1.3.26 Targeted nanostructures tailored to *in vivo* delivery

Development of efficient nanostructures for drug delivery tailored to in vivo use requires careful engineering of different aspects (targeting unit, nanoparticle composition, encapsulation method) that must be optimized separately but synergistically to provide best performances. At NEST laboratory we focused both on development of innovative targeting sequences tackling the transferrin receptor pathway and on innovative formulation strategies to improve encapsulation efficiency of small molecules and proteins.

Development of nanostructured carriers for delivery of therapeutic payloads represents an assessed research line at NEST. The focus in this area is on the assembly of nanostructures that can perform efficient and targeted delivery of delicate therapeutic payloads (e.g. enzymes) without compromising the activity of the payload itself. Particular attention has been paid to the perspective use *in vivo* of these systems. This involved development of nanostructures able to avoid, or selectively interact with, serum proteins. The activity can be divided in two separate optimizations: design/evolution of targeting units, and engineering of the nanoparticle scaffold. These two activities are shortly described in the following.

Development of binding sequences for targeted delivery

Targeted delivery requires engineering of structures able to recognize biomolecules either critically involved in the pathologic process or playing a pivotal role in crossing of biologic barriers. Transferrin pathway is, in this sense, an extremely interesting target. In fact, transferrin receptor is overexpressed (up to 100 folds) in high-proliferating cells such as in tumors. Additionally, it is expressed on the surface of the blood brain barrier, where it constitutively delivers iron to the brain. These two features make it an ideal target for drug delivery in cancer treatment and in all those pathologies that involve the central nervous system.

Transferrin receptor is saturated, in physiologic conditions, by endogenous transferrin. Thus, therapies based on transferrin-mediated delivery have little chance of success. Recent advances in this field took in consideration alternative targeting molecules such as oligonucleotide and peptide aptamers. In the framework of the ongoing research activity in nanomedicine, a rational optimization of an oligonucleotide aptamer was performed on the basis of a previously published structure. This reported anti-TfR aptamer undergoes a series of conformational equilibria leading to the formation of a major, inactive conformer at physiologic temperature. Conformational equilibrium of the reported aptamer was evaluated in silico and confirmed experimentally at different temperatures; punctual mutations were performed to obtain a mutated form (DW4) which is completely stable at 37 °C, with nanomolar affinity towards human and murine transferrin receptor [1]. This aptamer is also able to promote internalization in cells of large nanoparticles, allowing selective delivery of liposome-encapsulated doxorubicin to living cells [2].

Capability of oligonucleotide aptamers to perform combined delivery of two therapeutic payloads in cells was evaluated (Fig. 1, A). To this end, an anti-transferrin receptor oligonucleotide aptamer was exploited in the selective co-delivery of two therapeutic agents: a small drug (Doxorubicin) and an oligonucleotide decoy against NF-kB (an anti-apoptotic protein activated in tumor

cells). This strategy allows performing two therapeutic actions at the same time, i.e. cytotoxic activity of the drug and inhibition –induced by the decoy- of natively constituted anti-apoptotic mechanisms. This construct shows high cytotoxicity (comparable with free doxorubicin) in target cells, with negligible off-target effect (Fig. 1, B) [3].

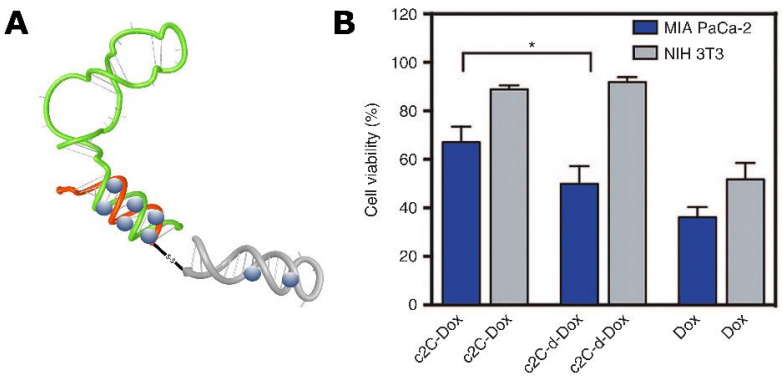

Figure 1. *A:* Scheme of aptamer-based nanostructure for co-delivery. The TfR aptamer (green) is partially hybridized with an antisense oligonucleotide (red) to provide anchor points for doxorubicin (blue dots). A disulphide bond connects this architecture to the NF-kB decoy (grey). The whole system readily disassembles during cell internalization leading to release of cytotoxic (doxorubicin) and antiapoptotic inhibitor (NF-kB decoy) agents. *B:* Combined effects of these molecules and selective uptake triggered by NF-kB leads to highly selective cytotoxic activity that does not affect control cells, while exerting an important effect on tumor cells *in vitro*.

