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Effect of physicochemical and surface properties on *in vivo* fate of drug nanocarriers

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ABSTRACT

Over the years, a plethora of materials – natural and synthetic – have been engineered at a nanoscopic level and explored for drug delivery. Nanocarriers based on such materials could improve the payload's pharmacokinetics and achieve the desired pharmacological response at the target tissue. Despite the development of rationally designed drug nanocarriers, only a handful of such formulations have been successfully translated into the clinic. The physicochemical properties (size, shape, surface chemistry, porosity, elasticity, and many others) of these nanocarriers influence its biological identity, which in presence of biological barriers *in vivo*, could significantly modulate the therapeutic index of its cargo and alter the desired outcome. Further, complexities associated with developing effective drug nanocarriers have led to conflicting views of its safety, permeation of biological barriers and cellular uptake. Here, in this review, we emphasize the effect of physicochemical properties of nanocarriers on their interactions with the biological milieu. The review will discuss in depth, how modulating the physicochemical properties would influence a drug nanocarrier's behavior *in vivo* and the mechanisms underlying these effects. The goal of this review is to summarize the design considerations based on these properties and to provide a conceptual template for achieving improved therapeutic efficacy with enhanced patient compliance.

Contents

Abbreviations: ANP, atrial natriuretic peptide; CARG, cyclic amino peptide; CPB, cylindrical polymer brushes; ECM, extracellular matrix; EPR, enhanced permeation and retention; gp60, glycoprotein 60; HA, hyaluronic acid; ICAM-1, intracellular adhesion molecule 1; IFP, interstitial fluid pressure; MMP-9, matrix metalloproteinase 9; MOFs, metal-organic frameworks; MPS, mononuclear phagocyte system; NPs, nanoparticles; NOD, non-obese diabetic; PAP, pulmonary arterial pressure; PDGF, platelet-derived growth factor; PEGDA, poly(ethylene glycol) diacrylate; PEI, poly(ethylene imine); PLGA, poly(lactic-co-glycolic) acid; PLL, poly(L-lysine); PSNs, porous silicon nanoparticles; QbD, Quality by Design; QDs, quantum dots; QTPP, Quality Target Product Profile; RBC, red blood cell; RES, reticular endothelial system; SAP, systemic arterial pressure; Tf, transferrin; TIMP-1, tissue inhibitor of metalloproteinase-1.

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1. Introduction

Over the past few decades, nanotechnology has emerged as a significant contributor to improving the quality of life, with nanomedicines serving a pivotal role in the treatment of life-threatening disorders. Nano-meter scaled systems offer unique surface properties as compared to the bulk materials which gives them the ability to interact with other systems on the nano-scale, which in-turn gives rise to interesting applications. One such application is the loading of therapeutic moieties to nanocarriers and their delivery to a target site. Loading of the drug into the nanocarrier can prevent the unwanted interaction of the cargo with off-targets present in the biological milieu. In doing so, this strategy offers a degree of controlled release and the promise of site specificity, which neither the free drug nor the parent bulk material from which the nanocarrier is derived, offer [1]. 'Nanocarriers in drug delivery' thus has been an active area of research in the past two decades, with an average rate of >100 publications per month since 1995 (Fig. 1A), according to PubMed (accessed on 26th October 2018). Nanocarrier-based drug delivery has not only been limited to academia, but has been successfully translated to clinics with several products like Doxil®, Onivyde®, Vyxeos®, Abraxane® for cancer therapy, Definity® for MRI contrast imaging, Ferraheme® for iron replacement therapy, Diprivan® for general anesthetic application and so on [2]. Nanoformulations like Doxil® and Abraxane® have been commercial blockbusters since they entered the market and are tested in over hundreds of clinical trials for a range of indications even today [2].

Despite the academic and commercial successes of nanocarrier based drug delivery systems, their complete therapeutic potential seems far from being achieved. In many of the drug loaded systems, nanocarriers do not seem to offer a significant improvement in the therapeutic benefit over the free drug counterpart and this continues to be a challenge. As a result, significant scientific effort has been put in trying to solve this problem [3,4]. One such effort has been an article by Chan and co-workers, where the authors carried out a comprehensive study of the different types of nano-systems that have been tested on different solid tumor models between the years 2005 and 2015. The authors concluded that <1% of the injected dose reaches the desired site of action and this is true for all types of nanocarriers analyzed (Fig. 1B) [5]. This and many other studies [4,6] point out that the sub-therapeutic efficacy of nanocarriers has been due to the presence of biological barriers to the

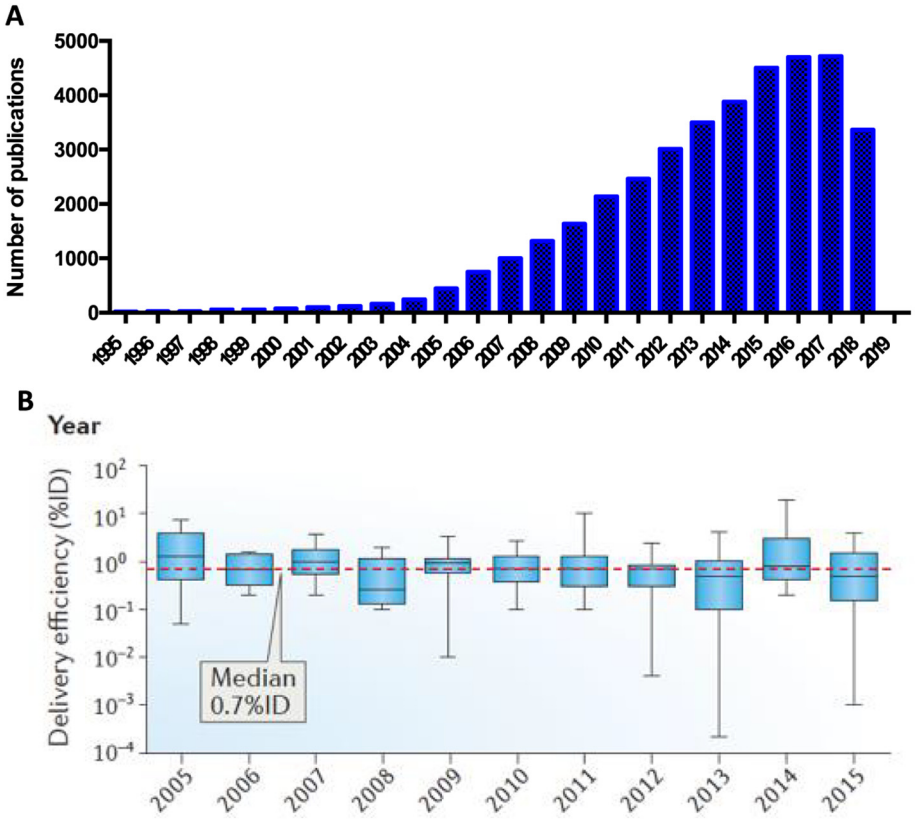


Fig. 1. Nanocarriers in drug delivery. (A) The number of publications with the keywords "Nanocarriers in drug delivery" from 1995 through 2019, indicative of the scientific efforts being put in the field (accessed from PubMed on 26th October 2018). (B)Analysis of median delivery efficiencies of nanoparticles (different types, different physicochemical properties) in terms of %ID or %ID/g in various solid tumor models from 2005 to 2015, highlighting major concerns in the field of nanocarrier mediated drug delivery.[Adapted with permission from [5].]

injected nanocarriers. Also, the extent of interaction of these biological barriers with nanocarriers seems to be dependent on their physicochemical properties. In other words, to understand the *in vivo* fate of nanocarriers, one needs to understand the physicochemical properties of the carrier and its interaction with the biological systems.

In the literature, physicochemical properties like size, shape, surface chemistry, porosity and elasticity and their *in vivo* effect have been extensively studied, but often in isolation. This review aims to consolidate scientific efforts made in order to understand how physicochemical properties affect the *in vivo* behavior of nanocarriers. The mechanisms and rationale underlying the use of specific property for addressing a certain question are mentioned in the respective sections. A major focus of this review is to understand the biological barriers like mononuclear phagocyte system (MPS) existing to the injected nanocarriers and how physicochemical properties may be altered to tweak the biological identity of the nanocarriers to facilitate their barrier avoidance. The review also discusses the potential toxicities that occur *in vivo* when any of the physicochemical properties is modified beyond specific thresholds. Lastly, the review also provides some recommendations for the future studies to be carried out in understanding the *in vivo* behavior of nanocarriers. The aim of this review is to provide the reader with a conceptual framework of design parameters for physicochemical properties of nanocarriers to alter their *in vivo* behavior with certain thresholds that have been empirically established.

2. *In vivo* biological barriers encountered by intravascularly injected nanocarriers

To understand the effect of physicochemical properties on tweaking the biological identity of a nanocarrier, we follow its path from administration to the site of action and understand the biological barriers confronted by it in a progressive manner. The major barriers to the injected nanocarriers include the mononuclear phagocyte system (MPS), physiological hemodynamics, site specific extravasation and cell membrane trafficking and endosomal compartmentalization (Fig. 2). While, a majority of nanoparticle (NP)-based formulations encounter the first three barriers, nuclear or cellular drug delivery systems combat additional intracellular obstacles. This section aims to describe the impact barriers have on the fate of administered nanocarriers *in vivo*.

2.1. The mononuclear phagocyte system (MPS)

Nanomedicine often fails to achieve therapeutic levels of drugs at the disease site. This is primarily due to non-specific uptake of nanocarriers in healthy tissues and is facilitated by a network of phagocytic cells. These cells – bone marrow progenitors, blood monocytes, tissue macrophages, and dendritic cells – constitute MPS which marks the carriers for clearance from circulation and the body, thereby affecting its pharmacokinetics and tissue biodistribution. This system is also referred to as the Reticular Endothelial System (RES).

Following intravenous (i.v.) administration, the path of nanocarriers can be divided into circulation, margination, firm adhesion and cell internalization [7]. In circulation, plasma proteins such as serum albumin, complements, immunoglobulins and apolipoproteins adsorb onto its surface and form a corona. This process is referred to as opsonization. The "protein corona" defines the physiological character of the drug-carrier and is dependent upon the particle' size, shape, zeta potential, hydrophobicity/hydrophilicity and surface functionalities like targeting ligands. The nanocarrier with its protein corona then binds to specific receptors on phagocytes, undergoes internalization and is transported to phagosomes prior to lysosomal fusion. This process of sequestration is dominantly carried out by macrophages of the spleen, liver and lymph nodes, thereby influencing its biological half-life and efficacy. Opsonization is also known to negate the effect of active

