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Engineering approaches in siRNA delivery

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Graphical abstract

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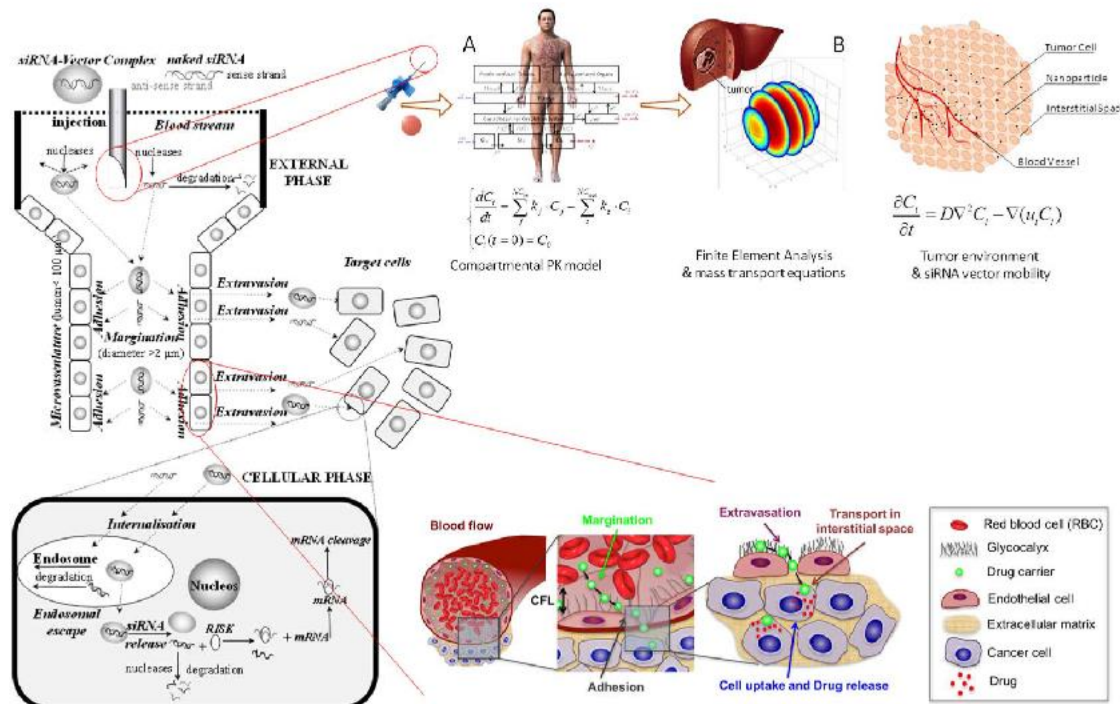

$$\begin{cases} \frac{dC_i}{dt} = \sum_j^{NC_{in}} k_j \cdot C_j - \sum_z^{NC_{out}} k_z \cdot C_i \\ C_i(t=0) = C_0 \end{cases}$$

$$\frac{\partial C_i}{\partial t} = D\nabla^2 C_i - \nabla(u_i C_i)$$

Abstract

siRNAs are very potent drug molecules, able to silence genes involved in pathologies development. siRNAs have virtually an unlimited therapeutic potential, particularly for the treatment of inflammatory diseases. However, their use in clinical practice is limited because of their unfavorable properties to interact and not to degrade in physiological environments. In particular they are large macromolecules, negatively charged, which undergo rapid degradation by plasmatic enzymes, are subject to fast renal clearance/hepatic sequestration, and can hardly cross cellular membranes. These aspects seriously impair siRNAs as therapeutics. As in all the other fields of science, siRNAs management can be advantaged by physical-mathematical descriptions (modeling) in order to clarify the involved phenomena from the preparative step of dosage systems to the description of drug-body interactions, which allows improving the design of delivery systems/processes/therapies. This review analyzes a few mathematical modeling approaches currently adopted to describe the siRNAs delivery, the main procedures in siRNAs vectors' production processes and siRNAs vectors' release from hydrogels, and the modeling of pharmacokinetics of siRNAs vectors. Furthermore, the use of physical models to study the siRNAs vectors' fate in blood stream and in the tissues is presented. The general view depicts a framework maybe not yet usable in therapeutics, but with promising possibilities for forthcoming applications.

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Keywords : siRNAs; delivery vectors; in vitro models; mathematical modeling; physical modeling

1. Introduction

Small interfering RNAs (siRNAs) consist of two short RNA molecules named, respectively, antisense and sense strand (Posocco et al., 2015). These double-stranded constructs represent a powerful and versatile gene-silencing tool as they trigger the sequence-specific cleavage of mRNA transcripts (Gao and Huang, 2013). Due to the short length, siRNAs can be easily synthesized via chemical pathways and used to target the mRNAs of genes causing disease, thus showing their therapeutic potential (Farra et al., 2011; Lang et al., 2008; Werth et al., 2010).

However, unfortunately, siRNAs use in clinical practice is limited because of their *in vivo* instability. Indeed, not only the double-stranded nature may trigger the innate immune system (Gao and Huang, 2013), but, if administered systemically (*e.g.*, via the vascular route) (Figure 1), they have to deal with: (i) blood nucleases, which can rapidly induce their degradation and (ii) the elimination by the reticulo-endothelial system (RES) and by kidney filtration (Huang et al., 2011). In addition, the crossing of the vascular wall before reaching the diseased tissue represents another obstacle. The efficiency of this process is strongly influenced by the size of the endothelium fenestration, a feature that can vary considerably among tissues. For instance, siRNAs accumulation in the liver is favored due to the fenestration size of the liver endothelium (Wisse et al., 2008). Moreover, once in the desired tissue, siRNAs have to overcome another obstacle that is represented by the cell membrane crossing. This step is unfavorable due to the negatively charged surface of cellular membranes, which tends to repulse the negatively charged phosphate groups present in siRNAs. Additionally, the hydrophilic nature of siRNAs hinders the crossing of the hydrophobic layer of the cell membrane. Last, but not the least, inside cells, siRNAs are (i) susceptible to further degradation by cellular nucleases and (ii) are subject to the cellular trafficking issue, *i.e.* the sequestration by cytoplasmic vesicles, named *endosomes*, in which they remain entrapped with no possibility to reach the cell cytoplasm (Brown et al., 2001; Kaneda, 2001), where the target mRNA resides. Finally, studies performed in the last ten years, have revealed the presence of off-targets effects that can be due to an incorrect delivery (Aagaard and Rossi, 2007; Raemdonck et al., 2008; Snove and Rossi, 2006).

All the above considerations clearly indicate that naked siRNAs have no chance to exploit their therapeutic action. This is the reason why two main strategies have been undertaken to overcome siRNAs delivery obstacles (Grassi et al., 2010; Scaggiante et al., 2011): (i) introduction of chemical modifications into their structure to make them resistant to nucleases-mediated degradation, (ii) complexation with/binding to different materials that protect the double stranded construct and improve siRNAs vascular wall and

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cellular membrane crossing. Although also viral vectors have been considered (typically, retroviruses, lentiviruses, baculoviruses, and adenoviruses) (Grassi et al., 2006; Yla-Herttuala and Alitalo, 2003), mainly due to safety concerns, nowadays non-viral vectors play the predominant role as siRNAs vectors. Non-viral vectors can be subdivided into three classes on the basis of their characteristic length (Putnam, 2006), *i.e.* nano, micro, and macro scales vectors. Usually, nanoscale vectors are represented by polycationic polymers or lipids that self-assemble with siRNA to form polyelectrolyte complexes (poly- or lipo-plexes, respectively). Other nano-vectors comprehend liposomes, mesoporous silica nanoparticles, polymeric micelles, dendrimers, cyclodextrins, metallic (gold, silver) nanoparticles and superparamagnetic iron oxide (Grassi et al., 2010; Licciardi et al., 2015). Microscale vectors can be represented, for instance, by microbubbles, where siRNAs reside on the surface, and by polymeric particles entrapping siRNA within the polymeric network. Macroscale vectors are two/three dimensional scaffolds or matrices (such as, but not limited to, polymers) hosting the desired siRNA. Obviously, it is possible to embed nanoscale vectors inside micro- or macro-scale vectors to get a chimeric system (Phua and Leong, 2010; Venturoli, 2012) in order to optimize the release kinetics of nano-complexes at the desired site of action.

Delivery of drugs, vectors' design and production, pharmacokinetics of siRNAs are the fundamental steps that have to be considered in designing a therapy based on siRNAs, once the correct molecule and the correct target have been identified. Modeling is a useful tool to understand the underlying phenomena and to facilitate the design of novel vectors and production processes (Lamberti, 2015; Siepmann and Siepmann, 2008).

Aim of this review is to analyze the available literature and to investigate the complex approaches used to describe the delivery of siRNAs. Future work might need to use difference equations for the typical logistic growth equation (i.e. eq.7) which has been used in biological and pharmaceutical phenomena (Dokoumetzidis and Macheras, 1997; May, 1976). In this case, the dynamics of the system can be really rich and in some cases unpredictable (Dokoumetzidis et al., 2001). The first section will focus on a mathematical model that describes the fate of siRNAs once administered, and emphasizes the importance of the use of carriers for the drug delivery. The following section focuses on the production of suitable nano vectors for siRNAs delivery, with a particular emphasis on the mathematical modeling of production processes. The use of hydrogels as delivery matrices, and the related mathematical problems, is the subject of the subsequent section of the review. Once these vectors are released in the blood stream , their fate is accounted for, with particular emphasis on the so-called margination phenomenon. In this case, the 'modeling' tool has to be considered as an in-vitro model, which is a different approach respect to the proper in-silico models proposed in the other sections of this review. The same in-vitro approach is adopted in the following section, where the use of bio-reactors is reported, as a potential source for pharmacokinetics of siRNAs

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administration. Last section is devoted to the mathematical modeling of pharmacokinetics of siRNAs vectors in the human body.

Altogether, this paper provides a full view of the siRNAs delivery problem, which is the last obstacle to be overcome before gene therapies become a reality for the wellness of human beings. The last word about siRNAs delivery is still to be spoken, however – in our opinion – the present review shows how the research is directed towards the right direction.

2. The role of mathematical modeling

2.1 siRNA delivery

siRNAs can be systemically or locally administered to different tissues/organs. Among them, skin, lungs, eyes, nervous system, digestive system, vagina, and the inner coronary wall are potential targets for local administration (Vicentini et al., 2013). In particular, quite often, *siRNA*s are intended to hinder cells hyper-proliferation as it occurs in tumors (Farra et al., 2010; Farra et al., 2011) and in artery restenosis (Dapas et al., 2009; Werth et al., 2010). This is a pathological condition that may occur after the percutaneous transluminal angioplasty (*PTCA*) treatment. Restenosis consists in the partial/total re-occlusion of the artery lumen mostly due to smooth muscle cells hyper-proliferation (Davia et al., 2009).

