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ENHANCEMENT OF COLLOIDAL STABILITY OF DRUG NANOCARRIERS IN COMPLEX BIOLOGICAL ENVIRONMENT

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ABSTRACT. In recent years the development of novel approaches for the production of nano-formulations (nanocarriers) for efficient transport of drug molecules in living systems offers a wide range of biotechnology applications. However, despite the remarkable developments of recent synthetic methodologies, most of all nanocarrier's action is associated with a number of unwanted side effects that diminish their efficient use in nanomedicine. This highlights some critical issues in the design and engineering of nanocarrier systems for biotechnology applications, arising from the complex environment and multiform interactions established within the specific biological media. Many questions still remain open for what concerns the way to deal with the complexity of the biological processes involved. What is the minimal number of key parameters (and their related key factors) required to describe behavior of nanomaterials without sacrificing the complexity of the identified process? In other words, what is the "minimum level of complexity" to assume in the theoretical and experimental models that may satisfactorily describe the nanocarriers (and nanomaterials) interaction with biological systems. Herein, we analyze relevant open questions with the aim of offering possible perspectives for the development of next-generation nanomaterials that are able to overcome the critical issues during their action in complex biological media.

1. Introduction

Complex biological systems (and humans in particular) have emerged from billions years of evolution and result from the long adaptation process to their specific environment. During their evolution time, they naturally developed different strategies to create (nano)materials with high structural complexity and high level of functionality. In contrast, the development of synthetic nanomaterials with controlled properties are limited by our current understanding of the biology of living systems. The development of nanocarriers technology for the efficient delivery of therapeutic drugs has experienced considerable expansion in recent years (Bozzuto and Molinari 2015; Chen *et al.* 2016). The design and engineer of novel functional nanomaterials has generated a variety of smart nano-carriers for the encapsulation and controlled delivery of therapeutics (Ishida *et al.* 2001, 2002; Lombardo *et al.* 2016a). More specifically, a variety of strategies developed in the last decades

employ engineered nano-carriers with desired physico-chemical properties that, exploiting a combination of a number of suitable soft interactions (Kiselev *et al.* 2013; Lombardo *et al.* 2019a), facilitate the transit through the natural barriers of biological systems from the point of administration up to the site of action the therapeutic drug (Ceresa *et al.* 2013; Bourgaux and Couvreur 2014; Lombardo *et al.* 2018). However, the intrinsic complexity of biological environments strongly influences the functionality of nanomaterial, and often complicates the effective use of nanocarriers (and nanomaterials) for therapeutic treatment (Allen and Cullis 2013; Chow and Ho 2013). This means that to a precision of the synthesis protocols very often do not correspond to a precision in the specific tasks to be performed. Furthermore, the understanding of nanocarriers interaction in complex biological systems still represent a big challenge in the research field of nanotechnology (Lombardo 2014; Lee *et al.* 2015; Alibakhshi *et al.* 2016). Theoretical and experimental investigations of real nanocarriers adopt interpretation models of that present a minimal complexity that often look unrealistic if compared with the complex environments of biological systems. This represent a critical issue in the design and engineering of functional materials in the field of biotechnology and nanomedicine.

Herein, we analyze some open questions with the aim of offering possible perspectives for the development of next-generation nanomaterials that are able to overcame the critical issues during their action in complex biological media.

2. Nanocarriers in nanomedicine and biotechnology applications: Achievements and critical issues

Recently, the design and fabrication of nanoparticle-based materials (or nanodevices) with integrated and enhanced properties have gradually gained a strategic importance in the field of biotechnology. One of the biggest challenges for the use of nanocarriers (and nanomaterials) for therapeutic treatment consists in the enhanced performance in diseased tissues and in the potential reduction of the side effects (Bozzuto and Molinari 2015). Various nanomaterials provide important benefits and new opportunities for the smart nanocarriers, including micelles, liposomes, dendrimers, solid nanoparticles, nano-emulsions and large variety of other nanostructured materials that are able to interact with cells and biological systems thus offering a great versatility in designing different functional (and targeting) concepts.