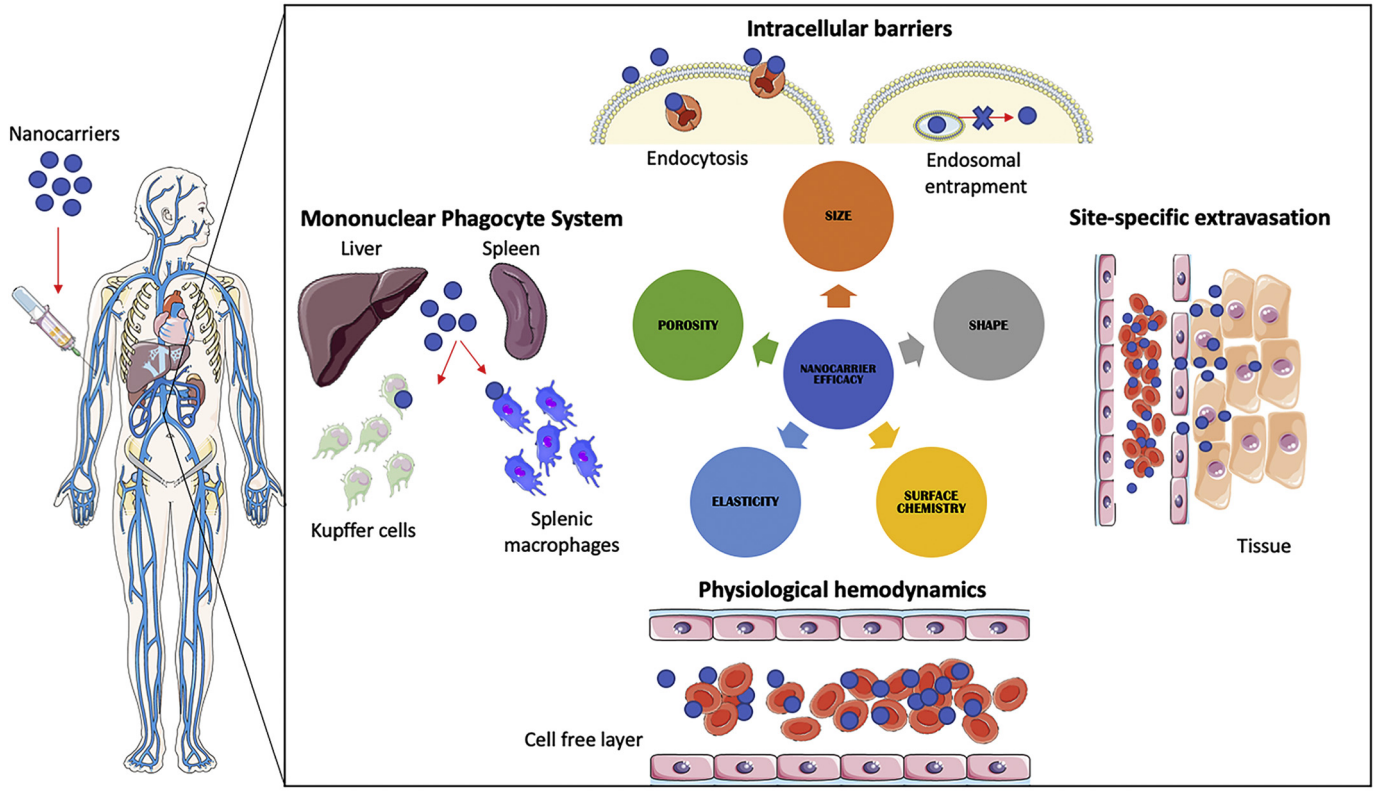


Fig. 2. Biological barriers encountered by intravascularly administered nanocarriers- the uptake by Mononuclear Phagocyte System, lack of margination into the cell free layer due to physiological hemodynamics, difficulty in site specific extravasation and tissue accumulation and intracellular barriers to nanocarriers. The extent of the interaction of the nanocarriers with the biological barriers is affected by its physicochemical properties- Size, Shape, Surface chemistry, Elasticity and Porosity.

targeting ligands, causing lack of specificity. Dawson et al. showed how opsonization could shield the targeting ligands from binding and cause loss of specificity. The transferrin (Tf) functionalized silica particles did not bind to its targeted Tf receptors present on A549 cells or its soluble form [8]. Other studies have also shown the effect of the physicochemical properties on protein corona and fate of nanocarriers *in vivo*, such as size and surface charge [9,10]. For example, a larger surface area to volume ratio permits more protein to bind onto smaller particles in contrast to large-sized particles [11,12]. In terms of surface charge, highly cationic carriers are eliminated promptly from circulation. Neutral or anionic carriers circulate significantly longer, resulting in increased accumulation at the target site like tumor and improved efficacy [13,14].

In a variety of scenarios, MPS does not represent a biological barrier but can actually be used as a target. For instance, macrophages are largely involved in inflammation and consequently diseases such as cancer and atherosclerosis. These cells could therefore, serve as potential targets for treatment purposes. Kreuter et al. engineered poly (butyl cyanoacrylate) nanoparticles coated with polysorbate 80. Adsorption of apolipoproteins to the polysorbate 80 surface, enabled these to act as lipoprotein particles and traverse the blood-brain barrier *via* low-density lipoprotein-mediated endocytosis. In another study, Wentworth et al. surface functionalized CdSe/ZnS quantum dots (QDs) with the inflammatory metabolite cholesterol 5,6-secosterol atheronal-B. Consequently, a conformational change was induced to the structure of apolipoprotein B that formed the corona. This in turn enhanced QD uptake in macrophages [15].

2.2. Nanocarrier hemodynamics and hemorheology

Engineering nanocarriers with extended circulatory properties and low blood clearance rate can assist in site-specific delivery. The particles have to sustain extended periods of circulation and laterally drift through the blood cell core layer before adhering to the vascular wall. This would allow targeted carriers to either bind with cells of interest and promote favorable receptor-ligand interactions or penetrate through the leaky vasculature of tumors. The hemodynamics or the movement of blood cells in circulation is unsteady and can vary with the size and location of the blood vessel [16,17]. Studies have shown that hemodynamic properties such as flow pattern, rheology, shear stress and velocity contribute to the initiation and enhancement of platelet adhesion to the vascular wall [18]. Leukocyte or platelet adhesion was found to be larger under high shear stress and steady flow than in pulsatile flow [18–20]. By having a clear understanding of the blood vessel and the blood flow dynamics relative to the disease pathology, it is possible to manipulate the carrier's physicochemical properties and achieve optimal adhesion to the endothelial wall. Studies have shown that carriers with a spherical geometry exhibit minimal lateral drift and are less likely to marginate to vessel walls, thereby binding less to the endothelial cells. Such carriers tend to stay within the cell-free layer region found between the red blood cell (RBC) core and the vascular wall and do not come in contact/bind to endothelial walls, hindering site-specific delivery [21–23]. Thus, an optimal carrier would break from the red cell core, accumulate in the cell free layer, and move in close proximity to the vessel walls with an increased lateral drift to establish contact with endothelial cells and receptors of interest. In this regard, the non-spherical counterparts are capable of a much complex motion such as tumbling and rolling. They can achieve margination without the aid of an external force. In addition, lateral drifting velocity has been shown to have a direct relation to the nanocarrier's aspect ratio. Rod-shaped particles can rotate and oscillate from one wall to the other, tending to marginate more than spherical particles [7].

One other factor that governs the adhesion to the blood vessel walls is the balance between the carrier's ligand density, the number of cell-surface receptors and the shear stress at the vessel walls. In addition, size and shape can also impact particle adhesion. Although the hydrodynamic force is small for submicron particles, the interaction area at the particle/cell interface is small, resulting in minimal number of ligand-receptor bonds that can withstand shear stress. In case of larger particles, the number of ligand-receptor bonds increases, although the hydrodynamic force grows along with it [24]. Chareonphol et al. demonstrated that spherical particles at size 2–5 μm displayed better margination to the wall at intermediate high shear rate and channel height than sub-micron sized particles when tested using parallel plate flow assays [25].

2.3. Nanocarrier site-specific extravasation and tissue accumulation

While nanocarrier site-specific extravasation and accumulation is valid for all kinds of diseases including neurodegenerative, infectious and inflammatory diseases, the concept is better outlined with regards to treating cancers. Following sustained circulation and selective, yet firm adhesion to the vascular walls, nanocarriers are found to accumulate at different regions of the tumor. These then cross the endothelial layer and penetrate deep within the interstitial space of tumors prior to being taken up by the cancer cells. In contrast to normal blood vessels, the tumor vasculature is characterized by the presence of complex, disorganized leaky vessels with heterogeneous blood flow [26,27]. This could facilitate the passive targeting or preferential accumulation of nanocarriers at the tumor site, also termed as the enhanced permeation and retention (EPR) effect as coined by Maeda et al. [28,29]

The endothelial surface of tumor neovasculature is characterized by the presence of overexpressed receptors such as VEGF, α_v β_3*etc.* Nanocarriers have often been functionalized with ligands specific to such receptors for targeted adhesion and localization at the desired location [30]. In addition, it is important to note that the permeability of the nanocarriers across the tumor vasculature also depends on physiological characteristics of the vessels itself. Tumor heterogeneity could result in vasculature that's not leaky at all places. This could affect the vascular permeability of the nanocarriers and in turn its tumor accumulation rates. Studies have also shown the effect of tumor microenvironment on the vascular permeability of nanocarriers and its tumor accumulation rates. One study showed that EPR depended on the type of tumor and the tissue in which it is located [31]. The rate of liposome accumulation was assessed in various tumor xenografts including 4 T1 (breast cancer), 3LL (lung cancer) and CT26 (colon cancer) cells at different primary and metastatic sites. This was found to correlate with the relative ratio of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1), suggesting that increased levels of MMP-9 indicated increased vascular permeability. Thus, MMPs could potentially be used as biomarkers for EPR and thereby identify patients who could respond to nanoparticle-based therapy. In a different study, the effect of collagen on the diffusion rate of particles of varying size was examined with tumor models and analyzed *via* computational modeling [32]. A direct correlation was found to exist between the collagen content in tumor vasculature and nanocarrier permeability, thereby suggesting the potential for collagen as a new biomarker in nanocarrier mediated cancer therapy.

The composition of the interstitial compartment of tumor tissues is notably different from a normal tissue. The absence of an anatomically well-defined functional lymphatic network along with unregulated cancer cell proliferation, widespread fibrosis and dense extracellular matrix (ECM) elevates the interstitial fluid pressure (IFP) in tumors. While EPR could promote increased vascular permeability and accumulation of nanocarriers at the tumor site, elevated interstitial fluid pressure (IFP) could impede its extravasation into sites of interest. This could have significant implications in diagnosing and treating tumor growth and metastases using nanocarriers [33].

Over the years, various approaches have been tested to surmount the high IFP in tumors. A balance between proangiogenic and antiangiogenic factors attributes to normal vasculature. However, in tumors, elevated levels of proangiogenic factors such as VEGF, basic fibroblast growth factor and platelet-derived growth factor (PDGF)

contribute to the highly tortuous and heterogenous network of newly formed blood vessels. In an attempt to restore the balance and normalize the tumor vasculature, Jain and co-workers explored the use of antiangiogenic agents. This enable diffusion of drugs, nanoparticles and polymer-drug conjugates to the tumors [34–36]. The penetration rate of drugs and nanocarriers could also depend on the density of ECM. As a result, strategies that involve the reduction of collagen or glycosaminoglycan density in tumors were tested and found to increase nanocarrier accumulation within tumors.

2.4. Cell membrane trafficking and endosomal compartmentalization

In the case of intracellular drug delivery, nanocarriers are required to get endocytosed by cells and release its cargo, following site-specific extravasation and accumulation. Unlike hydrophobic molecules, which are capable of diffusing through the cell's lipid bilayer membrane, macromolecules require an active cell-uptake mechanism. With respect to charge, cationic nanocarriers were internalized more compared to their anionic counterparts when tested in different cell types [37–39].

On endocytosis, nanocarriers form membrane invaginations and intracellular vesicles (phagosomes or endosomes) that fuse with highly acidic lysosomes [40]. While clathrin-mediated endocytosis is the classical pathway for a majority of nanocarriers, phagocytotic-mediated endocytosis is prevalent for cells of the MPS. In case of clathrin or receptor-mediated endocytosis, particles functionalized with ligands bind specifically to cell surface receptors. The ligand-receptor complex then forms clathrin-coated pits which cleave from the membrane to form clathrin-coated vesicles. The vesicles then fuse with early endosomes and route to lysosomes or recycle back to the plasma membrane. The lysosomes degrade the particles and its cargo prior to inducing its activity. There also exists an alternate pathway known as the caveolae-mediated endocytosis for macromolecules. In this case, integral membrane proteins called caveolins and peripheral membrane proteins called cavins form 50–60 nm membrane invaginations called caveolae. The caveolae pinches off from the membrane and fuses with caveosomes of neutral pH. Unlike the phagosomes or the endosomes, caveosomes are known to bypass lysosomes in some instances.