In case of systemic administration (vascular route), *siRNAs* targets can be the same but the delivery process is much more complex due to the necessity of getting to the proper site of action from the systemic circulation. In this case, the delivery process can be subdivided into two distinct phases as depicted in Figure 1: the external one and the cellular one (Bartlett and Davis, 2006). While systemic administration involves both of them, local administration and *in vitro* delivery are affected only by the second phase.

One of the most important aspects of the external phase is represented by the size of the *siRNA*-vector complex (*SVC*). When *SVCs* are smaller than 6 nm, they are rapidly eliminated by renal excretion, while bigger SVSs (150–300 nm) are taken up by cells of the monomolecular phagocytic system, mainly present in the liver and in the spleen. *SVCs* with size falling in the range of 30–150 nm are found in the bone marrow, heart, kidney, and stomach (Gao and Huang, 2013). In addition, it was reported that red blood cells (*RBCs*) affect the transport and distribution of micro-particles in human microvasculature (diameter < 100 μm) (D'Apolito et al., 2015). Indeed, due to collisions with *RBCs*, micro-particles are pushed towards the artery wall where *RBCs* are almost not present. This radial movement, called “waterfall effect”, is at the basis of the phenomenon known as margination, the mechanism by which particles distribute along vessel radius to the wall in a size and shape depending manner. In particular, D’Apolito (D'Apolito et al., 2015) showed that 3-µm spheres marginated more efficiently than 1-µm spheres and that smaller spheres did not significantly marginate. Although *SVCs* extravasation (Figure 1) implies *SVCs* contact with the artery wall and the

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subsequent wall adhesion (these are two distinct phenomena occurring in series), it is clear that extravasation cannot occur unless *SVCs* undergo margination. In light of these considerations, it is clear that a reliable deliver strategy should rely on chimeric systems where *siRNA* nano-vectors are embedded into micro vectors. The idea is that the micro-vector allows margination and once the process is completed, it should break into nano-vectors to allow efficient extravasation.

Once the external phase has been successfully completed, *SVCs* have to be internalized by crossing the cellular membrane (Figure 1). At this step, nano-vectors have the very important tasks : (i) shield the effect of the electrostatic repulsion between siRNA/cell membrane and (ii) bypass the hydrophilic nature of *siRNAs* that impedes the crossing of the cell membrane hydrophobic layer. In case of lipoplexes, *SVCs* get to the cytoplasm by a partial cellular membrane fusion whereas polyplexes are internalized by unconventional endocytic pathways not requiring dynamin or caveolin (enzyme and protein responsible for endocytosis in eukaryotic cells) (Gao and Huang, 2013). As soon as *SVCs* reach the cytoplasm, they are sequestrated by endosomes and their escape from such vesicles may be a limiting step in the efficiency of the whole gene silencing process.

For instance, the use of pH-sensitive polymers (Sardo et al., 2015), nano-gels, or carbonate apatite and the application of photochemical internalization can enhance endosomal escape. Once escaped from endosomes, *SVCs* have to release the *siRNA* into cytoplasm where the antisense strand is up-taken by the cellular protein complex termed *RISC* (*RNA*-induced silencing complex), while the sense strand is discarded and does not take part to the silencing process (Posocco et al., 2015; Scaggiante et al., 2011). The antisense strand drives *RISC* to the target *RNA* that is bound via a perfect complementarity and, subsequently, degraded.

2.2 Mathematical modelling

2.2.1 Release mechanisms

On the basis of the mechanisms outlined in Section 2.1 regarding siRNA delivery, it is evident that, among the different aspects that can rule drug release kinetics (Grassi and Grassi, 2014), drug (= *siRNA*) diffusion inside the vector and drug dissolution (*i.e.* transition from drug solid phase to drug solution) are of limited relevance. On the contrary, drug – vector interactions, vector swelling/erosion and vector shape/size can play a predominant role.

siRNa-vector interactions can be due , for example, to electrostatic effects, hydrogen bonding, lipophilic interactions and other not specific non-covalent interactions (Grassi and Grassi, 2014). Whatever the specific kind of interaction, the development of a mathematical model can take advantage of the approach proposed by Singh and co-workers (Singh et al., 1994), who studied the drug polymer-interaction inside

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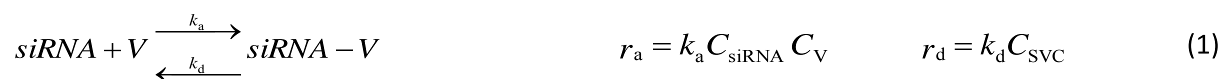
matrix systems. It is reasonable to assume that the formation/destruction of the *siRNA* – vector complex (*SVC*) obeys to the following general equation:



$$siRNA + V \underset{k_{\mathrm{d}}}{\overset{k_{\mathrm{a}}}{\rightleftharpoons}} siRNA - V \qquad r_{\mathrm{a}} = k_{\mathrm{a}} C_{\mathrm{siRNA}} C_{\mathrm{V}} \qquad r_{\mathrm{d}} = k_{\mathrm{d}} C_{\mathrm{SVC}} \qquad (1)$$

where k_a and k_d are the association and dissociation constants, respectively, *V* indicates the vector, *siRNA-V* is the complex (*SVC*), r_a and r_d arethe association and dissociation rates, respectively. C_{siRNA}, C_v, and C_{SVC} are the concentration of *siRNA*, vector, and *siRNA*-vector complex, respectively. k_a and k_d are, typically, temperature dependent but for *in-vivo* conditions is not so relevant, since temperature is essentially constant in both the external and cellular phases of the *siRNA* delivery (Figure 2). What is more important is that k_a and k_d may depend on small local pH variations, so that, for example, association can prevail in the external phase (where *SVC* has to be stable), whereas dissociation prevails in the cellular phase as it is well known that the endosomes environment is more acid than the external and cytoplasm environments. Moreover, *SVC* stability can be seriously compromised by the presence of albumin, the most abundant blood protein that can lead to *SVC* dissociation (Cavallaro et al., 2014; Grassi et al., 2010; Sardo et al., 2015).

Despite the swelling process is very important for delivery systems based on polymeric matrices as it represents the real engine of the drug release kinetics, in case of siRNA delivery the swelling role is essentially connected to the erosion of micro/macro-vectors. Erosion, due to chemical and/or physical reasons, can take place on the surface (surface or heterogeneous erosion) or it can affect the whole vector body (bulk or homogeneous erosion) (Miller-Chou and Koenig, 2003; Siepmann and Göpferich, 2001b). Bulk erosion is usually due to a rapid swelling caused by the body fluids uptake that, in turn, can cause a hydrolytic or enzymatic degradation of the vector structure (typically a three-dimensional polymeric network). On the contrary, surface erosion can be due to chemical (hydrolytic or enzymatic) and/or physical reasons (hydrodynamic conditions of the external physiological aqueous fluid) and it is associated to a slow swelling process. In particular, for physically cross-linked polymeric systems, surface erosion simply implies chains disentanglement (amorphous polymers) that is preceded by crystal unfolding in case of semi-crystalline polymers (Mallapragada and Peppas, 1997). Thus, surface erosion is usually connected to a slow nano-vectors release while bulk erosion gives origin to a fast nano-vectors release. In this context, Knipe and co-workers (Knipe et al., 2016) showed an interesting example of a chimeric delivery system for the oral release of siRNA targeting *TNF*-α, an inflammatory cytokine that is a common clinical target of inflammatory bowel diseases. The chimeric delivery system consists of micro-gels (size < 30 μm) composed of poly(methacrylic acid-co-N-vinyl-2-pyrrolidone) (PMANVP) crosslinked with a trypsin–degradable peptide linker. PMANVP micro-gels contain *siRNA* loaded poly-cationic nano-gels (2-(diethylamino)ethyl metacrylate) (size ≈ 120 nm) that proved to facilitate both cellular uptake and endosomal escape. PMANVP

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matrix was designed to collapse around nano-gels in order to protect them from degradation in the stomach (pH 2 – 4). In the intestine (pH 6 – 7.5), on the contrary, PMANVP matrix swells allowing matrix degradation due to the uptake of intestinal fluids containing different enzymes such as trypsin. Consequently, nano-gels can be released and internalized by cells thus resulting in a considerable *TNF*-α knockdown in murine macrophages.

Recently, Soares and Zunino (Soares and Zunino, 2010), dealing with drug release from a polymeric stent coating, built up an interesting mathematical model able to discriminate between bulk and surface erosions via the evaluation of the Thiele modulus (Λ):

$$\Lambda = \frac{D^{\infty}}{L^2 \tilde{k} \rho_w^{\infty}} \qquad (2)$$

where ρ_w^{∞} is the water density, $\tilde{k}$ is the hydrolysis rate constant, L is a characteristic length of the micro/macro vector (diameter) and D^{∞} is the diffusivity of water into an intact and dry polymer. High Λ values mean that bulk erosion is prevailing (sigmoidal mass loss), whereas low Λ values denote that surface erosion becomes the leading mechanism (zero-order mass loss).

2.2.2 *In vivo* and *in vitro* experiments

Malphettes and Fusseneger (Malphettes and Fussenegger, 2006), relying on mass balance and kinetic rate laws, converted biochemical *RNA* interference pathways into a set of ordinary differential equations that describe the dynamics of *siRNA*-mediated *mRNA* degradation in mammalian cells. In particular, they assumed that degradation can be properly described by the Michaelis-Menten scheme (Michaelis and Menten, 1913):

$$SRC + mRNA \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} SRC - mRNA \xrightarrow{k_{cat}} SRC + CmRNA \qquad (3)$$

where *SRC* is the *siRNA-RISC* complex, *SRC-mRNA* is the activated complex formed by *siRNA-RISC* complex and *mRNA*, *CmRNA* is the cleaved *mRNA* (reaction product), k_1 and k_{-1} are the direct and the inverse reaction constants while k_{cat} is the cutting constant. It is evident from eq. (3) that the authors assumed a multiple turnover mechanism *i.e.*, once released from the cleaved *mRNA* target, *SRC* can process further targets. In addition, they supposed that k_{-1} is negligible while *mRNA* has *n* different *siRNA*-specific target sites sharing the same value of k_1 and k_{cat}. Thus, they got the following powerful expression for the *mRNA* degradation rate R_{deg}:

$$\frac{\mathrm{d}[CmRNA]}{\mathrm{d}t} = R_{\deg} = k_{cat} \frac{nk_1 [siRNA_0][mRNA]}{k_{cat} + nk_1 [mRNA]} \qquad (4)$$

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$$\frac{\mathrm{d}[mRNA]}{\mathrm{d}t} = -R_{\mathrm{deg}} - k_{\mathrm{adeg}}[mRNA] \qquad (5)$$

where the square brackets represent the molar concentration of species, k_{adeg} is a kinetic constant related to the unspecific *mRNA* degradation, and $siRNA_0$ is the initial *siRNA* concentration in the reaction environment.

