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Walking the line: the fate of nanomaterials at biological barriers

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Abstract

Biological systems have developed an efficient multi-tiered defense system to block foreign substances such as engineered nanomaterials (NMs) from causing damage. In a pathological scenario, the disease itself may also pose additional barriers due to the imbalance between abnormal cells and their surrounding microenvironment, and NMs could behave similarly or differently to classic foreign substances, depending on their unique characteristics. Thus, understanding the mechanisms that govern the fate of NMs against these biological barriers, including the strategies that can be used to shift their fate between access and blockage, become key information for NMs design. In this manuscript, we first describe the biological barriers that NMs may encounter, and further discuss how these biological barrier interactions could shift the fate of NMs between toxicity and therapeutic potential. A list of effects that may influence NMs access at nano/bio interface are presented and discussed, followed by personal insights on the important nano/bio topics that require additional research for a better understanding of NM/biological barrier interactions.

Keywords

Biological barriers; nano/bio interface; nanomaterials; nanosafety; nanomedicine

Introduction

Biological systems, including the human body, have developed a highly efficient defense system by which foreign species/substances such as bacteria, viruses, fungi, and chemicals — some of them nanoscale in size — are usually blocked, preventing any harm [1]. The defense system includes physiological barriers, such as skin [2], air-blood lung barrier [3], barriers in circulation [4, 5], blood-brain barrier [6], and barriers in the reproductive system

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[7], etc., all of which not only protect their respective organ systems, but also provide selective access to certain substances such as nutrition and messenger molecules [8, 9]. These barriers largely rely on cells to serve as the basic functional unit, where various cellular organelles (e.g. cell membrane, endosome and lysosome) control the extracellular and intracellular access and trafficking of foreign substances [10, 11]. Engineered nanomaterials (NMs), usually within a size range of 1~100 nm in at least one dimension, now feature widely in medicine, diagnostics, cosmetics, food, and consumer and industrial products, which subsequently emerge in the environment [12, 13]. The growing use of NMs has caused concerns about their safety due to the complexity of their behavior, which cannot necessarily be translated and predicted from the information known of their bulk substances [14–16]. Biological barriers often use familiar mechanisms to regulate access of NMs, and can even develop new mechanisms for blockage of NMs, adding further complexity to their behavior. Compared with bulk materials, NMs exhibit complicated physicochemical properties, which include size, shape, surface area, charge, chemical composition, crystallinity, mechanical property, colloidal stability, defect and porosity, etc., which may individually or in combination impact NMs interaction with a specific biological barrier. For example, NM/cell interactions are usually comprised of the nanoparticle surface, particle-liquid interface with biological molecules, and interface with cellular compartments. In a given system, the key characteristics that define the NMs surface are chemical composition, surface functionalization, shape, porosity, surface crystallinity, and hydrophobicity/hydrophilicity. These physicochemical properties actively determine the nature of NMs interactions with biological fluids, which may include the formation of a protein corona, electrical double-layer, dissolution or surface restructuring. These dynamic interactions resultantly affect interactions with cellular compartments (e.g. cell membrane receptor, lysosomes, mitochondria) or biological molecules (e.g. proteins, lipids, collagen and nucleic acids), leading to a vast number of cellular events including cellular internalization of certain NMs [11]. In the case of nano therapeutics, the disease itself could add additional barriers due to the pathological imbalance between abnormal cells and their surrounding microenvironment, as seen in a tumor microenvironment [17].

Given this background, understanding the underlining principles of biological barriers becomes critical knowledge to improve nano safety and efficacy for nanomedicines [15, 17, 18]. For the latter, while we will primarily focus on nano therapeutics in this manuscript, it suffices to say that many of these principles can be applied to nano diagnostic and theranostic platforms. In this article, we first delineate the biological barriers (physiological, pathological, and cellular) that NMs may encounter, with a focus on the role of NMs properties in the nano/bio interactions. We also discuss the strategy to shift the fate of nanomaterials between toxicity and therapeutic potential. We further provide perspectives that aim to highlight the important future research areas for better understand NM/barrier interaction.

Biological barriers – a primary defense system at the border

When NMs interact with biological systems, they are met by barriers such as the skin epithelial barrier, gastrointestinal tract (GIT), and the air-blood barrier, all of which serve as part of the primary defense system preventing NMs access into the deeper levels of organs

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(Figure 1). Take skin for example, the safety and skin penetration of NMs have been widely evaluated in different particle types, such as polymeric particles, lipid based particles, metal/metal oxide particles, carbonaceous materials, and semiconductor nanocrystals [2]. Depending on size and surface property, NMs access can be achieved via the intercellular pathway involving the lipid channels between corneocytes to reach greater internal depth, or via the appendage route with the assistance of hair follicles and sweat glands [2]. Since the stratum corneum serves as the primary interface and barrier to NMs, the size and surface characteristics of NMs are key parameters that determine NMs penetration or rejection. In certain cases, dermal exposure to NMs could give NMs access to the blood stream and secondary organs, possibly resulting in sub-lethal injuries such as the induction of oxidative stress, inflammation, and photo sensitization [2, 19].

Another major barrier that NMs encounter is the GIT, where approximately 10^{12} micro- or nanoparticles per day per person will come in contact with mucosa (e.g. epithelium, lamina propria, muscularis mucosae) and non-cell defense systems in the form of low pH, enzymes in saliva and bile, IgA-containing mucus, and health-enhancing intestinal bacteria [20–23]. The mechanisms of NMs access through gastrointestinal mucosa are complicated. Minimally, it involves i) endocytosis through epithelial cells, ii) transcytosis via the M-cell rich layer of Peyer's Patches, iii) persorption through gaps in the gut lumen, and iv) paracellular uptake under pathological conditions [20, 23]. While many particles do not lead to damage in the gut, there is evidence suggesting that morphological abnormalities (e.g. blunted microvilli) and compromised GIT function may be resultant from particulate exposure in food [21, 22, 24], and more recently, possibly associated with toxic ion shedding metal oxide NMs or long aspect ratio NMs (nanoparticles with a length many times that of their width) [25–28].

