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A Focus on "Bio" in Bio–Nanoscience: The Impact of Biological Factors on Nanomaterial Interactions

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Bio–nanoscience research encompasses studies on the interactions of nanomaterials with biological structures or what is commonly referred to as the biointerface. Fundamental studies on the influence of nanomaterial properties, including size, shape, composition, and charge, on the interaction with the biointerface have been central in bio–nanoscience to assess nanomaterial efficacy and safety for a range of biomedical applications. However, the state of the cells, tissues, or biological models can also influence the behavior of nanomaterials at the biointerface and their intracellular processing. Focusing on the "bio" in bio–nano, this review discusses the impact of biological properties at the cellular, tissue, and whole organism level that influences nanomaterial behavior, including cell type, cell cycle, tumor physiology, and disease states. Understanding how the biological factors can be addressed or exploited to enhance nanomaterial accumulation and uptake can guide the design of better and suitable models to improve the outcomes of materials in nanomedicine.

1. Introduction

The development of next-generation medicines is underpinned by research into nanomaterials that are designed to carry therapeutic agents and transport them to their intended target cells or sites.[1] The encapsulation, complexation, or loading of drugs within nanomaterials (e.g., synthetic and natural polymers, lipids, and inorganic materials) assembled into stable particulates broadly defined as nanoparticles (NPs), can lead to longer circulation times that improve drug bioavailability, drug protection from degradation, drug stability, and enhanced tissue penetration, and cellular uptake (**Figure 1**). Prior to reaching its target cell and to act on its extracellular or intracellular target, the encapsulated drug encounters biological barriers such as recognition by the immune system and restricted access to the interstitium within the

target organ. At the cell surface and inside the cells, the dynamic phospholipid bilayers of the outer and organelle membranes present additional barriers that need to be traversed for cargo delivery. The precise design of NPs aims to address these barriers and facilitate their accumulation at the target site. For example, Doxil® is a liposomal formulation of the chemotherapeutic drug doxorubicin that has a reduced toxicity profile compared with the free drug and has been surface modified with polyethylene glycol (PEG) to prolong its circulation time.[2] The non-PEGylated version of liposomal doxorubicin (Myocet®), which differs from Doxil® in size, composition, and drug loading, has a reduced circulation time but avoids the cardiotoxicity that is associated with Doxil®, highlighting how NP formulation can impact interactions with the biointerface.[3] In addition, the small-interfering RNA (siRNA) therapeutic in the gene silencing therapeutic Patisiran® (Alnylam), approved by the US Food Drug and Administration, for polyneuropathy in hereditary transthyretin-mediated amyloidosis is formulated in lipid NPs, which serve to protect siRNA from degradation and facilitate its entry into cells.[4] Lipid NPs are also central to the ground-breaking messenger RNA vaccines approved against SARS-CoV-2.[5] Nanomaterials have therefore demonstrated capacity to improve drug delivery in therapeutic applications, including cancer therapy, vaccines, and infectious diseases.[6]

Despite recent advances, significant challenges and limitations remain to realize the full potential of nanomedicines and expand their clinical impact to a wider range of diseases and medical conditions. Some of these challenges include premature uptake and clearance by the liver and inefficient delivery to target tissues, where on average, less than 1% of injected NPs reach the target tissues.[7] To address these key challenges, attention has focused on the understanding of how biological systems (e.g., cells, organs, and organisms) interact with and process nanomaterials, which is often referred to as bio–nanoscience. Bio–nanoscience relates how properties, structures, and processes at the nanoscale affect the interactions

between nanomaterials and biological structures (bio–nano interactions). From the nanomaterial standpoint, the physicochemical properties of materials or particles, including size, shape, composition, rigidity, and surface chemistry, can affect how the nanomaterial interacts with biointerfaces and consequently its transport, function, and fate at the single cell, multi-cellular, and whole organism level. For example, materials with a cationic surface show enhanced cellular uptake and particle diameter can influence the intracellular distribution of gold NPs (AuNPs).[8] At the tissue level, particle size and rigidity influence the penetration of particles through tissue or three-dimensional (3D) models of organs. An extensive list of nanomaterials have been the focus of bio–nano studies, from well-studied model NPs, such as gold, silica, and liposomes, to novel polymeric and hybrid structures with demonstrated potential for biological applications. Advances in materials science and nanoscience have expanded the toolbox of strategies that can be employed to manipulate or engineer particles to improve biological outcomes.

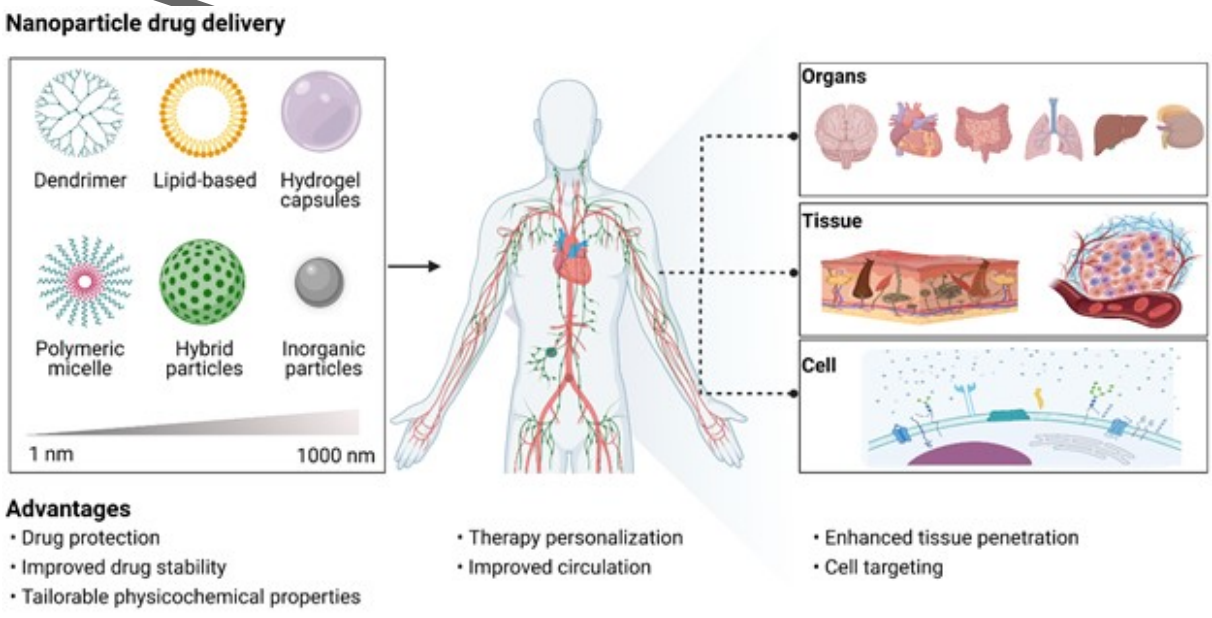

Figure 1. NP-mediated delivery of therapeutics can offer advantages from drug protection to personalized therapy. Biological factors at the whole organism, organ/tissue, and cellular level can influence bio–nano interactions and inform particle design. Created with BioRender.com.

From the biological standpoint, the transport, function, and fate of the nanomaterial can also be influenced by the biological model itself and its state/condition and properties.[9] Before a nanomaterial can interact with its target cells to exert its function, it must navigate complex biological systems and the local physiological environment of the target site. It is well known that successes in animal studies have not effectively translated into clinical trials. This can be assigned to differences in the physiology of animal models and humans; however, even organisms of the same species can display different phenotypes, as demonstrated in common laboratory mouse strains,[10] and hence, may react differently to nanomaterials. At the organ or tissue level, the physiological state of the organ, including tumor physiology and tissue microenvironment, can influence its ability to retain nanomaterials. Recent advances in stem cell technologies have developed a variety of 3D models that mimic organs and tissues in fabricated devices, which have served as versatile models for studying bio–nano interactions in vitro. At the single cell level, different types of cells (e.g., epithelial, endothelial, neurons, and immune cells) interact and process nanomaterials in different ways. Differences between immortalized cell lines and primary cells can be stark, particularly if the desired application is not tumor related. The cell cycle and the activation levels of the cells also change the condition of the cells that can alter the way they process materials.[11]

In this review, the "bio" in bio–nano interactions is discussed. Instead of focusing on how nanomaterial properties influence biological outcomes, we discuss how biological factors affect the outcomes of nanomaterials. The review begins at the single cell level and discusses how the properties of various cell types can influence the intracellular uptake of NPs (Section 2). We highlight how the state of the cell, including its phase of growth and growth conditions, changes the amenability of cells to particle uptake. The bio–nano interactions at the organ and tissue level are then discussed, including highlights of how fabricated 3D models of organs have been used in bio–nanoscience (Section 3). Finally, recent findings of

biological effects at the whole organism level, including gender, strain, and the proteome, on the performance of nanomaterials are covered (Section 4). Understanding how differences in the biological system change the response to nanomaterials can potentially lead to personalized and precise engineering of nanomaterials (Figure 1) to enable better outcomes in clinical trials and transformational advances in biomedicine.