Bartlett and Davis (Bartlett and Davis, 2006) realized a mathematical model, composed by twelve ordinary differential equations, to describe the entire process of *in vivo siRNA* delivery and action at the cellular level (*mRNA* degradation). In particular, the authors, focusing the attention on *siRNA* vectors represented by cationic lipids or polymers, proposed a model able to describe both *in vivo* and *in vitro* siRNA release. This model relies on five compartments, three of which represent the external phase of *in vivo siRNA* release, the remaining two are connected to the cellular phase (see Figure 2). Once administered by injection, siRNA-Vector complexes (*SVC*) can bind to plasma components (such as albumin) according to the binding and un-binding constants ($K^{\mathrm{P}}_{\mathrm{b}}$, $K^{\mathrm{P}}_{\mathrm{u}}$), can be eliminated according to the elimination constant ($K^{\mathrm{P}}_{\mathrm{el}}$) or it can be transferred to the tissues "*in the local vicinity*" of the target cells such as tumor cells. Interestingly, this compartment resembles, for its functionality, the gastrointestinal circulatory system compartment (GICS) introduced by Di Muria and coauthors for the simulation of small organic drugs with any administration route oral absorption (Di Muria et al., 2010).

The flux of *SVCs* towards the tissues *in the local vicinity* depends on the kinetics constant K_{t} and the quantity "*partition*" expressing the fraction of the initial dose that can arrive to the tissues in the local vicinity. The *SVCs* passage from the tissues *in the local vicinity* to endosomes indicates the shift from the external phase to cellular one and K_{int} is the internalization constant. Once crossed the cellular membrane, the model becomes, necessarily, much more complex as it has to describe the siRNA-mediated gene silencing mechanism. Inside endosomes, *SVCs* can be diluted due to cell division, can unbind ($K^{\mathrm{E}}_{\mathrm{u}}$) to get free *siRNA* (*S*) and free vector (*V*) or it can go to cytoplasm ($K^{\mathrm{E}}_{\mathrm{esc}}$). *S*, in turn, can undergo degradation ($K^{\mathrm{E}}_{\mathrm{S\text{-}d}}$), dilution due to cell division or it can be transferred to the cytoplasm ($K^{\mathrm{E}}_{\mathrm{S\text{-}esc}}$). Inside cytoplasm, *SVCs* can be diluted due to cell division and they can get free *siRNA* (*S*). *S* can bind to *RISK* to get the activated *RISK* (*RISK**) according to the binding and un-binding process governed by the constants $K^{\mathrm{C}}_{\mathrm{RISK\text{-}b}}$ and $K^{\mathrm{C}}_{\mathrm{RISK\text{-}u}}$. *RISK** can be diluted due to cell division, can undergo degradation according to the kinetics constant $K^{\mathrm{C}}_{\mathrm{RISK\text{-}d}}$ or it can react with the target *mRNA* to give *mRNA* cutting according to the classical Michaelis-Menten scheme working in multiple turnover mode. *Rm* is the activated complex between the *RISK** and the target *mRNA*, P indicates the reaction product, *i.e.* the cut *mRNA*. The model is completed by the two equations describing the reduction of the target protein *Pr* (eq. (6)) and the variation of target cells number *Z* (eq. (7)):

$$\frac{\mathrm{d}[Pr]}{\mathrm{d}t} = K^{\mathrm{Pr}}_{\mathrm{f}}[mRNA] - K^{\mathrm{Pr}}_{\mathrm{d}}[Pr] \qquad (6)$$

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$$\frac{\mathrm{d}Z}{\mathrm{d}t} = K_g Z\left(1 - Z/Z_{\max}\right) \tag{7}$$

where $K^{Pr}{}_f$ and $K^{Pr}{}_d$ are, respectively, the protein formation and degradation constants, K_g is the cells growth constant and Z_{max} is the maximum number of sustainable target cells. Eq. (7) represents the typical logistic growth equation used to model solids tumors growth.

One of the most important aspects of this model consists in recognizing that, due to cells division, the concentration of all the *siRNA*-associated species decreases at each division. In particular, dilution is set equal to the ratio of new cells divided by the total number of cells. Thus, if the cell number doubles in one day, the dilution would be equal to 0.5 and the concentration of all *siRNA*-associated species would be likewise reduced by 50%. In other words, *siRNA* associated species are diluted equally between the daughter cells after each cell division. Obviously, dilution does not affect other intracellular species as they are produced intracellularly by both of the daughter cells. This model proved to describe very well experimental data concerning *siRNA*-mediated gene silencing in cell lines (mice subcutaneous tumors generated by Neuro2A cells, mouse liver) expressing the luciferase gene.

3. Production of micro and nano vectors for siRNAs delivery

Previous section emphasized the relevance of vector properties in the siRNAs delivery. Therefore, production of suitable delivery vectors is the mandatory first step to successful therapies based on siRNA' uses (Barba et al., 2015).

3.1 Vector size

Usually, the vectors size is in the order of nanometers to assure their absorption through biological membranes. In principle, large vectors (in the order of microns) embedding nano vectors can be produced to provide a further shield to the nano vectors. In addition, this allows the increasing of the shelf life of pharmaceuticals and makes viable the administration via oral route (*i.e.* the most used one for drug administration, even if it is not very likely that it is used for siRNAs administrations due to their sensitivity to external stimuli, unless further protection is given to nano vectors containing siRNAs). The micro and nano vectors, in turn, can be embedded in a larger matrix (such as hydrogel-based matrices) with the aim of realizing well-defined drug release kinetics. However, the ultimate vector size is on the nano scale, since it has to travel through the blood stream in capillaries (diameters less than one micron) towards the therapeutic targets.

3.2 Vector nature

Vectors can be obtained working with polymeric excipients as well as with lipid molecules. Vectors obtained using polymers are known as PNPs (Polymeric Nano Particles), and they are produced both as

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microcapsules/matrix systems and as polyplexes (complexes between polymers and siRNAs). Vectors based on lipid molecules are known as LNPs (Lipid Nano Particles), and they can be lipid-siRNAs aggregates or vesicles made of a double layer of lipids with an aqueous core in which the siRNAs can be contained. These structures are also known as liposomes, and currently they are the most adopted vectors for siRNAs delivery (Bochicchio et al., 2015).

3.3 PNPs production via solvent evaporation from emulsions

The most adopted process to produce PNP is the solvent evaporation from (multiple) emulsion(s) (Bochicchio et al., 2017). In this process, the first step consists in the production of an emulsion (usually O/W = Organic phase in Water phase), with polymer and its solvent (organic solvent), and water with a suitable surfactant (to promote the micelle formation). Drugs can be embedded in the organic phase if they feature a hydrophobic behavior or in the water phase if they are hydrophilic. In case of siRNAs, they have hydrophilic structures, therefore they would be in the water phase. Further emulsions can be carried out to get multi-layered particles as final product.

Once the emulsions are produced, they are subjected to an evaporation step, during which the organic solvent evaporates causing the polymer solidification and then the production of particles embedding the drug. The design of the subsequent layers and the choice of materials (polymers, solvents, tensioactives), along with the selection of the operative conditions (energy supply for emulsions, solvent evaporation conditions), allowtailoring to tailor not only the structure of the particles but also their drug loading/release properties.

Processes based on solvent evaporation from emulsions are largely used in the production of pharmaceuticals and nutraceuticals. However, a deep understanding of their fundamentals still lacks. The success of their application relies on the experience of operators and improvements are often obtained by trial- and -error procedures. The mathematical modeling of the two main steps described above can help both increasing the process knowledge, by defining suitable correlations between operative variables and product quality, and designing the production protocols more efficiently by minimizing energy supply and solvents waste.

3.3.1 Emulsions production

The first step is the obtainment of micelles (or emulsions). Modeling this step requires the knowledge of the phase thermodynamics and the description of the kinetics of micelle formation. Several models are available in literature. One modeling approach is based on Population Balances (PBs) (Shchekin et al., 2016; Starov et al., 2010), which take into account the aggregation/disaggregation of tensioactive molecules to produce a stabilized layer (with the hydrophobic tails of the molecules into the organic phase and the hydrophilic heads into the water phase). Another approach is based on the Molecular Dynamics Simulation (MDS), which, however, is still inadequate to give a useful view of what happens, since problems with

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reasonable computational costs have length-scales limited to nanometers and time-scales limited to microseconds (the integration time-step being of the order of femtoseconds, at least initially) (Shillcock and Lipowsky, 2007). There is an uprising method for the simulation of these systems, defined as Dissipative Particle Dynamics (DPD), which considers small fluid elements as its fundamental units. As a result, the dynamical behavior of micron-sized amounts of matter can be simulated for microseconds and, sometimes, for milliseconds. For example, the behavior of a nanovector-DNA complex to be used against cancer was studied with the DPD method (Ding and Ma, 2013).

All these modeling approaches, to the best of our knowledge, are still far to be used in process design and management, since they are not yet validated against experimental data and are not in easy-to-be-used form for practical applications (*i.e.* too many equations, too many parameters and consequently too much computational time).

3.3.2 Solvent evaporation

In principle, the solvent evaporation step, during which the PNPs are formed by polymer solidification and the drug is (or should be) embedded in the particles, is much easier to be modeled. Indeed, the well-known transport phenomena of solvent diffusion and evaporation, polymer solidification, particle shrinkage or swelling, water and drug diffusion – hopefully limited – are involved. In the past, some attempts were made to describe the solvent evaporation process (Li et al., 1995a; Li et al., 1995b). The approach, even if appears promising, was not pursued lately, most probably because of the lack of experimental data suitable for model validation. A more recent review on the topic (Li et al., 2008) reports both the mechanistic approach, already mentioned, and the use of engineering correlations, which has weak physical foundations but it is really easy to be used. Therefore, the most used approach for the description of the solvent evaporation from multiple emulsions is to correlate the parameters of the product (encapsulation efficiency, drug loading, particle size) to the process parameters (energy supply, amount of tensioactive) via engineering correlations, which , however, are predictive only within the investigated range of parameters (Dalmoro et al., 2010).

3.4 LNPs production via breaking of double layer lipid film

Liposomal structures, on micro and nano scale, can be produced by a vast array of traditional and novel procedures. However, the most common method involves the preparation of a thin lipid film made of two layers of lipids that mimic the biological membranes, and the breaking of this film, which causes the fragments closing to give vesicles. The reforming to give curved structures is due to the balance between elastic energy of the bilayer and the excess energy of the curved edge. The modeling approach to describe the bilayer behavior consists in the energy balance accounting for external supplied terms (stirring, ultrasonic energy, and so on) (Barba et al., 2014). A properly tuned model allows estimating (and potentially predicting) the vesicles size as effect of the rate and amount of energy supply.