Compared to the abovementioned skin and GIT barriers, the air-blood barrier in the lungs has been studied in greater depth [1, 29]. It has been shown that the distribution of particles in the lungs is dependent on their physiochemical properties, most notably, size and charge [1, 29–31]. The size-dependent effect on distribution can be partially explained by the NMs interaction with lipids and proteins in the alveolar capillary membrane, which responds efficiently to micro-sized particles recognized by alveolar macrophages [1]. This is in contrast with relatively less efficient phagocytosis, which opens the possibility of NMs escape and targeting of the secondary organs through the blood stream [1]. Though the nanoparticles in question varied in composition, shape, size and surface charge, it was shown that non-cationic nanoparticles of hydrodynamic size <6 nm can rapidly enter into lymph nodes and the bloodstream, and are ultimately removed via renal elimination [32]. New focus on the impact that NMs have on the renal system has illuminated adverse *in vitro* and *in vivo* effects, such as increased oxidative stress and renal interstitial fibrosis, but the detailed mechanisms of toxicity are still being identified [33].

In the mammalian system, organ-specific barriers are developed in vulnerable and functionally important organs, such as the blood-brain barrier (BBB) for central nervous system protection, the blood-testis barrier, and the placental barrier (Figure 1). The highly selective functionality of the BBB is largely due to endothelial tight junctions that are further supported by interactions with astrocytes and pericytes, resulting in an effective lack of

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fenestration and influx to the brain [6]. This tight influx control is complemented by the efflux transport system, which rapidly eliminates classic xenobiotics and NMs buildup in the brain, and together, these mechanisms protect the function of the central nervous system from external disruption [6]. While the BBB blockage is generally efficient, the exception indeed exists [34], and NMs are known to gain access across the BBB via a transcytosis-mediated route [35]. A well-known example is the transport of transferrin (Tf) across the BBB [36], which is similar to transcytosis processes reported with other protein types such as insulin [37] and immunoglobulin G [38]. Accordingly, these molecules were frequently used as surface modification for the delivery of therapeutic payloads crossing the BBB [39, 40]. In the case of traumatic brain injury and brain tumors, BBB permeability was increased putatively due to the compromised endothelial lining [41, 42]. Interestingly, researchers have found that drug delivery via olfactory mucosa is another efficient way to bypass the BBB and increase drug distribution in the brain, therefore serving as an alternative means for drug delivery to the brain [43]. While appealing due to its non-invasive nature, intranasal delivery to the brain requires comprehensive biosafety assessment including the experimentation on NMs bio-accumulation. It has demonstrated that carbonaceous NMs have exhibited an enhanced access to the brain via the facilitation of olfactory mucosa and olfactory nerve, but this phenomenon is associated with increased hazardous potential [44].

In terms of the biological barriers of the reproductive system, specialized cells and structures act as dynamic barriers against harmful substances. Sertoli cells of the male reproductive system facilitate spermatogenesis as it occurs inside the seminiferous tubules, and also serve as an important protective barrier to prevent harmful substances from reaching germ cells [1]. It was shown that repeated intravenous (IV) administration of water-soluble multi-wall carbon nanotubes into male mice led to reversible testis damage (e.g. induction of oxidative stress and pathological morphology of the seminiferous epithelium) at day 15 without affecting fertility [45]. Although NMs were able to maintain contact with spermatozoa, in the case of gold nanoparticles, mobility and penetration capability were compromised [46]. A recent study looking at the nanotoxicity of magnetic nanoparticles on bull sperm cells reported that the maghemite nanoparticles had little to no effect on the structure or function of the sperm cells, nor did the nanoparticles have an effect on cell kinetics [47]. Barriers of the female reproductive system include mucosal and epithelial barriers, and in pregnancy, the placental barrier is a complex and vital part of reproductive defensive systems. The permeability of the placental barrier depends on the stage of pregnancy, with permeability gradually increasing from the first to third trimester [48, 49]. Thus, NMs may have a higher chance to cross the placenta at later stage of pregnancy [1]. In a study that used an *in vitro* model of the placental barrier, the results showed that surface chemistry and size affected NMs uptake and transport. Despite the influence of these characteristics, both iron oxide and silica nanoparticles were able to cross the placental barrier, with iron oxide nanoparticles showing greater toxicity than the silica nanoparticles [50]. This suggest that reproductive impact of NMs should be considered in terms of clinical use of these imaging nanoparticles [51].

While the availability of various therapeutic NMs is an exciting development, it is important to point out that these early-stage NMs are still far from perfection, which can be partially explained by the presence of biological barriers. For example, intravenous (IV) injectable

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cancer drug nanocarriers have gained hope as “magic bullets” with the capability of selectively bypassing normal tissue while actively targeting pathological sites and cells [52]. The reality is, however, that majority nano particulates are recognized and effectively captured by the mononuclear phagocytic cells of the reticuloendothelial system (RES) in the liver and spleen. This sequestration is often increased by the surface coating of nanoparticles with a corona of proteins that lead to opsonization and enhance phagocytosis by the RES, underscoring the double-edged sword of surface modifications for targeting ability [53]. Potential safety concerns regarding nanotherapeutics are growing as work to develop solutions to the problem of their nonspecific distribution is still ongoing [54, 55]. While nanoparticles are frequently found in the liver, but their path, biological effect, and fate are not fully understood. Tissues of the mononuclear phagocyte system are well-known terminals of NMs after therapeutic of inadvertent exposures, but there is still a gap in understanding the biological effects that NMs directly have on the liver, which requires further investigation.

NMs/cell interactions have been intensively reviewed in the literature [12, 13, 56], but a brief discussion regarding cellular barriers that NMs may encounter is worthwhile as nanomedical research continues to grow. There are a series of cellular barriers preventing NMs access, as cells frequently serve as the basic functional unit in various barriers [10]. The fate of NMs/cell interaction can be roughly divided into particle/cell association (as seen with fumed and cationic silica nanoparticles [57]) and NMs internalization (as is the case with gold [58], mesoporous silica [59], and iron oxide [60] nanoparticles). The interactions between nano particulates and cells are commonly mediated by the formation of the protein corona when in biological environments, rather than its original manufactured surface, and these interactions are critical in determining NMs bioactivity [61]. There are a huge number of interactions when NMs make contact with biological aqueous solutions, phospholipids, nucleic acids, proteins, cellular compartments and nanometer-scale biological components [56]. Fortunately, none of these nano/bio interactions have been known to have pathological consequences for humans, but there is experimental evidence of membrane damage, DNA cleavage, mitochondrial damage, frustrated phagocytosis, induction of oxidative stress, and inflammation related to the interaction of NMs with biological processes [56].

How do biological barriers shift the fate of nanomaterials/nanomedicines between toxicity and therapeutic potential?

Knowing that NMs access is frequently determined at the nano/bio interface, the key tasks become (i) understanding of the principles that govern the NMs access through a biological barrier, and (ii) determining whether it is possible to reconcile imposing aspects of certain barriers to improve NMs access or vice versa. Although it is too early at this moment to summarize a detailed blueprint with respect to both tasks, the field is beginning to appreciate the infrastructural requirements to perform such research at the nano/bio interface, including the development of quantitative structure–activity relationships (QSARs) to design the next generation of NMs.