2. Single Cell Level

The cell is the basic unit of a living organism. The human body is comprised of different types of cells, which have specialized roles in the body and are therefore specifically equipped to perform their role. For example, the primary role of macrophages is to patrol and rid the body of foreign materials. They are specially designed to phagocytose (engulf) foreign materials that have been marked for degradation and are rich in enzymes that degrade proteins, nucleic acids, and other macromolecules. In nanomedicine, materials are often designed to target a specific cell or group of cells implicated in the disease of interest. For example, CD4+ T cells are targeted for human immunodeficiency virus (HIV) infection and tumor cells or immune cells are targeted in cancer nanomedicine. However, different types of cells interact and process nanomaterials in different ways. Cells commonly internalize particles via mechanisms including macropinocytosis, phagocytosis, caveolae-mediated endocytosis, and clathrin-mediated endocytosis, as discussed in more detail in other reviews,[12] but not all pathways are active in all cell types. For example, HepG2 cells (hepatocytes) are unable to internalize NPs by caveolae-mediated endocytosis because caveolin-1 is poorly expressed on HepG2.[13] There are more than 200 different types of cells in the body,[14] but in this section, we focus on several cells that are studied widely in bio–nanoscience and highlight the properties and barriers at the cell level that can influence their

interaction with nanomaterials. The influence of cell properties, including the growth phase, and growth conditions on nanomaterial internalization and function will also be discussed.

2.1. Effect of Cell Type

The cell type has been shown to influence the interactions of various shapes of NPs.[15] The main types of cells that are studied for bio–nano interactions and NP studies in general are listed below. The properties of the cells that could influence NP uptake or processing are discussed and are illustrated in **Figure 2**.

2.1.1. Endothelial Cells

Endothelial cells line the blood vessels and lymphatic vessels in a continuous cell monolayer known as the endothelium. The endothelial lining is in direct contact with blood or lymph and acts as a selective barrier that presents a challenge for NP transport.[16] The glycocalyx, composed typically of glycoproteins and glycolipids, covers the surface of endothelial cells. It is proposed that the glycocalyx plays a role in regulating NP internalization by endothelial cells.[17] In addition, adhesion molecules are present on the endothelial cell surface, including P-selectins, E-selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1.[18] The expression of adhesion molecules is either inducible or constitutive in endothelial cells, which is important in the context of NP targeting. For example, ICAM-1 expression is not constitutive and is induced by cytokines.[19] It is expressed in certain pathological conditions such as inflammation, oxidative stress, and ionizing radiation. Other molecules expressed by endothelium such as growth factor receptors, integrins, and selectins also present as potential targets for NPs. The vascular endothelial growth receptor (VEGFR) family, comprising VEGFR1, 2, and 3, have been exploited for endothelial cell targeting.[20,21] VEGFR3 is largely expressed by the lymphatic endothelium, whereas VEGFR1 and 2 are confined to the vascular endothelium.[19]

Junctions between endothelial cells vary in different tissues. Tight junctions allow small molecules, such as oxygen and nutrients, to easily pass through, greatly limiting the passage

of NPs. Discontinuous endothelium has 100–200 nm wide fenestrations.[16] Fenestrated endothelium in the endocrine glands, digestive tract mucosa, and the kidney peritubular capillaries possess large, transcellular pores of about 50–60 nm.[16] In contrast, the blood–brain barrier (BBB) in the central nervous system (CNS) has the tightest endothelium but also low levels of pinocytic activity compared with the systemic endothelium.[22] The tight junctions also limit paracellular (in between cells) transport of molecules.[23,24] Small lipophilic molecules can easily cross the endothelium by passive diffusion. In contrast, the passage of polar metabolites is regulated by transport proteins and the majority of the molecules are transported via cellular transcytosis.[23] This includes large, hydrophilic and charged molecules such as glucose, amino acids, and drugs, which are delivered via active transport. Endothelial cells in the CNS also express adenosine triphosphate-binding cassette transporters (ABC transporters), which can actively pump out drugs that cross the BBB.[25] Under certain pathological conditions, such as cancer and atherosclerosis, the endothelium can differ significantly. Tumors exhibit high vascular permeability with pore sizes of 380–780 nm.[23] This leakiness causes the extravasation of NPs from the vasculature, and the lack of lymphatic drainage causes retention of NPs within the tumor interstitium, a combination of factors known as the enhanced permeability and retention (EPR) effect.[26] In addition to physical pores, transendothelial channels, made up from vesiculo-vacuolar organelles, are prominent in endothelial cells of the tumor and are also involved in macromolecule extravasation.[27]

NP interaction and uptake by endothelial cells can be influenced by cell growth conditions and tissue properties. For example, the well-studied cell line human primary umbilical vein endothelial cell (HUVEC) exhibits reduced NP uptake in confluent compared with sub-confluent cell cultures, owing to the differential expression of endocytic markers in HUVEC during stages of growth.[22] Gromnicova et al. reported that the uptake of NPs in kidney

vascular endothelium (ciGENC) was four times higher than that in the brain endothelium (hCMEC/D3).[28] The different rate of internalization was attributed to the higher endocytosis rate in kidney endothelium and the different composition of glycocalyces, which are known to play important roles in NP uptake.[17a] Degradation of endothelial glycocalyx increases the exposure of endothelial adhesion and signaling molecules, hence promoting the interaction between NPs and endothelium.[29] Therefore, the choice of in vitro system and the factors affecting NP uptake must be carefully considered and the results interpreted with these limitations in mind.

2.1.2. Epithelial Cells

Tightly connected epithelial cells form the linings of outer and inner surfaces of the body such as skin and the respiratory, gastrointestinal, reproductive, and urinary tracts. They are classified based on their shapes (i.e., cuboidal, squamous, and columnar), which in turn determines their function. Epithelial cells are predominantly involved in forming a protective barrier and are specialized for secretion and absorption.[30]

Epithelial cells cover an extensive surface area, with up to 300 m^2 in the human gut mucosa and 150 m^2 in the respiratory tract—a vast surface not just for uptake of nutrient and gas exchange but also for NP association.[31] The apical surface of epithelial cells typically faces the external environment[32] and is covered in mucus that forms a physical and immune barrier by trapping pathogens, toxins, and particles from the environment, preventing entry into the epithelium. Mucus therefore presents a major barrier for NP internalization and particle engineering strategies that promote either mucoadhesiveness or mucopenetration have been investigated.[33–35] The main component of mucus is mucins, which are high molecular weight glycoproteins that are cross-linked to form a polymer network with a mesh size ranging from 100 to 2000 nm that can impede the diffusion of particles.[36] Mucins can form hydrophobic interactions, hydrogen bonds, and be involved in electrostatic interactions

with diffusing particles, potentially acting as a site of attachment for NPs that can lead to enhanced drug absorption or retention.[37] Neutrally charged NPs, e.g., those coated with PEG, have shown the most rapid penetration of mucus.[38] In addition to mucus, intestinal epithelial cells have surface glycocalyx, which presents an additional barrier to efficient NP penetration.[39] Using Caco-2 cells as an intestinal epithelium model, it was demonstrated that microparticles can bind to Caco-2 cells that lack glycocalyx but fail to adhere to glycocalyx-containing epithelial cells.[40] The thickness of the glycocalyx also influences particle uptake. Antigen-coated AuNPs ~28 nm in diameter failed to adhere to enterocytes (intestinal absorptive epithelial cells) but could adhere to epithelial microfold or M cells of the intestines, which had a thinner glycocalyx layer than enterocytes.[40] In the alveolar epithelium, pulmonary surfactant presents a barrier to NP uptake.[41] The pulmonary surfactant is composed of 92% lipids (consisting predominantly of zwitterionic and dipalmitoylphosphatidylcholines) and protein[42] and forms a 0.2–0.5 μm thick layer on the surface of epithelium cells. The proteins, notably surfactant proteins SP-A and SP-D, can mediate interactions with immune cells and facilitate phagocytosis presenting a barrier that can be avoided or exploited to achieve effective drug delivery.[43]

Epithelial cells also possess specialized structures, such as cilia and microvilli, which aid in absorption or secretion and protection. Cilia function as a mucus clearance mechanism and are responsible for the removal of mucus and any particles entrapped in the mucus, resulting in poor tissue permeability of particles.[44] Microvilli are specialized finger-like projections found on the apical surface of intestinal epithelial cells. They increase the surface area of apical membranes to provide maximal absorption and diffusion of molecules in the gut.[32] However, microvilli have an electrostatic barrier that inhibits microbial adhesion and may also impede NP association. Epithelial cells generated with reduced number of microvilli displayed an increased uptake of microbial particles compared with microvillus-positive

cells, which was shown to be caused by electrostatic repulsion.[45] In the skin, epithelial cells are present under the skin upper layer, the stratum corneum, which presents a barrier to NP penetration to the epidermis for cutaneous topical delivery.[46] The stratum corneum consists of dead cells (keratinocytes) in a protein- and lipid-milieu, which may restrict the diffusion of drugs and NPs to the epidermis. Both chemical (e.g., NP carriers, excipients) and physical methods (e.g., ultrasound, microneedles) have been investigated to enhance delivery to the epidermis or transdermally for a wide range of applications.[47] Readers are referred to selected studies and reviews for more detailed discussions.[47,48]