In a nutshell, these biological barriers exist to maintain homeostasis, in order to prevent any foreign substance/pathogen from colonizing any specific cell or tissue. Nanocarriers, being foreign objects often encounter and are affected by the presence of these barriers. Therefore, strategies have been and are being further developed to minimize these interactions by altering the physicochemical properties.

3. Physicochemical properties of nanocarriers and their effects *in vivo*

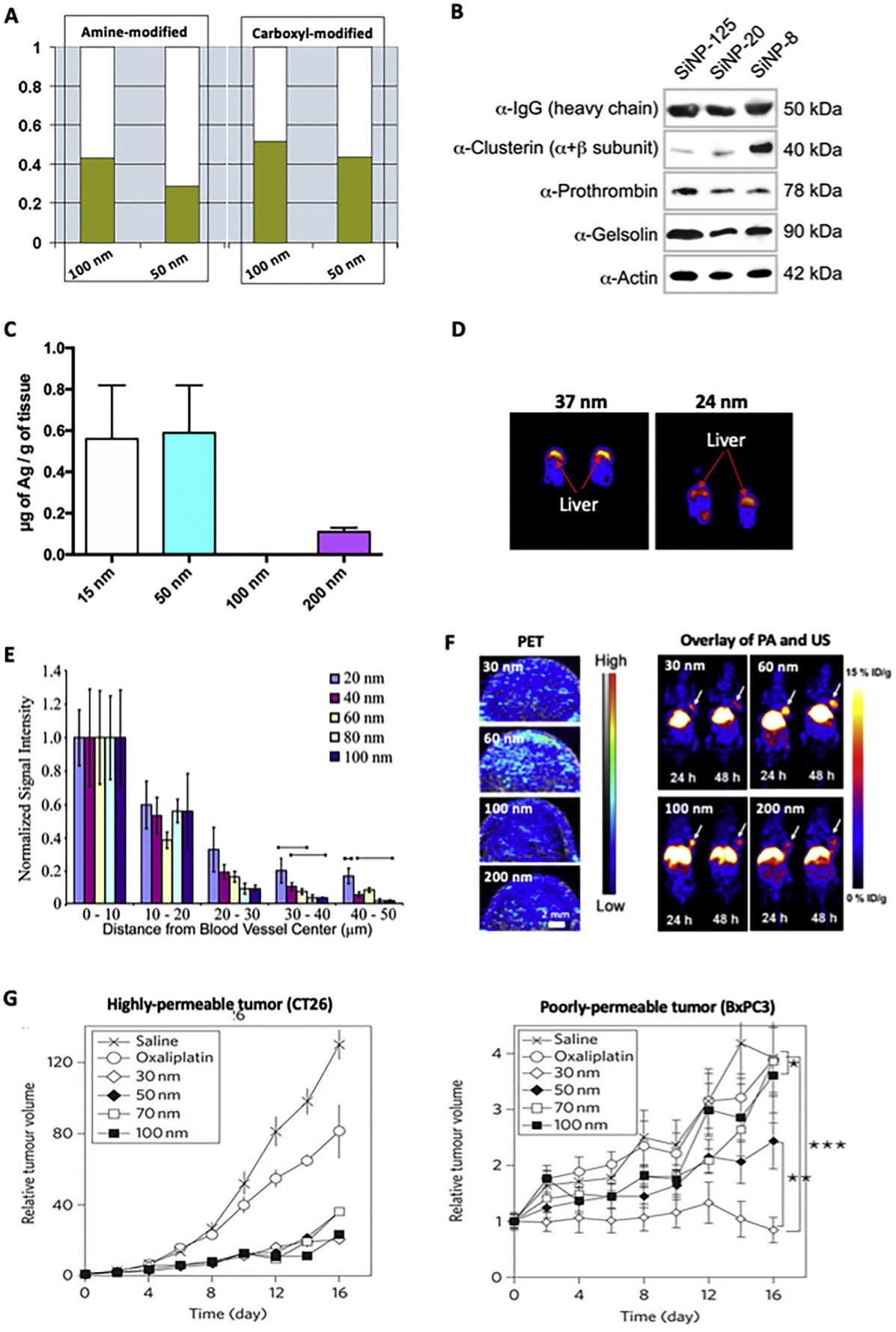
Physicochemical properties (size, shape, surface chemistry, porosity, elasticity, et al.) are the "synthetic identity" of nanocarriers. Studies have revealed that physicochemical properties are critical determinants of the *in vivo* fate of nanocarriers, by influencing multiple *in vivo* processes like protein corona formation, blood circulation, biodistribution, and cellular interaction *in vivo* [41–43]. In this section, we introduce major physicochemical properties of nanocarriers and literature evidences showing their impact on the *in vivo* fate. Emphasis are mainly focused on *in vivo* fate of nanocarriers following intravenous administration.

3.1. Size

Size directly determines the surface area of nanocarriers available to interact with biological environments, thus being a critical physicochemical property influencing the *in vivo* fate of nanocarriers. Plasma proteins adsorb onto nanocarriers to form a protein corona, which gives a unique "biological identity" to the nanocarriers and determines their interactions with the phagocytes [44,45]. Studies have revealed that nanocarrier size significantly influences protein corona formation [46,47]. Lundqvist et al. studied the effect of size on protein corona formation using polystyrene nanoparticles as a model system [48]. They found the homology between the protein coronas of 50 and 100 nm carboxyl- or amine-modified polystyrene nanoparticles is <50% (Fig. 3A), revealing that the corona around nanoparticles are size-dependent. Similarly, Stauber and co-workers studied the effect of size on plasma derived corona using monodispersed amorphous silica nanoparticles having different sizes (20, 30, and 100 nm) [49]. Results from their study revealed that the nanoparticle size affects the protein corona rather quantitatively than qualitatively. Particularly, smaller nanoparticles (20 nm) attracted more lipoproteins like clusterin while the larger nanoparticles (100 nm) showed higher affinity towards complement-related proteins like prothrombin and gelsolin (Fig. 3B).

Size significantly influences the blood circulation time and the biodistribution of the nanocarriers. Blood clearance and biodistribution of nanocarriers are the outcomes of filtering within the organs and barriers between the organs and their surrounding fluids [50]. Kidney has a cutoff size of around 6 nm and nanocarriers smaller than 6 nm can be quickly filtered out by the kidney [51,52]. Although there is no certain cutoff size for the liver and splenic filtration, nanocarriers larger than 200 nm can be rapidly captured by the liver and spleen due to the activation of complement [50,53,54]. The impact of size on blood circulation and biodistribution has been widely studied and a smaller size seems to result in a longer circulation time and reduced accumulation in the liver and spleen [50]. As an example, Sonavane et al. compared the blood circulation and biodistribution of gold nanoparticles with different sizes (15, 50, 100, and 200 nm) [55]. They found that smaller nanoparticles (15 and 50 nm) exhibited a longer circulation time (Fig. 3C) and a higher accumulation in all organs. Particularly, they were even able to pass through the blood-brain barrier. However, larger nanoparticles (100 and 200 nm) showed much shorter circulation time (Fig. 3C) and more accumulation in the liver and spleen. Similar finding was reported by Sun et al. for shell-cross-linked nanoparticles. The authors demonstrated that the smaller particles (24 nm) exhibited a longer circulation time and lower accumulation in the liver than the larger ones (37 nm) (Fig. 3D), and the nanoparticle size did not influence the accumulation in the spleen significantly [56].

Size also plays a critical role in the accumulation and penetration of nanocarriers at the diseased sites, especially in the case of tumors. Increase in the size of nanocarriers is known to affect their vascular permeation negatively. [57–66] Accumulation of nanocarriers in the tumor sites is fulfilled by the EPR effect which is generally applicable to nanoparticles of the size range between 30 and 200 nm [50,67,68]. Accumulation and distribution of nanocarriers within the tumors are determined by their circulation time, local gradient and local penetration [4,59,69]. Nanocarrier size can influence the tumor accumulation and penetration separately. As an example, Chan et al. studied the effect of nanocarrier size on passive targeting of tumors using PEGylated gold nanoparticles with different sizes (10–100 nm) [59]. They found that accumulation of 40–100 nm particles is exclusively dependent on the blood half-life while accumulation of the particles in the range of 20 nm depends on both the size and the blood half-life, with 60 nm showing the most accumulation in the tumor. Moreover, they also found that smaller nanoparticles rapidly penetrated through the tumor matrix while larger nanoparticles only stayed near the vasculature (Fig. 3E). Similar finding was found by Chen et al. in a study which shows 60 nm perylene diimide nanoparticles are the best for tumor accumulation compared to the nanoparticles of the sizes 30, 100, and 200 nm (Fig. 3F) [58]. Noticeably, the effect of nanocarrier size on tumor accumulation also depends on the physiological properties of tumors like tumor type, tumor stage, and tumor permeability [62,66]. As an example, Kataoka and colleagues compared the accumulation and antitumor efficacy of long-circulating, drug-loaded polymeric micelles with different sizes (30, 50, 70, and 100 nm) in tumor models with different intrinsic permeability. Findings from their study


A
Amine-modified
Carboxyl-modified
100 nm
50 nm
B
SiNP-125
SiNP-20
SiNP-8
α-IgG (heavy chain)
α-Clusterin (α+β subunit)
α-Prothrombin
α-Gelsolin
α-Actin
50 kDa
40 kDa
78 kDa
90 kDa
42 kDa
C
μg of Ag / g of tissue
15 nm
50 nm
100 nm
200 nm
D
37 nm
24 nm
Liver
E
Normalized Signal Intensity
20 nm
40 nm
60 nm
80 nm
100 nm
0 - 10
10 - 20
20 - 30
30 - 40
40 - 50
Distance from Blood Vessel Center (μm)
F
PET
High
Low
Overlay of PA and US
30 nm
60 nm
100 nm
200 nm
24 h
48 h
2 mm
15 % ID/g
0 % ID/g
G
Highly-permeable tumor (CT26)
Poorly-permeable tumor (BxPC3)
Saline
Oxaliplatin
30 nm
50 nm
70 nm
100 nm
Relative tumour volume
Time (day)

suggested that micelles of all sizes exhibited similar, high tumor accumulation and comparable antitumor efficiency in the tumors with high permeability. However, only the 30-nm micelle could penetrate into the poorly permeable tumors to achieve therapeutic efficacy (Fig. 3G) [66].

3.2. Shape

Shape is a pivotal physicochemical property of nanocarriers that largely determines the *in vivo* fate. Shape of nanocarriers has significant effects on multiple *in vivo* processes including macrophage uptake, blood circulation and biodistribution, margination and extravasation, and disease targeting. Although most of the current nanocarriers, under preclinical or clinical studies, are of spherical shapes, the unique properties of non-spherical nanocarriers may provide a new window for rational design of nanocarriers for specific purposes.

Macrophage uptake or phagocytosis is the first barrier after intravascular administration of particles. Early studies on particle shape effects suggested that particle shape plays an important role on macrophage uptake [11,12,70]. Mitragotri and *co*-workers prepared polystyrene nanoparticles of different shapes and investigated the effect of particle shape on phagocytosis [11]. Results from their study revealed that particle shape, not size, plays a dominant role in macrophage uptake. More specifically, local particle shape at the point of initial contact, rather than the overall particle shape, determines the complexity of actin structure necessary to initiate phagocytosis, and thus dictates whether particles are phagocytosed or simply spread on cell membranes. They demonstrated that the internalization velocity is inversely correlated with tangent angle (Ω). When $\Omega < 45°$, particles can be successfully internalized *via* forming actin-cup and ring. However, when $\Omega > 45°$, particles can only spread on membranes but not be internalized easily. In a follow-up study, they further proved that particle shape affects the attachment and internalization to macrophages separately [71]. Prolate ellipsoid particles attached to the macrophages more efficiently than the oblate ellipsoid or spherical particles. However, the oblate particles exhibited higher internalization efficiency than the spherical or prolate ellipsoid particles.