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In order to understand the principle that determines NMs access, the 1st requirement is the need for the use of standard reference NMs that can be tested in tissue culture and *in vivo* models to assist mechanistic research for quantification of NMs bioavailability. In our view, it is more effective and practical to establish QSARs at the cellular level, and to then use the resulting information on NMs dosimetry, structural and behavioral properties, and *in vitro* performance to prudently plan experimentation *in vivo*. Standard reference NMs that vary systemically in their properties can be tested with an intention to explore the transportation mechanisms [62]. We advocate the combined use of *in vitro* high throughput/content discovery and *in vivo* validation to establish nano-SARs, which would allow ranking of biological effects, *in silico* data transformation/modeling/mining, and informed decision-making. This notion is consistent with pharma's drug development approach, as well as the report from the US National Academy of Sciences, "Toxicity Testing in the 21st Century: A Vision and a Strategy", which advocates the transition from classic descriptive animal testing to quantitative, mechanistic and pathway-based toxicity testing in tissue culture using high throughput approaches [12, 56]. Before the execution of expensive experiments of pharmacokinetics/pharmacodynamics (PK/PD) and biodistribution in rodent and big animals, it may be useful to consider the use of advanced tissue culture models (co-culture and 3D spheroid culture, or an *in vitro* perfusion model) and less expensive *in vivo* models (e.g. zebrafish and chicken embryo models). These models will allow the researcher to evaluate the biodistribution, ADME (absorption, distribution, metabolism, and excretion), PK/PD, toxicokinetics and toxicodynamics, primary and secondary targets, bioaccumulation, tissue and intra-tissue distribution, and biocompatible/biohazardous effects. For example, important evaluative information on polyethyleneimine(PEI)-functionalized nanoparticles was collected when delivered to zebrafish embryos, and were found to be the most toxic when compared to succinic acid-functionalized or PEG-functionalized nanoparticles [63]. This is in line with the idea that positively-charged particles are more toxic than neutral or negatively-charged particles, likely due to their increased cellular uptake and cell membrane damage. The impact that increased cellular uptake has on cell viability reiterates the delicate balance between toxicity and therapeutic potential, given that effective drug delivery is reliant on efficient cellular uptake [63]. Being able to confirm these conjectures using quantitative high throughput methods is essential to fundamentally understanding the determinants of NMs biological access.

The 2nd important requirement to control the fate of NMs is the practice of "safe-by-design" approach. This is relevant to both non-biologically used NMs and the safety aspect of nanomedicine. The implementation of the safe-by-design approach begins with nano-Environmental Health & Safety (EHS) research to proactively incorporate the health and safety of NMs into the design process, rather than considering EHS as a post-facto add-on issue [64]. Although it is difficult to use a single parameter design feature to ensure overall safety, useful approaches that might contribute to this area are being identified, including the experimental evidences of improved access or blockage of NMs at biological barriers [12]. These include the design of a safe surface coating to influence cellular NMs trafficking and *in vivo* distribution (e.g. cationic NMs [57], up-conversion NMs [65], rare earth NMs [66]), adjusting nanoparticle size to alter the biodistribution and bioavailability [32], use of doping technology (e.g. ZnO by iron doping to reduce Zn^{2+} dissolution [67], and PdO doping to

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tune band-gap and Fermi energy levels [68]), hydration/rehydration to impact cellular membrane access and toxicity (e.g. fumed silica and graphene oxide [69]), shape design to tune the cellular uptake mechanism (e.g. silica and gold NMs with shape design to manipulate uptake mechanisms [70, 71]), among others. However, it should be noted that tuning NMs properties may influence their performance if changing the electrical, magnetic, thermal functions that are essential for their application; therefore, certain compromises may be needed [64].

An example of compromises made when tuning NMs involves the use of ZnO nanoparticles, where it was hypothesized that a slower nanoparticle dissolution rate would decrease its bioavailability and toxicity [26]. Since mixed Zn-Fe oxides are more resistant to acidic pH than pure ZnO nanoparticles, using a “flame spray pyrolysis” process [72], a library of ZnO nanoparticles was synthesized with an increased atomic % of Fe element and the particles were observed to be uniformly distributed throughout the crystal matrix [72]. Abiotically, it was demonstrated that crystal field splitting by the 3d orbital of the substituted Fe^{2+} augmented particle stability in biological solutions. When tested in biological systems, Fe-doped ZnO showed a significant reduction of intracellular ion release, cytotoxicity and pro-inflammatory cytokine release in macrophages. The biocompatibility was confirmed in murine lung [26]. While the toxicological endpoints differ, when testing the same ZnO nanoparticle library in zebrafish embryo and larvae, a similar protective effect was observed with Fe doping [26]. More recently, this doping strategy was tested using other metal oxide nanoparticles, such as CuO in which the dissolution potential was reduced by iron doping [73]. Another example is long aspect ratio NMs. By using different types of cancer cells and a mesoporous silica nanoparticle library that contains spheres and rods of different aspect ratios, it was demonstrated that the aspect ratio determines the rate and abundance of particle uptake by a macropinocytosis process. The rods with an appropriate aspect ratio, such as 2.5, efficiently triggered the cell membrane sensing mechanism by induction of the maximum number of filopodia, actin polymerization, and activation of small GTP-binding proteins for cytoskeleton assembly [70]. Realizing the effect that shape has on influencing NMs access through the cellular barrier has led to the enhancement of efficiency of drug delivery carriers for cancer treatment [70]. In a nano-EHS study, CeO_2 nanoparticles with aspect ratios >100 led to longer retention in fish gut, resulting in significant injury in the epithelial lining in the GIT and disrupted digestive function more so than shorter rods or spheres [24]. In a comparative analysis using single wall carbon nanotube (SWCNT), graphene, and graphene oxide, it was shown that graphene oxide, particularly large particle sizes, was the most pro-fibrogenic NMs in mouse lung [31]. This is likely due to the unique characteristics of graphene oxide itself as well as its colloidal stability and aggregation status in biological system [31, 74]. Researchers further showed that Pluronic PF108 polymer dispersed SWCNTs and small graphene failed to exert pulmonary fibrogenic effects, inspiring the use of size regulation and Pluronic coating as important safety design features in designing carbonaceous NMs [31, 75].

