yet, it remains critically important to optimize and validate those pre-modifications, in terms of cohesion and shape of the nanocarrier core. Indeed, electrostatic and hydrophobic forces between the condensing agent and the nucleic acids uphold the nanocarrier core. Chemically modifying the condensing agent may thus decrease the density of these interactions, as discussed below.

Ideally, as for chemical grafting, reagent moieties would already be available on the surface of the nanocarrier to be modified. A good example is the pendant primary amino groups resulting from the encapsulation of nucleic acids by polycations such as branched polyethylenimine (PEI) or poly-L-lysine (PLL), which are called polyplexes. Yet, for some applications, chemical moieties such as protected thiols have to be conjugated to the nucleic acid condensing agent, with little impact on the core properties [8, 9]. Such strategy was used by Roy *et al.* to increase the TAT peptide bioavailability on the surface of polyplexes, which would otherwise be limited due to a strong interaction between TAT peptides and DNA [9]. Polycations such as PEI have been conjugated with small biomolecules, e.g., saccharides [10, 11] or peptides [12-14], but also with more sizeable biomolecules [15, 16] and polyethylene glycol (PEG) chains (pre-PEGylation) [12, 14, 17], prior to nanocarrier formation. Such modifications strongly altered the nanocarrier size, shape and cohesion [17-19]. In the case of polyplexes, incorporation of PEG prior to surface formation has been hypothesized to cause a decrease in the density of core electrostatic interactions that has been reported to confer diffuse, shapeless [17], even worm-like [18] unfit constructs [20]. Similarly, lipid-containing nanocarriers featuring lipid layers at their surface are often PEGylated through the addition of lipid-PEG conjugates – such as PEG-DSPE [21-23], PEG-DOPE or PEG-STR [24] – directly in lipid layer formulations. Several comparative studies reported similar limitations of pre-PEGylation compared with post-PEGylation for lipid-containing nanocarrier formulations [22, 25]. Most recent works on polyplexes now include limited pre-PEGylation [16] in order to maximise the modification efficiency by decoupling the nanocarrier surface and core integrity.

Once a primary population of nanocarriers has been formed from the first building blocks, pre-modified or not, additional components may be added to the structure, either by auto-assembly, or chemically. This phase of surface modification can be followed by a purification step if the integrity of the nanocarrier can be preserved [21, 26, 27]. Another strategy to circumvent purification steps is to minimize the amount of unwanted chemicals in the reaction mixture of modified nanocarrier and to use the latter as is [16].

2.3 SURFACE COATING

Surface coatings include surface modifications designed to control the entire surface physicochemical identity of the nanocarrier. The aim of this type of modification is mostly to gain stability in the blood circulation (steps 1 to 4 Figure 1), but also to mediate cellular entry and escape from endosomes (steps 1 and 3 Figure 2, respectively).

2.4 MONOVALENT TETHERING

A monovalent surface coating corresponds to the functionalization of a substrate through a single bond between the surface and each biomolecule in order to yield an efficient physical envelope separating the core of the nanocarrier from the exterior. The most prominent example of this technique is PEGylation, although monovalent coatings with other hydrophilic agents such as polysaccharides, poly amino acids or poloxamers have also been investigated [28-30]. For a more detailed review on monovalent PEGylation and the subsequent characterization and evaluation of coated nanoparticles, see [31]. The density of such grafted hydrophilic chains can be tuned so as to yield so-called mushroom or brush conformations. The latter has been reported to promote a better shielding of nanocarriers, yet it proved harder to achieve for longer PEG chains [31]. Several

geometrical features related to both the nanocarrier and the shielding chain influence the threshold surface density for the brush conformation, making it a subtle feature to harness. The extensive data collected on PEGylation over the last decades have shed light on limitations to the use of PEG for stealth coating, *e.g.*, accelerated blood clearance (ABC) after repeated systemic administrations, which may be related to anti-PEG antibodies, and the non-biodegradability of PEG [29]. For more details about PEG alternatives for the stealth coating of nanocarriers, see [28].

Another issue specific to monovalent surface coating is the phenomenon coined as core parts "drawing", whereby hydrophilic PEG chains are thought to gradually escape the nanocarrier surface, taking away the molecule it is grafted on [32]. Destabilization of the nanocarrier core integrity has also been hypothesized to be related to an internal rearrangement of post-PEGylated nanocarriers over time [33].

2.5 MULTIVALENT TETHERING

2.5.1 MULTIVALENT POLYMERIC COATING

A multivalent surface coating refers to the use of coating agents bearing multiple anchor sites able to dock onto the pre-formed nanocarrier. This approach addresses the issue of core parts "drawing" raised against monovalent PEGylation [32]. Furthermore, such coatings have been reported to form more consistent nanocarrier outer envelopes, thus enabling a better decoupling of its core and surface properties. Anionic polymers form the most widely used class of multivalent coating agents (see Table 1). This stems from the fact that a large fraction of non-viral nanocarriers feature a positively charged surface, which has repeatedly been associated with poor *in vivo* biodistribution, specificity and

toxicity profiles [3, 31, 34]. Through multiple electrostatic interactions, anionic polymers are thought to auto-assemble onto positively charged nanocarriers, yielding shielded hydrophilic nanocarriers with increased salt stability and enhanced serum resistance. There is however a persisting drawback to this technique. Primary nanocarrier populations formed by the mixing of nucleic acids and cationic materials often yielded free remaining cationic material in solution. Thus, upon addition of polyanion, a subpopulation of nanoparticles without nucleic acid cargo is generated; it was coined as "ghost particles" by Hönig *et al.* for their PEI-based polyplexes [35]. Although their presence yields poorly defined nanocarrier suspensions, it is not necessarily a liability for the overall transfection process. The authors hypothesized that "ghost particles" and nanocarriers cointernalization in target cells helped reduce the toxicity associated with free PEI while yielding a productive proton sponge effect. The formulation ratio is therefore a key parameter to optimize in the design of electrostatic polymer coatings.