Shape has a significant impact on the circulation time of nanocarriers. On one hand, particles of different shapes can be phagocytosed at different rates and thus have a different blood circulation time, on the other hand, non-spherical nanocarriers have a deviating hydrodynamic behavior compared to the spherical ones [43,72]. As a result, they have a different behavior in aligning with the blood flow and therefore a distinctive circulation time. Literature evidences suggest that particles deviating from spherical shapes like filomicelles [73,74], nanorods [75–77], nanoworms [78,79], and nanodisks [80] have extended circulation time compared to their spherical counterparts. In one study, Geng et al. synthesized filomicelles to compare their transport and trafficking to those of spheres of similar chemistries [74]. They found that filomicelles were in the circulation for up to one week which is ten times longer than the spherical counterparts. Further mechanistic study revealed that the extended circulation time could be attributed to its orientation under flow and a subsequently lesser cellular uptake. Similar findings were also found for the nanocarriers with other shapes like rods and disks. For instance, Ghandehari and colleagues reported that gold nanorods had a significantly longer circulation time than their spherical counterpart. Specifically, nanorods persisted in the circulation for 24 h while nanospheres were in the blood for 6 h only [76]. In another study, Hu et al. fabricated amphiphile polymer based nanostructures with four different shapes including spheres, disks, large compound vesicles, and staggered lamellae [81]. Results from their study suggested that all the three shaped nanostructures exhibited a significantly extended circulation than the spheres. Particularly, staggered lamellae and disks had longer circulation half-life than the large compound vesicles and spheres (Fig. 4A). Apart from influencing the circulation time, shape also affects the biodistribution of nanocarriers. Compared to spherical nanocarriers, aspherical nanocarriers that show reduced phagocytosis generally exhibit reduced liver accumulation. This phenomenon has been observed for multiple nanoparticle shapes, such as filomicelles [82], nanorods [76], and nanodisks [75]. For example, Ghandehari and colleagues found that the gold nanorods accumulated significantly lesser in the liver as compared to the gold spheres of similar size and surface chemistry (Fig. 4B) [76]. In addition to the differences in the liver accumulation, particle shape can have a significant impact on the nanocarrier distribution in other organs. More specifically, particles of specific shapes may show enhanced specific accumulation in certain organs. As an example, Decuzzi et al. compared the biodistribution of silica nanoparticles of four different shapes including spherical, hemispherical, cylindrical and discoidal [83]. Findings from their study suggested that the cylindrical particles showed the highest accumulation in the liver compared to all the other shaped particles. In contrast, the discoidal particles accumulated higher in majority of the organs but the liver. Enhanced accumulation was especially observed in the highly vascular organs like lung, spleen, and heart for the discoidal particles. In addition, hemispherical particles showed similar biodistribution to the spherical particles, except them showing more accumulation in the spleen and less in the lung (Fig. 4C). More interestingly, subtle changes in particle shape may significantly influence the biodistribution of nanocarriers. For example, particles of similar shapes but different aspect ratios have been reported to have dramatically different organ distributions [75,84]. In a study conducted by Huang et al., the authors compared the biodistribution and clearance of a long-rod mesoporous silica nanoparticle (aspect ratio 5) and a short-rod one (aspect ratio 1.5). They found that short-rod nanoparticles mainly accumulated in the liver while the long-rod nanoparticles preferably accumulated in the spleen. In addition, PEGylation resulted in increased accumulation in lungs for both particles and more significant accumulation was observed for the long-rod nanoparticles (Fig. 4D). Moreover, the short-rod nanoparticle exhibited a more rapid clearance rate than the long-rod counterpart [75].

Shape of nanocarriers has profound impacts on accumulation at the target disease sites. Especially, specific shapes, such as discoidal and rod, have been leveraged to target the vascular endothelium, based on the fact that particle shape significantly impacts the margination and adhesion process [41]. Non-spherical nanoparticles have distinct motions with tumbling and rolling and thus result in unique margination dynamics. On the other hand, elongated particles usually show reduced drag and higher surface area than spheres. As a result, certain non-spherical nanocarriers can adhere to the endothelial walls more strongly due to enhanced multivalent bonding [84–86]. Extensive flow

Fig. 3. Effect of nanocarrier size on its *in vivo* fate. (A) Nanocarrier size impacts plasma protein binding qualitatively. The homology of protein coronas around polystyrene nanocarriers of 100 and 50 nm was <50%, indicating nanocarrier size influenced the species of plasma proteins bound to nanocarriers. (B) Nanocarrier size influences plasma protein binding quantitatively. Smaller silica nanoparticles (SiNP-8) attracted more lipoproteins (clusterin) while the larger counterpart (SiNP-125) attracted more complement-related proteins (prothrombin and gelsolin). (C) Size affects the circulation time of nanocarriers. Smaller gold nanoparticles (15 and 50 nm) exhibited longer circulation than the larger counterparts (100 and 200 nm). (D) Size impacts biodistribution of nanocarriers into RES organs. A smaller shell-crosslinked nanoparticle (24 nm) was less captured by liver compared to its larger counterpart (37 nm). (E) Size critically determines the penetration rate of nanocarriers into tumor sites. Smaller sized gold nanocarriers were found to penetrate deeply into tumors while the larger sized counterpart only stayed near the vasculature. (F) Size largely impacts the accumulation of nanocarriers in tumor sites. A 60-nm perylene diimide nanoparticle showed the highest tumor accumulation as compared to its other sized counterparts (30, 100, and 200 nm). (G) Size effect on tumor targeting and treatment depends on the permeability of tumors. Smaller sized drug loaded micelles demonstrated enhanced tumor inhibition efficacy only in a poorly-permeable tumor model (BxPC3) but not in a highly-permeable tumor model (CT26). [Adapted with permissions from [48,49,55,56,58,59,66].]

chamber studies have revealed that the non-spherical nanoparticles have a greater degree of lateral drifting and enhanced adhesion towards the vessel walls and thus can have a better chance to interact with the endothelium [87–89]. For example, Cooley et al. studied the margination and adhesion behavior of polystyrene particles of four different shapes (spheres, oblate, rod, and prolate) using microfluidic devices [90]. They found that ellipsoidal particles exhibited much higher margination probability than the spheres. Compared to the spheres, oblate and rod particles demonstrated the higher adhesion at different shear flow conditions in the presence of RBCs. Shape effects of nanocarrier on the endothelium targeting have also been studied. As an example, Mitragotri and *co*-workers designed antibody-coated polystyrene nanoparticles of spherical and rod shapes [91]. Their microfluidic study indicated that rod-shaped nanoparticles exhibited higher specific and lower nonspecific accumulation under flow at the target site as compared to the spherical counterpart. Moreover, by changing shape from spherical to rod, enhanced vascular targeting was observed *in vivo* in lungs and brains for nanoparticles coated with anti-intracellular adhesion molecule 1 (ICAM-1) and anti-transferrin receptor antibodies (Fig. 4E). Similar findings were also observed by Muzykantov and co-workers. They found that disk nanoparticles exhibited extended circulation time and more specific endothelial targeting to the lungs [80]. Apart from vascular endothelium targeting, shape has shown to significantly influence the targeting to other disease sites like the tumor. Extensive studies have revealed that nanocarriers with specific non-spherical shapes exhibited enhanced tumor targeting and accumulation, such as nanotubes, filomicelles, rods, and disks [79,92–99]. The enhanced tumor accumulation can be attributed to 1) longer circulation time; 2) better margination, adhesion, and extravasation; and 3) less return of particles out of the tumor microenvironment [42,84]. As an example, Ghandehari and co-workers demonstrated that gold nanorods were accumulated more significantly in the tumor than gold nanospheres in an orthotopic A2780 human ovarian cancer model [76]. Similar findings were found in a study conducted by Christian et al. demonstrating that paclitaxel loaded filomicelles convected more into the tumor sites as compared to the spherical micelles of the same copolymer [73]. It should be noticed that shape not only impacts tumor targeting but also has potential effects on the intratumoral distribution of nanocarriers. Black et al. investigated the tumor uptake and intratumoral distribution of gold nanoparticles of four different shapes (nanosphere, nanodisk, nanorod, and cubic nanocage). Results from their study showed that nanospheres and nanodisks accumulated in the tumors more significantly than the nanorods and nanocages. However, nanospheres and nanodisks were distributed only on the surface of the tumors while nanorods and nanocages were distributed throughout the tumors (Fig. 4F) [100].

3.3. Surface chemistry and properties

Surface chemistry refers to the properties of nanocarrier surface that are in direct contact with the biological microenvironment, such as surface charge, surface hydrophobicity, targeting ligands, and many others. Surface chemistry directly determines the interaction between nanocarriers and physiological environments and thus significantly impacts the *in vivo* fate of nanocarriers.

3.3.1. Surface charge

Surface charge affects the *in vivo* fate of nanocarriers by influencing the opsonization process. Surface charge density has both qualitative and quantitative impacts on the plasma protein adsorption. Qualitatively, surface charge impacts the species of adsorbed plasma proteins.