Achieving the capability to manipulate certain biological barriers to control NMs access (the 3rd requirement) is the least developed area of understanding the fine line between nanotoxity and therapeutic potential, yet it plays an increasingly significant role in the success of sustainable nanotechnology. Due to the multifunctionality of the nano platform, it

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is imperative to consider the use of a nano-engineered approach to address the physical and functional components of biological barriers that result in either unwanted access for potentially toxic NMs, or limited access for therapeutic NMs. For nanomedicine, innovative ideas and experimental data are emerging that promote the use of step-wise, multistage or combination therapies to provide an impact on various biological barriers, i.e. blood vessel permeability, blood vessel integrity, and drug-metabolizing enzymes at the site of interest, ultimately leading to improved efficacy of NMs [76, 77]. At this time, it is reasonable to speculate that an engineered approach may also allow researchers to manipulate other physicochemical barriers (hypoxia, high interstitial fluid pressure, pH), heterogeneous cellular barriers (e.g. pericytes, fibroblasts, and immune cells), functional barriers (e.g. immunosuppressive environment) and non-cellular barriers (e.g. collagen, matrix metalloproteinase, cytokines).

Future scientific developments for an integral understanding of biological barrier/NMs interactions

Given the important roles of various biological barriers, we have discussed how biological barriers affect the fate of NMs, and have noted that the sustainable development of NMs demands an in-depth and comprehensive understanding of the interactions between biological barriers and NMs. While there have been plenty of studies that describe nano/bio observations in the literature, mechanistic studies are still lacking; in particular, the practical ways to manipulate biological barriers are largely unexplored [63]. Here, we highlight a few biological barriers that require further in-depth exploration.

(i) Understanding the enhanced permeability and retention effect and the role of tumor stroma in cancer nanomedicine

Currently, most nanomedicines are designed for cancer therapy, and many of them are administered systemically to target a solid tumor site via the enhanced permeability and retention effect (EPR effect). The EPR effect refers to the fact that substances in the nano size range (e.g. macromolecule and NMs) tend to extravasate in tumor tissue more so than normal tissue, due to the large fenestrated vasculature found in the majority of tumor tissues [78, 79]. The use of a nano-engineered approach has directed more focus on optimizing the EPR effect for tumor targeting (Figure 2). Although the EPR effect has been comprehensively reviewed [79–81], there is still continuous debate regarding the effectiveness of EPR effect, especially in the clinical setting. The effectiveness of EPR varies case by case, and the diversity partially stems from the involvement of the tumor stroma, a physical and functional barrier preventing NMs access. For a classic liposomal nanocarrier, it is generally believed that the EPR effect is involved to improve the delivery and intratumoral drug concentration compared to the delivery and distribution of the free drug administered in animals and patients. While promising pre-clinically, the EPR's effectiveness for early stage engineered NMs is highly dependent on cancer type, and generally speaking, the so-called "magic bullet" effect has not yet been achieved in patients [82]. In fact, the working principle for "leaky tumor blood vessels" is still not fully understood from a cancer biology perspective. Vascular abnormalities that contribute to the EPR effect include enlarged tumor fenestrations, irregular angiogenic branching, and

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abnormal vessel density, and have frequently been demonstrated in various solid tumor models. Researchers have also demonstrated that for cancer types with a thick dysplastic stroma (e.g., pancreatic cancer), the vasculature is associated with low perfusion caused by pericytes and collagen deposition that cover the tumor vasculature. Thus, in addition to structural abnormalities, damage from lymphatic drainage, stromal effects, and non-cellular factors are collective influences on the performance of the EPR effect, and should considered when evaluating its effectiveness. For non-leaky tumor types, it remains to be determined whether nanoparticles gain access primarily via tumor fenestrations or an independent process. Our study was able to show that a nutrition-dependent transcytosis mechanism may potentially serve as a major mechanism of access for drug delivery nanoparticles, rather than the EPR effect (Figure 3) [83–85]. We demonstrated that transcytosis-inducing iRGD peptide can enhance nanoparticle access and efficacy in pancreatic cancer. The mechanism of action of iRGD is initially mediated by interactions with tumor-associated integrins, followed by peptide cleavage and the release of the C-terminal end that engages neuropilin 1 (NRP-1). Results revealed differences in carrier uptake during iRGD treatment between phenotypically paired patient tumors with differential NRP-1 expression in tumor vasculature, thus shining a light on possibilities for enhancing nanoparticle efficacy using a personalized approach with iRGD administration [83].

In addition to transcytosis activation, several approaches have been used to overcome the stromal barrier, especially in stroma-rich pancreatic cancer. Stromal depletion molecules have been developed to essentially obliterate the dense stromal environment to improve drug delivery [86]. The results of an ongoing clinical trial have demonstrated that the combination of a chemotherapeutic agent with PEGylated hyaluronidase (PEGPH20) can ablate hyaluronan and overcome the stromal barrier, allowing the chemotherapeutic drug access to the cancer site [87]. Another strategy is to use pharmacology to mediate access across the stromal barrier. The Food and Drug Administration (FDA)-approved use of an albumin-bound paclitaxel nano-complex (Abraxane®) combined with gemcitabine has demonstrated capabilities in suppressing stromal density and reducing expression of a gemcitabine-degrading enzyme (cytidine deaminase) at the tumor site [88, 89]. In order to obtain optimal synergy and stroma depletion *in vivo*, we designed a single carrier to co-deliver gemcitabine with a sub-cytotoxic dose of paclitaxel in a ratiometric-designed fashion, defined as the *in vivo* release of a drug combination from a nanocarrier with the purpose of establishing a fixed drug ratio at the target site [18, 90]. Our results showed a log-fold increase in drug delivery efficacy in a subcutaneous model, as well as a significant inhibitory effect on primary tumor growth and elimination of metastatic foci in an orthotopic model [18]. Another practiced method of gaining access across the stromal barrier is the modification of vasculature to improve NMs access to tumor sites. One option is to target the transforming growth factor beta (TGF-β) pathway, which promotes pericyte coverage of vascular fenestrations, among its pluripotent biological effects (Figure 4) [91]. TGF-β inhibition by a kinase inhibitor or monoclonal antibody has shown promising results for enhancing vascular access and delivery of cancer drugs and nanocarriers to the tumor site [92, 93]. While the work to identify viable targets to influence stromal barrier permeability at tumor sites has been ongoing, there is still much that needs to be ascertained and explored, and it may be

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worthwhile to turn attention to other possible mechanisms, including antifibrogenic drugs [94], Hedgehog signaling inhibitors [95], and the lowering of the interstitial fluid pressure prior to systemic administration of NMs [96].