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4. siRNAs delivery from hydrogels

4.1 Why hydrogels?

Gene therapy is conquering more and more room in the pharmaceutical field due to its proved *in vitro* ability to cure diseases otherwise untreatable. However, the *in vivo* delivery of small interfering RNA (siRNA) has to deal with and overcome several extracellular and intracellular barriers to successfully lead siRNAs to the "active site" in the cell (RNA interference), where they can conduct their therapeutic effect. The use of vectors capable to carry, protect, and target these molecules is therefore essential. Nowadays the vectors can be classified in viral and non-viral based vectors, the latter being safer than the first but still less efficient in delivery (Yin et al., 2014). Whatever siRNA-vector system is chosen, it can be useful to either obtain a sustained release or localize the delivery. This can be achieved by entrapping, physically or chemically, the siRNA-vector system in polymers forming hydrogels (Nguyen et al., 2013; Segovia et al., 2015). Hydrogels can be formed by crosslinking or by self-assembly from hydrophilic polymers of natural, synthetic, or semi-synthetic origin. The release of encapsulated nucleic acid (NA) can be controlled via the hydrogel pore size, network degradation rate, or through physical interaction or chemical conjugation of the vector system with hydrogel components (Germershaus and Nultsch, 2015).

4.2 Materials

4.2.1 Natural polymers

Natural polymers (such as collagen, chitosan, alginate, hyaluronic acid, gelatin, or fibrin) are widely used to produce NA delivery systems. These systems can be based on agarose, which is a FDA approved polysaccharide extensively used as matrix system to deliver pharmaceuticals, i.e. it was used as matrix to deliver a siRNA-liposomal transfection complex to non-delimited cutaneous wounds (Thanik et al., 2007). Collagen is a biocompatible and safe material and, due to these properties, it is a favorable matrix for in-site drug delivery. An example of its application is the collagen use as carrier to test the feasibility of localized and sustained delivery of siRNA for in vivo gastric cancer inhibition (Peng et al., 2016). Collagen efficacy as injectable hydrogel system for localized and sustained delivery or siRNA was demonstrated, also in comparison with other biodegradable materials (Krebs et al., 2009). Among the natural polymers, chitosan is the most used in siRNA delivery, because it is particularly attractive for clinical and biological applications due to its low toxicity, biocompatibility, and biodegradability. Chitosan is characterized by long term retention and was used as hydrogel for sinus injections to deliver siRNAs to silence the expression of VEGF (vascular endothelial growth factor) (Cao et al., 2015). Chitosan hydrogels were used as local delivery systems of siRNAs in several applications, ranging from tumor growth therapy (Han et al., 2011) to periodontitis prevention (Ma et al., 2014). An excellent critical review summarizing the recent progresses on biodegradable and injectable hydrogels produced from natural polymers for biomedical application is

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proposed by Li and coworkers (Li et al., 2012). The gelation and biodegradation, which are two key factors to affect the cell fate or drug delivery, are highlighted.

4.2.2 Synthetic polymers

The main advantage in the use of synthetic polymers as carrier for siRNA is the possibility to improve the polymer characteristics by chemical or composition modifications, or by functionalization, which usually reduces the risks of immunogenicity, too. Therefore, it is not surprising that a series of synthetic polymers were used to either encapsulate or deliver nucleic acids. An application of an engineered biodegradable system is the covalent incorporation of cationic linear polyethyleneimine (LPEI) into photocrosslinked dextran (DEX) hydrogels through a biodegradable ester linkage (Nguyen et al., 2013). The main novelty of this system consists in the ability to control the release of the LPEI/siRNA complex over time. In fact, LPEI could electrostatically interact with siRNA, which is maintained within the hydrogels; and the degradation of the covalent ester linkages determines the siRNA release rate. A biodegradable polymer composed by poly-D,L-lactic acid with randomly inserted dioxanone and polyethylene glycol (PLADX-PEG) was tested as a delivery system for siRNAs to study a safe method for siRNA to permeate through the cytoplasmic membrane into cells (Manaka et al., 2011). Other examples of synthetic polymers used in siRNA delivery are: a cell penetrable nanopolyplex assembled injectable poly(organophosphazene) hydrogel to induce sustained intra-cellular delivery of siRNAs with high gene silencing efficiency via cell penetration (Kim et al., 2013); polymers obtained by the incorporation of a cationic polymer into a D,L-lactide-co-glycolide (PLGA) matrix to produce nanoparticles loaded with siRNAs (Patil and Panyam, 2009); and hydrogel scaffold based on polyamidoamine (PAMAM) dendrimer cross-linked with dextran aldehyde to increase the nanoparticle (containing siRNA) stability (Segovia et al., 2015). An alternative approach to the localized and sustained delivery of siRNA at the target site is the production of nanofiber scaffold. In fact, the efficacy of siRNA-PCLEEP (poly(caprolactone-co-ethylethylene phosphate) nanofibers in controlling fibrous capsule formation was investigated (Rujitanaroj et al., 2013).

4.2.3 Bioresponsive hydrogels

As mentioned above, the release of siRNA from hydrogels is controlled exclusively by the hydrogel or vector properties, such as the diffusion or the degradation process. Recently, a new kind of delivery systems was proposed: the bioresponsive-hydrogel based systems (Germershaus and Nultsch, 2015). These systems are characterized by the use of polymers whose properties change in response to external stimuli, such as pH or temperature variations, light or enzymes presence. Due to this characteristic, the siRNA release from these matrices can be modulated by changing the external conditions in order to reach the desired release rate. One of the first examples of localized and long-term delivery of siRNA using hydrogel with thermosensitivity and biodegradability properties is the Kim approach, which uses an injectable polyplex hydrogel composed by a low molecular weight polyethyleneimine conjugated with a thermosensitive and

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cationic polymer that has a cleavable ester linkage able to form an hydrogel from a solution at body temperature to control the release rate (Kim et al., 2012). Bioresponsive hydrogels, used for the siRNA release, are those proposed by Huynh (Huynh et al., 2016), which are photodegradable PEG–DPA (poly(ethylene glycol)–di(photolabile acrylate)) macromere synthesized to produce a hydrogel system that can activate the release of siRNA by UV light exposure. This result was achieved by inserting, during the polymerization process, two ortho-nitrobenzyl (ONB) photolabile groups. An interesting application of bioresponsive hydrogels in siRNA release in that proposed by (Zhao et al., 2013), who prepared a transdermal patch containing siRNA for the treatment of a fibro-proliferative disorder of the dermis characterized by the presence of a pressure-sensitive adhesive hydrogel.

4.3 Mathematical modeling

Depending on the hydrogel type and concentration, as well as on the interaction between the siRNA-vector and the hydrogel matrix, the release mechanism can be governed by diffusion, erosion, or a combination of both. The mathematical modeling of these complex drug delivery systems can aid to discriminate among the relative importance of such phenomena, and reach a deeper understanding and speedup of the design phase.

4.3.1 Diffusion driven release

When the siRNA-vector is not chemically bond to the hydrogel and the mesh size of this last is large enough to allow molecular movements, the siRNA-vector can be released by diffusion, which is driven by concentration gradients. The diffusion-based processes can be relevant in particular when the siRNA-vector systems are small in size (nano-particles) and the hydrogels mesh size is large (more than 100 nm). Furthermore, even if this scenario is unlikely to happen, the mathematical treatise of the diffusion process is still of interest since the model is the basis also for the most common approaches to describe erosion based processes (see the next section).

It is quite common in literature to model diffusion in hydrogels with simple semi-empirical models rather than much more complex phenomenological-based models. This is because the mathematics of diffusion in hydrogels is quite complex since the diffusant has to move in a polymeric network whose mesh size depends on the hydration level. The hydration level in turn influences the system state (glass or rubber) and the internal stresses that can affect the molecules movements.

For this reason the Peppas' equation (Peppas, 1985), and its following modifications (Peppas and Sahlin, 1989), have gained a lot of attention:

$$\frac{M_t}{M_\infty} = kt^n \qquad (8)$$

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M_t is the mass of drug released at time t, M_∞ is the total drug loading; k and n are constant to be determined. Due to the approximations of this equation, it is valid for the first 60% of the normalized drug release (Siepmann and Peppas, 2011). With this very simple equation, it is possible to describe most of the experimental release data, but not many phenomenological consideration can be done. However, a theoretically based hypothesis to explain the capability of this equation of describing the majority of drug release profiles from HPMC based systems was formulated by Rinaki et al. (Rinaki et al., 2003). This hypothesis was based on the observation that the displacement of the solute in disordered media is not proportional to the time and the percolation model for non-classical diffusion effects, which is essentially built for disordered media, was successfully used to describe the release kinetics from hydrogels' matrices.

Another alternative for the description of release profiles is based on the empirical use of the Weibull function:

$$\frac{M_t}{M_\infty} = 1 - \exp(-a \cdot t^b) \tag{9}$$

a and b are the model parameters. b represents the so-called "shape parameter" (Langenbucher, 1972), because its value determines the curve's shape. This was presented as an empiric model, in which the parameters are not physically based, thus at the beginning it has been criticized and its use has been limited in the establishing of in vivo/in vitro correlations (Costa and Lobo, 2001). Later, as a result of the study of Papadopoulou et al. (Papadopoulou et al., 2006), who related the exponent of the Weibull function (Eq. 9) to the exponent of the power law (Eq. 8), a link between its value and the diffusional mechanisms of the release was found: $b < 0.75$, the release follows Fickian diffusion; $0.75 < b < 1$, indicates a combined mechanism, frequent in release studies; $b = 1$, the concentration gradient in the dissolution medium is the driving force for the release (first-order release); $b > 1$, indicates that a complex mechanism governs the release process (Papadopoulou et al., 2006).

A mechanistic model based on diffusion (coupled with surface erosion) in swellable matrices was proposed by (Siepmann et al., 1999) and, in its wake with several improvements, other models have been devised (Caccavo et al., 2015; Chirico et al., 2007; Kaunisto et al., 2013; Lamberti et al., 2011). All these approaches consider the mass transport equations for water (i=1) and drug (i=2) in a swellable polymer (i=3), accounting for the network sieving effect using water concentration dependent diffusivities (D_i) and relating the deformations, swelling and erosion, to mass balances. The system of partial differential equations describing these phenomena has the following formulation:

$$\begin{cases} \rho \dfrac{\partial \omega_i}{\partial t} = \boldsymbol{\nabla} \cdot \left(\rho\, D_i\, \boldsymbol{\nabla} \omega_i\right) \quad i = 1{,}2 \\ \omega_3 = 1 - (\omega_1 + \omega_2) \end{cases} \tag{10}$$

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Where ρ is the system density, ω_i the species mass fraction. The eqs. (10) have to be solved, numerically, with proper Initial and Boundary Conditions (IC and BCs). The swelling (and surface erosion) description can be achieved by either considering the system mechanics (i.e. (Xu et al., 2013)) or using a mathematical artifice capable to relate mass balances to volumetric deformations.

4.3.2 Erosion driven release

When the siRNA-nanovector is chemically linked to the hydrogel structure, its release occurs following the hydrogel degradation. In particular, hydrogels can erode following two mechanisms, *i.e.* the surface and bulk erosions. The former interests the outer layer of the system, while the latter happens homogeneously inside the matrix.

For both surface and bulk erosions some semi-empirical models exist (reported in (Siepmann and Siepmann, 2008)), similar to eq. (8). However, also in this case phenomenological-based models should be preferred.