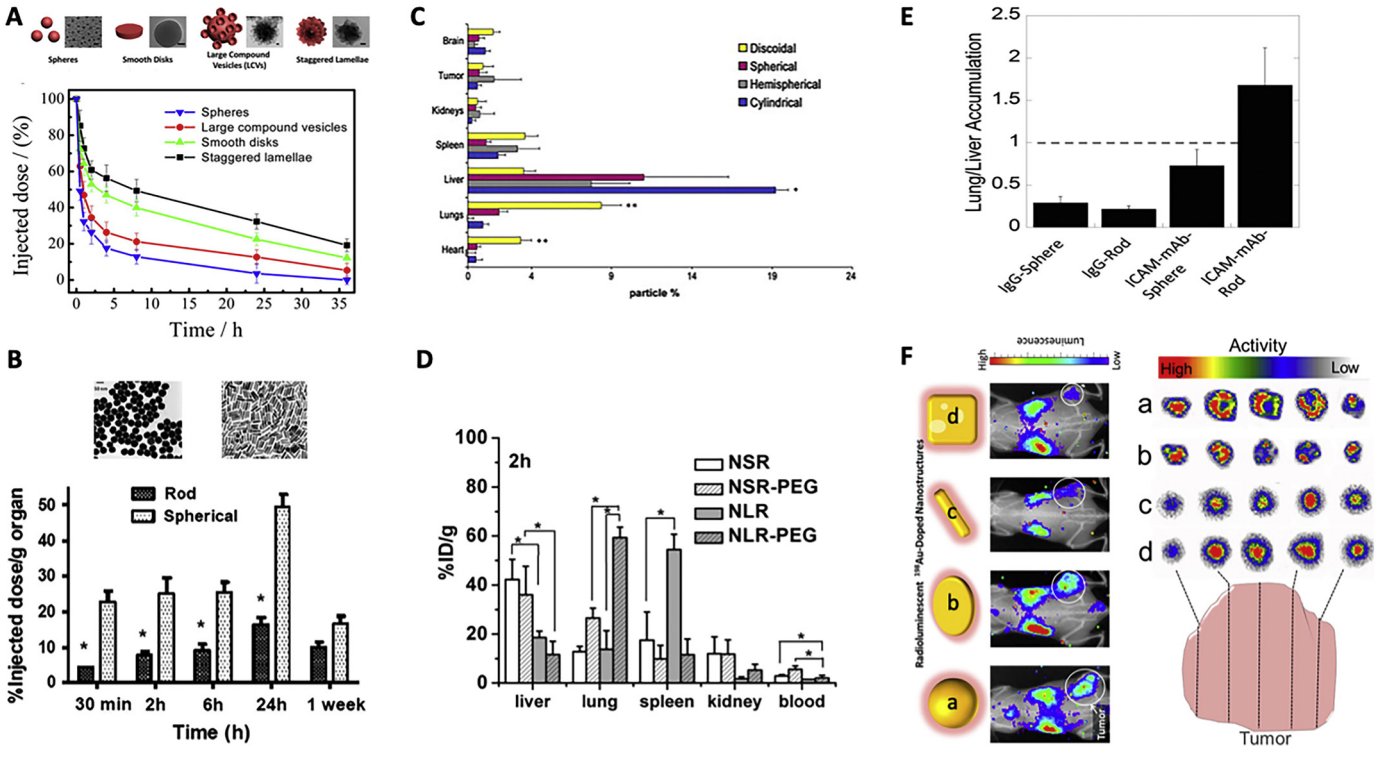


Fig. 4. Impact of particle shape on the *in vivo* fate of nanocarriers. (A) Shape affects the circulation time of nanocarriers. Staggered-lamellae-shaped and disk-shaped amphiphilic polymeric nanocarriers circulated significantly longer than their sphere-shaped counterpart. (B) Non-spherical nanocarriers may exhibit different RES capture behaviors compared to their spherical counterpart. For example, gold nanorods were found to accumulate significantly less in the liver than gold spheres. (C) Specific nanocarrier shapes can enable targeted accumulation in specific organs. (D) Subtle changes in particle shape can result in dramatically different biodistribution of nanocarriers. For example, compared to the short-rod silica nanocarriers (aspect ratio 1.5), the long-rod ones (aspect ratio 5) showed more accumulation in the lung and spleen but less in the liver. (E) Shape effect could be exploited to enhance specific targeting to endothelium. For example, anti-ICAM-1 antibody conjugated rod-shaped polystyrene nanocarriers exhibited more specific targeting to the lung endothelium as compared to their spherical counterparts. (F) Particle shape can impact the tumor accumulation and intratumoral distribution of nanocarriers separately. [Adapted with permissions from [75,76,81,83,91,100].]

Using polystyrene nanoparticle as a model, Muller and co-workers illustrated that cationic and anionic nanoparticles majorly bind plasma proteins with a pI <5.5 (such as albumin) and >5.5 (such as IgG), respectively [101]. Quantitatively, nanocarriers with higher surface charge densities adsorb more proteins than those with a neutral charge. As an example, Gessner et al. fabricated a series of polymeric nanoparticles with different negative charge densities [102]. They found that increasing the negative charge densities resulted in an increased amount of the adsorbed plasma proteins. However, no significant differences were detected in the species of the adsorbed proteins. Semple et al. reported similar findings for liposomes with different surface charge densities [103]. They formulated liposomes with different surface charges by changing the composition of the lipids and found that the cationic liposomes bind significantly more plasma proteins than the neutral counterpart.

Extensive studies have proven that surface charge has a critical impact on the blood circulation and biodistribution of nanocarriers: high surface charge densities usually result in faster blood clearance and RES capture while the neutral charges contribute to the extended blood circulation and reduced RES clearance [42,47,104–106]. As an example, Xiao et al. fabricated PEG-oligocholic acid nanoparticles with a range of surface charges (−26.9 to 37.0 mV) and studied their effect on the *in vivo* biodistribution [107]. Results from their study revealed that the highly positive (>15 mV) or negative (<−15 mV) nanoparticles were phagocytosed more by the liver macrophages (Kupffer cells) and thus resulted in higher accumulation in the liver. In contrast, nanoparticles with a slightly negative charge (−8.5 mV) exhibited significantly reduced Kupffer cell uptake and liver accumulation. Similar findings were observed by He et al. for chitosan grafted polymeric nanoparticles [108]. Results from their studies suggested that positively charged nanoparticles had a shorter blood circulation time and higher liver and spleen accumulation than the negatively charged nanoparticles. Moreover, higher negative charge densities have resulted in higher liver accumulation and less retention in the blood. Apart from effects on biodistribution, surface charge even affects the sub-organ distribution of nanocarriers. In a recent study, Vachet and co-workers demonstrated that gold nanoparticles with neutral, positive, or negative surface exhibited dramatically different sub-organ distributions. Particularly, positively or negatively charged nanoparticles accumulated extensively in the red pulp of the spleen while the neutral counterpart accumulated more in the white pulp and marginal zone of the spleen. In addition, the neutral nanoparticles were associated more with Kupffer cells in the liver. In contrast, positive nanoparticles accumulated more in the liver hepatocytes and the negative counterpart showed a broader distribution in the liver (Fig. 5A) [109]. Surface charge also influences the intracellular trafficking of nanocarriers. Developing methods to fabricate nanocarriers or macromolecular drugs that escape the endosomes has been one of the current focuses in the field. Incorporating cationic polymers such as poly(ethylene imine) (PEI) or poly(L-lysine) (PLL), within the carrier framework, enables interaction with the negatively charged endosomal membrane causing it to destabilize instead ('flip-flop' mechanism) [110]. An alternate strategy is to synthesize particles made out of polymers (PEI, histidine) with large buffering capacity. The presence of secondary/tertiary amine groups facilitates the polymers to absorb protons resulting in an influx of water to cause endosomal swelling and rupture [111].

Surface charge also plays a significant role in targeted accumulation of nanocarriers in the disease sites. To take cancer as an example, accumulation of nanocarriers in the tumor sites is the sum of blood circulation and local interaction with tumor associated cells, both of which are affected by surface charge [4,112–116]. Literature evidence shows that nanocarriers with different surface charges exhibited dramatically different tumor accumulation and penetration [105,117–119].

Studies by Xiao et al. showed that the amount of PEG-oligocholic acid nanoparticles accumulated in the tumor was inversely correlated with their surface charge densities (either positive or negative) [107]. Particularly, they demonstrated that the highest tumor accumulation could be achieved by making the surface slightly negative. Although not being good for longer circulation and tumor accumulation, positive surface charge has been reported to lead to better tumor extravasation and penetration. As an instance, Yin and co-workers found that surface charge density had different impacts on tumor accumulation for negatively and positively charged nanoparticles [108]. Higher surface charge densities caused lower tumor accumulation for negatively charged nanoparticles. However, for positively charged particles, nanoparticles with higher charge densities exhibited higher accumulation in the tumor. The authors attributed this to the fact that nanoparticles with a higher positive charge density could more efficiently depart from the interstitial space and be internalized by tumor cells and the associated endothelium. Similar findings were reported in a recent study by Wang et al. [117] They found that compared to the neutral and anionic counterparts, the cationic PEG-b-PLA based nanoparticles exhibited a shorter blood circulation and a lower tumor accumulation but a significantly higher tumor extravasation, penetration and cellular uptake. Moreover, the cationic nanoparticles loaded with docetaxel resulted in much better antitumor efficacy in multiple tumor models over the neutral and anionic ones (Fig. 5B). The enhanced antitumor efficacy of positively charged nanocarriers can be attributed to that they showed increased preferential binding and permeability in angiogenic tumor vessels when compared with their anionic/neutrally charged counterparts and tested with normal vasculature [120]. More interestingly, based on the understanding of how surface charge influences tumor targeting and accumulation, studies have been done to design surface-charge-switchable nanocarriers to achieve enhanced antitumor efficacy. For example, one study showed that doxorubicin-containing zwitterionic nanoparticles could circulate long enough to extravasate into the tumor microenvironment where pH was ~6.8. This led the zwitterionic particles to shed its anionic coat and expose its cationic charge for enhanced tumor cell uptake and therapeutic response [119,121].

3.3.2. Surface hydrophobicity

Surface hydrophobicity not only influences opsonization but also determines the pharmacokinetics and biodistribution of nanocarriers. Nanocarriers with a more hydrophobic surface tend to absorb more plasma proteins after *in vivo* administration and therefore undergo faster blood clearance and capture by the RES [43,122]. Over the past decades, many strategies have been explored to reduce the undesired RES clearance by changing the surface hydrophobicity of nanocarriers. Attaching hydrophilic polymers/moieties, such as PEG, poloxamer, dextran, chitosan, poloxamine, pluronic F127, poly(oxyethylene), and many others, has been proven one of the most promising approaches [47,123–132]. Especially, PEGylation has been most widely exploited to mask surface hydrophobicity and to make "stealth" nanocarriers. The mechanism of PEGylation in extending nanocarrier circulation time is based on its capability to mask the surface charge and hydrophobicity of nanocarriers and hinder the opsonization process, thus preventing recognition by the RES and extending the circulation time [42,133]. This was best exemplified by PEGylating liposomal doxorubicin where half-life was increased from minutes to hours [134,135]. Another example was shown by Huang et al. where a low liver uptake and an increased accumulation of the injected dose in NCI-H460 tumors was seen for PEGylated polycationic DNA liposomes [136]. Previous studies revealed that the efficiency of PEGylation is largely dependent on its properties, such as its length, density, and conformation [42,43,133,137]. PEG conformation ("mushroom" and "brush") on the nanocarrier surface is closely related to the PEG length and density (especially PEG density). At a lower density, PEG exists on the nanocarrier surface with a "mushroom" conformation. On increasing the PEG density, the space between each PEG molecule as well as their degree of freedom is reduced, which in turn causes a conformation switch from the "mushroom" to "brush" [42,43]. Brush-like and intermediate