From the physicochemical perspective, hydrodynamic size of NMs for optimally utilizing the tumor EPR effect is around 50–200 nm, and can vary depending on cancer types; sizes greater than 500 nm or less than 6 nm are not typically considered. Negatively charged or neutral NMs show relatively less non-specific interaction with negatively charged biological membranes, however, a slightly positive charge on the NMs surface could be used to facilitate cellular uptake and improve lysosomal escape without the induction of toxicity. Modifications, most notably PEGylation (e.g. Pegylated Doxorubicin or Doxil®), are important design features for the reduction of non-specific uptake by macrophages, but their immunogenicity and other performance limitations should be considered. As a work around, newly developed polysaccharides including natural or synthetic/reconstituted cellular membranes are emerging as promising alternatives for RES organ evasion [97–99].

As more nanomedicines are reaching clinical trials and vying for FDA approval, it is more important now than ever to study the EPR effect in patients. In a meta-analysis of the efficiency of nanoparticles in drug delivery over the past 10 years, the authors concluded that only 0.7% (median) of the injected nanoparticle dose was accumulated in solid tumors, with little improvement over time [100]. Other experts feel that the EPR effect is still an important principle in nanoparticle drug delivery, and that the previously reported efficiency rate of 0.7% was an unfair assessment in determining nanomedicine efficiency [101]. Although it might appear that a low accumulation rate in the tumor indicates low efficiency, researchers argue that nanoparticle accumulation at the tumor site does not necessarily mean that the active pharmaceutical ingredient is not being delivered and imparting its effects on tumor cells. For example, liposomal doxorubicin has low detectability in the tumor itself, but is still higher than the equivalent dose of free drug, and results in greater therapeutic outcomes because of its increased tumor exposure, PK and reduced cardiotoxicity [101]. While acknowledging the growing number of FDA-approved nano drugs that are currently available for cancer therapy, we recognize that translation in nanomedicine is still a challenge, not solely because of issues associated with delivery and the reliance on the EPR effect, but more so due to the lack of novel and translatable NMs formulations [101]. There is still much hope for progress in the field, and many researchers have focused their efforts on the characterization of nanoparticles to guide their specialized development [102]. Nanomedicine researchers have also begun looking at ways to evaluate and manipulate the EPR effect across different types of tumors to determine its effect in patients, with recent preclinical evidence demonstrating the prediction potential of superparamagnetic iron oxide nanoparticles and magnetic resonance imaging (MRI) in determining the effectiveness of the EPR effect *in vivo* [103].

(ii) Understanding the effect of the protein corona

In biological fluids, NMs tend to immediately adsorb a range of proteins, forming a “protein corona” that is physically associated with the NMs surface, and acts as the “real surface” that physically or biologically interacts with barriers encountered (Figure 5) [74, 104]. The

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dynamic composition and stability of this protein corona are sophisticated and can have profound impacts on NMs/barrier interactions. Their resulting effects on the biological barriers and biocompatibility of the NMs are still not completely understood and historically underestimated. Attachment of different surface proteins can lead to various and contrasting NMs fates, such is the case with opsonization—where NMs surfaces are coated with opsonins via van der Waals, electrostatic and hydrophilic/hydrophobic interactions, effectively marking them for phagocytosis ("eat me" signal)—versus coating NMs surfaces with CD47 glycoprotein ("don't eat me" signal). The NMs coated with opsonins, particularly immunoglobulin G (IgG), were ultimately taken up and cleared by macrophages, whereas NMs coated with CD47 and other self-peptides were able to bypass phagocytic recognition and clearance [105]. To bypass phagocyte-mediated cellular barriers, "stealth" brushes (e.g. PEGylation) are an important design feature that can be used to mimic a cell's glycocalyx, preventing opsonization and phagocytic clearance, thus increasing NMs blood circulation time and likelihood of effective disease targeting [106]. Conversely, the NMs surface itself may alter the structure and function of the adsorbed proteins and therefore impact recognition and cellular uptake of nanoparticles by cells [63, 74]. An NMs own protein corona profile can also have effects on the proteome of different cells and organisms, beyond the initial biological barrier [107]. How this "soft" or "hard" protein corona affects cellular responses such as NMs internalization, intracellular transport, signaling pathway activation, and exclusion, has yet to be fully understood. This exploration may largely rely on various high-resolution experimental techniques for quantitative assessment of protein/NMs interactions. Theoretical modeling, such as molecular dynamics simulation, could be a powerful tool for predicting interactions and aiding mechanistic understanding [74, 108]. For example, researchers demonstrated that the π-π stacking interactions between carbon nanotube and aromatic residues (Trp, Phe, Tyr) are the key factors in determining the adsorption capacity of the protein corona [109]. The immediate binding of biological proteins to the nanotubes may significantly reduce the cytotoxicity in an acute stage, whereas the sub-acute and long-term outcomes need to be studied further, due to competitive and dynamic alterations of the protein corona.

(iii) The "Trojan horse" mechanism

Previous studies have mainly looked at the biological NMs/barriers interactions; however, the access of NMs via induced or triggered secondary mechanisms is still not well understood. The triggering of secondary mechanisms to bypass a biological barrier is much like the concept of the "Trojan horse", where NMs are disseminated into intercellular communication systems via a biological vehicle. This means that even though NMs themselves are blocked at the abovementioned barriers, the induction of NMs through a "Trojan horse" mechanism could potentially lead to activation of cascade responses and biological outcomes at a particular site [110]. Our study revealed that exosomes could serve as an important signal conveyor for nanoparticle-induced systemic immune responses, bypassing the alveolar-capillary barrier; this is a new mechanism that has not been identified in the past [110]. Pulmonary exposure to magnetic iron oxide nanoparticles (MIONs) in mice generated exosomes in the alveolar space, and these extracellular membrane vesicles acted as "Trojan horses" for the nanoparticles, allowing their distribution through intercellular communications [110]. These exosomes were translocated through the air-blood

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barrier into circulation, and were subsequently involved in dendritic cell maturation and T-cell activation [110]. Given this information, it is reasonable to speculate that exosomes or other unidentified "Trojan horse" mediators may act as conveyors for pulmonary signal transduction in nanoparticle-induced immune systemic responses, which can ultimately contribute to an *in vivo* effect [110]. A similar mechanism was reported involving cancer drug delivery, where cancer cells that took up cisplatin-laden nanoparticles were capable of releasing the active drug following their own apoptosis, effectively inducing intake of the drug and apoptosis in the surrounding tumor cells that were previously unaffected [111].