More specifically smart vehicles like lipid-based (Sackmann 1995; Lombardo *et al.* 2016b; D'Angelo *et al.* 2017) and synthetic polymer-based nanoparticles (Kopecek and Yang 2007; Hruby *et al.* 2015; Zhou *et al.* 2018), carbon-based (Bianco *et al.* 2005) materials and metallic nanoparticles (Adeyemi and Sulaiman 2015)have great potential in altering biological functions, as well as for drug delivery, gene transfection and (in vitro and in vivo) imaging applications. Finally, mesoporous silica nanoparticles (MSNPs), with their high surface area and the ability to modify pore size and surface chemistry, represent a new generation of "smart nanomaterials" for the development of innovative prototypes for the delivery of a variety of drugs combinations and other cargos to cells (Bonaccorsi *et al.* 2009; Li *et al.* 2012; Pasqua *et al.* 2016). Moreover the noncovalent interactions (such as the electrostatic, van der Waals, and hydrogen-bonding interactions) of the drugs with the nanocarrier's internal surface cause preferential adsorption of cargo to the MSNP, with

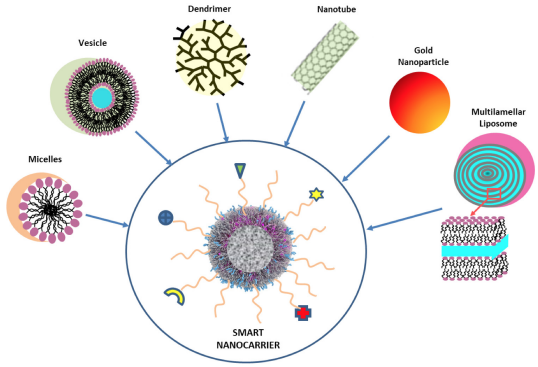

FIGURE 1. Sketch of the main nanocarriers systems for smart application in biotechnology.

Name	Treatment	Drug Formulation
Doxil	Breast cancer, Ovarian cancer, AIDS-related Kaposi's sarcoma, Myeloma	PEGylated liposomal DOX
DaunoXome	HIV-associated Kaposi's	DaunoXome
AmBisome	Visceral leishmaniasis, Fungal infection, Cryptococcal Meningitis	Liposomal amphotericin B
Myocet	Metastatic breast cancer	Non-PEGylated liposomal DOX
Marqibo	Acute lymphoblastic leukaemia	Liposomal vincristine

TABLE 1. Some liposome based nanocarriers approved by the U.S. Food & Drug Administration FDA.

loading capacities exceeding those of other more common drug delivery carriers (such as liposomes or polymer based conjugates)(Aiello *et al.* 2002; Morelli *et al.* 2011; Watermann and Brieger 2017). A sketch of the main nanocarriers employed for smart nanomedicine and biotechnology application is reported in Figure 1. Although recent development of novel nanocarrier systems with longer blood circulation time, only a limited number of them have been translated into clinics for applications (Anselmo and Mitragotri 2016; Liu *et al.* 2016). In 1995 the United States Food & Drug Administration (FDA) approved the first formulation (Doxil) encapsulating the cancer drug doxorubicin within a lipid-based nanocarrier. After more than 20 years the FDA in U.S. and the European Medicines Agency (EMA) in the European Union have approved in clinic several other nano-carriers formulations including liposomes, nano-suspension, polymer nanoparticles, nanocapsule, micelles, etc.. These approved formulations have been adequately evaluated and deeply optimized over the years. However, some critical issues connected with low efficiency and biocompatibility restrict the translation of many other proposed nano-carriers based medical approaches into the clinic (Liu *et al.* 2016). In table 1 some of the most important liposome based nanocarriers approved by FDA are reported.