The surface erosion, as previously reported, can be introduced in a mass transport model (eq. 10) through mathematical artifice (i.e. sequential layer model (Siepmann and Peppas, 2000) or by the Arbitrary Lagrangian-Eulerian (ALE) method (Caccavo et al., 2015; Kaunisto et al., 2013)) usually driven by mass constraints.

The bulk erosion is dealt with diffusion-reaction based models, since the bulk erosion is often generated by reactions inside the matrix that lead to the polymer degradation. An example of these models is presented by Dokoumetzidis et al. (Dokoumetzidis et al., 2008), where the dissolution is considered as a reaction between the undissolved species and the molecules of the dissolution medium. Under this hypothesis the dissolution rate is determined by the concentration of the undissolved species and the solubility can be seen as the concentration at the reaction equilibrium. This model was proved to be more flexible and particularly suitable to simulate the in vivo conditions (Dokoumetzidis et al., 2008). Later, a modification of this model, where the kinetic constant is considered time-dependent, was used to describe the behavior of supersaturated dissolution curves, where the solubility is higher at the beginning and drops with time (Charkoftaki et al., 2011).

The mass conservation for the i-th species, similarly to eq. (10), in which the rate of mass production of the i-th species (r_i) is considered, has the following form:

$$\rho \frac{\partial \omega_i}{\partial t} = \boldsymbol{\nabla} \cdot \left(\rho \, D_i \, \boldsymbol{\nabla} \omega_i \right) + r_i \tag{11}$$

The main difference of eq. (11) with respect to eq. (10), aside from the reaction related term (r_i), is in the diffusion coefficient D_i. Indeed, beside the dependence of D_i on the water content (polymeric mesh size variation), a dependence on time has to be considered. This is because the bulk erosion increases the

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porosity of the matrix, thus allowing for easier molecules transport. The time dependence can be either assigned (usually with exponential functions) or estimated with a Monte Carlo based approach (Grassi and Grassi, 2005; Lao et al., 2011; Siepmann and Göpferich, 2001a). The time-dependence of the parameters is the drastic consequence of the non-classical kinetics of diffusion limited reactions, which is called “fractal kinetics” (Dokoumetzidis and Macheras, 2011). Processes like dissolution and release, which are based on diffusion principles, have been modeled with this type of kinetics (Dokoumetzidis et al., 2004; Macheras and Dokoumetzidis, 2000).

An example of the behavior of diffusion-controlled, diffusion plus surface erosion controlled, and diffusion plus bulk erosion controlled processes is depicted in Figure 3.

The diffusion in a swelling system has been modeled according to eq. (10) and following the approach of (Caccavo et al., 2015): the swelling velocity of the domain boundaries, as previously mentioned, can be derived from local mass balances.

The surface erosion can be accounted for by considering a shrinking velocity, which can be modeled with the introduction of an additional parameter: $k^{surface}$ [m/s]. This represents the inward velocity of the system boundaries: the greater this value, the faster the surface erosion. As it can be seen from Figure 3 (left) the erosion surface contributes positively to the drug release, reduces the diffusion path-length (increasing the concentration gradient) and therefore increases the rate of drug release. The limiting case is $k^{surface}$=0, where the system is dominated by species diffusion without the surface erosion contribution.

On the other hand, the bulk erosion, in Figure 3 (right), can be modeled with the same equations (eq. (10), disregarding the presence of reactions, for the sake of simplicity), imposing $k^{surface}$=0 (no surface erosion) and considering diffusivities depending on water (i=1) concentration (network sieving effects) and time (bulk erosion effect) of the type: $\mathrm{D_i = D_{i0}(\omega_1) \times \exp(k^{bulk}t)}$. As for the surface erosion, also the bulk erosion, with respect to a simple diffusion driven process ($\mathrm{k^{bulk}} = 0$ in Figure 3 (right)) contribute to the drug release. In this case, the erosion does not affect the diffusion path-length (excluding system breakage) but it increases the structure permeability and therefore the release rate.

5. siRNAs vectors in blood circulation

In last years, substantial efforts have been made in the design and functionalization of nano therapeutics for drug delivery capable of overcoming all the biological barriers present in human microcirculation. In fact, after the injection within the blood stream and before reaching the diseased cells, drug carriers have to penetrate the barriers present in the body, undergoing a long journey, even defined as an *odyssey* in a recent paper (Nichols and Bae, 2012). This odyssey deals with the transport by blood flow via the microcirculatory network where the drug carriers interact with blood cells, margination, interaction with

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endothelial glycocalyx, adhesion, extravasation, transport in tumor interstitial space and, eventually, cellular uptake and drug release (Figure 4). The use of nano-scale systems as a powerful tool to deliver drugs in the treatment of diseases such as cancer and other inflammation-related diseases (Blanco et al., 2015) has been driven by the process known as enhanced permeability and retention effect (EPR) (Matsumura and Maeda, 1986). This effect refers to the increased permeability of the vessels wall due to signals from pathological tissues, which leads to a preferential passage of circulating drug carriers, and to an increase of the retention inside tissues, caused by impaired lymphatic drainage. Nevertheless, the enhanced transport due to the EPR effect is not sufficient to overcome all the barriers to drug transport.

The first step of the journey is the drug carrier transport in blood flow in microcirculation. After injection, a drug carrier travels through the circulatory system, where it can be blocked before reaching the disease site by several competing processes, such as opsonization (*i.e.* the molecular mechanism by which a pathogen is chemically modified to be suitable for ingestion) and uptake by phagocytosis, clearance in organs such as kidneys and liver, and extravasation into non-diseased tissues (Arruebo et al., 2007; Blanco et al., 2015). Furthermore, blood proteins can damage drug carriers. To mitigate all these problems, the carrier surface can be modified by using hydrophilic polymers, such as poly(ethylene glycol) (PEG), widely used in drug delivery strategies to make long-circulating “stealth” carriers (Nag and Awasthi, 2013). The second step is particle margination, the mechanism according to which particles migrate along the vessel radius to the wall, in analogy with the behavior of platelets, which concentrate in the cell-free layer (CFL) near the wall, due to their rigidity, allowing a rigid-body flipping motion near the wall (Zhao et al., 2012). Independently of the targeting mechanism (*i.e.* in passive targeting the EPR effect promotes drug delivery while in active targeting drug carriers are functionalized with receptors specific for tumor cells), drug carrier margination propensity is essential to maximize the particle contact and adhesion to the vessel wall, adhesion step being strongly dependent on margination. It is worth highlighting that, even if margination and adhesion are two distinct phenomena, adhesion is sometimes considered the hallmark of margination. This results in the measure of margination in terms of particles adhered to vessel walls under flow (Namdee et al., 2013), not taking into account the flow-induced migration and distribution of particles, which are key factors in margination mechanism. Another difference between the processes is that while margination is mainly affected by bio-mechanical and geometrical properties (D'Apolito et al., 2015; Tan et al., 2013), adhesion may depend on other factors such as the target, biochemical signaling, and receptor/ligand density (Doshi et al., 2010). In light of these remarks, the two phenomena should be independently investigated, taking into consideration the relevance of margination as a fundamental prerequisite to achieve effective particle adhesion. Concerning margination, it is worth considering the peculiar nature of blood. In fact, at the micro-scale of carriers, blood cannot be considered as a homogeneous fluid but as a concentrated suspension of deformable red blood cells (RBCs), which represent the large majority of blood cells. In the capillary flow occurring in microvessels, RBCs are subjected to wall-induced lift enhanced by their

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deformability. A shear-induced diffusion due to cell-cell collisions counterbalances this transport, leading to the formation of a CFL in proximity of the wall and a RBC-rich core in the center of the vessel (Kumar and Graham, 2012a). In turn, the flow of drug carriers in the RBC-rich core is affected by their collisions with RBCs. This results in a two-steps process: (i) the shear-induced slow diffusion (D'Apolito et al., 2015) and (ii) the “waterfall effect”, *i.e.* a fast lateral displacement in the proximity of CFL (Vahidkhah and Bagchi, 2015), likely due to the inhomogeneity of RBC spatial distribution that creates bypass regions through which particles can rapidly reach the CFL. The combination of the slow shear-induced diffusion and the fast waterfall effect leads to the size and shape dependent phenomenon of margination (Kumar and Graham, 2012b; Vahidkhah and Bagchi, 2015). The big majority of the experimental studies investigated micro- and nano-particle margination in microcirculation-model systems, but they have been carried out in absence of RBCs (Gentile et al., 2008; Lamberti et al., 2014; Toy et al., 2011). In recent years, some progress has been made in the direction of more physiologically relevant conditions, both in commercially available systems and in home-made devices. However, despite the former (*e.g.*, Cellix, Fluxion, and Ibidi) requires a large amount of sample and does not reproduce the flow conditions present *in vivo* (large vessel model *vs* microvasculature), the big majority of the home-made devices are not able to mimic microvasculature geometrical features, such as bifurcations and diverging/converging vessels (Dokoumetzidis and Macheras, 2003; Kwak et al., 2014; Lamberti et al., 2014). The development of microfluidics techniques has contributed to the understanding of blood flow in human microvasculature, providing more physiologically relevant models, and creating a novel research field known as *Blood-on-chip*. Microfluidics shows a number of advantages, such as the ability to use very small quantities of samples and reagents, and to carry out separations and detections with high resolution and sensitivity, low cost, short times for analysis, and small footprints for the analytical devices. In microfluidic channels the flow is completely laminar, allowing one to model fluid transport in a simple way and offering new capabilities to control concentration in space and time (Whitesides, 2006). Microfluidics is based on soft lithography techniques (Bryan et al., 2009) and allows creating glass and silicone rectangular microchannels (Tomaiuolo et al., 2011), which mimic the passage of RBCs in the microvasculature network (Tomaiuolo et al., 2011). A microfluidic-based physiologically relevant model of microcirculation was recently used to investigate the effect of RBC flow on particle margination as a function of particle size and shape (D'Apolito et al., 2015) and surface charge (Figure 5). It was found that margination is strongly enhanced by the presence of RBCs and that micro-particles outperform nano-particles in terms of margination, confirming previous literature results and the optimal dimension for margination (*i.e.* 2–3 µm) (Mueller et al., 2014; Namdee et al., 2013). The effect of drug carrier surface charge was investigated by using liposomes (i.e. LUV -large unilamellar veiscles). To date, LUVs are the most successful FDA-approved drug-carrier systems, thanks to the similarity of their morphology to that of cellular membranes, to their ability to incorporate different substances, and to the coupling with site-specific ligands (Bozzuto and Molinari, 2015). For instance, LUV are optimal candidates

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for the delivery of drugs based on nucleic acids (NABDs) such as siRNAs for gene therapy. It was found that neuter and cationic LUVs show a comparable margination propensity. This result does not exclude that the surface charge can play a significant role in the subsequent steps of carrier journey to their target (D'Apolito et al., 2017).