Some selected polymers for the multivalent electrostatic coating of non-viral gene delivery vectors are listed in Table 1. The reader is here referred to two reviews discussing electrostatic [36] and polymeric [32] surface modifications for nucleic acids delivery. Note that the polymer coating itself can be used as a targeting agent. For example, chondroitin sulfate C and hyaluronic acid have been used to target the CD44 receptor, a cancer biomarker. Polymer coatings have also been designed to promote endosomal escape. For example, polypropyl acrylic acid (PPAA) is an anionic polymer at physiological pH that converts into a neutral, more lipophilic one upon endosomal acidification, thereby displaying membrane disruptive abilities [37]. Surface modifications of gene nanocarriers can even play a role at the ultimate step of the

delivery, *i.e.*, gene transcription (see step 6 Figure 2). Amphoteric polymer coatings have been designed to activate gene transcription, thus mimicking the role of natural high mobility group (HMG) proteins. For instance, Yoshihara *et al.* reported the development of an amphoteric PEG derivative, bearing a combination of primary amino groups and carboxylic acids. The authors finely optimized the moieties content of their PEG derivative so as to maximise mRNA titers related to their PEI/pDNA polyplex-mediated *in vitro* transfection assays [38].

2.5.2 OTHER SURFACE CROSS-LINKING AGENTS

Further decoupling the multiple properties of a nanocarrier can be achieved by addressing lateral and steric stabilization separately. Steric stabilization refers to PEGylation and related hydrophilic polymer coatings, whereas lateral stabilization refers to the surface reticulation by chemical or electrostatic bonds designed to strengthen the assembly against disruptive agents such as heparin.

Also called nanoparticle caging, surface cross-linking is often based on stimuli-responsive bonds such as disulfide bridges [39, 40] or acid-labile ketal linkers [26]. Taratula *et al.* developed such a strategy and performed thorough studies on their polypropylenimine (PPI) dendrimer-based siRNA delivery system (see **Error! Reference source not found.**) [39]. Free primary amines from their nanocarrier surface were cross-linked with a homo-bifunctional disulfide-bridged reagent. Remaining free primary amines were used for post-PEGylation (see step 3 **Error! Reference source not found.**A). Caging efficiently prevented siRNA release *in vitro* upon addition of poly(methacrylic acid), a competing polyanion, where PEGylation only had a poor outcome (see **Error! Reference source not found.**B). The silencing ability of the

nanocarrier upon caging was fully preserved *in vitro* (see **Error! Reference source not found.**C), and subsequent *in vivo* biodistribution performed 3 days after systemic injection in nude mice bearing a human lung cancer xenograft proved outstanding (see **Error! Reference source not found.**D), which strongly suggested that the temporality of the reducible caging was adequate. Russ *et al.* used dithiobis(succinimidyl propionate) (DSP) in a similar cross-linking strategy: their reducible cross-linking yielded an increased and prolonged pDNA-mediated gene expression after intratumoral injection in mice compared with a non-reductive cross-linking and with no cross-linking. Systemic delivery resulted in an increased tumor-specific gene expression and a decreased off-target to other organs [40].

2.6 POLYMER/LIPID HYBRID SYSTEM

Through numerous attempts to combine the advantages of both polymer- and lipid-based nanocarriers, a new class of hybrid nanocarriers has emerged. The multiplicity of these hybrid architectures is illustrated by the varied nomenclature found in literature: multifunctional envelope-type nano device or MEND [24, 41], nanoplex [42], lipid polymer nanoparticle or LPN [43], or the more general CSLPHN for core-shell-type lipid-polymer hybrid nanoparticles [7]. Polymer/lipid hybrid systems materialize an advanced way to decouple core and surface properties of the nanocarrier, most often in order to combine the optimized mechanical properties of a polymeric core with a more biomimetic surface made of lipid. Polymer/lipid hybrid systems have been thoroughly reviewed recently [7]. One step further in biomimicry is the use of actual erythrocyte membranes to coat nanocarriers, as first described by Hu *et al.* [44].

One of the main methods used to form these systems is to prepare polymeric nanocarriers in a first step, which are then co-incubated with either pre-formed liposomes or with their precursor monolayer while the latter is being rehydrated. Numerous variations on a single step method have also been reported. Temperature, solvent type or pH, mechanical energy input, compounds and formulation ratios are the main control levers that can be used to adjust the size, surface charge, shape and polydispersity of the nanocarrier distribution [7].