3. Clearance process of nanocarriers and strategies to overcome biological barriers

It is known that although a main portion of nanocarriers have preferential accumulation in the tumor area, due to the EPR effect, the presence of biological (or physical) barriers in the body can affect the accumulation of therapeutic nanoparticles into the tumour tissues. Therefore a question arises in the scientific debate: how effectively current nanoparticles target drugs to diseased tissues ?. In this respect, a recent paper reviewed the literature about the nanoparticle drug delivery from the past decade and estimated that the median delivery efficiency is low - only 0.7% of an injected dose of nanoparticles ends up in a tumor (Wilhelm *et al.* 2016). Interaction of nanocarriers with blood proteins plays a crucial role in the tissue distribution and clearance process of intravenously injected liposomes. Nanocarrier clearance process involves, in fact, the absorption on the surface of naocarrier of the plasma *opsonins proteins* (that include various protein such as immunoglobulin, fibronectin, lipoproteins) and their recognition by the MPS, followed by excretion of the cargo at the hepatic level and its subsequent metabolism by Kupffer cells. In a second alternative way, liposome nanocarriers are metabolized by splenic macrophages, and after their accumulation, they are metabolized and eliminated by the target tissues. Size, surface charge and colloidal stability (Ishida *et al.* 2001, 2002) are the main factors affecting clearance process (by the MPS) via proteins opsonization. Generally, large negatively charged liposomes are eliminated more rapidly than small, positive (or neutral) charged nanocarriers (Bozzuto and Molinari 2015). A second clearance process, involved in liposome-based nanocarriers, is based on the action of the high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) contained in the blood. These lipoproteins interact with liposomes nanocarriers and causes lipid transfers (lipid depletion) and changes on the structure of liposomes surface and reduction of their colloidal stability, which is followed by the liposome destruction and release of the cargo to the plasma (Ishida *et al.* 2001, 2002). In order to hinder the clearance process caused the interaction of nanocarriers with blood proteins, antifouling surface (protein resistant) ligands such as poly(ethylene glycol) (PEG) and zwitterionic molecules are widely employed for to avoid nonspecific protein adsorption and cell adhesion before nanocarries reach the tumour sites. The aforementioned processes identify the key role of the physico-chemical properties of nanocarriers during the clearance processes. In this respect the design and engineering of the physico-chemical properties of novel nanocarriers allow a proper control over the structure-function relationship thus minimizing the RES sequestration of therapeutic compounds and unwanted side effects during drug delivery processes.

4. Modes of interactions of nanocarriers with biological systems

Upon their insertion in biological fluids nanomaterials undergo transformation that may profoundly alters their structure and properties. The main interactions of nano-materials with biological systems can be classified into three basic modes: chemical, mechanical, and electronic.

Inclusion of nanomaterials in biological environments often creates non-equilibrium systems that may lead to high chemical reactivity (chemical interaction) and adsorption capacities that may lead to phase transformations that include oxide formation, sulfidation,

degradation, and dissolution (driven by oxidation or hydrolysis) (Wang *et al.* 2016). Particularly important are the oxidative and reductive dissolution processes that cause the release of soluble ionic species that are often the primary drivers of adverse biological responses. Chemical interactions between nanocarriers surfaces and biological fluid phases include chemical adsorption of ions, small molecules, proteins and ligand exchange. Biomolecular adsorption, including protein corona formation, is expected to be particularly important. The subsequent physical transformations such as aggregation, dispersion, and deposition are also important. Among the possible transformations, dissolution is particularly significant for the biological response, since soluble dissolution products that co-exist with the solid phase have been implicated in the toxic responses for many nanomaterials (Wang *et al.* 2016).

Physical and mechanical interactions between nano-materials and soft biological structures are of special importance for high-aspect-ratio nanostructures, which can mechanically perturb soft cellular substructures such as plasma and lysosomal membranes. For example, long nanotubes have been implicated in adverse biological responses associated with cytotoxicity and frustrated cellular uptake (Wang *et al.* 2016). The presence of sharp edges can cause spontaneous penetration of cell membranes with low energy barriers and can lead to lipid extraction and membrane damage. Low-dimensional materials may cause mechanical stress, deformation, and damage when cells attempt to package large, stiff plate-like or fibrous structures into soft spherical lysosomes during cellular uptake.

Finally, nanomaterials can perturb biological process through electronic and surface redox interactions. Electronic and redox surface reactions can alter the electronic, optical, and magnetic properties of molecules and their ensembles by adding or removing electrons. Within a naonocarrier system those processes can be exploited if they are associated with correlated molecular reorganization processes such as assembly/disassembly (Fukino *et al.* 2017), transformation of ensembles, geometric changes, and molecular motions that are designed to be redox-responsive. In those cases, the permissive electron (or H) transfers between material surfaces and biomolecular redox couples in cells and tissues can perturb some essential biochemical pathways (or initiate new pathways) that lead to adverse outcomes (mediated by reactive oxygen and nitrogen species). One of the great advantages of redox-responsive devices and nanocarriers and nanomaterials is that they have the potential to be readily integrated into existing electronic technologies.

In Figure 2, the modes of interaction between nano-carriers and biological systems is reported. The arrows highlight the bi-directionality of the interactions, as the nanocarriers (and/or their nanostructure transformation) products induce biological responses while the biological environment induces chemical or physical material transformations.