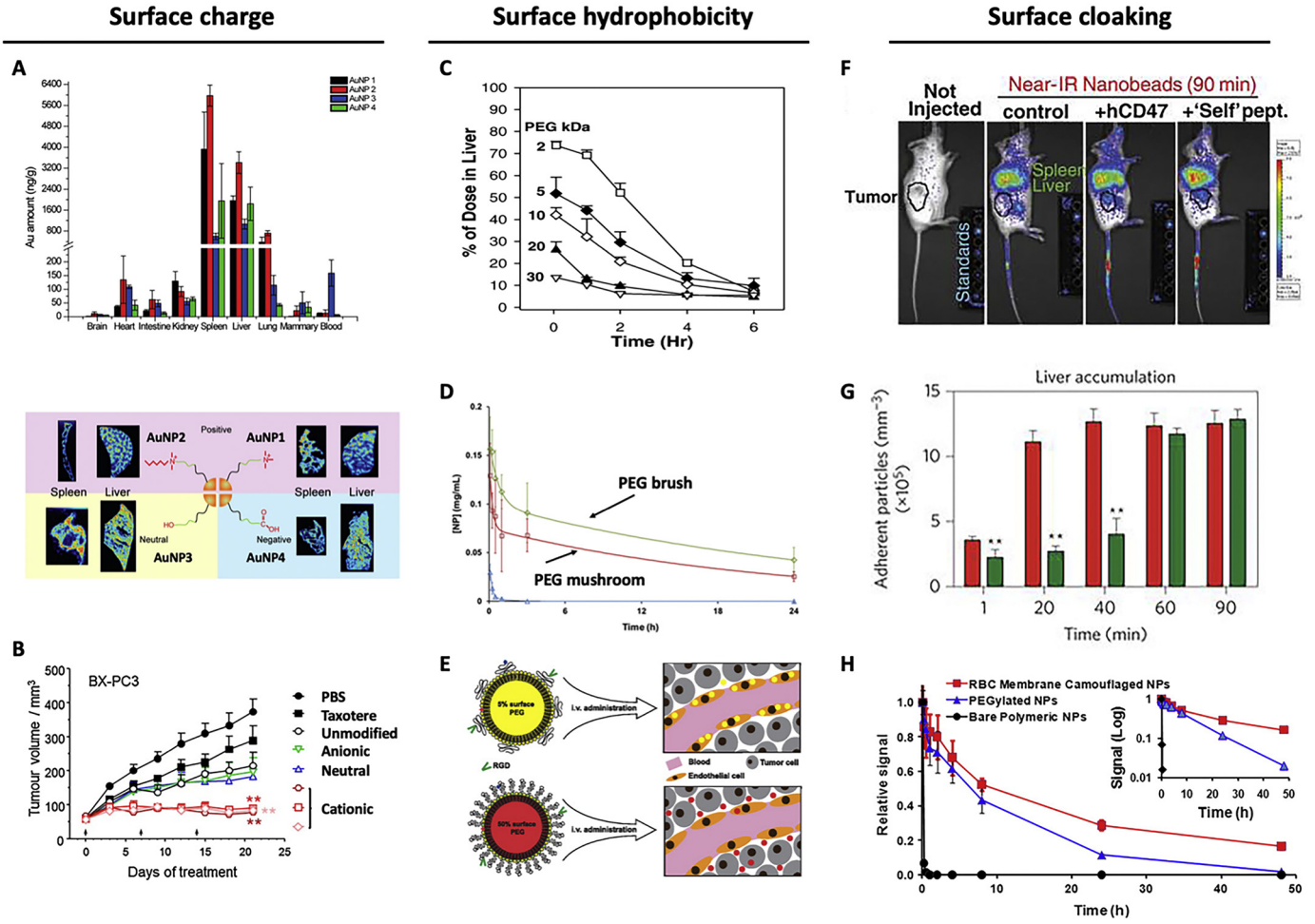


Fig. 5. Effect of surface chemistry and properties on the *in vivo* fate of nanocarriers. (A-B) Surface charge. (C-E) Surface hydrophobicity. (F-H) Surface cloaking. (A) Surface charge not only impacts the circulation and biodistribution of nanocarriers but also influences the sub-organ distribution of nanocarriers. (B) Cationic PEG-b-PLA based nanocarriers resulted in better antitumor efficacy than their neutral and anionic counterparts in a BX-PC3 tumor model. (C) and (D) PEG length and density have a significant impact on extending the circulation half-life of nanocarriers. (E) PEG density can impact the tumor accumulation of drug nanocarriers and their interactions with tumor-associated cells. (F) Conjugation of a CD47 "self" peptide to nanobeads significantly improved their accumulation in an A549 tumor model. (G) Coating nanocarrier surface with leukocyte membranes could remarkably reduce non-specific uptake of nanocarriers by the liver. Red bars: uncoated nanocarrier; green bars: leukocyte membrane coated nanocarrier. (H) A RBC membrane coated nanocarrier exhibited much longer circulation time than the conventional PEGylated counterpart. [Adapted with permissions from [109,117,143,146,149,156,164,166].]

configurations for PEG reduced the complement activation and phagocytosis as compared to the mushroom-like conformations [138].

The effect of length and density of PEG on the *in vivo* fate of nanocarriers has been widely studied. Although there is not a universal optimal PEG length and density existing for all nanocarriers, literature evidences suggest that a relatively longer length and a higher density has better capability to mask the nanocarrier surface hydrophobicity/charge and thus to prevent the plasma protein from binding, which subsequently extends the circulation time of nanocarriers and reduces accumulation in the RES organs [43,139–142].

In a systematic study, Khargharia et al. fabricated polyacridine peptide nanocarriers with a range of PEG lengths (2, 5, 10, 20, or 30 kDa) and studied their effect on the pharmacokinetics and biodistribution of the nanocarriers [143]. Results from their study suggested that with PEG lengths increasing, the circulation time of the nanocarriers was extended and the liver accumulation was reduced. Particularly, the longest PEG length (30 kDa) could maximally block the liver uptake up to 13% of the injected dose (Fig. 5C). It should be noticed that the dogma "longer is always better" does not apply to all cases. For examples, Madliney et al. reported that PEG length (ranging from 5 to 20 kDa) showed a negligible impact on the *in vivo* biodistribution of a silicate-based nanocarrier [144]. Similarly, Kaminskas et al. demonstrated that the circulation and biodistribution of poly-L-lysine dendrimer nanocarriers were mainly determined by the total molecular weight of PEGylated dendrimer rather than the PEG chain length alone [145]. These different findings of the effects of PEG length can be attributed to the differences in material properties and other physicochemical properties.

Compared to PEG length, PEG density has a stronger effect on the *in vivo* fate of nanocarriers, in the sense that PEG density has a larger impact on the coverage of the nanocarrier surface and its accessibility to plasma proteins [133]. Effect of PEG density has been widely studied. As an example, Barratt and *co*-workers studied the effect of PEG density on the pharmacokinetics and biodistribution of poly(rac-lactide) nanocapsules [140]. They found that increasing the PEG density from 10% to 30%, the circulation time of nanocarriers significantly decreased and the liver accumulation was remarkably reduced. In another study, DeSimone and colleagues systemically investigated the impact of PEG density on protein binding, macrophage uptake, pharmacokinetics and biodistribution of nanocarriers fabricated by PRINT technology [146]. Results from their study revealed that compared to the counterpart with a lower PEG density, nanoparticles with a higher PEG density absorbed fewer albumin proteins, circulated in the blood for a longer time (Fig. 5D), and accumulated less in the liver. Due to their effect on the circulation time and biodistribution, PEG length and density have

been reported to have significant effects on the targeting and accumulation of nanocarriers to the disease sites, such as tumor (Fig. 5E) [147–149]. It should be noticed that a too high PEG density may not be desirable for achieving long circulation or enhancing accumulation in target sites [43]. Such a PEG density can cause the imbalance between the hydrophobicity and hydrophilicity of nanocarriers, leading to particle instability and aggregation in circulation and thus a faster clearance by RES organs [43,150,151]. On the other hand, because PEG usually reduces the interaction between nanocarriers and cells, a very high PEG density may result in insufficient cross-talk between the nanocarriers and the cells at the target sites [42,152,153].

3.3.3. Surface cloaking

Apart from using synthetic polymers like PEG, researchers have used biological membranes [154,155] or derived peptides [156] to cloak the nanocarriers in order to perform unique functions. The use of biological cloaking [157–162] is an extensive field of research by itself and is beyond the scope of this paper to be discussed at length. However, we have included a few representative examples that elicit how a certain biological surface cloak can help escape a barrier.

Inspired from naturally circulating red blood cells in the body, Discher et al. computationally designed, synthesized and attached the 'don't eat-me' marker CD47 'self' peptides to 160-nm nanobeads. The peptides would facilitate an active stealth mechanism to evade the MPS. When tested in NSG non-obese diabetic (NOD) severe combined immunodeficient *IL2rγ*^{null}) mice, the nanobeads circulated significantly longer with delayed phagocytic clearance by the liver and spleen. In addition, an increased accumulation of nanobeads with paclitaxel (Fig. 5F) and as a consequence, substantial shrinkage was observed for A549 tumors *in vivo* when compared with conventional Cremophor EL formulation [156]. Certain peptides like INF7, H5WYG, and GALA have membrane destabilization property which can be utilized for achieving endosomal escape.

The use of membranes derived from blood cells, namely erythrocytes [163–165], leukocytes [166] and platelets [161,167] have been documented for multiple applications. Tasciotti and co-workers functionalized the surface of particles with cell membranes isolated from leukocytes [166]. A tenfold decrease in the opsonization (IgG and albumin) and a significantly lower uptake in murine J774 macrophages (~75% decrease) and human THP-1 phagocytic cells (~50% decrease) was seen for nanoparticles coated with membranes isolated from the same species. In a mouse model of melanoma, the biomimetic nanoparticles showed >2-fold accumulation at the tumor site, and less in the liver (Fig. 5G). Although a time-dependent increase in liver accumulation of the nanoparticles was seen, this was primarily due to its association with the liver endothelium and not on account of phagocytic effects of the Kupffer cells [166]. The same strategy was used elsewhere for achieving endosomal escape. TEM revealed that the absence of coated particles in lysosomes, while uncoated particles were compartmentalized within the endosomes after 24 h [166]. In a different study, Hu et al. showed that fabricating poly(lactic-*co*-glycolic acid) nanoparticles with membranes (both lipidic and protein components) isolated from red blood cells could extend its circulation for as long as 72 h (Fig. 5H) [164].

Apart from using blood cell membranes, binding or hitchhiking drugs to serum proteins has shown as a promising approach to promote long circulation. Drug- serum protein interactions have been attributed as one of the important factors that affect the drug's circulation time and tissue distribution. A classic example of this would be the FDA approved Abraxane, which is serum albumin functionalized paclitaxel. Not only did this help in improving the circulation time but also caused the construct to bind to glycoprotein 60 (gp60) and facilitate the cellular internalization *via* the caveolae-mediated pathway, facilitating transvascular movement into and across the tumor interstitial space, deep into the distal regions of the tumor. Consequently, a better safety/toxicity profile was seen in patients treated for metastatic breast cancer [168].

3.4. Elasticity

Elasticity refers to the ability of a material to be deformed. This ability of a material to be elastic is quantified using certain physical quantities like Young's moduli. Elasticity of nanocarriers has been thought to be a very important factor governing the fate of nanocarriers *in vivo*. This largely arises due to the fact that most of the cells in the body are deformable and have mechano receptors present on their surface, which helps them respond differently to materials of different stiffness [169]. Apart from influencing the *in vitro* uptake, elasticity has been shown to have effects on particle endocytosis [170]. Due to this hypothesis, the effect of elasticity of nanocarriers has been systematically investigated in the literature [171–178].

One of the early studies was carried out by Merkel et al., where they investigated microparticles made from 2 – hydroxyethyl acrylate crosslinked using poly(ethylene glycol) diacrylate (PEGDA). By varying the crosslinker density, the authors tuned the elasticity of particles from ~ 64 Pa to ~ 8 Pa. The authors found that this 8-fold decrease in the elastic modulus correlated to a 30-fold increase in elimination half-life of these particles [171]. A similar effect was observed by Müllner et al. when they altered the rigidity of cylindrical polymer brushes (CPB) and concluded that stiffer CPBs show a decreased plasma residence time [176]. Zhang and *co*-workers investigated the effect of stiffness on poly(carboxybetaine) nanogels and found that softer nanoparticles are able to pass through the splenic filtration more easily than their stiffer counterparts, thus leading to a longer circulation half-life (Fig. 6A) [174]. A systematic study of the effect of elasticity on circulation time was demonstrated by Anselmo et al. The authors concluded that the softer particles (~10 kPa) offered enhanced circulation as compared to the harder particles (~3000 kPa) (Fig. 6B). The authors also found that softer particles exhibited reduced uptake in macrophages, endothelial cells and cancer cells during *in vitro* experiments [172]. Addition of targeting peptides to elastic nanocarriers can generate biomimetic drug carriers. For instance, Anselmo et al. attached peptides involved in platelet aggregation to elastic nanoparticles fabricated using the layer-by-layer assembly of poly (allylamine hydrochloride) and bovine serum albumin, thereby mimicking the structure and surface of natural platelets. As compared to untargeted nanoparticles, targeted platelet-like nanoparticles accumulated at the wound site in a tail transection model, inducing a 65% reduction in the bleeding time [175].

Elasticity of nanocarriers has also been explored in tumor targeting applications. Recently, Guo and co-workers demonstrated that soft nanolipogels (lipids encapsulating alginate core) (~45 kPa) show a 2.6-fold increase in the tumor uptake than their stiffer counterpart (~19 MPa), with stiffer counterpart showing high accumulation in the liver (Fig. 6D) [177]. Key et al. investigated the effect of stiffness on PLGA nanoparticles altered by varying amounts of crosslinker in mice bearing non orthotopic brain/skin cancers (Fig. 6C). The authors found that softer particles accumulate upto~20% of the injected dose per gram tumor and when tagged with radioactivity, the labeled particles can be used to detect malignant tissues as small as 0.1% the weight of animal *via* PET imaging [173]. The effect of softer nanocarriers accumulating in the tumor tissue more than the harder counterparts was also observed by Hui et al. and Zhang et al. using liquid filled silica nanocapsules and lipid surrounded PLGAs respectively [179,180]. Elasticity of nanoparticles can also be combined with external stimuli like ultrasound. Chumakova and co-workers used 200 nm PLGA encapsulating air bubbles with the idea of limiting the oscillations due to the elasticity of the PLGA shell. In combination with ultrasound, an 8-fold increase in the transfection efficiency was observed with little to no cell death caused by ultrasonication [178].