3 SURFACE DECORATION

Complementary to surface coating, the decoration of nanocarriers with various types of biomolecules has been envisioned and tested for a range of applications in order to better control the fate of the nanocarrier *in vivo*. Decoration differs from coating as it is not designed to change physical properties such as surface charge or hydrophilicity, but rather to add a specific biological function. Active targeting of nanocarriers by means of a ligand specific to a relevant biomarker is the main implementation of surface decoration.

3.1 ACTIVE TARGETING

This concept enables the selective delivery of nanocarriers to a target-bearing subpopulation of cells. To date, a broad variety of molecules has been used as targeting agents. It includes oligopeptides [12], glycoproteins [5, 45], saccharides [10, 11, 46], antibodies – whole [21] or fragments [47], – aptamers [48], other small molecules [49, 50], and their derivatives [30, 51, 52]. Typically, numerous types of cancer cells have been reported to over-express cellular receptors that have been exploited as target

biomarkers [51, 53], in order to limit off-target gene transfer to healthy cells (step 1 Figure 2). For instance, recent phase I clinical trials of siRNA gene therapy on human melanoma patients involved transferrin-decorated nanocarriers to target transferrin receptors [54].

Ligand-mediated targeting is thought to contribute to the overall process of nucleic acid delivery mainly in terms of nanocarrier uptake by target cells [45, 55] (step 2 Figure 2). The resulting organ and tumor biodistribution of targeted nanocarriers may be significantly improved when the EPR effect is limited [39] (see Figure 4D), but that is not always the case [45]. As reviewed by Heidel *et al.*, in several reported cases active targeting did not change the overall biodistribution of nanocarriers to the tumor, but, rather, it increased the fraction of nanocarriers inside cells [55]. The function of ligand-mediated targeting is thus quite focused, yet a serious limitation may arise when the target receptor activation is not desired. For instance, the epidermal growth factor receptor (EGFR) – a cancer biomarker of the Erb receptor family – is correlated to mitotic activity; EGF-mediated targeting may activate the receptor signalling pathway, which may work against the treatment itself [12, 56]. Here, a solution was recently evidenced by Schäfert *et al.*, who reported that the EGFR-specific peptide GE11 avoided unwanted subsequent signaling upon ligand-induced internalization of targeted PEI-based polyplexes while mediating effective targeting to EGFR-over-expressing cells [12].

Active targeting is now an established surface modification to enhance nanocarriers, although most reports on receptor-mediated non-viral gene delivery do not present any clear optimization of their ligand decoration parameters, such as ligand density,

orientation or spacer length. Optimization studies focused on such parameters may yield valuable insight to potentiate active targeting strategies.

3.2 CELLULAR ENTRY

Without any surface modification, most nanocarriers display an efficient cell uptake [4]. The latter is caused by non-specific interactions that promote a close contact between the nanocarrier and the cell surface. Those interactions are mainly electrostatic: globally positive nanocarriers are attracted to the negatively charged cell membrane. The roughness of the nanocarrier surface modulates that process of cell adherence and uptake [4]. Shielding strategies aim at reducing those non-specific adherence phenomena in order to improve both systemic half-life and targeting specificity. As a result, cellular entry can be substantially hampered, making room for surface decoration strategies addressing the latter [57], as a complement to surface coating strategies that were discussed earlier.

A decoration strategy addressing cell entry can be included within a targeting strategy. For example transferrin has been reported to be useful both for active targeting and cellular uptake [27, 54]. Yet some targeting molecules do not promote an adequate cellular entry, even though they provide specificity through receptor-binding on target cells. The internalization pathway of a given cell receptor may prove unfit to the nucleic acid delivery strategy, for instance if preferential routing towards lysosomal pathway occurs, as illustrated in Figure 2. In order to control the internalization of the nanocarrier separately from the active targeting, the nanocarrier can be decorated with agents promoting cell entry, such as the helper lipid DOPE in lipid layer-containing nanocarriers, or fusogenic cell-penetrating peptides (CPP). This peptide family can

interact non-specifically with cell membranes through several mechanisms that are still partially understood, making them a potent tool to improve the delivery of nanocarriers. Fusogenic CPP have several different origins, from natural protein transduction domains – *e.g.* the TAT peptide – to rational design – such as octaarginine, – and have been thoroughly reviewed [58, 59]. Huang *et al.* recently reviewed the innovative strategies implemented to optimize the use of cell-penetrating peptides. As CPP are non-specific, their use may result in a higher off-target cell entry. Thus, the CPP fusogenic activity should remain inactive until the nanocarrier gets in close vicinity to its target cells in order to mitigate off-target uptake and toxicity in healthy tissue [57, 59]. Thus, the emerging environment-responsive nanocarriers arguably represent a way to capitalize on CPP.

Endocytosis has recently appeared to be a determinant pathway for the CPP-mediated uptake of non-viral nanocarriers; concomitantly, several fusogenic CPP-mediated uptake strategies have been reported to be limited by the next hurdle in the route towards cargo delivery, that is endosomal escape [58].