5. Strategies to improve nanocarrier colloidal stability and circulation time

When injected into the blood circulation nanocarriers rapidly interact with the complex biological environment encountered. The clearance of circulating nanocarriers from the bloodstream, coupled with their high uptake by the MPS, represent an obstacle to any attempt at targeting to tumors. In order to preserve the optimal nanocarriers efficiency, the organism defenses must be circumvented by avoiding the nanocarrier recognition and

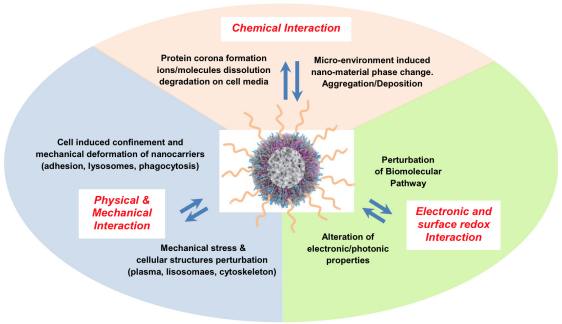

FIGURE 2. Modes of the synergistic interaction between nano-carriers and biological systems.

the consequent neutralization and elimination of the invading active drugs. The physicochemical properties of nanocarriers, such as size (and morphology), surface functionality (charge typology, stiffness), are the main parameters that can affect their biological clearance (Moore *et al.* 2015). Different strategies has been adopted to prolong the circulation time, while the relevant mechanisms of stabilization can be exploited to improve colloidal stability in the biological media (Lombardo *et al.* 2004a; Casadonte *et al.* 2010; Pasqua *et al.* 2019).

A first approach to prolong the release rate of entrapped drugs consists in the choice of drugs with enhanced hydrophobic character or by incorporating cholesterol lipids (Geng *et al.* 2014; Bozzuto and Molinari 2015). Due to its hydrophobic character, cholesterol preferentially interacts with the core region of the neutral membrane of liposomes nanocarriers, thus inducing a dense packing of phospholipids. (bilayer-tightening effect). This causes a reduction of their permeability and increases in vivo and in vitro stability, thus inhibiting their transfer to high-density lipoprotein (HDLs) and low-density lipoprotein (LDLs).

A second approach to stabilize liposome nanocarriers in solution consists in the inclusion of charged components that create a sensitive electrostatic surface charge (ζ-potential) that promote the interaction of liposomes with cells and prevents their aggregation and flocculation in solution. Some investigations indicate that negatively charged liposomes are less stable than neutral and positive liposomes when injected into the blood circulation, as they rapidly interact with the biological system subsequently to their opsonization with circulating proteins, thus inducing a rapid uptake by the MPS and possible toxic effects (Bozzuto and Molinari 2015).

Another important approach for the improvement of circulation times consists in conjugation to the surface of the liposome nanocarrier of natural (e.g. dextran, alginate, chitosan) or synthetic (e.g. poly(ethylene glycol) PEG; poly(vinyl alcohol), PVA; poly(vinyl pyrrolidone), PVP) polymers (Allen and Cullis 2013). This approach allows to overcome most of the challenges in drug delivery processes such as the low blood circulation half-life, toxicity, interception by the immune system, biocompatibility and antigenicity issues. Among the hydrophilic polymers, PEG represents the most widely used polymer conjugate (Jain 2010). PEG creates, in fact, a concentration of *highly hydrated polymer brushes* (hydration shell) around the nanocarriers surface with extended crosslink (Caccamo *et al.* 2017; Caccamo

and Magazù 2017). This process sterically inhibits both hydrophobic and electrostatic interactions with plasma proteins or cells, thus reducing liposomal uptake process by the MPS. PEGylated nanocarriers are not opsonized and are able to escape the capture by the cells phagocytic systems (so called "*stealth liposomes"* effect). Many studies demonstrated that PEGylated liposomes were able to improve the stability and blood-circulation time, together with low plasma clearance and low volume of distribution (with minimal interaction with non-tumoral tissues) (Jain 2010).

Finally, the enhancement of the colloidal stability of nanocarriers in solution may be obtained by the inclusion of charged components that confer a net electrostatic repulsive forces (Bozzuto and Molinari 2015; Lombardo *et al.* 2016a). It is worth stressing that combined steric and electrostatic interaction generated by drugs inclusion may induce phase transitions in liposomes that strongly influences the structural stability of the nanocarriers as demonstrated by different studies (Kiselev *et al.* 2008; Kohlbrecher 2016; Yang 2016). A detailed study of the interactions occurring between drug nanocarriers and biological systems should become a prominent task of the design and characterization of new drug delivery systems. Moreover different scattering techniques can be applied by employing artificial membranes as simplified models for cell membranes (Katsaras and Gutberlet 2000; Wanderlingh *et al.* 2014; Kiselev and Lombardo 2017). Those studies have given a strong input to the understanding of the complex combination of soft interactions that a biomolecule can develop toward biological systems.