Despite these barriers, opportunities to traverse the epithelium include passive diffusion, receptor-mediated, carrier-mediated, transcellular, or paracellular transport. In transcellular transport, particles are endocytosed from the apical surface and transported to the basolateral surface of intestinal epithelial cells, which mainly occur in enterocytes and M cells in the intestine. An intestinal in vitro triple culture (M cells, goblet cells, and Caco-2 cells) permeability model was used to compare the uptake of polystyrene particles by these intestinal epithelial cell types. It was demonstrated that the penetration of particles was facilitated by M cells,[49] which is consistent with their normal function of delivering antigens from the environment to the lymphoid follicles via transepithelial transport.[50] In human alveolar epithelium, the alveolar type I (ATI) epithelial cells were proposed to be responsible for the uptake of NPs because ATI cells cover 95% of the alveolar surface and possess highly active clathrin- and caveolin-mediated pathways critical for transepithelial transport.[51] ATI internalized NPs whereas alveolar type II (ATII) epithelial cells were unable to. Although both ATI and ATII possess endocytic capability, ATII cells are mainly involved in the secretion of surfactants.[51]

In epithelial cells and other cell types, expression levels of the receptor(s) involved in the targeted mechanism of NP uptake (i.e., receptor-mediated endocytosis) need to be carefully

assessed. Dawson and co-workers studied the effect of altering the expression of scavenger receptors in non-phagocytic A549 lung epithelial cells. Scavenger receptors have been implicated in NP uptake in various settings and expression of these receptors may affect the endocytosis of NPs. Paradoxically, higher NP (10, 50, and 200 nm silica) uptake was observed for cells with siRNA-silenced scavenger receptors, likely owing to the upregulated expression of one or more other scavenger receptors.[52]

2.1.3. Antigen-Presenting Cells

Antigen-presenting cells (APCs) are cells that process and present antigens on major histocompatibility complex (MHC) molecules on their cell surface to promote an immune response against the foreign antigen.[53] APCs include dendritic cells, B cells, and macrophages and play a crucial role in the adaptive immune response and are therefore targets for particulate vaccines and immunomodulatory agents. APCs are found in the circulation and lymph nodes but are also prevalent in tissues including skin, spleen, and liver. The primary role of APCs is to process foreign materials; hence, it is not surprising that APCs use various mechanisms to internalize NPs, including clathrin- and scavenger receptor-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and phagocytosis. Pattern recognition receptors (PRRs), such as toll-like receptors, are present on APCs and recognize specific pathogen-associated molecular patterns or damage-associated molecular patterns.[54] In dendritic cells (DCs), activation of PRRs and initiation of an immune response can be triggered by binding of the NP itself or an immunostimulatory molecule delivered by the NP.

APCs also have other endocytic receptors, such as CD80, DC-SIGN, CD11c, and DEC205, expressed by DCs, that can be exploited for internalization. Even within APCs, different cell types vary in the receptors and processes they use for internalization. Phagocytosis is principally carried out by macrophages, although DCs also phagocytose large particulates.

Both DCs and macrophages possess receptors such as mannose receptor and Fc receptors, whereas the DEC205 receptor is expressed specifically in DCs. C-type lectin receptors are found most abundantly in DCs. DCs also express aquaporins, which allow them to undergo micropinocytosis constitutively unlike macrophages, which undergo micropinocytosis upon stimulation. Compared with DCs and macrophages, B cells possess lower capacity to take up antigens for presentation to CD4+ T cells. However, antigen recognition via B cell receptors (BCRs) is up to 10000-fold better for the presentation of antigens to CD4+ T cells. The recognition of antigens by BCRs leads to internalization via clathrin-coated pits and loading on MHC class II molecules.[55] A major barrier to B cell targeting, however, is accumulation in the sinus of the lymph node for translocation to the B cell follicles.

NP engineering strategies, including tuning size and surface properties, have been assessed to address uptake in APCs, particularly in lymph nodes.[56,57] Hardy et al. used 50 and 500 nm inert non-toxic polystyrene particles (PS50G and PS500G) to show that all APC subtypes, identified as CD11b+ CD103+ DCs, B cells, and macrophages, preferentially take up smaller particles (PS50G).[58] In terms of surface charge, it was reported that cationic NPs preferentially associate with CD103+ and CD11b+ DCs. The association of NPs to certain DC populations such as CD103b and CD11b modulates subsequent immune responses such as translocation to the lymph nodes and T cell priming.[59] Foged et al. found that a positive surface charge can increase the uptake of 1 μm particles by DCs in vitro.[60] Furthermore, combined with a small particle size (270 nm), coating of the NPs with antigen (ovalbumin) induced a stronger immune response in DCs, as demonstrated by the increased secretion of inflammatory cytokines interleukin 1b and tumor necrosis factor-α.[61]

2.1.4. T Cells

T cells or T lymphocytes are a type of white blood cells that play an important role in immunity and repair. They mediate immune responses against microorganisms and

neoplasms but are also targets of viral infection, including HIV. T cells are therefore attractive targets for the treatment and prevention of various diseases. The delivery of NPs to T cells, however, is challenging because of the non-phagocytic nature of the cells. Hence, strategies to improve particle internalization by T cells, including ligand-based targeting, have been investigated.[62]

NPs can interact with T cells directly by targeting a surface marker or indirectly by the uptake and presentation of DCs. Each T cell subset presents distinct phenotypic markers, anatomical location and functions, and hence the surface marker to target may differ depending on the subset of T cell and the application. T cells have been targeted with drug-loaded NPs as treatment strategies for unhealthy T cells, and other agents are delivered to T cells to modulate the T cell response, migration, number, and activation, including in immunotherapy and chimeric antigen receptor T therapy. A goal for T cell targeting is the delivery of nucleic acid therapeutics and latency reversing agents to latently infected T cell populations harboring the HIV genome, which presents the biggest challenge to an HIV cure. Although anti-retroviral therapy (ART) against HIV can reduce viral loads to manageable levels, the cessation of ART can induce the activation of latently infected T cells and reproduction of HIV in CD4 T cells. Hence, the delivery of agents that can eliminate the latent T cell population would present a potential functional cure for HIV. However, besides the non-phagocytic nature of T cells, other challenges for NP-mediated delivery to T cells include lack of specific receptors for latently infected cells and difficult access to T cell-rich regions in the body. T cells are present in the periphery (T cells constitute up to 30% of cells in human peripheral blood) and reside in tissues, including gut, brain, lungs, and lymph nodes. Lymph nodes are particularly rich in T cells and a better understanding of lymph node delivery of NPs is guiding future design of nanomaterials that could enable uptake by T cells in these sites.[63,64]

2.1.5. Neurons

Neurons are electrically excitable cells in the nervous system that function in receiving, processing, and transmitting signal to other cells via the synapses. Neurons generate an electrical impulse or an action potential that causes the electric potential of the axonal membrane to jump from −60 mV in resting cells to 50 mV at the peak of action potential. The electrical signal is converted into a chemical signal via the secretion of neurotransmitters to the neighboring cell at the synapse.[65,66] Studies suggest that the neuronal activity can affect the interaction between NPs and neuronal cells. Dante et al. observed selective binding of NPs to neuronal membranes driven by the neuronal spiking activity in which the binding is only observed in negatively charged NPs.[67] In contrast, NPs with positive or neutral charge showed negligible interaction with the neuronal membranes. The negatively charged NPs were also shown to bind exclusively to excitable neuronal cells and not to non-excitable glial cells. Another study also demonstrated preferential uptake of quantum dots with negatively charged CL4 coating by neurons.[68] In stem cell-derived sensory neurons, Czuba-Wojnilowicz et al. found higher particle association of positively charged silica particles compared with negatively charged particles, which might be due to the material itself or difference in the excitability of the cells.[69] When sensory neurons were organized in a 3D structure in the form of neurospheres, particle size had a more pronounced effect than charge, although charge was found to influence cell binding and internalization.[70] Both positively charged and negatively charged particles with sizes below 280 nm were found to penetrate deeper into neurospheres than particles >400 nm.[70]

Axoplasmic transport is observed in neurons and can be categorized into either anterograde or retrograde transport. The former refers to the transport of synthesized proteins and molecules in vesicles from the cell body to the axon terminal and the latter refers to the trafficking back to the cell body. Hence, the axoplasmic transport can become a potential

pathway for nanodrug delivery. NP design has exploited the neurotrophic property of nontoxic fragment C of tetanus toxin, which can be retrogradely transported by the neurons for cell targeting.[71] Increased expression levels of a reporter gene in dorsal root ganglia neurons was reported following in vivo delivery of plasmid DNA via a nanocarrier grafted to the toxin fragment, indicating exploitation of the retrograde pathway.[71b] NP delivery to neurons in the brain is impacted by the BBB and has been reviewed thoroughly by Furtado et al.[22] Even within the CNS, the cell type can impact NP uptake. For example, AuNP uptake was found to be higher in microglial cells (2- to 17-fold) than neuronal cells in culture and it was suggested that the higher differentiation status and low cell division (mitotic activity) may result in lower numbers of intracellular NPs.[72]

2.1.6. Stem Cells

Stem cells are known for their ability to self-renew and to differentiate into mature functional cells. They can be categorized into embryonic (ES), adult stem cells (ASCs), and induced pluripotent stem cells (iPSC) based on their origin[73] and are classified based on their cell potency (pluripotent, totipotent, unipotent, multipotent, or oligopotent).[74] ES are pluripotent, which can give rise to different cell types, and are derived from embryos, thus often deemed as controversial.[75] Similar to ES, iPSC, which are derived from somatic cells, possess the proliferation and differentiation capacities into many types of cells.[76] ASCs, also known as tissue-specific stem cells, have been found to reside in different tissues. ASCs can be classified according to their localization within the body, including hematopoietic stem cells, mesenchymal stem cells (MSCs), neural stem cells, adipose-derived stem cells (ADSCs), and intestinal stem cells.