An alternative approach to TfR targeting was developed by considering the complex environment represented by blood. It is accepted that immediate solvation of exogenous materials by serum proteins leads to a substantial change of surface properties of administered nanostructures. This translates in the shift from the "chemical identity", i.e. the surface functionalization obtained during nanoparticle synthesis and isolation, to the "biological identity", that is the nanoparticle solvated with one or more layers of serum proteins. This layer, which is at the interface between the nanoparticle and the organism, ultimately drive the fate of the nanoparticle itself. There are two not mutually exclusive strategies to overcome this effect: avoiding nonspecific adsorption by using antifouling coatings or exploiting surface functionalization to selectively bind a specific serum protein, which in turn can steer the destination of the nanoparticle.

This last approach was exploited by developing a peptide aptamer against transferrin. Unlike the classic aptamer selection process, which is exquisitely experimental and thus with unpredictable binding site on the protein, a combined modelling/evolutionary algorithm (Fig. 2) was adopted to rationally design a small peptide (Tf2).

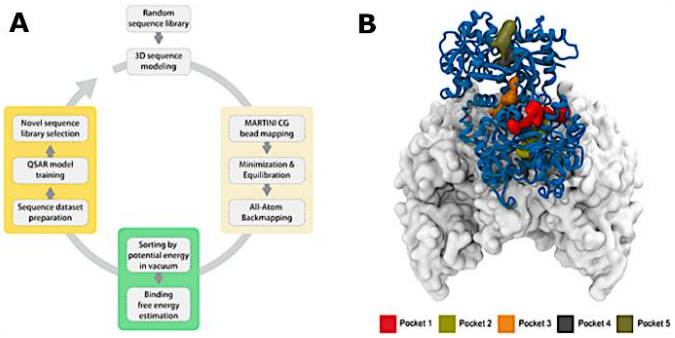

Figure 2. *A* Schematization of *in silico* selection process for Tf2. A suitable pocket is identified on the transferrin in a position which is not proximal to the binding site to either TfR or iron binding sites. An iterative evolutionary algorithm is performed, screening a random peptide library for binding energy in the pocket, introducing random mutations and selecting at each iteration the most affine candidates. Multivariate optimization allows identifying best candidates. *B In silico* identification of suitable pockets for aptamer selection (in this selection "Pocket 3", orange was chosen) on the docked transferrin-TfR complex.

This peptide recognizes transferrin in a pocket that does not interfere with transferrin binding to its receptor or iron ions. Other serum proteins interact only to a minimal level with Tf2, leading to selective protein adsorption on the surface of nanoparticles derivatized with Tf2 [4]. Controlled orientation of transferring on Tf2 functionalized nanoparticles leads to nearly 40-fold increase in cell uptake, compared with non-derivatized nanoparticles. Notably, the absolute amount of transferrin adsorbed on the surface of Tf2-derivatized and untargeted nanoparticles is similar, so the increased uptake is driven to a large extent by the proper orientation of transferrin adsorbed onTf2-derivatized nanoparticles. This strategy has also been validated using mesoporous silica nanoparticles with Tf2. The presence of this peptide significantly improves uptake in tumor organoids derived from MiaPaCa-2 cell line [5].

Development of nanodevices tailored to enzyme delivery in vivo

The identity of a nanoparticle scaffold significantly affects delivery efficiency of therapeutics. Design of a nanostructure for *in vitro* and *in vivo* delivery requires efficient encapsulation of the active principle, with minimal impact on conformation in the case of enzymatic payloads, whose activity is strictly connected with secondary and tertiary structure. Additionally, surface composition of the nanoparticle must be appropriately designed to modulate interaction with serum proteins. Finally, disassembly triggered by changes in physico-chemical properties of the surrounding environment (e.g. pH changes upon internalization) can be conferred by appropriate design of nanoparticle excipients. Nanoparticles were developed with the specific purpose of delivering delicate protein therapeutics across the blood brain barrier, addressing the complex issue of enzyme replacement therapy in lysosomal storage disorders.

Novel biomimetic, PEG-free scaffolds were engineered. These scaffolds are characterized by ultra-low protein adsorption and long residence time (>12h) in serum. The particles are based on a zwitterionic amino acid sequence (EKEKEKE) derivatized with a lipid tail to promote self-assembly and insertion in liposomes. These nanostructures outperform benchmark PEGylation reagents in terms of

protein interaction and can be conveniently modified with biorthogonal linkers for derivatization with targeting agents. Self-assembled particles loaded with doxorubicin and functionalized with DW4 aptamer efficiently internalize in cells demonstrating dose-dependent cytotoxicity Fig. 3 A), while nonspecific uptake of untargeted nanoparticles is completely negligible (Fig. 3 B) [2]. The same stealth architecture, modified with Tf2 transferrin-binding peptide, has been loaded with functional PPT1, and recently evaluated as a way to deliver protein palmitoyl thiotransferase (PPT1) enzyme *in vitro* to primary dermal fibroblasts derived from NCL-1 patients, leading to partial restore of biochemical (palmitoylation level) and proteomic parameters.