6. Engineering nanocarriers interactions and colloidal stability in complex biological environments

The experimental assessment of the colloidal stability of nanocarriers is a complex task due to the complex biological environment of pathological tissues. When nanocarriers are delivered into diseased tissues, in fact, their properties are strongly influenced by the high ionic content within the biological environment encountered (blood components, cytoplasm, nucleus, intracellular membranes and their enclosed structures). Moreover, possible ion complexation can modify the ionic strength and pH of the biological media thus influencing nanocarriers structural properties and their related functions. This circumstance is complicated by the fact that real biological systems exhibit heterogeneity, polydispersity and variations in surface properties within the different biological media, thus making the assumption of (idealized) model nanocarriers of identical nanoparticles unrealistic (Bozzuto and Molinari 2015). Finally the combination of self-assembly processes and synergistic effects can generate a structural and dynamic complex behavior of material systems at the nanoscale (Calandra *et al.* 2015a).

In Figure 3, the potential of the main soft interaction expressed by a nanocarrier (drug delivery) system is reported. The presence of an energy barrier resulting from the balance between repulsive and attractive forces prevents that two nanocarriers adhering together while approaching one another. In presence of additional charge screened effect that attenuate the electrostatic (ES) repulsion (or in case of particles collision with sufficient energy) nanocarriers are able to overcome that barrier thus favoring a decrease of their colloidal stability, followed by an aggregation of the nanocarriers. Control over the nanocarriers soft interactions represent, then, a crucial step for the engineering of the colloidal stability and

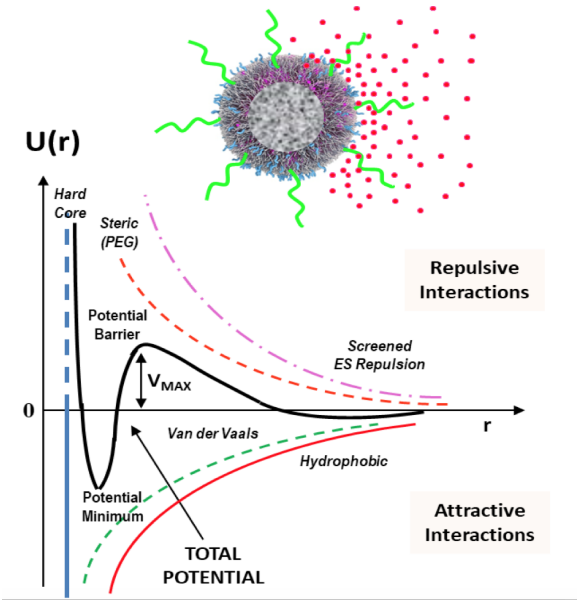

FIGURE 3. Example of the main soft interaction expressed by a nanocarrier (drug delivery) system.

biocompatibility of the therapeutic compounds, that are able to overcome obstacles and biological barriers to cellular and tissue uptake, and improving (both in vitro and in vivo) biodistribution of compounds to target sites (Ferrari 2015).

7. Supramolecular self-assembly and theranostic approach: Future perspectives and critical issues

Recent discoveries in nanoscience and nanotechnology highlight the powerful methods of supramolecular approaches for the construction of novel functional materials, with enhanced and emergent properties compared to those of the individual components (Ma and Zhao 2015). Molecular and supramolecular self-assembly are processes in which molecules (or basic building blocks) spontaneously self-assemble to form ordered aggregates (or macromolecules) usually in equilibrium states. Those nanostructures are based on the complex combinations of different non covalent forces acting at the molecular and supramolecular levels, and are able to create highly functional materials and devices with remarkable properties. Supramolecular self-assembly allows the fabrication of a large variety nanomaterials with emerging properties (Longo *et al.* 2006; Calandra *et al.* 2010) and various architectures (e.g. quantum dots, polymers, nano-stars, nanorods, nanodisks, nano-cages, Janus particles), chemical composition (organic/inorganic), and surface properties (e.g. decoration with specific ligands and charges).