3.3 ENDOSOMAL ESCAPE

Among the challenging series of barriers that make nucleic acid delivery complex, endosomal escape is one of the most pervasive for non-viral nanocarriers (see step 4 Figure 2), as a majority enter cells by endocytosis. For polymer-based nanocarriers, nucleic acid condensing agents such as PEI and other endosome buffering molecules have been tailored to promote endosomal escape [3]. Lipid layer-displaying nanocarriers also benefit from membrane-permeation features [60]. As regards surface modifications, the main endosome-disruptive biomolecules used for the surface decoration of

nanocarriers are endosomal escape peptides (EEP) [61]. They can act through two main mechanisms. One mechanism is the endosome bursting upon swelling, which is triggered by the proton-sponge effect driven by histidine-rich peptides such as H5WYG [61]. The cationic nature of such EEP can also be found in CPP. The other mechanism is based on conformational changes in the peptide secondary structure upon endolysosomal acidification. Cytotoxicity related to lipid bilayer destabilization provoked by such peptides has limited their use for *in vivo* studies [58]. Next generations of EEP implementations – *e.g.* activatable EEP – with reduced systemic activity and cytotoxicity may soon lift this barrier in the development of efficient non-viral gene nanocarriers.

3.4 NUCLEAR TRANSPORT

Impaired nuclear translocation (see step 5 Figure 2) is an inevitable issue for plasmid DNA delivery, for the nuclear pore complexes do not enable passive diffusion of assemblies larger than a few dozen nanometers [59, 62]. When fast-dividing cells are targeted for non-viral gene delivery, substantial nuclear translocation can happen upon nuclear membrane dissolution [3]. Otherwise, nuclear localization signals (NLS) emerged as chaperones enhancing nuclear entry through active translocation across the nuclear envelope. NLS are peptide-based, although by extension other types of biomolecules – *e.g.* maltotriose [63] – can be considered NLS. Of interest, clustered basic lysine and arginine residues form a classic NLS peptide feature that can occur in fusogenic CPP and EEP as well. Thus, the nomenclature used to describe the peptides used for non-viral gene delivery is equivocal.

Finally, the NLS ligands included in the nanocarrier need to remain connected to the nucleic acid payload to efficiently chaperone the latter from the cytosol to the nucleus.

That is why NLS are often directly part of the nucleic acid condensing agents themselves, yet in some cases NLS are conjugated onto the surface of nanocarriers [63], especially when they are included in a CPP like the TAT peptide [9, 14, 21, 23].

4 DESIGN CRITERIA FOR THE TETHERING STRATEGY

Many tethering strategies have been exploited to modify the surface of nanocarriers with specific molecules, such as PEG or receptor-targeting ligands. The key elements mediating their success are summarized in Box 1**Error! Reference source not found.**. First, the strength of the weakest link and its potential disrupting cue both have to be tuned to the bioactivity time frame of the molecule. For instance the shielding activity of a hydrophilic coating in blood circulation may turn into an impediment past cellular entry [28]. Conversely, a nuclear localization signal (NLS) has to be active only in the cytosol. Thus, labile chemical bonds such as disulfide bridges [64] or hydrazones [16] have been used for PEGylation (see Table 2), but a non-cleavable ether bond was used to display maltotriose – an NLS – into the lipid envelope of a nanocarrier by Akita *et al.* [63]. An important point in the design of a bioconjugation strategy is that the chosen releasing cue does not have to correspond to the grafting link *per se*: a responsive bond can be included along a spacer conjugated to the biomolecule beforehand. For example, Dash *et al.* included an enzymatically cleavable tetrapeptide sequence to their HMPA-based polymer and used the 4-nitrophenoxy group for surface conjugation to the pendant primary amino groups of PLL/DNA polyplexes [65]. Shim *et al.* engineered a linear PEI onto which a third of the secondary amines were functionalized with a ketal-including short spacer terminated by a primary amino group. The latter was used to graft amine-reactive gold

nanoparticles through a stable amide bond, thus effectively decoupling bioconjugation and controlled release [26].

Secondly, the length of the linker that separates a ligand from a nanocarrier surface may affect the bioavailability of the former. For instance, Ishitsuka *et al.* reported a 27-fold increase in gene expression upon switching from a 2-kDa down to a 0.9-kDa PEG spacer for the decoration of a nanocarrier with the cell-penetrating peptide IRQRRRR. The resulting assembly was injected *via* the tail vein of mice for transfection in the lung [24]. They hypothesized that the increase in gene expression was due to a thinner hydrophilic layer separating the nanocarrier lipid envelope from the endosomal membrane of transfected cells, thus enabling a superior endosomal escape. Guiding principles for the adequate length of PEGylating agents are complex to elicit from the wide diversity of nanocarriers and applications tested, spacer length being strongly case-dependant [51].

Thirdly, the ligand orientation upon tethering is of importance, especially for larger biomolecules. Algar *et al.* emphasized the prevalence of this issue when using carbodiimide chemistry for protein conjugation [66]: the probable presence of several reactive sites for carbodiimide cross-linking on the protein will statistically lead to one or more subpopulations of tethered proteins with an inactivated or unavailable bioactive site. The use of unnatural amino acids for the preparation of polypeptides *in vitro* or *in vivo* through non-sense or frameshift codons such as the ketone-bearing p-acetylphenylalanine is an alternative to unspecific chemistries, although its use is still limited to a narrow range of applications [67].