Various studies, predominantly in vitro and ex vivo, have been performed using NPs to modulate stem cells, e.g., to guide differentiation into a specific type of cell,[77] promote, regulate or maintain differentiation,[78,79] or drive proliferation and survival through various

signaling pathways.[80] NPs loaded with curcumin targeted to neural stem cells induced neurogenesis via the Wnt/β-catenin pathway.[81] NPs have also been developed to eradicate cancer stem cells (CSCs), which are involved in tumor initiation.[82] As Notch signaling promotes self-renewal in CSCs, NPs capable of transporting γ-secretase, an inhibitor of Notch signaling, were designed to target CSCs. CSCs demonstrated efficient uptake of the NPs, inhibiting Notch signaling and successfully reduced the CSC population.[83] Surface markers overexpressed on CSCs, including CD133 and CD44, have also been exploited for targeted therapies and imaging modalities.[84] Uptake of silica NPs in breast CSCs was found to be facilitated by scavenger receptors,[85] although other internalization mechanisms are active in stem cells. CSCs reside in the interior of a tumor mass and hence NPs must avoid clearance in the blood, extravasate from tumor vessels, and diffuse inside the tumor to access the CSCs or other cells in the tumor mass.[86]

Stem cells can migrate and home to tumor or injury sites. They also possess low immunogenicity owing to the lack of MHC class II molecules on their surface. This gives them the ability to cross biological barriers and enhance intratumoral distribution,[78] which can be exploited for therapeutic purposes. As carriers, drug-loaded particles are first incubated with stem cells in vitro. The stem cells are then administered to home to the tumor site. For example, in a proof-of-principle study, MSCs, which have a tropism for brain tumors, were used as carriers of polylactic acid NPs and lipid nanocapsules, displaying efficient NP uptake and migration of the NP-loaded MSCs toward a human glioma model.[87] ADSCs were also used to deliver paclitaxel-loaded NPs to brain tumors.[88] NPs have also been used to direct and track stem cell migration. A chitosan-based NP carrying stromal cell-derived factor 1, a chemokine that directs cell migration, was shown to promote stem cell trafficking.[89]

In general, the cellular uptake of NPs in stem cells is size dependent. It was reported that the optimal size for uptake is between 20 to 70 nm. AuNPs of 30 and 50 nm were preferentially taken up by ADSCs.[90] TiO_2 nanotubes of 70 nm in length were shown to be the ideal size for osteogenic differentiation of human ASCs compared with shorter (50 nm) and longer (100 nm) TiO_2 nanotubes.[91] It was also reported that the shape of NP influences the osteogenic differentiation of human MSCs (hMSCs).[92] In that study, 40, 70, and 110 nm AuNPs were used, and it was found that the spherical particles (40 and 70 nm) and the rods (70 nm) upregulated the expression of an osteogenic marker.[92] Furthermore, surface modification of NPs affects their interaction with stem cells. MSCs were treated with AuNPs of different surface charges. Positively charged NPs displayed enhanced uptake by hMSCs.[93] Both AuNPs and chitosan-based AuNPs induced osteogenic differentiation. However, it was shown that the AuNPs activated the p38 MAPK pathway in MSCs, whereas the chitosan-conjugated AuNPs activated the Wnt/β-catenin pathway in ADSCs.[94] This finding shows that different surface modification of NPs can initiate different signaling pathways in stem cells.

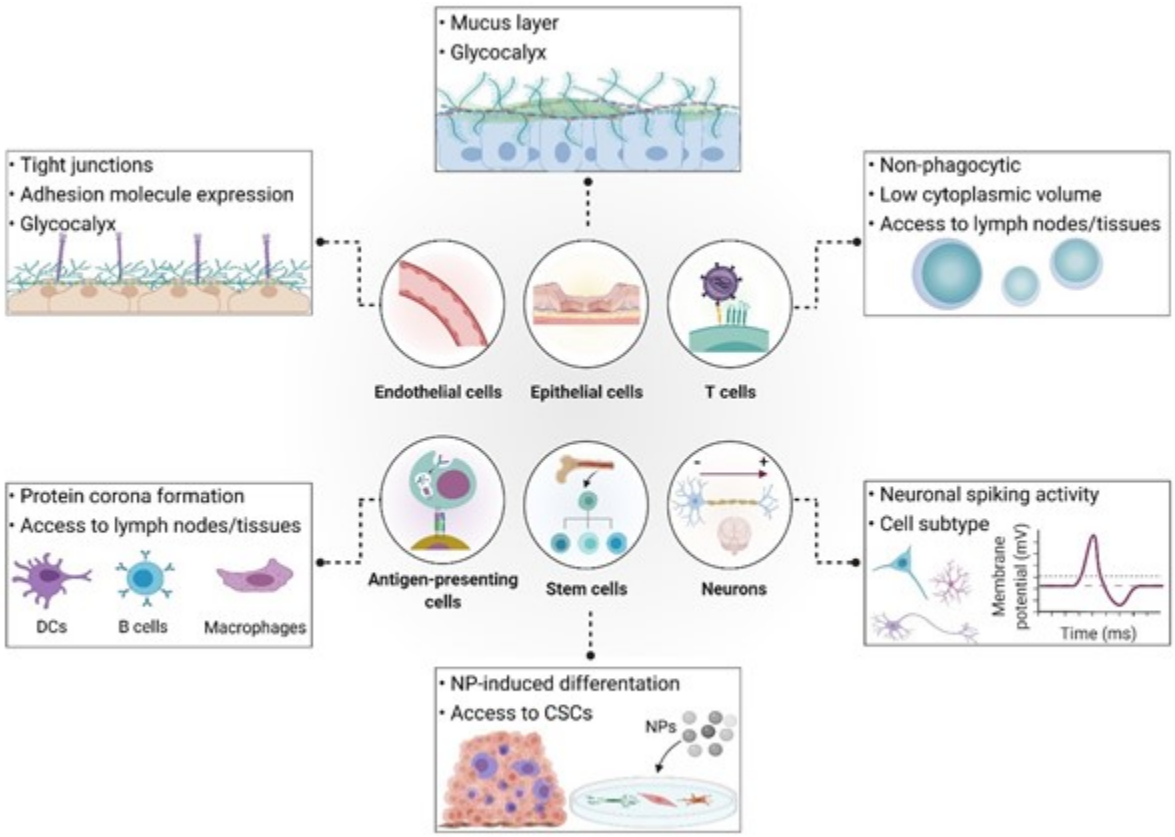

Figure 2. Biological barriers in different cell types. Surface properties, cellular pathways, cell morphology, and physical access to certain cell types can present barriers to effective NP uptake. DCs, dendritic cells; CSCs, cancer stem cells; NPs, nanoparticles. Created with BioRender.com.

In summary, variations in cell properties across different cell types can influence bio–nano interactions and hence, particle design. Access of the NPs to the cell surface itself must overcome immune clearance, tight endothelial barriers, and penetration through tissue, but at the cell interface, barriers including the glycocalyx and mucus present additional challenges (Figure 2). NP internalization mechanisms can also differ with cell types, with APCs, for example, readily internalizing particles, whereas specific pathways are either absent or more pronounced in other cells. Surface receptors play an important role in targeting strategies and internalization. The specificity of the receptor to the cell of interest, its expression (regulated versus constitutive), internalization, and recycling must be considered. In the case of neurons, excitability and electric potential of the membrane can also influence uptake, which further

highlights the differences between cell types and the need to design NPs specific to the cells and their location for effective and efficient delivery.

2.2. Effect of Cell Cycle

The cell cycle is a series of intracellular events that cover the stages of cell growth and division by actively dividing cells. The cell cycle is composed of two gap phases (G1 and G2), a synthesis (S) phase, in which the nuclear DNA is duplicated, and a mitosis (M) phase, where the duplicated nuclear DNA partitions and the cell divides (see **Figure 3**). G0 is an additional resting phase for cells that have temporarily stopped dividing. The M phase takes place in stages that include prophase, metaphase, anaphase, telophase, and cytokinesis as the cell divides into two daughter cells. Endocytosis is not affected during mitosis. When cells divide, the NP population is split between daughter cells leading to the dilution of the concentration of NPs in the cell.[95] Yan et al. showed that the mitotic partitioning of polymer NPs in the daughter cells is asymmetric, with the bias measured by an average segregation deviation of 60%.[95c] The mammalian cell lines commonly used in cell studies with NPs typically comprise a mixture of cells in different phases of the cell cycle. These cell lines are typically transformed cells and therefore will spend more time in the growth phase and mitosis than healthy cells. This may alter the interaction between NPs and cells and result in the dilution of NPs with culture time.