(iv) NMs dissolution in biological systems

Dissolution is a particularly important consideration for metal and metal oxide NMs due to their high surface reactivity [112–116]. For example, we have demonstrated that due to differences in surface area and reactivity, copper nanoparticles (23.5 nm) dissolve more rapidly in physiological conditions (e.g., stoma fluid) than their micron-sized countertypes. The resulting copper ions exhibit different penetration capabilities in the GIT barrier than nano particulates, leading to moderately acute toxicity to mice, pathological damage, heavy metal overload, alterations in serum ceruloplasmin, turbulence of pH homeostasis, and metabolic alkalosis *in vivo* (Figure 6A) [27], [28]. Dissolution can also occur within cellular compartments such as lysosomes. Dissolution of upconversion nanoparticles, which contain rare earth metals that are unstable through dissolution in lysosomes, led to lysosomal damage via an intracellular phosphate complexation and transformation mechanism [65, 66] (Figure 6B). Recognition and awareness of this dissolution mechanism prompted researchers to use a phosphonate coating as a "safe design" feature to ameliorate the instability of the particle surfaces. This effective coordination of organophosphates and rare earth atoms was able to prevent cellular damage and preserve imaging capabilities [65]. While dissolution is a common phenomenon in many metal/metal oxide NMs, precise control of the rate and abundance of ion release is still a challenging task. Future study is need to fully understand the fate of particles and ions, including their interaction with various biological barriers.

Summary

It is clear that the exposure of vulnerable organs to NMs is a significant cause of toxicity, and is further complicated by the deviations in biodistribution and penetrance, both of which are dependent on NMs interactions with biological barriers. Although flawed barrier permeability in target organs may allow engineered NMs to generate adverse effects *in vivo*, this vulnerability also gives NMs access through biological barriers to reach the disease site, and could potentially be tuned to produce therapeutic effects. Efficient biological barriers are not usually discerning when rejecting hazardous or therapeutic NMs, thus, a mechanistic understanding of the interactions between NMs and the three tiers of barriers is of great significance in the field. With this understanding, it is possible to envisage the use of a nano-engineered approach and safe-by-design techniques to precisely manipulate NMs access through imposing biological barriers, ultimately employing transformative nanotechnology and life-saving medicines.

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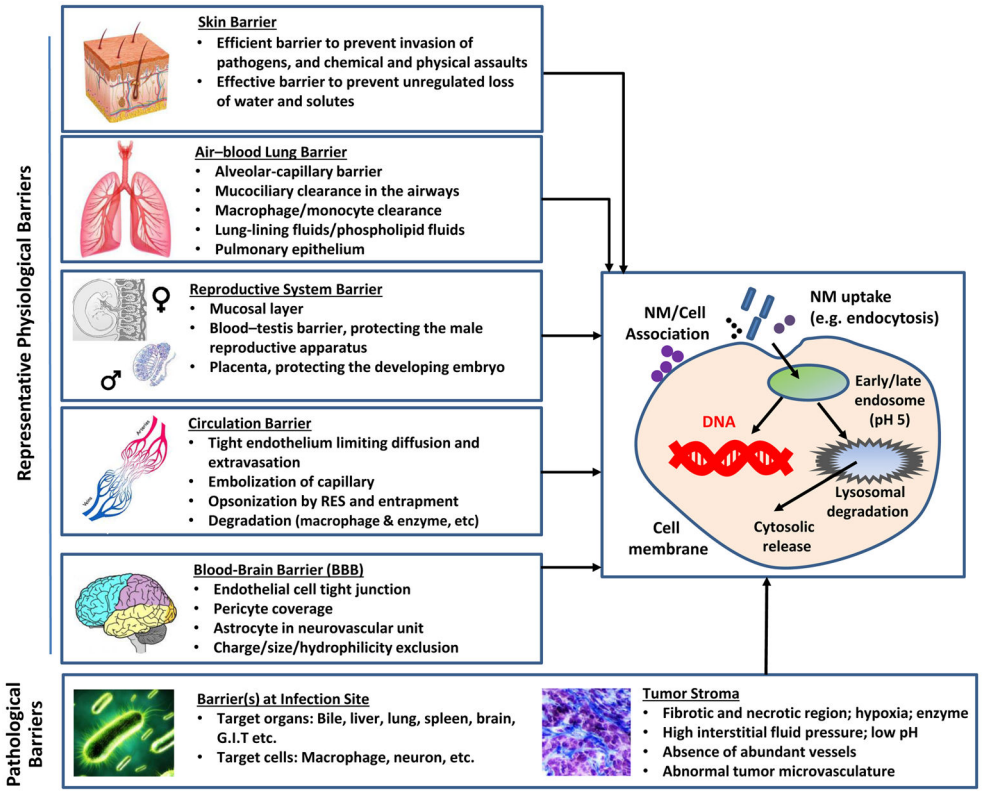

Figure 1.

Representative physiological, pathological, and cellular barriers that NMs may encounter in the biological system.

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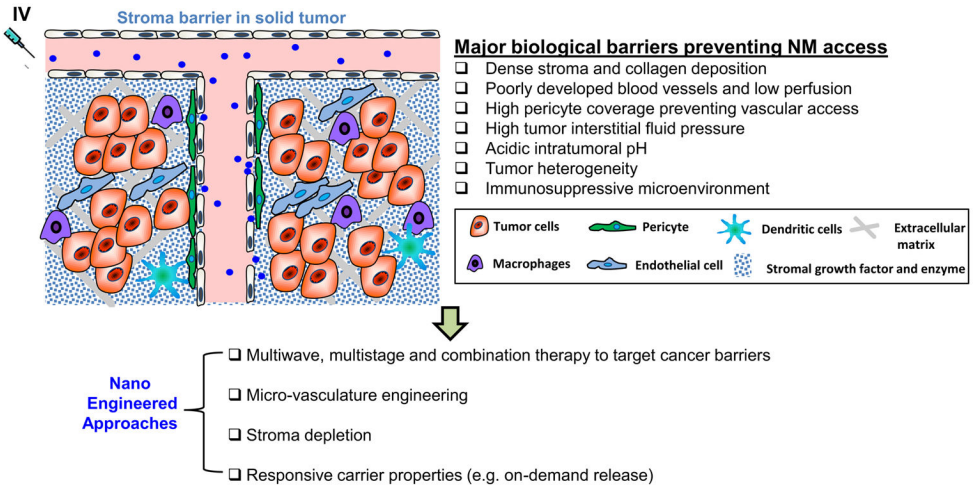

Figure 2.