Finally, the ligand density and its repartition on the surface of the nanocarrier have been reported to affect the outcome of the tethering strategy. First, improved receptor targeting and cell uptake of decorated nanocarriers both have been correlated to an increase in average ligand density [8, 68], but excessive density has also been reported to impair cell uptake. Furthermore, the repartition of ligands onto the surface is believed to be determinant. Lee *et al.* compared two well-defined ligand-decorated architectures with the same average density, but with different local densities [50]. Their nanocarrier triggered no significant gene silencing when ligands were more evenly distributed, and over 50% of silencing with a higher local ligand density. Cell uptake was similar for both nanocarriers; the authors hypothesized that intracellular trafficking was influenced by the ligand repartition. Other studies have proposed that appropriate ligand densities may yield a higher avidity, thus increasing uptake by cells [69, 70], and pointed at clustered subpopulations of ligand.

Thus, the four major aspects for the display of ligands on nanocarriers listed in Box 1 should be cooperatively optimized in order to efficiently enhance gene delivery overall. Algar *et al.* discussed the potential of bioorthogonal chemistry for the bioconjugation of several nanoparticles in order to meet with such demanding goals. Additionally, Stephanopoulos and Francis recently published comprehensive heuristics on protein bioconjugation strategy that they included in their review on the decision process for bioconjugation [71].

5 COMBINED SURFACE MODIFICATIONS

As reviewed so far, a series of distinct hurdles slow down the advancement of gene therapies that rely on the systemic injection of non-viral nanocarriers. A large empirical evidence-based knowledge of these well-identified barriers has been built over hundreds of publications for several decades. There is now a range of specific strategies that are available to specifically address each of these barriers. Some agents have demonstrated multiple effects. For instance, the TAT peptide is a CPP that includes an NLS [9], hyaluronic acid is a lateral stabilizer that targets CD44 receptors [72], the GALA peptide is a pH-responsive EEP that has an affinity for sialic acid-terminated endothelial cell surface sugar chains [41]. Otherwise, the elaboration of multifunctional nanocarriers relies on the combination of several agents, the latter being often applied as surface modifications of pre-designed vectors.

5.1 DECORATED COATING

Coating a nanocarrier yields stabilized assembly, but that is often at the expense of reduced nanocarrier/cell interactions. Decorating a coated nanocarrier with a ligand being able to facilitate targeting, uptake and even endosomal escape is a classical way to mitigate that reduced nanocarrier/cell interaction. For instance, decoration of PEGylated nanocarriers, especially for targeting purposes, is an efficient rationale that benefits from extensive empirical data [51], even though general formulation rules are very limited. Spacer length is a subtle parameter to optimize on a given construct in order to yield a sufficient bioactivity without compromising other aspects of non-viral gene delivery. The dilemma is that conflicting principles coexist (see Table 3).

Conversely, Stefanick *et al.* recently published a detailed study on the decorated coating of liposomes for targeting purposes. The authors reported that the length of the linker

used for decoration had a dramatic influence on cell uptake, whereby excessive length completely abolished the peptide targeting ability for a given PEGylation thickness (see Figure 5A). Furthermore, the cell uptake was found to plateau at around the same ligand density for two different target cell lines (Figure 5B) as well as for another disease model involving different peptides and target cells *in vitro*. Figure 5C illustrates the utter importance of optimizing these design parameters [68].

5.2 MULTIPLEX DECORATION

When several different decoration ligands are tethered onto the surface of nanocarriers, a multiplex decoration is obtained; the purpose of which can be to address sequential barriers to systemic delivery. For example, Akita *et al.* recently studied the synergistic effects of the fusogenic peptide GALA and the nuclear localization signal maltotriose by anchoring them both onto the lipid bilayers of their nanocarrier [63]. *In vivo* experiments were carried out on mice by intravenous injection of a small volume of highly concentrated nanocarrier suspension. Both GALA and maltotriose decorations sequentially yielded an increase by an order of magnitude in reported gene expression in the liver after 6 hours. The authors postulated that GALA and maltotriose only played a role for endosomal escape and nuclear transfer respectively in an independent manner. Cheng *et al.* observed a cross-talk between their two ligands – a CPP and folate – for the cellular attachment of their nanocarrier onto folate-receptor-bearing KB cells. The authors postulated that avidity effects emanating from both ligands yielded synergistic effects for cell binding and uptake both *in vitro* and *in vivo* [73].

Another purpose for multiplex decoration is to target several cell lines. Jing *et al.* recently reported a dual decoration strategy, whereby they managed to target two different cell

lines from a single homogenized nanocarrier injection in tumor-bearing mice. Transferrin was used to target HepG2, a human liver carcinoma cell line; and mannan was used to target Kupffer cells [74]. The dual decorated nanocarriers yielded transfection levels as high as both single decorated formulations did for their specific target cell populations.

Thirdly, reduced off-target effects can be achieved by dual ligand targeting to the same cell type. For instance, Kluza *et al.* targeted liposomes to newly formed endothelium by decorating their liposomes with both cyclic RGD and anginex peptides. The former targeted $\alpha_v\beta_3$ integrins, and the latter targeted galectin-1, both present on activated endothelial cells. This dual decoration strategy yielded synergistic targeting and uptake of liposomes *in vitro* by activated endothelial cells; thus it can potentially reduce off-target to cells bearing only one of the target receptors [75].