During the different stages of the cell cycle, the mass, size, surface area, shape, and activity of the cell changes, which can influence the interactions with nanomaterials at different phases. Cell changes during mitosis include shape, membrane tension, protein expression, cell volume, and viscosity. For example, during mitosis, cells become spherical and are more compact before cell division. In 2012, Dawson and co-workers reported that the cell cycle can influence the uptake of NPs.[72c] Using a human lung carcinoma cell line (A549) and ~40 nm carboxylated polystyrene NPs (PS-COOH), the rate of NP uptake was found to be similar

in cells in different phases of the cell cycle, but the concentration of the particles in the cells after 24 h of incubation was dependent on the cell cycle phase with G2/M > S > G0/G1. This trend was found to be consistent for different cells types and particles of different types and materials. The observed difference in the concentration of cells with cell cycle reflects the degree of cell division during incubation—for NP incubation times shorter than the full cell cycle, cells will contain more NPs as they have not yet divided, whereas cells that have divided during the incubation time will have a diluted NP content as NPs are passed to daughter cells. This has implications in cell cycle phase-dependent targeting. As discussed by Dawson and co-workers, targeting the S or G2/M phases could be favorable, because tumor cells would pass through such phases more often than healthy cells, thereby achieving a larger intracellular dose of NPs. Go et al. reported the same pattern of particle concentration G2/M > S > G0/G1 in their study using microspheres.[96]

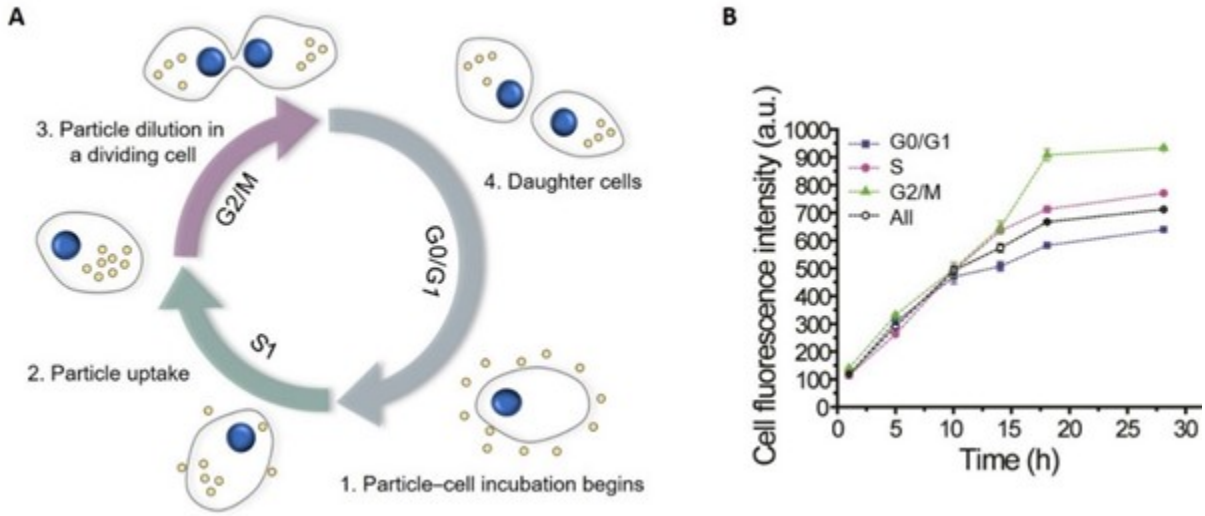

Figure 3. (A) Cell cycle and the effect of cell division on the dilution of NPs (yellow spheres). (B) Experimental data showing that concentration of PS-COOH in A549 cells during different stages of the cell cycle varies in the order of G2/M > S > G0/G1 after incubation of the particles with for 24 h. Reproduced with permission.[72c] Copyright 2012, Springer Nature.

The observation that cancer cells divide faster than healthy cells is being exploited to enhance the uptake of cytotoxic NPs inside cells. Kumar and co-workers[97] studied the effect of

different cell cycle phases on the uptake of 14 nm ZnO NPs. A431 cells were arrested at different phases of growth and the uptake of ZnO NPs was monitored. Higher uptake in the G2/M phase was observed followed by the S phase and subsequently the G0/G1 phase (i.e., G2/M > S > G0/G1) after incubation for 24 h This trend was found to be time dependent: at 6 h, the cellular uptake varied in the order of S > G2/M > G0/G1. Uptake at the G2/M has the advantage that dividing cells are targeted, especially if the particles can act to induce cell cycle arrest; this was shown to induce cell death by activating the reactive oxygen species.[97] Zheng et al. also reported that the rate of the uptake was cell cycle dependent using quantum dots. After 1 h incubation with various cells lines (HeLa, 293T, and QGY7701), the cells in G2/M had uptake rates 2–4 times higher than cells in G1.[98]

Changes in the plasma membrane during the cell cycle can also influence particle interactions. The expression of surface receptors on some cells can vary during the cell cycle,[53,99] which could have implications in active targeting and protein corona formation on NPs. The effect of the cell cycle on active targeting was studied by Zhou and co-workers.[100] The folate receptor is often exploited in targeted delivery owing to its overexpression on the surface of many tumor cells. The expression of the folate receptor was found to be highest during the S phase of the cell cycle (i.e., 93.2% expression of the receptor in the S phase compared with 80.0% in the G2/M phase) and consequently, the uptake of folate receptor-targeted polymer micelles was also highest at this S phase (i.e., 98.9% uptake in the S phase compared with 89.8% in the G2/M phase). Boucrot and Kirchhausen have reported that clathrin-mediated endocytosis is not affected by the cell cycle phase, although endosomal recycling back to the cell surface slows when cells become spherical during the prophase and metaphase stages of mitosis.[99d] Fielding and Royle, however, have reported that clathrin-mediated endocytosis is inhibited during mitosis. Nevertheless, the stage of the cell cycle

could affect the number of surface receptors on the cells that are available to engage with targeted particles.[101]

Many chemotherapeutic agents function by arresting the cell cycle. Hence, understanding the uptake of drug-loaded NPs as a function of the cell cycle is important. This can lead to strategies that seek to control the cell cycle (e.g., by pre-treatment with agents that drive cells to a specific cell cycle phase) to improve the uptake or increase the concentration of NPs and encapsulated drug inside the cells. In leukemia research, studies have shown that cells in the G0 phase of the cell cycle that are typically unresponsive to treatment become sensitive to the therapeutic (diphtheria toxin or doxorubicin) when pre-treated with granulocyte–macrophage colony stimulating factor—a glycoprotein that drove leukemia cells out of G0.[102] Similarly, estradiol has been used to increase the fraction of MCF-7 cells in the S phase to improve the sensitivity of the breast cancer cell line to an S phase-specific cytotoxic drug.[103,104] Roa et al. used glucose-capped AuNPs to improve the radiation sensitivity of DU-145 prostate cancer cells that are typically resistant to radiation. It was reported that the NPs induced the accumulation of the cells in the G2/M phase, which are more sensitive to ionizing radiation.[105] There are more recent studies that show the effect of NPs in arresting cells at specific phases[106] but few studies exist on how arrested cells improve the uptake of drug-loaded NPs.[72c] Some of the strategies to improve NP uptake by exploiting the cell cycle include pre-treatment with chemotherapeutics to induce cell cycle arrest followed by or in combination with NP treatment,[107,108] and exposure to ionizing radiation for early induction of G2/M arrest and subsequent NP incubation.[109] In the latter approach, higher uptake of doxorubicin-loaded iron oxide NPs was observed in G2/M-arrested osteosarcoma cells.[109] The cell cycle also has implications in gene delivery.[11] Although viral-mediated gene delivery (transduction) can occur throughout all stages of the cell cycle, gene delivery with non-viral systems (transfection), including polyethyleneimine[110] and liposomes,[111] has been

shown to be highest in the late S/G2 or G2/M phase when the nucleus is more accessible to internalized DNA, unlike in the G1 phase where the nuclear envelope is intact. In addition, the rate of pinocytosis, which is one mechanism of polyplex transport inside cells, was found to increase up to 70-fold from G1 to the middle of the S phase.[112] The delivery of antisense oligonucleotides using dendrimers also showed the highest nuclear accumulation in cells in the G2/M phase.[113] However, studies of cell cycle effects have their limitations, including the susceptibility of the chemical and physical methods of arresting cells in a specific growth phase to artifacts[114] and the potential distortion of the synchronous progression of the cell cycle.[115] Jandt and co-workers reported a method of synchronizing mammalian suspension cell cultures under near-physiological conditions, which were used to study transfection.[115,116] Using physiological synchronization, no obvious cell cycle dependency of transfection efficiencies of HEK293s cells using Lipofectamine 2000 was reported.[115]

2.3. Growth Conditions

2.3.1. Substrate

Differences in NP uptake can depend on the conditions of cell growth in two-dimensional (2D) cell cultures. The density of the cells, how close they are to one another, and the age of the cells can affect endocytosis and membrane properties.[117] The growth substrate can also affect NP uptake. Jhala et al. compared the uptake of bovine serum albumin gold nanoclusters (BSA-AuNCs) by cells grown on a biomimetic nanofiber substrate versus cells grown on non-coated polystyrene substrates. The cells grown on the biomimetic substrate showed higher NP uptake and this effect correlated with enhanced cell growth and extracellular matrix formation.[118]