Elasticity thus plays an important role in altering circulation of nanocarriers and their targeting with softer particles being able to escape biological barriers like the splenic filtration and selectively accumulate at certain sites like tumor tissue possibly because of their

ability to deform and resist movement out of the leaky vasculature as compared to harder nanoparticles.

3.5. Porosity

Porosity refers to the presence of internal microstructures [181]. One of the major advantages of having pores within the bulk is the ability of the material to have high surface areas which can be utilized for loading cargo and functionalization. These properties have also been explored for the purpose of drug delivery. Significant scientific effort has been put in understanding how different porous nanoparticles interact with cells *ex vivo* [182–185], but for the purpose of this paper, *in vivo* applications influenced by the porosity of nanoparticles are discussed in detail. As the idea of tweaking the porosity is relatively recent, studies investigating porosity as the sole parameter are fewer and the property is often used in combination with other physicochemical properties.

The most investigated material for effect of porosity on the *in vivo* fate is silica [184,186–200]. There have been multiple studies reported in the literature for use of porous silica nanoparticle for application in drug delivery, some using porosity to alter serum stability [195] or be stimuli responsive [192,197,198] others using it for drug loading applications in combination with targeting ligands [188,189,191,193,196,199]. A systematic study carried out by Yu et al. indicates that mesoporous silicon nanoparticles (PSNs) tend to accumulate in lungs following an intravenous injection as compared to their non-porous counterparts [195]. The authors attributed this to the difference in the serum protein- particle interactions as compared between the porous and non-porous particles (~22-fold difference in tissue affinity index) (Fig. 6E). Porosity of PSNs also results in interesting optical properties that are not fluorophore based. Photoluminescence of PSNs is on the order of 5–13 ms. This property has been used by Gu et al., where the authors improved the signal to background ratio 420-fold for *in vivo* imaging using these particles in mice (Fig. 6F) [192]. Previously Park et al. had demonstrated clinical safety of PSNs in mice [197]. The authors demonstrated that the PSNs self-destructed into components that are renally cleared without any toxicity. Attaching targeting peptides affects the biodistribution of these porous particles. A study carried out by Sarparanta et al. showed that atrial natriuretic peptide (ANP) modified PSNs showed 3-fold higher heart accumulation 10 min post intravenous injection as compared to the non-targeted counterpart in rats [188]. A similar effect had been previously observed by the authors in terms of mucoadhesion of targeted nanoparticles in rats [189]. Loaded particles with targeting ligands have been explored to combine the effect of encapsulating cargo with site specific delivery. A study by Coates and co-workers showed that the PSNs showed migration into livers, spleen, lungs and kidneys in mice as well as marmosets [191]. Further, PSNs when modified with CD11c peptide and loaded with $OVA_{323–339}$ and rapamycin showed 5-fold higher production of murine splenic regulatory T cells as compared to the control groups (i.v. treated with rapamycin or plain particles), 40 days post the injection. Recently, a study by Hussain and co-workers showed a 10-fold increase in vancomycin delivered by cyclic amino peptide (CARG) modified PSNs in the lungs as compared to free vancomycin injection in a murine *S. aureus* infection model [193]. Apart from these representative examples, modification with targeting antibody [199], combination with ultrasound [198] and *in vivo* delivery of siRNA [200] have

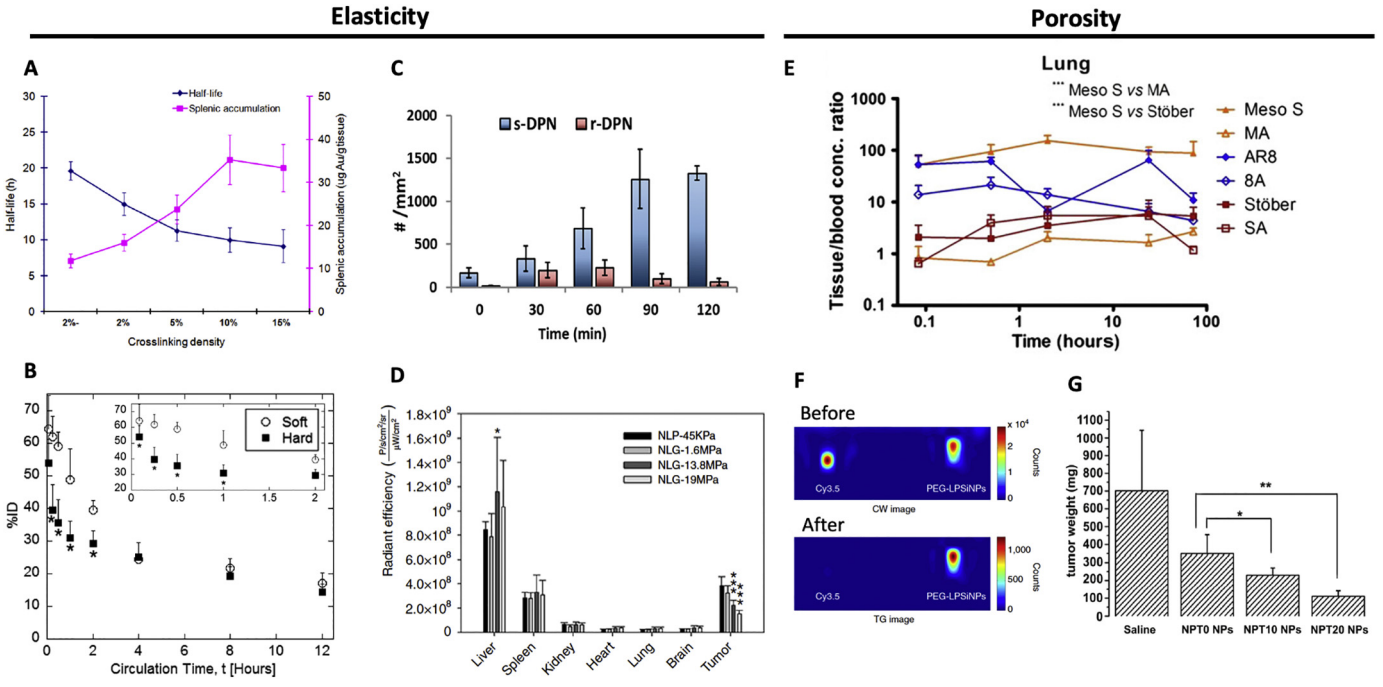


Fig. 6. Impact of elasticity and porosity on the *in vivo* fate of nanocarriers. (A) Stiffer particles result in a more splenic accumulation and a consequent decrease in the circulation half-life on increasing the crosslinking density from 2% to 15%. (B) Difference in the circulation half-life of soft (10 kPa) *versus* hard (3 MPa) nanocarriers, with soft nanocarriers exhibiting an extended circulation profile than their harder counterparts, the effect being most significant in first two hours post the administration of these nanocarriers. (C) Soft discoidal particles accumulate more than their harder counterpart in abnormal tumor vasculature within the first two hours after their administration thereby allowing soft discoidal nanoparticles to have a better diagnostic potential as compared to their harder counterparts. (D) Decrease in the tumor uptake with a consequent increase in the liver accumulation observed on increasing the elastic modulus of nanolipogels from 45 kPa to 19 mPa. (E) Improvement in the lung targeting efficiency of porous silicon nanoparticles as demonstrated by 22-fold increase in the lung/blood ratio for Meso S (porous silicon nanospheres) over Stöber (non-porous silicon nanospheres). (F) Diagnostic potential of porous silicon nanoparticles having longer photoluminescence as compared to conventional flurosphore based dyes like Cy 3.5. Signal from Cy 3.5 disappearing quickly after 18–19 ns because of its short-lived emission while the porous silicon nanoparticles showing negligible change in the signal intensity. (G) Improvement in the therapeutic efficacy of PLGA nanoparticle encapsulating docetaxel with increasing porosity as observed by an overall reduction in tumor weight on increasing the porosity. Porosity of PLGA nanoparticles was increased by varying the concentration of TPGS from 0% (NPT 0) to 20% (NPT 20) which resulted in a better drug diffusion profile at the tumor site, resulting in an improved efficacy. [Adapted with permissions from [195,172–174,177,192,202].]

been explored using PSNs. A recent study also described a non-crystalline form of silica, opal shale, being used for porous nanoparticle fabrication and its better tumor targeting capabilities compared to the administration of free drug [190].

Apart from silica nanoparticles, metal-organic frameworks (MOFs) [201] and poly(lactic-*co*-glycolic) acid (PLGA) nanoparticles [202] have been explored for *in vivo* applications. Horcajada et al. described the use of iron (III) carboxylate MOFs for encapsulation of four different kinds of drugs- Busulfan, Azidothymidine triphosphate, cidofovir and doxorubicin and the *in vivo* imaging of MOFs in rats for potential theranostic applications [201]. Zhu et al. described the use of porous PLGA particles to improve drug diffusion kinetics of docetaxel and the efficacy of treatment improved with increasing porosity in a xenograft nude mice model (Fig. 6G) [202].

Porosity thus affects biodistribution by virtue of difference in interactions with the cells and biological fluids. It also affects the drug diffusion kinetics *in vivo*. These two attributes form the basis of improved therapeutic efficacies observed in the abovementioned examples with porous nanocarriers.

4. Influence of physicochemical and surface properties on *in vivo* toxicity

Toxicity is an important consideration for design of nanocarriers for biomedical applications. Due to their extensive interactions with biological environments following their *in vivo* administration, nanocarriers can potentially have different degrees of toxicities to the body. Toxicity of the nanocarriers is mainly derived from the following aspects. First, nanocarriers interact with the blood cells and components as soon as they enter the blood stream, causing hematological toxicity [203]. Second, extensive amounts of nanocarriers can be accumulated in organs, especially the RES organs, which can lead to toxicity to specific organs, such as pulmonary toxicity, hepatotoxicity, splenic toxicity, and nephrotoxicity [203–205]. Moreover, nanocarriers can be recognized by the immune system as foreign substances, inducing multilevel immune responses against them and causing potential immunotoxicity [206]. Studies so far have revealed that molecular mechanisms of toxicity of nanocarriers involve the formation of free radical and induction of oxidative stress, the interaction with cellular components (such as mitochondria and nucleus), disruption and alteration of cellular functions, and creation of reactive oxygen species [203,205]. Especially, free radical formation can lead to the oxidation of cell components like proteins, nucleic acids, and lipids and therefore is believed to be the major contributor to the toxicity of nanocarriers [203]. Toxicological studies on nanocarriers suggest that the toxicity of nanocarriers is mainly dependent on their material properties. Depending on their biodegradability, biocompatibility, administration route and dose, nanocarriers made from various materials can lead to dramatically different *in vivo* toxicities [204]. There are not many reports investigating the effect of physicochemical properties on the *in vivo* toxicity of nanocarriers. However, the available limited studies suggest that the physicochemical properties of nanocarriers, especially surface chemistry, play an important role on determining the *in vivo* toxicity of nanocarriers [203].