In fact, once the carrier is entrapped in the CFL, it can interact with the endothelial glycocalyx (GCL), a negatively charged hairy layer of proteoglycans and glycoproteins located on the inner surface of the vascular endothelium. Despite the efforts made so far to measure GCL by using several approaches, ranging from electron to confocal and two-photon laser scanner microscopy both *in vivo* and *in vitro*, the thickness of this fragile layer is still not well established. In fact, data available in the literature are strongly dependent on the methodological approach, which results in a wide range of thickness, from 500 to 3000 nm (Kolářová et al., 2014). Moreover, it seems that GCL thickness is strongly dependent on shear stress and on gradient of shear stress (Kolářová et al., 2014). In physiological conditions, GCL acts as a protective barrier, determining vascular permeability, and plays a key role in the vascular mechanotransduction of biomechanical stimuli, such as shear stress, into biochemical signals on the endothelial cells (ECs). Pathological conditions, such as systemic inflammatory states (*i.e.* in tumor), lead to a degradation of the GCL structure, resulting in an impaired permeability barrier function and enhancing the drug carrier transport across EC layer. It is also likely that GCL affects the interaction between drug carriers and endothelial cells. However, the mechanisms governing GCL barrier function, both in physiological and pathological conditions, are still not well understood (Calderon et al., 2011). The next step is extravasation which, in physiological conditions, refers to the passage of white blood cells from microvasculature to the surrounding tissues. In some pathological conditions, such as solid tumors, the permeability of the EC layer constituting the vessels wall tends to increase in response to signals from damaged tissues, allowing preferential passage of circulating drug carriers, and the impaired lymphatic drainage system ensures the retention inside tumor spaces (according to the EPR effect) (Cho et al., 2008). The dominant mechanism of particle extravasation is transport through the fenestrated tumor walls (*i.e.* area of retraction of ECs) and can be modelled by the Starling's equation, which describes how fluid flow across EC layer depends on both hydrodynamic and osmotic pressure (see in the following). However, Starling's equation does not explicitly take into account the presence of the GCL, which opposes an additional resistance to the passage of drug carriers through EC layer (Alphonsus and Rodseth, 2014). Moreover, under pathological conditions, GCL properties, such as charge and layer thickness, can vary with respect to the physiological ones, likely affecting drug carrier extravasation efficiency. Extra barriers to drug carriers are represented by the properties (*i.e.* density, heterogeneity, and cross-linkage) of the tumor extracellular matrix (ECM) and by the degree of packing of tumor cells (Nichols and Bae, 2012). Thus, drug carrier transport in tumor interstitial spaces can be likely seen as a combination of diffusive and convective particle transport in a porous medium (Lieleg et al., 2009; Ramanujan et al., 2002). Moreover, the high pressure in the interstitial

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space of the tumor can hinder extravasation. The last two steps of the journey are cellular uptake and drug release. There are different mechanisms, either specific or nonspecific, by which carriers can adsorb onto cell surface or be subject to endocytosis. Drug carriers can also fuse with the tumor cell membrane or exchange lipid components. All the aforementioned processes are strictly dependent on the type and site of the tumor and on the drug carrier properties, such as size, shape, and surface charge (Calderon et al., 2011; Lieleg et al., 2009).

Taking advantage of microfluidics principles, organ-on-chip platforms are being developed as promising, ground-breaking technology, aimed at the fabrication of devices able to reproduce the geometrical, mechanical, and biochemical microenvironment of a certain tissue (Chan et al., 2013). The emerging technology of organ-on-chip is based on the idea of filling the gap between traditional 2D *in vitro* models of cellular tissues, which normally use a monolayer of a single cell type in a Petri dish, and animal models, that pay the price of being expensive, time consuming, complex to analyze, and often not able to correctly represent human physiology. The technologies at the base of organ-on-chip systems are microfluidics, bioMEMS (bio MicroElectroMechanicalSystems) and biomimetics. BioMEMS indicate miniaturized mechanical and electro-mechanical elements that are made using the techniques of microfabrication suitable for biological applications, while biomimetic concerns systems acting as imitation of nature elements with the aim of solving complex human problems (Patek, 2014). Recently, many steps have been done following the organ-on-chip philosophy, providing models for several organs, such as lung, liver, kidney, intestine, and blood-brain barrier.

6. siRNAs vectors transportation in tissues

''Bioreactors,'' a term generally associated with classical industrial bioprocesses such as fermentation, was initially used in Tissue Engineering (TE) applications to describe little more than simple mixing of a Petri dish. Over time, bioreactors used in TE research evolved, not only for the in vitro generation of various tissues (skin, tendons, blood vessels, cartilage, and bone), but also to serve as defined model systems supporting investigations on cell function and tissue development (Martin et al., 2004; Wendt et al., 2009). Moreover, in the context of cancer progression, invasion and metastasis, bioreactors have been utilized to miniaturize the natural counterparts, to introduce relevant forces, or to create a controlled environment to foster the assembly tumor-like tissues (Hutmacher et al., 2010). As a matter of fact, it has been underlined that culture in bi-dimensional (2D) or tri-dimensional (3D) systems differently affects sensitivity of cancer cells to compounds used in cancer treatment or to immune effector cells specific for human tumor associated antigens. For example, colorectal cancer (CRC) HT-29 cells were cultured on collagen sponges in static conditions or in perfused bioreactors and treated with 5-Fluorouracil (5-FU), a frequently used but often clinically ineffective chemotherapy drug (Hirt et al., 2015). In that case, it was observed that perfused

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cultures resulted in the rapid generation of Tissue Like Structures characterized by more homogeneous cellular organization and significantly higher cell yields, as compared to static cultures. Moreover, it was observed that the 5-FU effect was sensibly different between 2D and 3D cultures, in terms of number of apoptotic cells.

Hoffmann and coauthors utilized a miniaturized bioreactor based on a hollow fiber perfusion technology to study the effect of diclofenac on primary human liver cells compared with the same cells grown in monolayer 2D culture. Equally, a different behavior between the systems was found (Hoffmann et al., 2012). Bioreactor cultures displayed a period of stable diclofenac elimination and metabolite formation rates starting within the second or third day of diclofenac application. Diclofenac elimination and formation rates of the major metabolite 4'-OH-diclofenac were initially higher in monolayer cultures than in the bioreactor. However, metabolic rates in 2D cultures continuously decreased and showed generally lower levels towards the end of the diclofenac application period than in bioreactor cultures.

(Kirstein et al., 2008) fabricated a bioreactor able to allow the growth of cells in which it is possible to infuse chemotherapy to simulate concentrations measured in biological samples (*i.e.* human plasma). Throughout the described system, they tested the effect of infused gemcitabine on cell death for the anchorage-dependent MDA-MB-231 cell line.

However, to our knowledge, the drug effect is always evaluated directly on the target cells, without considering the drug transfer between plasma and tissue through vascularized tissues. In this context, a bioreactor able to simulate the physiological drug delivery process through capillary vessels would be an important tool to gather transport data *ex-vivo* and under controlled conditions for an effective model prediction of general drug delivery.

To this end, Carfì and coauthors developed a system able to mimic a vascularized biological tissue under physiological conditions (Carfì Pavia et al., 2017). The system is composed by a double flow perfusion bioreactor and a double structured scaffold (Figure 6). The double flow bioreactor is composed of a glass tube where the scaffold is located halfway in length. A recycled current of medium is able to perfuse the outer zone of the scaffold from the top to the bottom.

Externally, the prepared scaffold features a porous and interconnected structure for the 3D growth of the cells. Internally, it features a well-integrated tubular shape scaffold (vessel-like) for endothelial cells (ECs) growth. It was shown that the cells can grow into the porous scaffold, giving rise to a 3D tissue (Figure 7A). Moreover, it has been reported that if the endothelial cells are seeded into the vessel-like scaffold, they organize themselves into a well differentiated vessel structure (Figure 7B) (Carfì Pavia et al., 2010). The peculiarity of this construct, coupled with its insertion into a device, which allows cell growth under

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dynamic conditions, make it employable as a real route to mimic a vascularized biological tissue under physiological conditions.

By monitoring the drug in the inner fluid and the outer tissue uptake, it is possible to evaluate the parameters governing the drug transport from plasma to tissue, thus providing an essential element for drug delivery modeling in a cheaper and well controlled *ex-vivo* system.

7. Pharmacokinetics of siRNAs vectors

As abovementioned, the delivery of siRNAs to tumor site has shown to be ineffective because of the extremely rapid degradation of siRNA molecules both in the extracellular and intercellular environments. A further limitation to the efficacy of this treatment is that siRNA molecules do not have the ability to cross the biological membranes (Wang et al., 2010; Williford et al., 2014). Therefore, the delivery of intact siRNA molecules to the target site via direct systemic administration is practically unfeasible. The most investigated solution to this problem is the exploitation of vectors in which the siRNA molecules can be loaded. The original concept of *magic bullet* was coined long ago, at the beginning of the 20th century, by the Nobel-prize winner Paul Ehrlich who first introduced the notion of ideal vectors capable of selectively targeting the disease source within the body (Winau et al., 2004). Despite this *magic* has not yet been achieved, the ideal vector should be able to contain the siRNA molecules, protect them from degradation, and avoid their release in sites other than the target one. It should cross all the biological membranes to get from the blood circulation to the extracellular matrix, and finally to the target tumor cells.

Often, it is not sufficient to reach and be selective for a specific target site, specifically when the siRNA treatment targets a tumor. The tumor mass has very peculiar physical properties and an extremely heterogeneous structure. This is described in a number of publications (Au et al., 2001; Au et al., 2016; Baxter and Jain, 1989, 1990; Sefidgar et al., 2014). These papers discuss how the physical characteristics of the tumor hamper the siRNA vector distribution within the tumor mass. After the tumor generation, the first critical stage in the tumor growth is the formation of the blood supply network. Without it, the tumor cell ensemble would not be able to grow beyond a 2 mm diameter (Nishida et al., 2006). After the angiogenesis, the growth can be quite rapid and the tumor physical properties deeply differentiate. Just to mention few concepts, which are not within the purpose of this review, tumors have an irregular and abnormal vasculature (Nagy et al., 2009) and a high interstitial fluid pressure (Heldin et al., 2004). Furthermore, with tumor expansion, the core tends to become necrotic due to the absence of working blood vessels in the region. These issues, together with several other sources of tumor heterogeneity, are responsible for physical obstruction to drug delivery within the tumor mass.

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At the target, vectors should release siRNA at the optimal rate requested by the specific disease under treatment. It is therefore necessary to consider the pharmacokinetics (PK) at two levels: (i) the vector pharmacokinetics, which is important at the systemic level and targets the tumor cells, and (ii) the siRNA pharmacokinetics, which is mainly intracellular and targets the loading of siRNA on the RISC (*i.e.* RNA-induced silencing complex).