2.3.2. Incubation Conditions

The effect of incubation conditions on cell internalization of NPs has also been studied in vitro.[119] The uptake of cationic NPs was found to increase when cells were incubated under biomimetic dynamic conditions in a microfluidic channel versus in static conditions but this

was dependent on the cell type and shear stress.[120] It was suggested that the dynamic conditions stimulated endocytosis and promoted cellular uptake. The degree of cell association under dynamic conditions was also found to be particle shape dependent.[121] Epithelial cells were also found to be more affected by shear stress than endothelial cells. Sedimentation and well format are also important factors that can affect NP uptake.[122] In addition, it has been found that the method of NP administration, e.g., as a concentrated bolus, a bolus followed by mixing, or pre-mixed in cell culture media, can alter particle–cell interactions in 2D cell cultures. Particles administered in a concentrated dose were found to interact more with cells compared with particles pre-mixed in culture media. This was attributed to the higher protein adsorption (2-fold) observed on the concentrated bolus than that obtained with the pre-mixed particles after 24 h incubation, suggesting that the initial formation of the protein corona from the biological media has a lasting effect on subsequent particle–cell interactions.[123]

2.3.3. Cell Population

The heterogeneity of the cell culture can also influence cell uptake, and differences in uptake have been observed in single versus mixed cultures.[119] The uptake of NPs can be different in commonly studied immortal cell lines in vitro, including HEK293 cells, in comparison with primary cells or cells derived from tissues of patients. Table 1 shows differences in uptake in immortal and primary cells and highlights the particle dependency of this variance; however, in this example, the cell lines and primary cells do not represent the same tissue.[119] Although primary cells present a more relevant model for studying NP uptake, access to primary cells and specific growth conditions prevent their widespread use in bio–nano studies. Primary cells from blood, however, are more readily available following human ethics approval of their collection and use and have been applied in several blood cell association assays to study NP uptake by immune cells.[124–126]

Cells, including tumor cells, naturally grow in the presence of other cells that may have influence on their function, proliferation, and differentiation. In an in vitro model of the gut, where typically a single culture of Caco-2 cells are used for permeability studies, Schimpel et al. found that a triple culture consisting of M cells and mucus-secreting goblet cells in addition to Caco-2 cells were a more representative model for studying NP transport across the intestinal mucosa.[49] It was concluded that goblet cells and M cells strongly impact NP uptake in the intestine by creating a mucus barrier that lowers the penetration of particles (goblet cells) and identifying cells that are more likely involved in internalization (M cells).

Table 1. Internalization of ~200 nm poly[(methyl methacrylate-*co*-methacrylic acid)] (PMAA) and poly[(methyl methacrylate)-*co*-(2-dimethylamino ethylmethacrylate)] (PDMAEMA) in immortal and primary cell lines[a)]

	Cell line	3% PMAA	13% PMAA	20% PDMAEMA
Immortal	HEK 293	−	+	+
	L929	−	+	++
	HepG2	−	−	+
Primary	Primary muscle cells	−	+	++
	Differentiated muscle cells	+	++	+++
	HUVEC	−	+	++
	Mϕ	−	+	++

[a)]The mean fluorescence intensity (MFI) of the cells was measured via flow cytometry after 24 h incubation and rated as low (−, MFI <15), medium (+, 15 < MFI < 50), high (++, 50 < MFI < 150), and very high (+++, MFI > 150). Reproduced with permission.[119] Copyright 2015, Elsevier B.V.

Cell confluency (cell surface coverage on a growth substrate) is also a factor.[72b] Serdiuk et al.[72b] found that increasing cell confluency from 500 to 1300 and 2500 cells mm^{-2} lowered particle uptake in fibroblasts (3T3-L1) and epithelial healthy cells (SG cells). This work demonstrates that cell proliferation, which is more active at low cell confluency as the cells are growing, can strongly impact the uptake of NPs.

2.3.4. Effect of Serum and Biomolecular Corona

The presence of serum in the growth media, which is typically at 5% or 10% compared with 100% in blood, has been shown to affect particle–cell association and has been particularly pronounced in transfection studies with DNA and RNA nanoformulations.[127,128] Serum biomolecules in growth media can adsorb onto NPs to form a biomolecular corona that can mediate the interactions of NPs and cells. The presence of a biomolecular corona lowers the surface energy of the particles and has been shown to reduce the adhesion of silica[129] and carboxylated polystyrene[130] NPs, leading to a decrease in particle uptake.

2.3.5. Healthy versus Diseased Cells

The state of the cell may also influence NP uptake. The uptake and cytotoxicity of AuNPs in normal (L02) and cancerous (HepG2) hepatocytes was studied by Xia et al.[131] The uptake of 20 nm AuNPs was observed to be approximately 4-fold higher in cancer cells than in normal cells (**Figure 4**B), and approximately 7-fold higher with 50 nm AuNPs. This has been attributed to the presence and overexpression of multiple receptors on the membrane of cancer cells. It was found that in the cancer cell line, the AuNPs induced both apoptosis and necrosis, whereas the particles mainly induced necrosis in normal cells, suggesting potential cell-dependent toxicity effects and pathways.

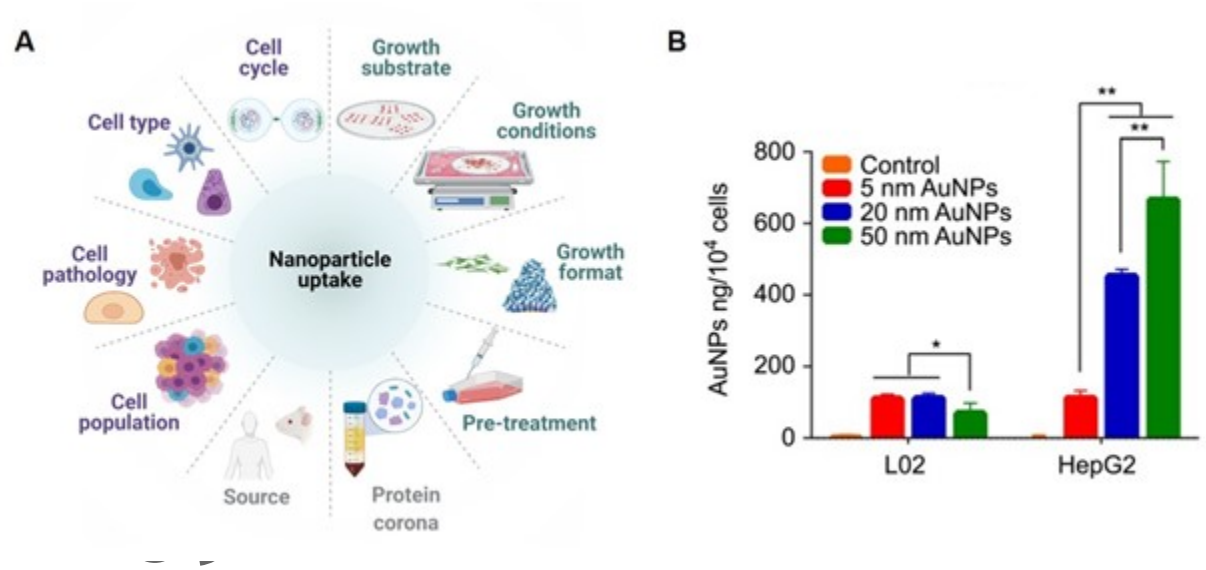

Figure 4. (A) Overview of factors at the single cell level that can influence NP uptake. Created with BioRender.com. (B) Uptake of AuNPs of different sizes by normal cells (L02) and cancer cells (HepG2) after 24 h as measured using inductively coupled plasma–mass spectrometry. * $P < 0.05$, ** $P < 0.01$, when compared with control. Reproduced with permission.[131] Copyright 2019, Dove Medical Press Limited.

2.3.6. Pre- and Co-treatment Strategies

Pre-treatment or co-treatment of cells with complimentary activated therapies has been shown to improve the uptake of NPs by cells. Radiation therapy, for example, can prime cells and tumors for NP uptake by changing the permeability and architecture of the blood vessels or mediating changes in pressure in the tumor microenvironment.[132] Yang and co-workers applied X-ray radiation immediately prior to NP administration and found that X-ray radiation increased tumor cell uptake of different types of NPs, including PEGylated melanin/copper sulfide (CuS@Melanin-PEG) NPs, AuNPs, liposomes, and silica NPs (SiNPs) (**Figure 5**).[133] This was attributed to X-ray-induced cell cycle change and upregulation of caveolin-1, which is involved in caveolin-dependent endocytosis pathway. The increased uptake of NPs as a result of co-treatment with X-ray radiation led to an enhanced anti-tumor effect. A greater reduction in tumor size in mice was observed after in vivo administration of 35 nm doxorubicin-loaded PEGylated melanin/copper sulfide NPs

compared with doxorubicin alone, NPs alone, or doxorubicin-loaded PEGylated melanin/copper sulfide NPs without X-ray.