5.3 MULTI-LAYERED CONSTRUCTION

Another level of surface modification can be achieved through multi-layered assemblies. An advantage of multi-layers is the increased loading capacity available per carrier. For example, Li *et al.* coated their positively-charged DNA/protamine core nanocarrier with an additional 15-nm thick plasmid DNA layer. On a subsequent step, a cationic lipid was auto-assembled onto the surface, and the latter was ultimately coated by o-carboxymethylated chitosan, a pH-sensitive hydrophilic polymer [76]. The authors' layer-by-layer technique yielded well defined nanocarriers. *In vitro* and *in vivo* studies provided encouraging results on the specific role performed by each component: a hydrophilic coating for a long circulation time, fusogenic lipids for endosomal escape, and NLS for nuclear transport.

Such multi-layered assemblies have also been designed to co-encapsulate a chemotherapeutic agent and nucleic acids. The motivation for this rationale is to address multi-drug resistance (MDR) often encountered in cancer chemotherapy. Nanoscale combination therapy applied to address MDR in cancer treatment has recently been reviewed by Khan *et al.* [77]. A property worth harnessing when dealing with multiple cargos is their controlled release, so that each pharmaceutical can better act on its own target.

5.4 ADVANCED CONTROLLED RELEASE SYSTEMS

As numerous non-viral gene delivery barriers are sequential (see Figure 1 and Figure 2), the corresponding nanocarrier features that address them may only be needed for a specific time frame. Hence, controlled uncoating strategies have been envisioned in order to either trigger the release of already used materials from the surface of the nanocarrier, or to set on another bioactive compound. This paradigm is now implemented in most recent PEGylation strategies (see Table 2). Huang *et al.* reviewed this rationale for the optimized decoration of nanocarriers with CPP, as the latter non-selective cell-penetrating abilities are to be avoided until target cells are reached.

DePEGylation is not the only surface controlled release application. Taratula *et al.* developed a reducible caging strategy for their nanocarrier but did not implement any dePEGylation [39]. In their experiments, the nanocarrier uncaging in endolysosomal compartments, which was triggered by reductive species, was sufficient to release the cargo siRNA in the cytosol for subsequent gene silencing (see Figure 4). Thus, the authors managed to optimize the biodistribution of their nanocarrier *in vivo* owing to their highly serum stable construct.

A broad range of cues have been explored for controlled release. Cues can be of environmental origin. That includes acidification, reduction and specific enzymes. Conversely, external cues applied locally have also been developed. They include light, ultrasound, heat and magnetic fields. Nanocarriers responding to stimuli have been reviewed by Fleige *et al.* [78].

6 FUTURE PERSPECTIVES

There is a duality in the progress of non-viral nucleic acid nanocarriers. Particularly complex assemblies with multiple features but having no clinical future coexist along frugal formulations with which clinicians struggle to make the awaited gene therapy revolution a reality.

On the one hand there is the upstream fundamental point of view: the numerous barriers to be overcome in gene delivery all have specific strategies addressing them. A methodical optimization should be carried out through an advanced decoupling of every single nanocarrier feature in order to gain indispensable knowledge and understanding of both specific and global governing principles and processes. An insightful example of methodical optimization is the recently published study of Stefanick *et al.* on the decorated coating of liposomes described earlier [68].

On the other hand, the focus also has to be directed on regulatory requirements, clinical relevance and treatment accessibility. Efforts in simplicity, robustness, cost-effectiveness and clinical efficacy have to be made in order to go towards applicable nanomedicines. An elegant outcome of these principles is the recently initiated clinical trials based on a

liposome-vectorized microRNA for liver cancer: the adequate formulations of four different lipids forms liposomes with a negative surface charge at physiological pH, which becomes positively charged in acidic tumor environment, thus enhancing local uptake by cancer cells [79]. The breakthrough works of Davis *et al.* on the first clinical trials for siRNA therapy through the use of active targeting are also remarkable tangible steps towards clinical success of non-viral nucleic acid delivery [54].

Both directions should be actively pursued as they are intrinsically bound together even though their specific outcome is of a different nature.

Apparent paradoxes can also be found among contradictory opinions towards the use of targeting ligands [80]. This stems from the fact that generalizations made over a myriad of therapeutic applications are likely to include numerous exceptions. For example, the EPR effect for the passive targeting of solid tumor tissue may actually be hampered by the active targeting mediated by ligand decoration. The latter may indeed compromise the nanocarrier shielding, or mediate off-target to cognate receptor-bearing healthy cells. Both effects may in turn reduce the nanocarrier systemic half-life that directly fuels the EPR effect [80, 81]. Conversely, active targeting may prove capital for other, more critical applications such as metastasized carcinomas, or when no significant EPR effect can be tapped [80]. Similar divergences have been discussed regarding nanotheranostics, that is nanomedicines combined with diagnostics modalities [81]. We believe that given the variety of the need for nanomedicines, all these prospective designs have their range of applications tied with specific criticalities,

Finally, complexity does not have to be systematically avoided in non-viral gene delivery. Rather, future systems with increased complexity will have to bring about substantial enhancements in clinical efficacy. This stems from the fact that some levels of complexity are inherent to potentially clinically relevant strategies, with the example of combination therapies addressing MDR in cancer treatment. The limitations of monotherapies and the substantial advantages of coupling drug and gene therapies in a single formulation is an exciting new paradigm that will probably be the subject of outstanding research in the near future [80-82].