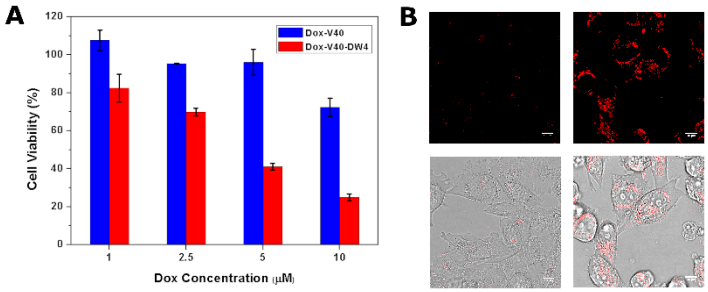

Figure 3. A Dose-response curve of doxorubicin encapsulated in untargeted (blue) and targeted (red) stealth liposomes administered to Mia PaCa2 cells. Untargeted liposomes are not internalized and hence show negligible cytotoxicity. *B* confocal fluorescence microscopy images of untargeted (left) and targeted (right) stealth liposomes to Mia PaCa2 cells.

Efficient loading of functional enzymes in nanoparticles is knowingly difficult, due to the harsh environment encountered by encapsulated proteins (very lipophilic, with important unbalance of saline content, ultimately leading to partial or complete enzyme denaturation). This leads to either poor encapsulation yield, or partial unfolding of the protein, often with almost complete loss of activity. Encapsulation in liposomes, which is usually obtaining by freeze-thaw or sonication, has a profound effect on both enzyme activity, that decreases by 30-40%, and on encapsulation yield, usually limited to less than 10-20%. These limitations can be circumvented by using chemically linked enzyme aggregates (CLEAs), produced by reaction of the enzyme with reversible chemical crosslinkers. This controlled aggregation makes the enzyme itself lipophilic and induces aggregation and precipitation from aqueous environment in small (<20nm) aggregates. These aggregates can be encapsulated by simple nanoprecipitation techniques in a matrix of PLGA, with excellent yields (>90%) and excellent activity retention (Fig. 4 A, B). Pristine enzyme can be easily restored by spontaneous hydrolysis of the crosslinker under mildly acidic aqueous conditions. At the same time, PLGA carrier easily decomposes leaving only enzyme. Overall, this approach represents an efficient strategy to perform delivery of lysosomal enzymes. PLGA-CLEAs nanoparticles functionalized with Tf2 transferrin binding peptide have been demonstrated to restore enzymatic activity in vitro with different enzymes, including GALC and PPT1 [6]. Appropriate modification of the nanoparticle design using proton sponges (polyethyleneimine, PEI) as nanoparticle excipients promote endosomal escape of the enzyme allowing cytoplasmic delivery of functional enzymes [7].

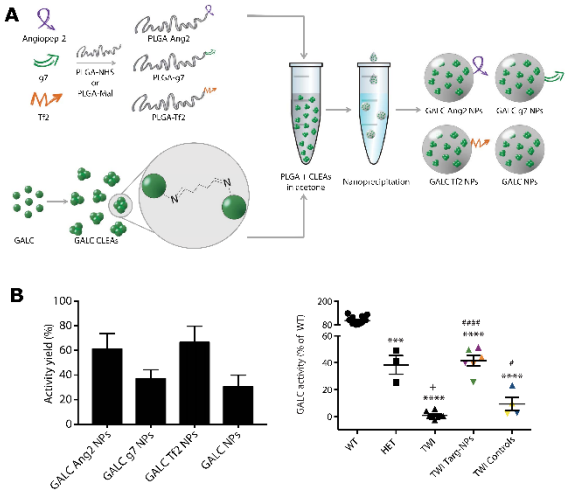

Figure 4. A Synthesis of targeted CLEAs PLGA nanoparticles. Enzyme aggregates are formed using glutaraldehyde and precipitate from water. After resuspension, CLEAs-PLGA nanoparticles are formed by conventional nanoprecipitation. Targeting sequences can be inserted by appropriate functionalization of PLGA. **B** Enzymatic activity (% respect to WT) of GALC after encapsulation (left) and at brain level (right) after intraperitoneal administration to Twitcher mice. Targeted nanoparticles (orange, green, magenta) lead to partial restore of enzymatic activity. Untargeted nanoparticles (yellow) and free enzyme (blue) fail to provide increase in enzyme activity.

CLEAs-PLGA nanoparticles loaded with GALC were functionalized with targeting sequences including: two peptides with reported capability to cross the blood brain barrier (Angiopep2 and glycosylated peptide g7), and the transferrin-binding Tf2 peptide for indirect targeting *in vivo* (Fig. 4 A). In all cases, intraperitoneal administration to mice restores physiologic enzymatic activity in the brain [8], demonstrating that Tf2-derivatized PLGA nanoparticles are effective in delivering functional, folded enzymes to the brain *in vivo* (Fig. 4 B).

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