4.1. Size

Effect of nanocarrier size on the *in vivo* toxicity has been investigated and most studies are focused on using model carriers like gold/silver nanoparticles [203,207–211], carbon nanotubes [212], and dendrimers [213]. It should be noticed that size effect on *in vivo* toxicity is nanocarrier-dependent and doesn't apply universally to every class of nanocarriers. Size effect of nanocarriers on their *in vivo* toxicity is derived from two major aspects. First, size directly determines the relative surface areas of nanocarriers, thus affecting the potential number of reactive groups on the particle surface [214]. Second, as discussed before, size impacts the distribution and accumulation of nanocarriers *in vivo* [4]. The interplay between these two factors determines that size has a complex effect on the *in vivo* toxicity. As an example, Zhang and coworkers synthesized PEG-coated gold nanoparticles (5, 10, 30, or 60 nm) and investigated the effect of particle size on their *in vivo* toxicity [207]. In this study, the authors injected 4 mg/kg of gold nanoparticles to mice and characterized multiple toxicity indicators. They found that nanoparticles of all sizes were present in the blood and bone marrow cells. Particularly, particles of 5 and 60 nm were found in these cells in an aggregated form. In addition, all sized nanoparticles increased the spleen and thymus indexes of animals, indicating potential effects to the immune system. Hematological results revealed that the 5 and 30 nm nanoparticles decreased the numbers of both white and red blood cells while the 10 and 60 nm counterparts showed an inverse trend. Moreover, in terms of the biochemistry assay, they found that the 10 and 60 nm nanoparticles led to liver damage, as indicated by a remarkable increase in aspartate as well as alanine transaminase. In another study, similar findings were observed by Chen et al. Size exhibited a complexed effect on the *in vivo* toxicity of naked gold nanoparticles [208]. Specifically, they found that out of a series of nanoparticles with different sizes (3, 5, 8, 12, 17, 37, 50, and 100 nm), gold nanoparticles in the range of 8–37 nm induced significantly higher *in vivo* toxicity than the other ones. Mice treated with nanoparticles of this size range exhibited severe sickness symptoms, including an increase in the Kupffer cells in the liver, loss of structural integrity in the lungs, diffusion of white pulp in the spleen, fatigue, loss of appetite, change of fur colour, and weight loss.

4.2. Shape

Particle shape may influence the *in vivo* toxicity of nanocarriers, but its exact effect has not been elicited yet. Based on the very limited numbers of reports, it seems that shape induced *in vivo* toxicity is highly nanocarrier-dependent [41,75,92,203,212,215,216]. It has been reported that aspect ratio significantly affects the *in vivo* toxicity of carbon nanotubes [212]. As an example, Donaldson and colleagues injected carbon nanotubes with different lengths (aspect ratios) into C57BL/6 mice [212]. Results from their study suggested that the long-nanotube exhibited remarkably higher *in vivo* toxicity than the short-nanotube. Specifically, compared to the short-counterpart, the long-nanotube resulted in elevated recruitment of inflammatory cells like polymorphonuclear leukocytes and foreign body giant cells, led to more severe asbestos-like pathogenic behaviors, and resulted in the increased formation of granulomas. However, this effect of aspect ratio does not seem to apply to other nanocarriers, such as silica nanorods [75]. For instance, Huang et al. investigated the shape effect on the biocompatibility of silica nanoparticles *in vivo* [75]. In this study, two differently shaped silica nanoparticles with aspect ratios of 1.5 or 5 were injected into mice and their toxicity was measured. Results from their study revealed that both particles exhibited potential induction of biliary excretion and glomerular filtration dysfunction. However, all of the hematological, biochemistry, and histological data indicated that the toxicity of the silica nanorods was not associated with the shape. As discussed before, some non-spherical shaped nanocarriers exhibit less or delayed uptake by macrophages. However, how the reduced or delayed macrophage uptake is related to the *in vivo* toxicity had yet been well understood for a long time. In a recent study, Wibroe et al. uncovered the shape effect of drug nanocarriers on the adverse cardiopulmonary reactions in pigs [216]. Drug nanocarrier-mediated cardiopulmonary responses are typically indicated by increased pulmonary arterial pressure (PAP) and decreased systemic arterial pressure (SAP). They found that compared to the spherical counterpart, the rod and disk polystyrene nanocarriers induced significantly less changes in PAP and SAP, indicating a less severe adverse cardiopulmonary reaction. Further mechanistic study suggested that this reduced adverse effect is due to delayed uptake of rod and disk nanocarriers by pulmonary intravascular macrophages.

4.3. Surface chemistry and property

The surface area and charge of nanocarriers play a critical role in their interaction at organ, tissue and cellular level and consequently the *in vivo* toxicity [105,112,217,218]. While surface charge is critical in achieving extended circulation and increased intracellular uptake, it may also cause toxicity. Studies have shown that change in surface charge could affect the *in vivo* distribution and toxicity of nanocarriers, irrespective of its size [219–222]. Positively charged NPs tend to induce more toxicity compared to negative or neutral nanocarriers. This is primarily due to the strong electrostatic interaction between the positive charge on nanocarriers and the negatively charged cell membrane composed of lipids and glycoproteins. In addition, the positive charge could also affect the cell cycle. It can bind strongly with the negatively charged DNA, causing damage and extended G0/G1 phase of the cell cycle resulting in cell apoptosis [223,224]. While the positive charge on nanocarriers could assist extensively in eliminating tumors, it will negatively impact the nanocarriers' *in vivo* pharmacokinetics, its dosing regimens and thereby, the therapeutic efficacy. As described earlier, positively charged nanocarriers promote opsonization and its subsequent clearance from the body. In addition, conformational changes to proteins adsorbed on nanocarriers could inhibit its function, disturb various biological activities and could potentially cause aggregation of amyloid-like fibrils resulting in diseases such as amyloidosis [225]. Thus, modifying the nanocarrier surface and its charge by grafting with polymers (PEG, PEI, PLGA, chitosan, or dextran *etc.*) or even encasing them within lipid vesicles and membranes isolated from leukocytes, RBCs *etc.* have reduced non-specific toxicity with improved diseased cell uptake [105,112,164,166,218]. Another way is to control the surface charge on nanocarriers with respect to its environment. By coating nanocarriers with pH-sensitive polymers, it is possible to switch the charge from being negative in physiological conditions to being positive in an acidic environment that is prevalent within the tumor microenvironment [119,226]. This could improve nanocarrier uptake and delivery of drugs to the targeted site.

4.4. Elasticity and porosity

Depending on its physicochemical nature, nanocarriers may be classified into 'hard' or 'soft' particles [227–229]. Hard nanocarriers include gold (Au) NPs, silver (Ag) NPs, metal oxide based NPs and quantum dots (QDs). Soft NPs include micelles, liposomes or polymer-drug conjugates *etc.* Although gold and Ag nanocarriers are relatively safe, their toxicity depends on surface functionality, dosage and also the type of cell line [230–232]. Ag nanocarriers have widely been used in dentistry, and as antimicrobial or an anticancer agent [233–235]. Metal oxide based nanocarriers such as those made of iron (Fe) or Titanium (Ti) have been used for theranostic purposes [236–238]. However, the particles have also been known to cause inflammation, DNA damage, genotoxicity, and affect the normal functioning of the liver, kidney, spleen, heart, immune system, glucose, and lipid homeostasis as well [239–247]. QDs have widely been tested for use in tumor detection or whole body imaging [248–252]. However, leakage of free ions of toxic heavy metals such as lead, arsenic, mercury, tellurium and cadmium from within its cores, could cause changes in cell morphology, oxidative stress, and organelle dysfunction [253–258]. Coating QDs with polymeric shell renders stability to the core and prevents the oxidative degradation and subsequent leakage. In contrast, soft materials such as PLGA, PEG, hyaluronic acid (HA) and other polymers have proven to be non-toxic, non-inflammatory, and non-immunogenic. They have been successfully used for targeted delivery of molecules [105,112,217,218]. Not much has been studied in terms of the effect of porosity on nanocarrier toxicity. However, porous silica nanocarriers have been known to generate dose-dependent ROS and subsequent oxidative stress damaging the cells [259–262].

5. Conclusion and future remarks

As discussed before, nanocarrier mediated drug delivery seems to be a promising technology that offers a degree of controlled release and the ability to limit the off-target effects in a way that the bulk material or the free drug is unable to. Despite this promise, nanocarriers have yet not achieved their full therapeutic potential. One of the major reasons for that is the biological barriers that exist to the intravascularly injected nanocarriers. The biological checkpoints and the mechanisms they employ to hinder the site-specific drug delivery of nanocarriers have been extensively reviewed in this paper. While the host body eventually is able to tag a foreign substance, and take care of it, this process of recognition by the host immune cells can be altered by manipulating the physicochemical properties of nanocarrier. The synthetic identity of the nanocarrier is defined by its constituent material and the emergent physicochemical properties. There are comprehensive reviews that discuss how material properties affect the *in vivo* toxicity [203]. However, this review particularly aims to encompass the work done by researchers in order to generate unique biological identities by only tweaking the physicochemical properties.

As the majority of the work in understanding how each parameter affects the *in vivo* fate was done in isolation, there exist no rigid thresholds on the values of any of the physicochemical parameter – size, shape, surface charge, surface hydrophobicity, surface functionalization, elasticity or porosity. However, there can be certain generalizations made about which parameter can be altered to avoid which barriers. Size has been and can be tweaked for avoiding the MPS or RES systems because phagocytosis and physiological filtrations *i.e.* renal, hepatic and splenic, appear to be working in a size-dependent manner. Aspherical or more generally anisotropic shapes can be used to affect margination in the blood flow and achieving tumbling, rolling and eventually extravasation into the tissue. Surface chemistries can be manipulated for a multitude of applications. One can practically evade the MPS by masking the hydrophobicity and altering the surface charge, be extravasated at a specific site using specific targeting ligands and also achieve improved cellular internalization. Surface chemistry represents the most versatile physicochemical parameter that can be altered. Elasticity can be tweaked to avoid the MPS as well as to achieve specific tissue accumulation in certain cases on the basis of deformability. Altering the porosity might help in changing the hemodynamic behavior of particles to achieve site specific delivery. All in all, manipulation of any of these parameters depend on what the final objective of the study is. One can select any parameter to be altered from the set of physicochemical parameters depending on the question they are trying to answer. Many of the changes to these physicochemical properties are often accompanied with toxicities. The effect of using wide ranges of these physicochemical properties and their associated toxicities are also summarized in the paper. In general, toxicities appear to be material and carrier specific and no definitive generalizations can be made.

Modulating the physicochemical properties can go a long way in helping nanocarrier based drug delivery achieve its complete potential. In this review, we have focused on the effect of changing physicochemical properties of intact nanocarriers, before they are administered *in vivo*. These properties are often subject to change upon degradation when the carriers are in the systemic circulation. An example of work highlighting these effects is a review by Parak and colleagues, where the authors discuss how the *in vivo* degeneration of inorganic nanoparticles change their physicochemical properties and in turn alter their *in vivo* fate [263]. A huge amount of scientific effort is going to be needed in order to completely understand how physicochemical parameters indeed affect the biological identity of a nanocarrier while addressing toxicity issues as and when they arise. In a general sense, the *in vivo* fate of nanocarriers is correlated to their individual physicochemical parameters. However, in many cases, nanocarriers differ in multiple physicochemical parameters rather than only one, which makes the prediction of their relative *in vivo* fate complicated [264,265]. To unravel

the correlation of entangled physicochemical properties of nanocarriers to their *in vivo* fate, a large enough library consisting of different nanocarriers differing in multiple physicochemical parameters is to be generated. Meanwhile, unique analysis methods should be used to analyze the data of physicochemical properties and *in vivo* fate of nanocarrier libraries. Such methods like hierarchical cluster analysis (HCA) can allow researchers to identify which nanoparticles have the most similar physicochemical properties and *in vivo* fate without having to specify individual physicochemical parameters [264]. Another way of approaching this conundrum can be using the Quality by Design (QbD) approach and scientific design of experiments to optimize of the physicochemical properties for a given nanocarrier and evaluating the desired therapeutic effect/targeting and the reduction in the associated toxicity as the Quality Target Product Profile (QTPP) [266]. This method also takes into account the interaction effects of varying parameters which are often missed when they are altered in isolation. The field of nanocarrier mediated drug delivery is exciting and largely interdisciplinary and efforts from diverse fields such as material science, chemistry, pharmacy, biological engineering coupled with the expertise of clinicians will help propel the field towards achieving its full potential in a clinical setting.

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