7 [BOX]EXECUTIVE SUMMARY

8 FINANCIAL & COMPETING INTERESTS DISCLOSURE

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CAPTIONS

Figure 1 – Extracellular hurdles encountered along the systemic delivery of nanocarriers for the treatment of solid tumors. ① Physiological salt concentration (small ions); ② Plasma proteins adsorption onto nanocarriers (opsonins); ③ Erythrocytes aggregation upon nanocarrier extracellular attachment; ④ Rapid clearance by the mononuclear macrophage system (MPS); ⑤ Fenestration of neo-vasculature at the tumor site; ⑥ Hindered diffusion through the extracellular matrix.

Figure 2 – Intracellular hurdles along the delivery of plasmid DNA. ① Off-target transfection in healthy tissue; ② Endocytosis by target cell; ③ Endosome routing towards lysosomes and subsequent pDNA degradation; ④ Escape from endosome and release into the cytosol; ⑤ Active or passive transport towards the nucleus and across the nuclear pore system; ⑥ Release of pDNA from its nanocarrier for subsequent transcription.

Figure 3 – The different contributing structures of a nanocarrier surface. A transient combination of these contributing structures may help conceptualize the actual surface behavior of a given nanocarrier for a better understanding of its interaction with the biological milieu.

Table 1 – Polymers used in vivo for the electrostatic coating of nucleic acid delivery vectors.

⊖ represents the polyanions charge bearers.

Polyanion	Mol. Wt. *kDa*	Charge density *⊖/kDa*	Charge bearer	Polycation or cationic lipid	Optimal ratio *⊖:N:P*	Results highlight	Year	Ref.
o-Carboxymethyl chitosan	50	2.8	$-COO^-$	protamine		↓ Cytotoxicity pH-triggered uncoating Similar expression in tumor	2012	[76]
Hyaluronic acid	1000	2.6	$-COO^-$	BPEI 25 kDa	12:12:1	Freeze-dry-proof Partial tumor suppression	2010	[83]
Poly(α,D-glutamic acid)	3.2	5	$-COO^-$	Poly(beta-amino ester) C32-117	33:33:1	↓ Uptake in lung ↓ Erythrocyte aggregation	2010	[34]
Chondroitin sulfate C		4.3	$-COO^-$, $-SO_3^-$	DOTAP	5.8:4:1	↑ CD44 targeting ↓ Uptake by tumor cells ↓ Erythrocyte aggregation ↓ Liver toxicity	2013	[72]
Hyaluronic acid	31	2.6	$-COO^-$	DOTAP	5.6:4:1	↑ CD44 targeting ↓ Off-target Rapid uncoating		
Poly(α,L-glutamic acid)	10.5	7.7	$-COO^-$	DOTAP	7:4:1	↓ Erythrocyte aggregation ↓ Liver toxicity		
PEG-succinic acid	9.7	3.7	$-COO^-$	LPEI 25 kDa	5:8:1	↑ Proton sponge effect ↑ Gene expression in liver, lung and tumor	2008	[84]
RGD-PEG-succinic acid	12	2.9	$-COO^-$	LPEI 25 kDa	4:12:1			
Dendrimer phtalocyanine	5.9	6.6	$-COO^-$	$C(YGRKKRRQRRRG)_2$	1:2:1	Photochemical internalization ↑ Colloidal stability ↓ Off-target ↓ Toxicity	2005	[85]

Figure 4 – Tumor-targeted siRNA delivery by a multi-functional nanocarrier. (A) Nanocarrier formation and sequential surface modification strategy. **(B)** siRNA release kinetics upon nanocarrier disruption by PMAA: Combined effects of PEGylation and caging. **(C)** *In vitro* silencing of BCL2: Impact **(Ci)** of PEGylation density and **(Cii)** of LHRH targeting **(D)** Biodistribution in mice **(Di)** of the siRNA condensing agent and **(Dii)** of siRNA in major organs 72 hours after injection.

*$p < 0.05$.

PPI: polypropylenimine; DTBP: Dimethyl-3-3'-dithiobispropionimidate; PEG-MAL: maleimidated PEG; LHRH: synthetic analog of luteinizing hormone-releasing hormone decapeptide; BCL2: mRNA target (B-cell lymphoma 2, a regulator protein involved in apoptosis); β_2-m: mRNA internal standard.

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Table 2. Range of interactions used in nanocarrier surface modification.