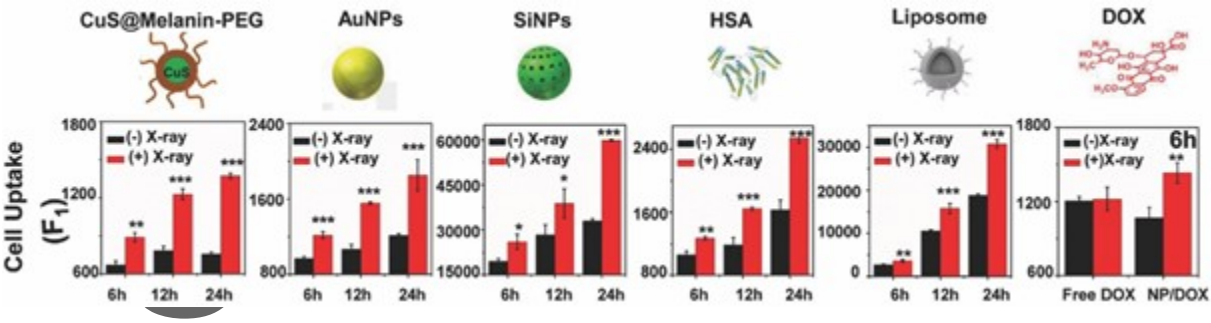

Figure 5. Uptake of different NPs by cells non-treated (black) or pre-treated (red) with X-ray irradiation. HSA, human serum albumin; DOX, doxorubicin. Reproduced with permission.[133] Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA.

Pre-treatment of cells with a therapeutic prior to NPs was also shown to increase NP uptake and accumulation in tumor cells.[107] After 24 h pre-incubation of U87 cells with docetaxel, higher NP uptake was reported when the NPs were subsequently added to the pre-treated cells. This was attributed to cell cycle effects as docetaxel acts by arresting cells in the G2/M growth phase. Further work is required in this area, with one therapeutic or agent potentially priming cells for uptake, pre-treatment or combination therapy has the prospect to enhance uptake of nanomedicine in target sites and facilitate the synergy of multiple therapeutics.

3. Tissue Level

Many studies on NP uptake and bio–nano interactions have largely been performed on 2D cell cultures. The last decade has seen advances in biological models, specifically 3D models that more closely resemble tissues in vivo for the study of bio–nano interactions and NP toxicity.[134] A variety of parameters differ between 2D and 3D models, including the microenvironment and heterogeneity of the cell population. Hence, 3D models may provide fundamental insight into particle interactions and better predict the success or failure of

nanomedicines. From 3D models in culture to tumor models in vivo, this section will describe how cells arranged in 3D structures can impact bio–nano interactions and NP uptake.

3.1. 3D Models/Tissue Mimics

3D models comprise a collection of cells, ranging from simple homogenous models to more complex heterogeneous population of cells arranged in a macro-sized cluster stabilized by cell–cell junctions, surface membrane projections, and extracellular matrices. 3D models include spheroids and organoids grown using various methods/platforms, including hanging drop, matrices and scaffolds, hydrogels, spinner flasks, biofabrication, and microwell liquid overlay.[11,135] Spheroids are usually composed of actively growing cells in the outer layer and nonproliferating cells in the center. The center can be necrotic because of poor oxygen supply, closely resembling the state of cells in actual tumors. All phases of the cell cycle are represented in spheroids, although in models of quiescent cells, cells in G0 are dominant.[11,136] The effect of the cell cycle in the context of drug delivery in spheroids has been reviewed.[11] As in 2D cells, cells in distinct growth phases (the cell cycle status) in 3D models might differ in how they respond to NPs and therapeutics, particularly with gene delivery systems, which may be highly cell cycle dependent.

The effect of cytotoxic drugs on 3D versus 2D cell cultures has been well studied.[137] The response to drugs has been shown to differ owing to limited penetration through the spheroids and differences in gene and protein expression and cell–cell communication.[138] In the context of NPs, comparison between 2D and 3D cell cultures has been studied using drug-loaded NPs, where the efficacy of the drug was greater in 2D systems than in 3D systems. In addition, control of the assembly process of 3D models can allow for long-term studies of bio–nano interactions over many weeks, potentially leading to a more accurate assessment of the long-term outcomes for particle interactions with biological systems, including degradation properties of nanomaterials.[136]

Studies of NP interactions with cells in 3D have been influenced by the properties of the model itself.[134] The composition of the model, that is, the constituent cells and whether composed of one type of cell or a heterogeneous population of cells can impact NP uptake. A 3D model composed of endothelial cells that reflects the role of the cells to form barriers in vivo was described by Salvati and co-workers.[139] A cell barrier mimic composed of human primary umbilical vein endothelial cells (HUVECs) arranged to form an endothelial barrier was found to partially inhibit NP uptake compared with confluent and subconfluent HUVEC cultures (**Figure 6**). The reduced uptake in the presence of cell barriers was attributed to lower expression of endocytic receptors.[139] Cell shape was found to have a significant influence on the cellular uptake of NPs.[140] Adherent cells in cultures are planar and have a stretched spreading morphology, with a greater amount of cell membrane exposed and susceptible to NP association, unlike cells tightly assembled into barriers and 3D mimics.

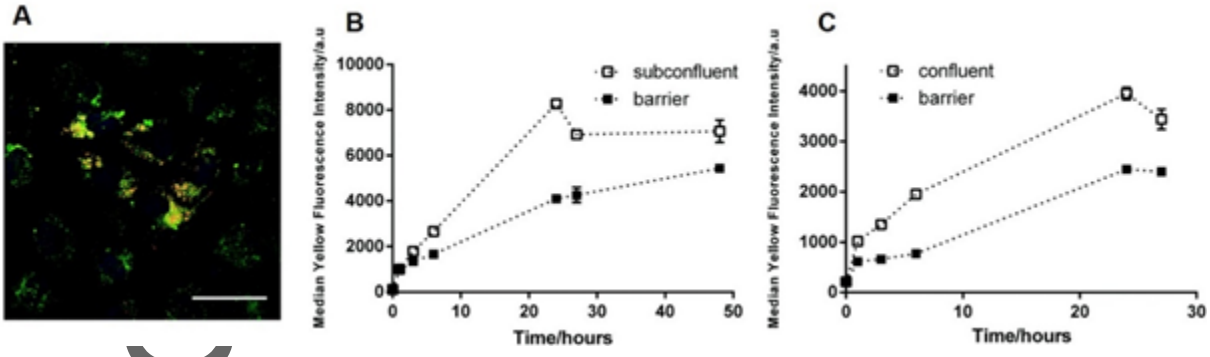

Figure 6. (A) Confocal microscopy image of a HUVEC barrier showing NP (red) uptake. Cells are stained with the lysosomal-associated membrane protein 1 (LAMP-1) (green). Uptake of NPs in endothelial cell barrier (3000 cells cm^{-2}) mimics compared with endothelial cells (HUVEC) in subconfluent (B; 3000 cells cm^{-2}) and confluent (C; 25000 cells cm^{-2}) cultures. Reproduced with permission.[139] Copyright 2018, Royal Society of Chemistry.

A reduction in particle uptake in 3D models (up to 80%) was also observed by Behzadi et al. using 30 nm quantum dots and LNCaP cells (a human prostate cancer cell line) arranged in a 3D scaffold.[141] The quantum dots distributed more evenly in cells in the 3D scaffold than the 2D-cultured cells. In contrast, Meng et al. found that the uptake of 170 nm polymeric

poly(glycerol adipate) NPs was higher in 3D models of brain tumors compared with a monolayer of cells when composed of a single cell type DAOY, a human cerebellar medulloblastoma cell line.[142] However, in 3D tumor models consisting of mixed brain cells, the monolayer of mixed brain cells exhibited higher uptake than the 3D model. This highlights the need to examine candidate particle systems with models that most closely resemble the architecture of the biological system or disease.