In addition to this, an ideal vector should be degraded and/or eliminated from the organism. An explicative example of this type of vectors is described in (Ceelen and Levine, 2015), chapter: "Development of Drug-Loaded Particles for Intraperitoneal Therapy". There, the authors describe a therapy successfully tested *in vivo* in animals for targeting peritoneal tumors. Polymer microparticles are able to penetrate the tumor, reach the inner region, and subsequently release the drug at different rates so to guarantee an ideal drug concentration versus time profile. Finally, the particles are degraded and eliminated.

The modeling of these processes is particularly complex as it requires a systematic approach. The vector PK can be described with a classic compartmental PK model or even a physiologically-based PK model. These models are based on the simplified representation of the body (or a specific sub-region of it) as a structure of perfectly mixed (*i.e.* homogeneous) and interconnected compartments. Mathematically, these models are based on a system of ordinary differential equations with suitable initial conditions (see also Figure 8A). The major issue dealing with these models is the equations parameterization, which can be obtained by non-linear regression of a set of experimental PK data (Abbiati and Manca, 2016).

The vector PK at the target compartment of the previous model can be used as input profile for a more sophisticated model of the tumor site. These models are particularly complex. They generally feature a system of partial differential equations (see also Figure 8B), which are solved with software for the finite element analysis as detailed in (Au et al., 2014). Here, the constitutive transport equations are based on the Darcy's and Starling law, and on the transport phenomena driven by diffusion (promoted by concentration gradients) and convection (promoted by pressure gradients). An interesting book useful for these studies, written by (Keener and Sneyd, 2009), focuses on the mathematical description of biological processes.

Once the vector concentration profile in space and time, within the target site, has been determined, the last step consists of modeling the siRNA pharmacokinetics. Intercellular trafficking processes are extremely complex and assorted, and occur at the micro- and nano-scales (Meneksedag-Erol et al., 2014; Wang et al., 2010). Consequently, they are still not fully understood and a number of studies are conducted to increase the knowledge of the endocytosis, cell membrane fusion, endosome transport, and exocytosis processes, just to cite some major areas of research. Despite some modeling papers are available in the literature (Ding and Ma, 2013; Dinh et al., 2007; Jorge et al., 2012), PK models capable of describing the intracellular path of the siRNA molecules up to the loading in the RISC complex are not yet available.

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8. Conclusions

The uses of siRNAs as novel drugs is gaining attention from the researchers over all the world because of their therapeutic potential. The contributions of engineers to the development of actual therapies are in the description, using physical (in-vitro) or mathematical (in-silico) models, of the behavior of living beings (in-vivo). The field is growing fast, and the availability of theoretical tools such as mathematical and physical models can foster the development of effective therapies based on siRNAs reducing the time requirements for bench-to-the-bedside evolution. The present review aimed to present the state of the art about this side of the problem. In the end, the main conclusion that can be drawn is that these tools (mathematical and physical models) can be useful and their development is worthwhile.

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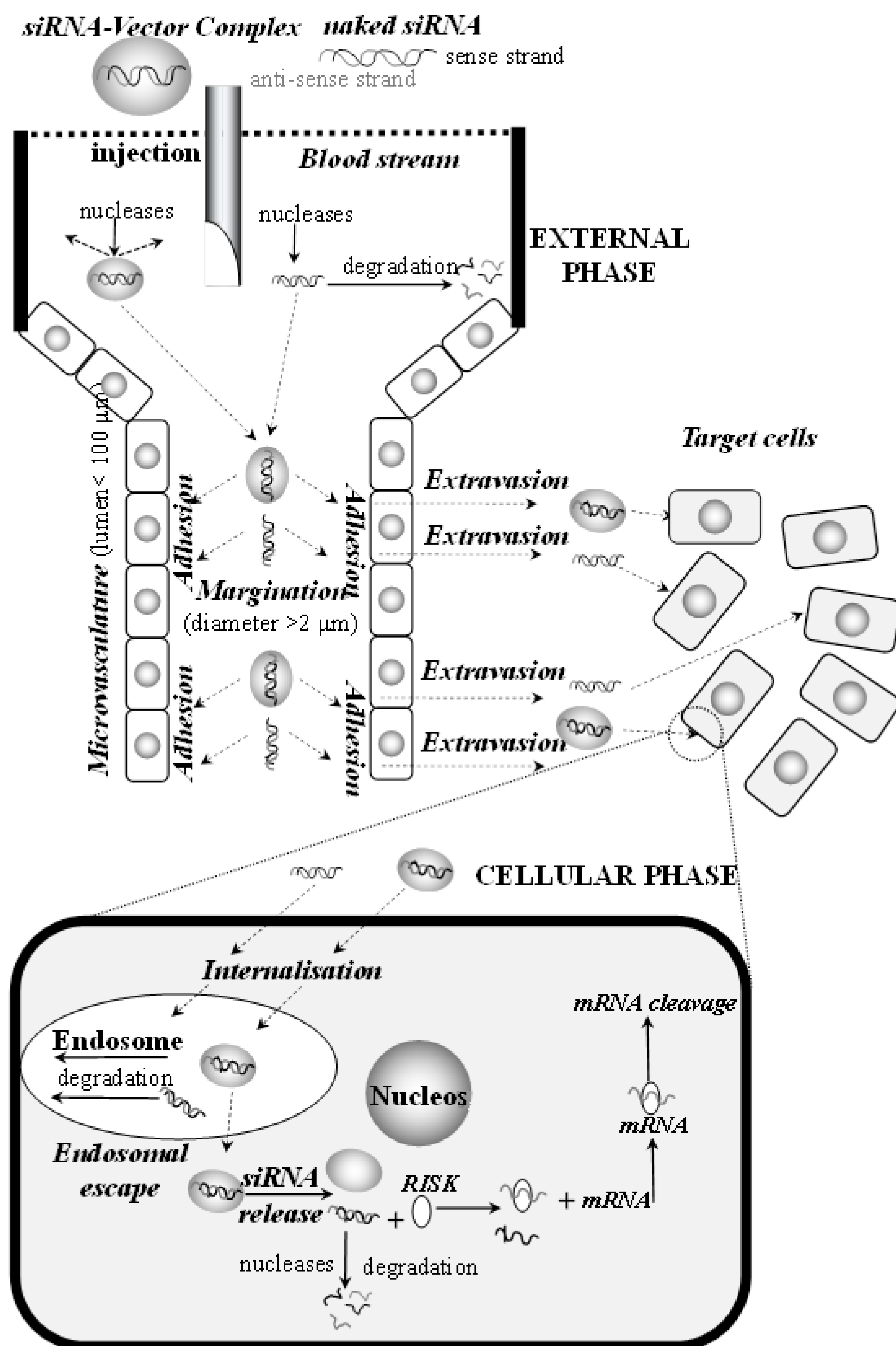

Figure 1. Schematic representation of the siRNA delivery process.

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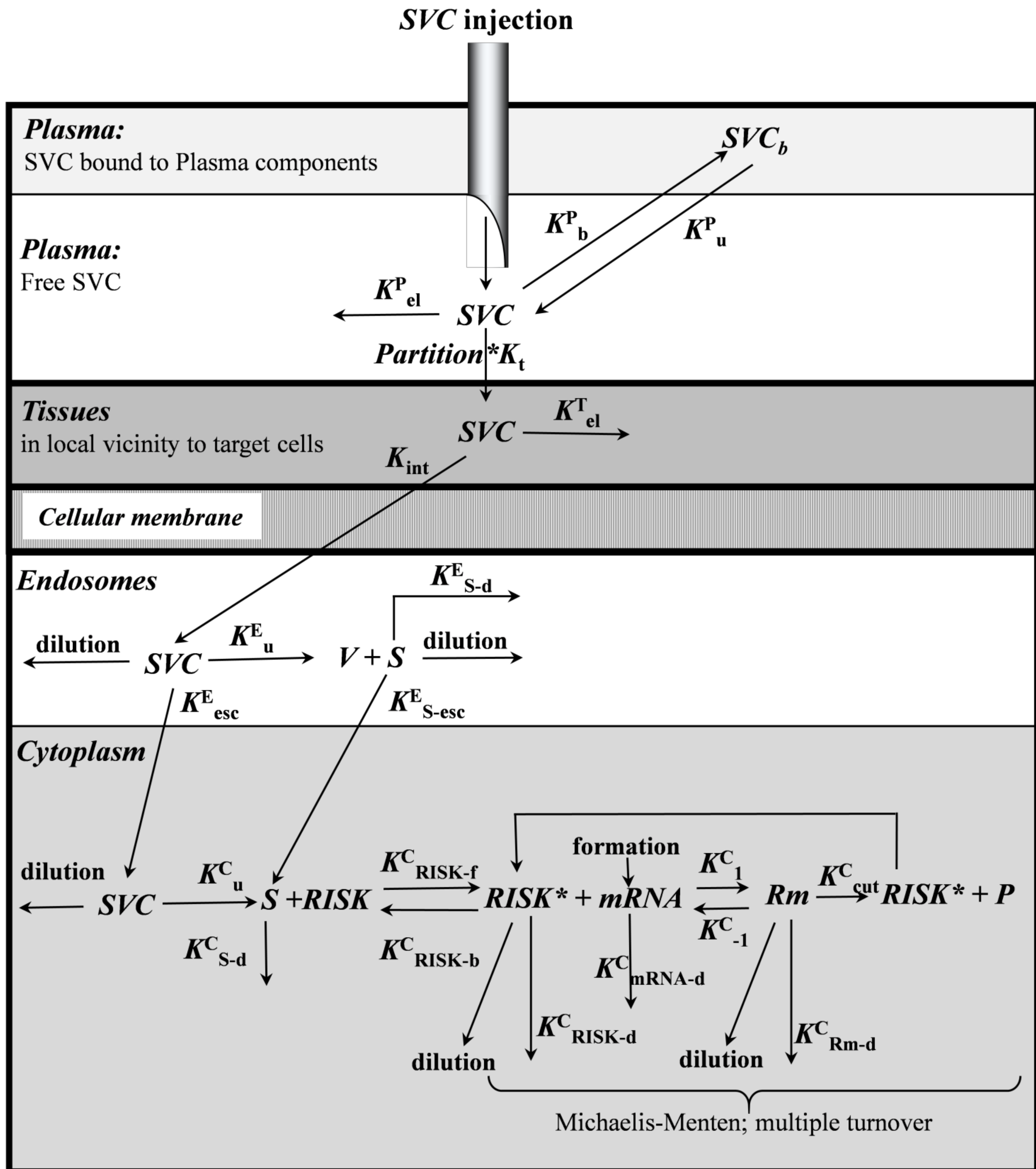

Figure 2. Schematic representation of the kinetics model for siRNA delivery and cellular action according Bartlett and Davis (Bartlett and Davis, 2006). Rm is the activated complex made up by the activated RISK and the target mRNA while P is the product of the Michaelis and Menten reaction, i.e. the target mRNA cut into two parts. This model can simulate both in-vivo (all five compartments) and in vitro (last three compartments) experiments.*