Schematic to show stroma barriers preventing therapeutic NMs access in solid tumor. The presence of dysplastic stroma is a physical and biological barrier. This includes collagen deposition, poorly developed blood vessels, pericyte coverage, high tumor interstitial fluid pressure, acidic pH, tumor heterogeneity and immunosuppressive microenvironment. We advocate an engineered approach using nanocarriers, which can address the stromal barrier or suppress the stromal abundance by the delivery of drugs that suppress the abovementioned challenges. A combination of these features is expected to achieve synergistic effects mediated by nanocarriers. Figures are adapted from our previous publication [117].

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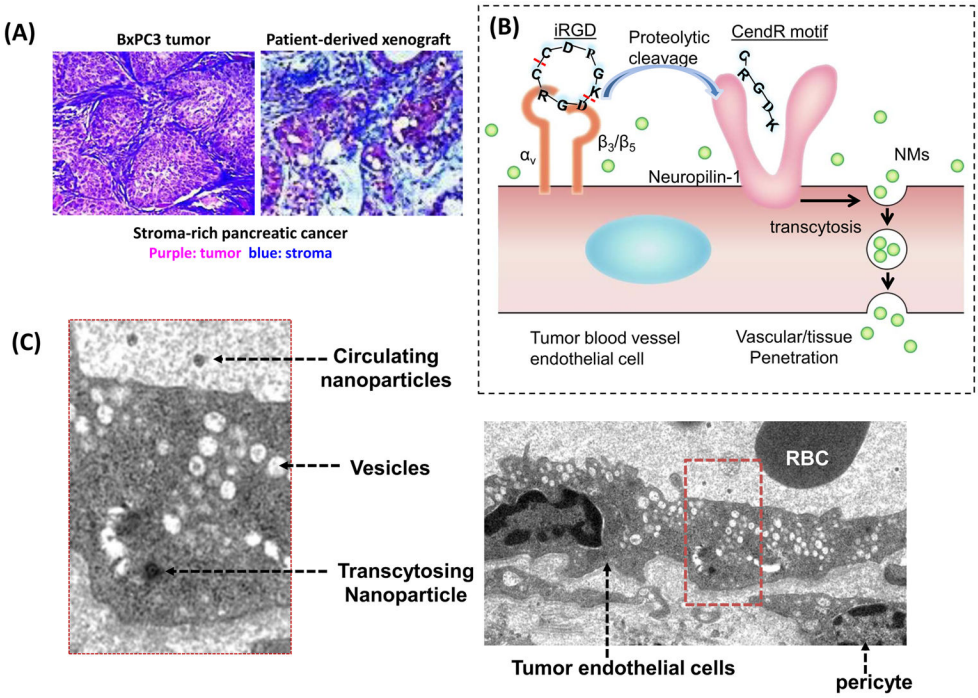

Figure 3.

Role of transcytosis in tumor access. Transcytosis is a complementary nanoparticle tumor access mechanism operating in a leaky vasculature-independent fashion. (A) Representative trichrome staining of tumor tissue section prepared from BxPC3 xenograft and pancreatic cancer patient-derived xenografts. Tumor and collagen (stroma) were stained in purple and blue color, respectively. In a stroma-rich solid tumor, tumor access and anti-cancer efficacy of nanocarrier can be enhanced by co-administration of an iRGD peptide that does not require covalent surface conjugation. (B) The iRGD effect is mediated by interaction with tumor-specific integrins, followed by peptide cleavage and the release of the C-terminal that further triggers neuropilin-1 or NRP-1 mediated transcytosis. (C) TEM visualization of tumor sample receiving iRGD plus nanoparticle demonstrates the appearance of a cluster of vesicles in endothelial cells, including a transcytosing nanoparticle from the blood vessel lumen [83]. A zoom-in picture with high magnification was provided (left panel).

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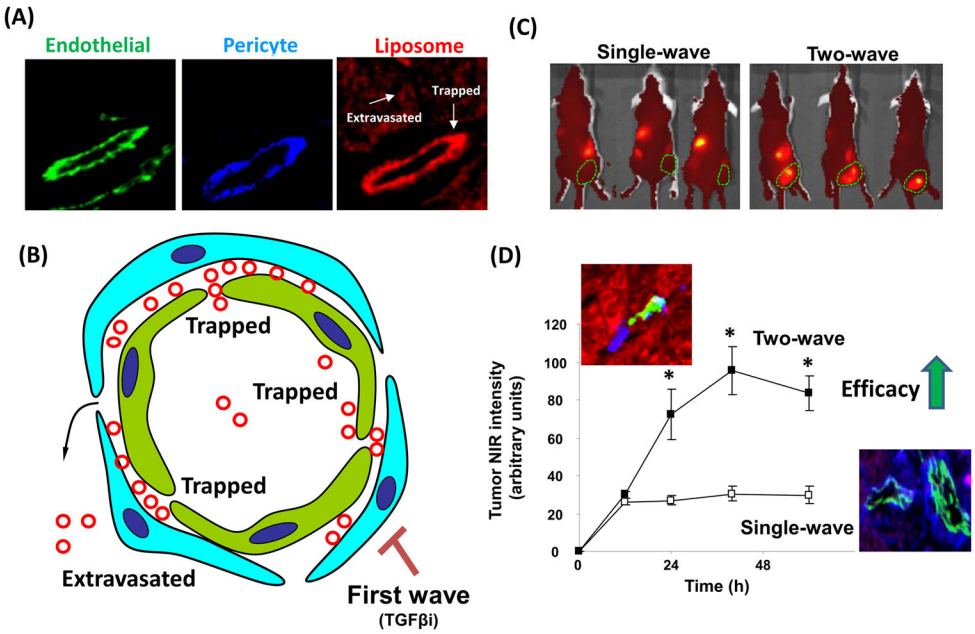

Figure 4.

Use of "Two-wave" therapy to target pericyte in tumor stroma and optimize drug delivery nanocarrier access and efficacy in pancreatic cancer. (A) A stroma-rich pancreatic cancer (BxPC3) bearing mice received IV injection of 50 mg/kg red labeled liposome (100 nm). Tumor tissues were harvested 48 hrs post injection and used for IHC staining of endothelial cells maker (CD31) in green and pericyte marker (NG2) in blue, respectively. Majority of liposomes were trapped in the tumor vasculature due to the high pericyte coverage. (B) We propose a two-wave nanotherapy, in which the 1st wave is used to overcome the pericyte coverage (by TGFβ inhibition nanoparticle) followed by the 2nd wave nanoparticle for drug delivery to improve the anticancer outcome. (C) A copolymer coated nanoparticle binds to the TGFβ inhibitor, LY364947 via a supramolecular chemistry process. The complexation is highly stable at pH 7.5 but can be disrupted in the acidic stromal environment (pH 5.5–6.5). Tumor bearing mice received IV injection of NIR-labeled 2nd wave particles with or without TGFβ inhibitor-delivering nanoparticle. The retention of 2nd wave particle was increased 10-fold by prior injecting mice with implanted BxPC3 xenografts with the LY364947 carrier. Use of a drug-loaded liposome to provide tumor killing in the same model confirmed the significant tumor shrinkage in mice compared with the controls. Figures are adapted from our previous publication [77].