Short bond type		Example	Cleaving/disrupting cue (example)	Application	Limitation	Ref.
Covalent, non-cleavable		Thioester		Long lasting PEGylation	Low uptake and gene expression	[64]
Covalent cleavable		Disulfide bridge	Reducer (glutathione)	dePEGylation after internalization	Premature dePEGylation, Nanocarrier aggregation	[64]
		Hydrazone	Acidification (pH≤5)	Endosomal escape upon dePEGylation	Premature extracellular cleavage	[16]
		4,5-dimethoxy-2-nitrobenzyl cage	Light (UV radiation)	Localized activation	Therapeutic window for tissue penetration	[86]
		Ester	Enzyme (esterase)	Separation of nanocarriers from targeted receptor	Slow cleavage rate	[87]
Non-covalent	Electrostatic	Poly propyl acrylic acid (PPAA)	pH-triggered neutralization (pH≤6)	Uncoating in early endosome, membrane disruptive activity	Limited nanocarrier systemic stability	[37]
	Hydrophobic and electrostatic	DOPE/CHEMS lipid bilayer	pH-triggered phase transition (pH≤6)	Protection from extracellular polyanions, pH-triggered fusogenicity	Low systemic stability, Decreased fusogenicity upon PEGylation	[88]
	Host-guest	Cyclodextrin-adamantane		Well defined and controlled conjugation	Need for host display on the nanocarrier surface	[69]

Box 1. Key elements in the display of biomolecules on nanocarriers.

- The strength of the weakest link and its eventual disruptive cue
- The spacer length separating the biomolecule from the nanocarrier surface
- The biomolecule orientation regarding the bioavailability of its active sites
- The biomolecule density and repartition on the surface

Table 3. Conflicting principles at stake for the decoration of PEGylated nanocarriers.

Optimization path	Advantage	Adverse effect
Spacer lengthening	▪ Ligand still bioavailable after physiological protein corona formation [5] ▪ Increased bioavailability away from the nanocarrier surface ▪ More flexibility to enhance binding to target	▪ Too intense ligand display may impair nanocarrier shielding and trigger opsonization [81] ▪ Additional spacer length may wrap around the ligand and bury it [68]
Spacer shortening	▪ Can be combined with triggered dePEGylation of longer chains in the vicinity of the target cells for a conditioned activation [16, 21, 23] ▪ Close vicinity between the nanocarrier and the endosomal membrane after endocytosis may promote escape to the cytosol [24]	▪ Ligand may be remain buried into the surrounding residual PEG chains and stay inactive ▪ Excessive steric hindrance from the nanocarrier surface may prevent binding to target

Figure 5 – Effect of liposomal PEG coating and peptide EG-linker length on the cellular uptake of HER2-targeted liposomes in two HER2-overexpressing cell lines. **(A)** Bell-shaped dependence of cell uptake with EG-linker length. **(B)** Evidence of a targeting peptide density threshold for cell uptake. Peptide density percentage corresponds to the molar fraction of lipid anchor in the lipid layer **(C)** Confocal microscopy images taken 3 h post transfection. Nuclei are in blue, liposomes in red.

EG: ethylene glycol; HER2: Human epidermal growth factor receptor 2;

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Executive summary

The nanocarrier surface

- Surface and core of nanocarriers bear specific properties and corresponding goals in gene delivery.
- Both are tied with different time frames, and thus need to be addressed separately.
- A nanocarrier surface is a dynamic and transient feature critically involved in the fate of the nanocarrier *in vivo*.
- Its modification can be carried out before, during, or after the formation of the surface.
- Whole surface coating and discrete ligand decoration form the two main surface modification approaches.

Surface coating

- The physico-chemical identity of the nanocarrier surface can be controlled by coating the latter with an additional agent.
- Monovalent coating is a well-characterized technique that provides steric stabilization, a critical feature involved in salt- and serum-stability.
- Multivalent coating confers lateral stabilization to the assembly by means of physical or chemical cross-linking on the surface of the nanocarrier.
- Several surface coatings additionally address delivery barriers including specific cell uptake, endosomal escape and nucleic acid cargo unpacking.
- A range of polymer/lipid hybrid systems capitalize on the attractive properties of surface lipid layers by anchoring them on a solid or semi-solid core that confers a greater stability.

Surface decoration

- The biological identity of the nanocarrier surface can be tuned by decorating the latter with bioactive molecules. Surface decoration can address four major types of applications:
- Active targeting to specific cell lines can be promoted by displaying molecules such as ligands that can bind to target cell receptors.
- Decoration with membrane-active compounds can favor uptake by cells.
- Impaired endosomal escape can be restored either with endosome-buffering or fusogenic agents.
- Intracellular trafficking and especially nuclear transport can be influenced by displaying signalling molecules such as NLS.

Design criteria for the tethering strategy

- Decoupling tethering and controlled release in surface modification strategies brings flexibility to the design.
- The length of the linker can influence greatly the activity of the tethered molecule in a case-specific fashion.
- The coupling method may affect the orientation of tethered molecules, which in turn modulates their activity in the biological milieu.
- Local higher tethered ligand densities promote avidity effects that can enhance targeting, uptake by cells, and intracellular trafficking.

Combined surface modifications

- Multifunctional surface modifications can include both decorations and coatings in a single to multiple layer assembly.
- Multiplex decoration can broaden cell targeting and uptake options.
- Advanced controlled release features enable the activation of several functions at relevant time frames.

Future perspective

- Decoupling the different nanocarrier features is critical to gain a better knowledge and understanding of the driving forces behind an efficient gene delivery.
- Conversely, efforts need to be made towards more scalable, economical, and clinically relevant formulations.