Through the appropriate manipulation of specific interactions it has been possible to design supramolecular nanostructured materials such as supramolecular receptors effecting

molecular recognition, signal processing and transport processes encountered in biological systems (Ferrari 2015). Chemical-physics investigation of complex associating properties highlight the preminet role of the interaction patterns (hydrogen bonding arrays, sequences of donor and acceptor groups, ion coordination sites, etc.), in the creation of more and more complex topology, architectures, structural transitions (Mallamace *et al.* 2001; Bonaccorsi *et al.* 2013a,b; Calandra *et al.* 2015b; Liveri *et al.* 2018; Lombardo *et al.* 2019b).

7.1. Stimuli-responsive nanocarriers and targeted drug delivery: perspectives and critical issues. The design of smart nanocarriers that can specifically respond to the tumour microenvironment is significant to reduce the side effect to healthy tissues (Allen and Cullis 2013; Dai *et al.* 2017). For example, it is known that the tumour region presents a complex microenvironment which is quite different from normal tissues, as it is characterized by unevenness of blood flow, hypoxia and acidic pH (Mura *et al.* 2013; Dai *et al.* 2017). Owing to these specific characteristics it is possible to exploit the physiology of diseased tissues for the development of *stimulus-responsive* therapeutic nanoparticles that are able to modulate their therapeutic action in response to an internal stimulus (Mura *et al.* 2013). For example the acidic pH microenvironment can be utilized to selectively trigger nanovehicles for enhanced cancer therapy efficacy. Different pH responsive (inorganic and organic) nanocarriers have been developed during the last decades, in order to modulate the tumour extracellular pH and combat these effects (Mura *et al.* 2013; Dai *et al.* 2017). It is worth noticing that exposure to electromagnetic fields may cause sensitive alteration on cell membrane components (Thakur and Sahu 2016; Calabró and Magazú 2018). For example, even to exposure to extremely low electromagnetic fields cause sensitive unfolding process in cell membrane proteins (Calabró *et al.* 2013). It has been demonstrated that shielding action of disaccharides may provide an interesting approach for the development of effective strategies to preserve proteins from electromagnetic fields (Magazú *et al.* 2013, 2016, 2018). In conclusion, the study of relevant model systems can be adopted as simplified models that mimic the relevant processes encountered in real cell membranes (Micali *et al.* 1998; Lombardo *et al.* 2004b). Those studies have given a strong input to the understanding of the complex processes driven by the interactions that a nanostructured material can develop toward biological systems. Furthermore, chemical-physics investigation of complex associating properties in nanomaterials highlight the prominent role of the interaction patterns in the creation of more and more complex topology, architectures and structural transitions.

8. Conclusions and future perspectives

In the past 20 years, the development of nanocarrier-based platform has led to significant progress in biotechnology and nanomedicine applications. Although these drugs show good performance against specific diseases, it cannot be ignored their inherent drawbacks, mainly connected with the limited absorption and request of frequent injection for patients. In this paper we provide an overview on some fascinating developments in the area of nanomedical research by addressing some relevant open questions and critical issues arising in the investigation of the interaction of nanomaterials (and nanocarriers in particular) with biological systems. Our aim is to offer some indications for the design of more efficient nanocarriers. As a matter of fact the complex microenvironment in living systems strongly

affect the functionality of nanomaterials. The interactions and structural changes induced in presence complex biological media may compromise the design goals (e.g. degradability of materials and biocompatibility issues). Therefore, a deeper knowledge and understanding of the real interactions involved in the diseased tissues is fundamental for the development of novel therapeutic approaches and protocols based on the employment of smart nanocarriers. However, many questions still remain open for what concerns the way to deal with the complexity of the biological processes involved. What is the minimal number of key parameters (and their related key factors) required to describe behavior of nanomaterials without sacrificing the complexity of the identified process? In other words, what is the *"minimum level of complexity"* to assume in the theoretical and experimental models that may satisfactorily describe the nanocarriers (and nanomaterials) interaction with biological systems. In our opinion, the investigation of a multiplicity of simultaneous factors and biological functionality may be replaced with the systematic study the effect few parameters at a time (such as surface charge density and/or nanoparticle size/topology). Finally, the difficulty to predict the behavior and responses of nanoparticles-based drug delivery systems is connected with the difficulty to fully describe (by mathematical equations) the complex structural and dynamic processes involved in biological systems. The identification of the key factors for the design of efficient nanocarriers represent then the fundamental (initial) step to channel, in the right direction, the research efforts of decipher the complexity involved in complex biological processes.

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