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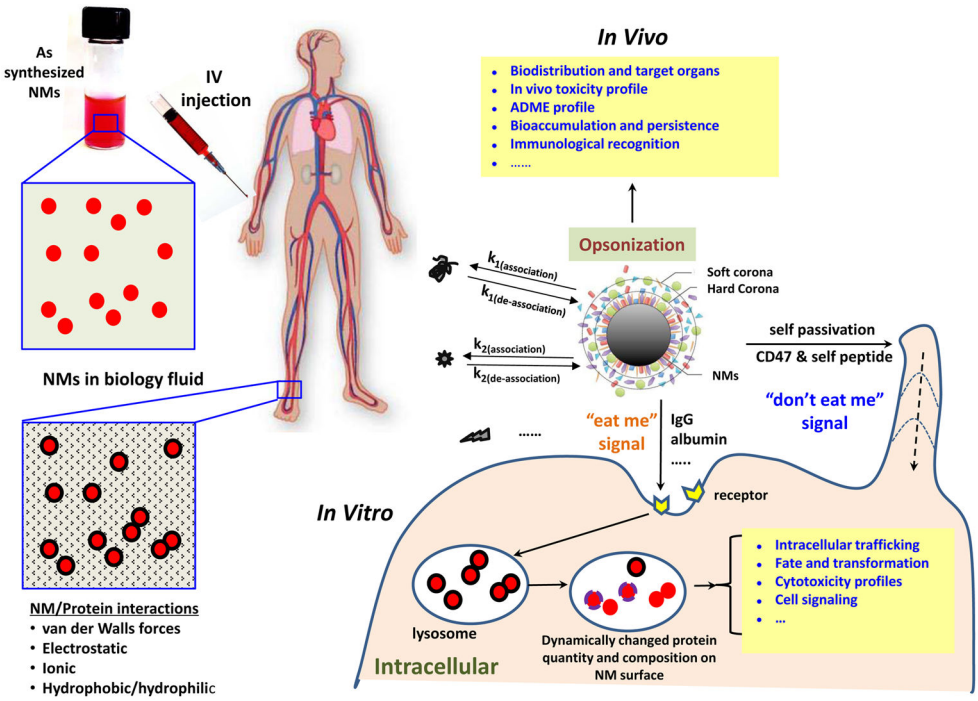

Figure 5.

Roles of the protein corona at the nano/bio interface. In the biological fluids, synthesized NMs can immediately associate with a range of proteins, forming a protein corona that is physically associated with the NMs surface. The protein corona becomes the "real surface" that physically and biologically interacts with barriers. The dynamically changed composition and stability of this protein corona could have a profound impact on the NMs interactive behavior since the adsorbed proteins would greatly affect the interactions of the NMs *in vitro* and *in vivo*. The composition and properties of protein corona determines biodistribution, ADME profile, bioaccumulation and persistence, and immunological recognition for a certain NM. At the cellular level (e.g. a macrophage), the fate of the NMs being internalized vs rejected largely depends on the characteristics of the protein corona.

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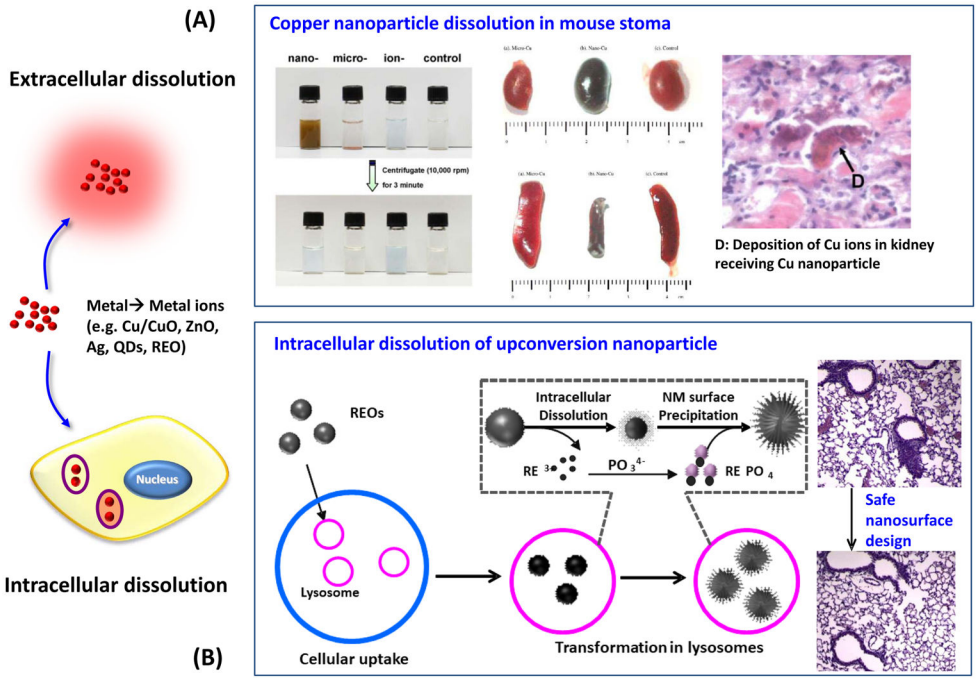

Figure 6.

Dissolution of metal/metal oxide nanoparticles. The ultra-high surface reactivity, which could lead to the generation of metal ions in target organs and cellular barriers, may add an additional level of complexity in the NMs/barriers interaction. (A) Copper nanoparticles rapidly dissolved in stoma fluid as compared to microparticles of the same type. The resulting copper ions exhibited different penetration capabilities in the GIT barrier than nano particulates, leading to moderate acute toxicity to mice, pathological damage, heavy metal overload, serum ceruloplasmin alteration, turbulence of pH homeostasis, and metabolic alkalosis *in vivo* [27], [28]. (B) Upconversion nanoparticles were unstable through dissolution in lysosomes, leading to lysosomal damage via an intracellular phosphate complexation and transformation mechanism. Phosphonate coating was used as a “safe design” principle to passivate particle surfaces, and had the ability to prevent cellular damage and preserve imaging properties [65] [66].