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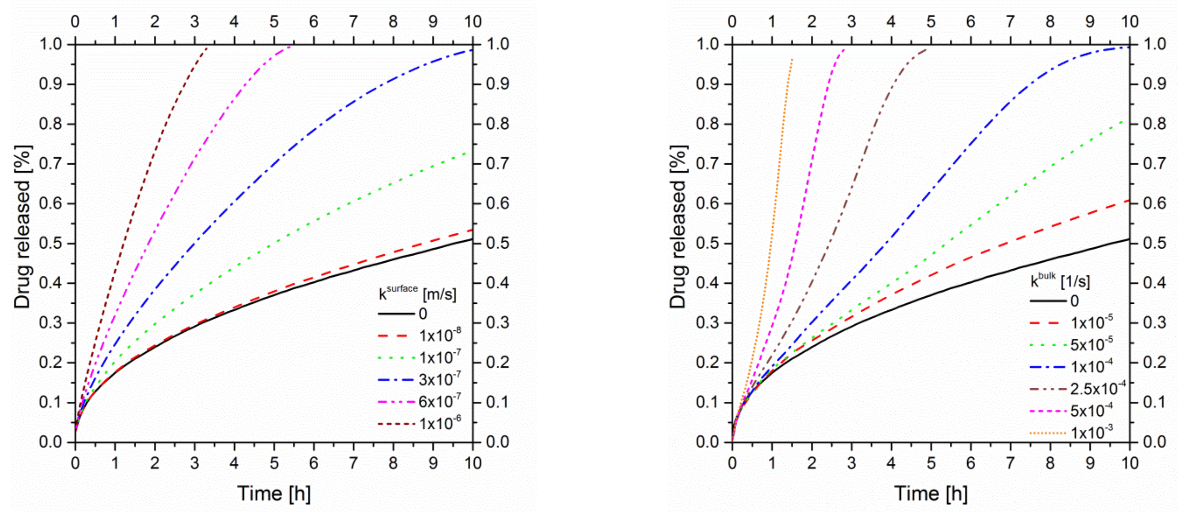

Figure 3. Diffusion (black solid line in both graphs), diffusion plus surface erosion (left), and diffusion plus bulk erosion (right) driven processes.

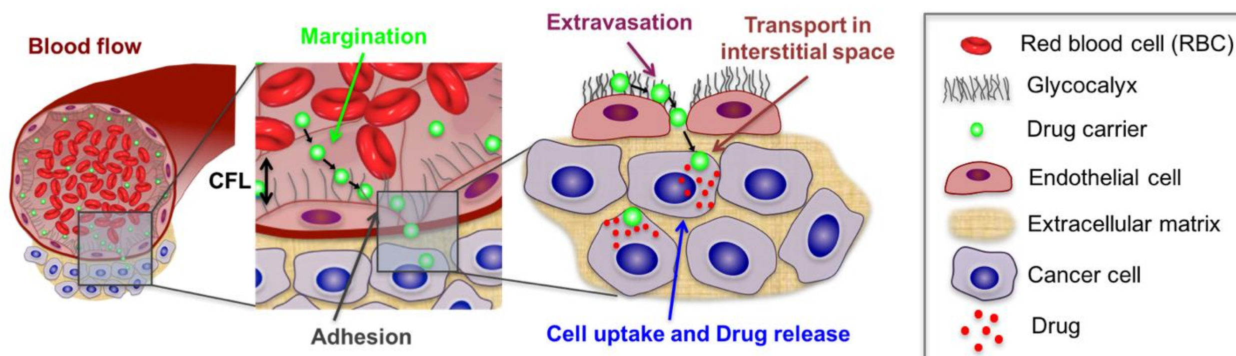

Figure 4. Cartoon of drug delivery pathway from blood flow to release.

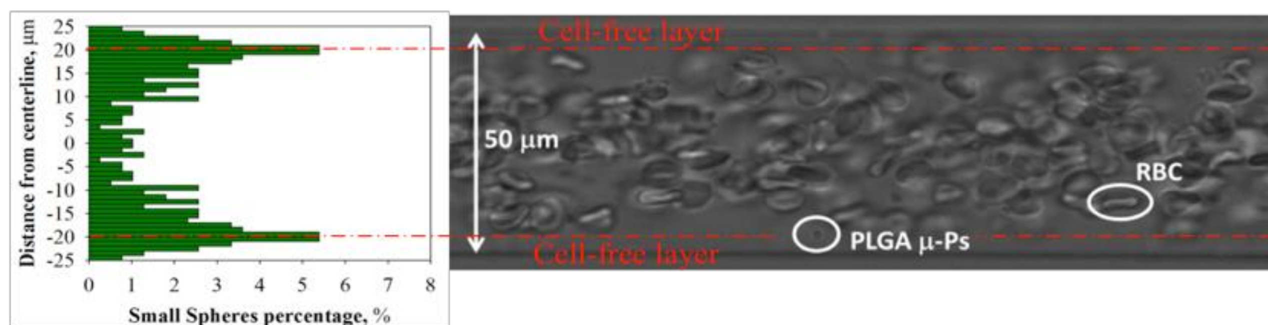

Figure 5. Margination of poly(lactic-co-glycolic acid)(PLGA) m-particles (spheres with diameter of 1 mm) in a suspension of RBCs flowing in a 50 mm glass microcapillary. Adapted from (D'Apolito et al., 2015).

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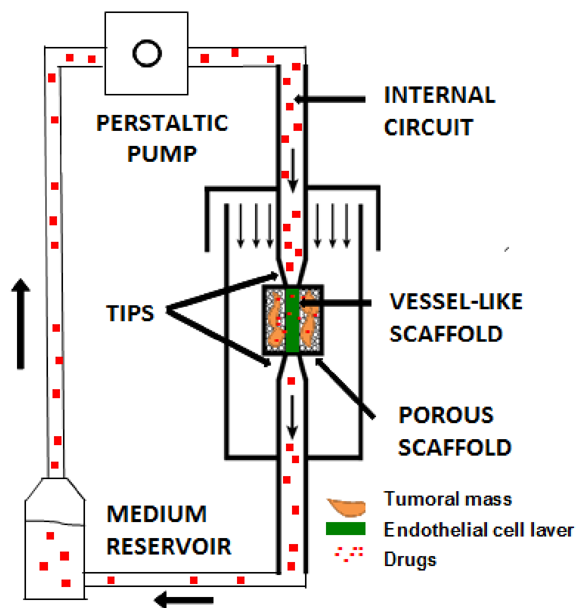

Figure 6. Schematic representation of the system

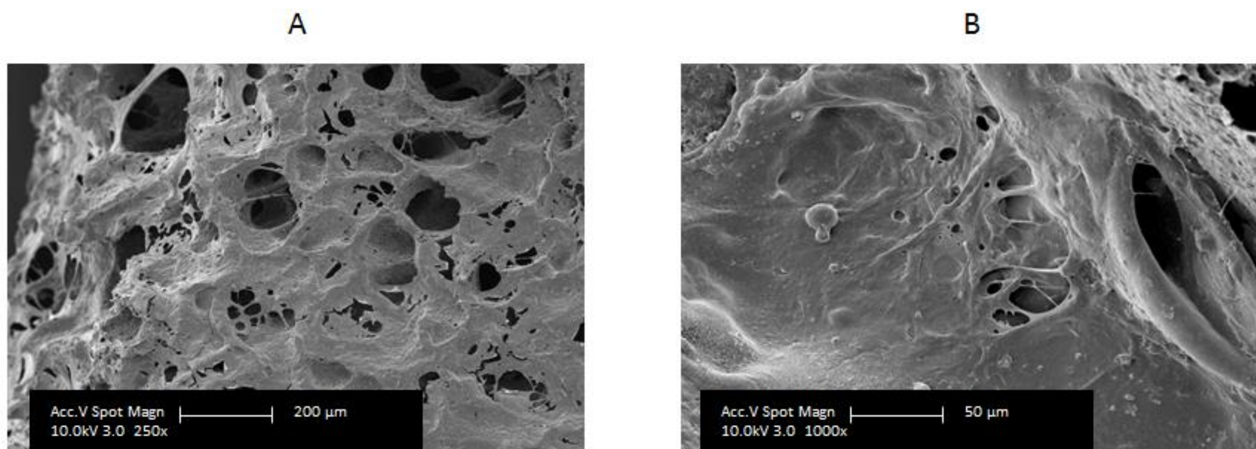

Figure 7. A) external surface of the composite scaffold with fibroblast grown 18 days; B) internal surface of the vessel-like scaffold with ECs grown 21 days.

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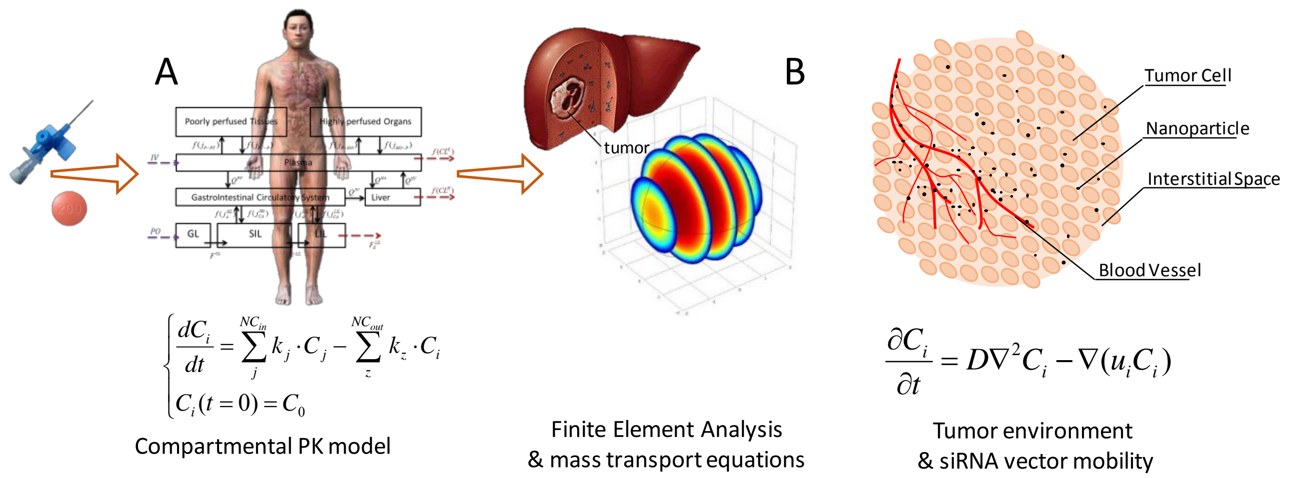

Figure 8: Sequence of the modeling approach for siRNA vectors. At the body scale (panel A), drug distribution is simulated with a compartmental PK model, based on a set of ordinary differential equations defining mass balances. At higher detail (panel B), the PK modeling of vectors in tumor masses can be carried out by finite element simulations. Mass transport equations allow simulating the drugs motility promoted by specific mechanisms (e.g., diffusion or convection). Tumor masses are extremely heterogeneous. siRNA vectors exploit blood vessels extravasation and can move in the interstitial liquid until cell internalization or elimination.