As in 2D, cells in 3D can also be influenced by growth conditions. In a 3D tumor model (MCF7 spheroids) grown on a microfluidic device, continuous administration of mesoporous silica NPs (45–300 nm resulted in higher uptake than transient administration.[143] There is typically reduced proliferation of cells in 3D culture, which could be affected by the growth substrate and conditions. The lower potency of cytostatic drugs in 3D has been attributed to their reduced proliferation compared with actively growing cells in 2D cultures.[134,137] The effect of size of the spheroids on NP uptake has not been studied as extensively as the effect of tumor size on particle uptake in vivo (the effect of tumor size on NP uptake will be discussed in Section 3.2). This is despite many studies that examine the effect of particle size on uptake by cells in spheroids.[70]

3.2. In Vivo Tumor Models

Tumors are complex structures made up of cancer cells, stromal cells, and vascular networks patrolled by immune cells and surrounded by the extracellular matrix (ECM).[144] The tumor microenvironment poses a challenge to nanomedicine penetration and uptake as the NPs need to avoid immune clearance, cross the vasculature, and pass through the interstitium and permeate through the ECM within the tumor. The vasculature supplying the tumor is disorganized with highly branched vessels of uneven diameters. The blood vessels in tumors are often fenestrated and discontinuous, and lack basement membranes, giving the tumor high vascular permeability.[145] Macromolecules that are large enough to avoid renal clearance can

readily traverse from the vessels into the tumor interstitium owing to its permeability. In human tumor xenografts, early studies observed that liposomes up to 400 nm in diameter could pass through the blood vessel pores, indicating that extravasation of particles is limited by the pore size.[146] In addition, the lack of lymphatic structures within the tumor reduces outflow of NPs. The combination of these two factors, the EPR effect, has been suggested as the main mechanism for NP accumulation in tumors in animals, although its existence in human tumor physiology has been debated.[147–149] Nevertheless, the lack of lymphatic drainage decreases the intravascular pressure and increases the interstitial fluid pressure (IFP), leading to venous stasis and collapse of blood vessels. The IFP in desmoplastic tumor is particularly high, at 75–130 mm Hg, compared with the IFP in normal tissue, which is 0–3 mm Hg. This feature further reduces blood flow to the tumor, disrupting the transport of drug and potentially NPs via the vasculature. Pressure within the solid structural components of the tumor (solid pressure) and that generated upon compression of cells and ECM around the vessels can also cause a reduction in blood flow.[23,26] Consequently, this can impair the delivery of nanomedicine via convective transport.[150] At the tumor periphery, the IFP is low, which generates a pressure gradient that can result in the diffusion of cells, growth factors and fluid out of the tumor, mediating the metastasis of tumor and inhibiting drug delivery.[151] The pressure gradient across the vasculature to the tumor means that transvascular transport relies on a concentration gradient of the diffused drug (or NP) between the blood and interstitial fluid[152] and can also be limited by cells (pericytes) that wrap around the blood vessels.[153]

Tumor-associated fibroblasts (TAFs) found near blood vessels can also limit NP uptake by sequestering NPs. This is because NPs are internalized by TAFs readily, taking up NPs 7-fold higher than other tumor cells post-intravenous injection.[153] TAFs promote increased ECM production, and have a high proliferation rate and unique cytokine secretion.[154] The ECM in

tumors is often high in collagen content, which is associated with poor prognosis and resistance to therapy.[155] In a study that compared tumor type and the anatomical location of tumor xenografts in mice, Pluen et al. demonstrated that the slower diffusion of macromolecules in tumors growing in dorsal chambers compared with tumors growing in the cranial windows is associated with the higher level of collagen type I, which correlates with a high density of fibroblasts in the tumor cells.[156] The hindrance of diffusion is associated with the tortuous diffusion pathway and narrow spacing (20–40 nm in width) formed by the collagen fibrils.[156] Coating NPs with the enzyme collagenase may be a strategy to address this—100 nm NPs coated with collagenase displayed an increased penetration into multicellular spheroid model, improving NP delivery into solid tumors.[155] Off-target uptake of NPs can also be observed in tumor-associated macrophages (TAMs), which have been implicated with promoting tumor growth. TAMs display phagocytic properties and a higher association with NPs compare with the cancer cells, which could prevent deep penetration of NPs and reduce the number of NPs into the tumor.[157] Targeting TAMs with NPs loaded with toxic drugs may be a strategy to eradicate TAMs and remove the immunosuppressive environment they create. Haber et al. demonstrated targeting of TAMs in mouse models of metastatic ovarian cancer by injecting anionic NPs in the intraperitoneal cavity.[158] Mass spectrometry analysis of the harvested tumors revealed NP uptake in 87% of the TAMs compared with 2% in cancer cells.

To address the fact that tumors at different stages of cancer progression vary in pathophysiology (cell density, vasculature, necrosis, and stroma), Chan and co-workers examined the effect of tumor volume on the accumulation and penetration of AuNPs of different sizes.[159] A steady increase in NP (15, 30, and 45 nm) accumulation with tumor volume was observed as the size of NP was reduced, and the NP uptake rate was generally constant regardless of tumor volume. The accumulation of 100 nm AuNPs in the tumors

studied was found to be low owing to their inability to diffuse through pores in the ECM that are smaller than the particle size. From their observations, Chan and co-workers proposed a method of selecting AuNPs according to tumor volume that might be translatable to personalized nanomedicine according to the disease state of a patient to enhance diagnostic and therapeutic outcomes (**Figure 7**).

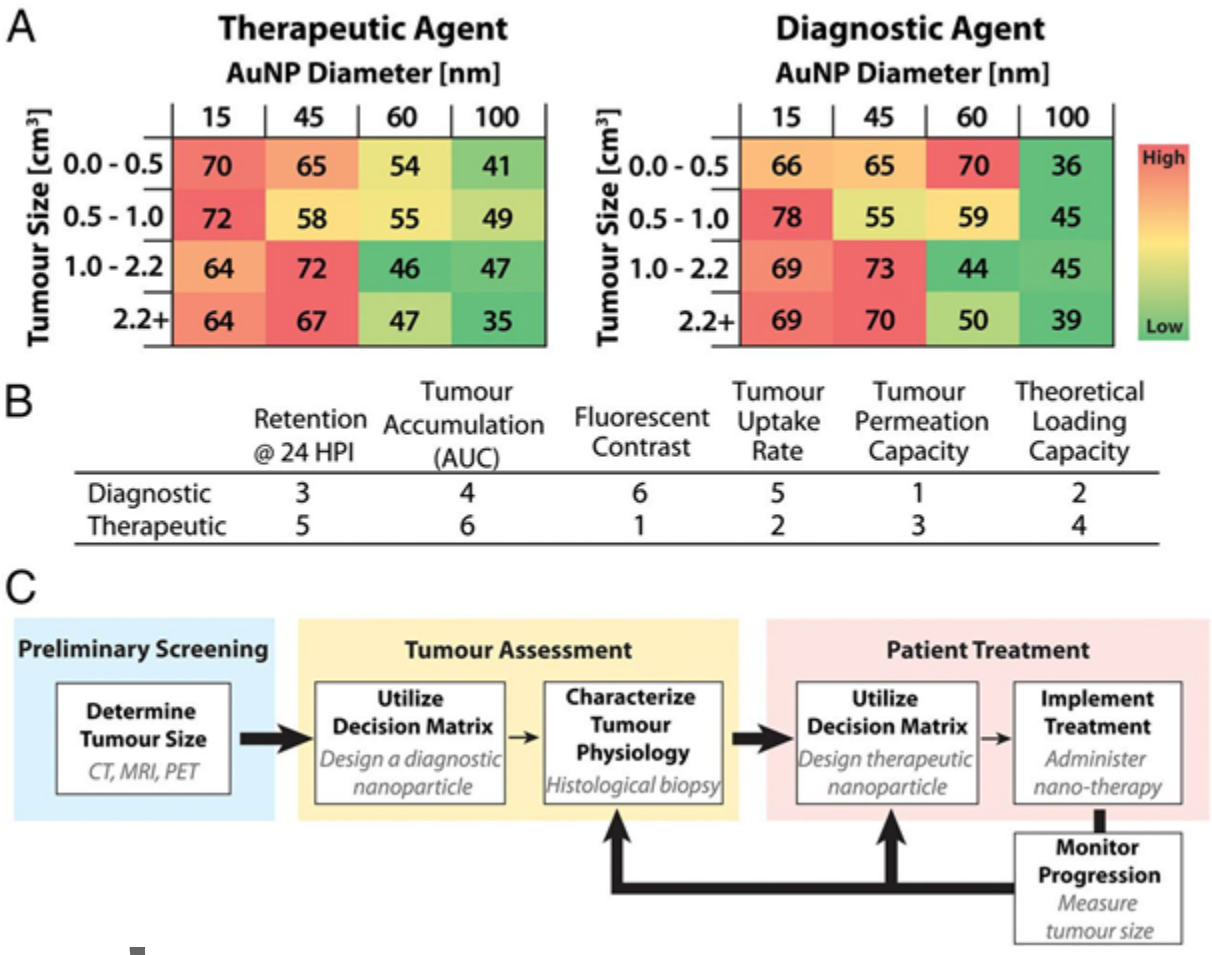

Figure 7. (A) Matrix for selecting therapeutic and diagnostic NP size based on tumor size/maturity: heatmap predicting NP utility for therapeutic (left) and diagnostic (right) applications for a given tumor volume. Values were calculated based on ranked experimental observations regarding parameters in (B) and the importance of each parameter for each application. (B) Weighted importance multiplier based on the parameter's importance for a given NP function. (C) Flowchart detailing a strategy for personalizing AuNP selection in the clinic for cancer detection and treatment. Reproduced with permission.[159] Copyright 2016, National Academy of Sciences.

4. Organism Level

Although 3D cell culture models in vitro and tumor and disease models in vivo offer more physiologically relevant systems to study NP uptake than 2D cell culture, they do not fully recapitulate the dynamic and complex environment of the whole organism, particularly in the human body. The biological barriers that exist in the body, including (i) opsonisation (marks foreign material for immune clearance), (ii) the mononuclear phagocyte system (clearance), (iii) endothelium (barrier to tissue penetration), (iv) interstitium (restricts movement within tissues), and (v) kidney filtration (removes small particles and macromolecules), undoubtedly influence the deposition patterns and uptake of NPs in a whole organism. Laboratory animals remain an important tool in preclinical studies of potential therapies, particularly with regards to safety. However, therapeutic outcomes in animal models often do not translate well in clinical trials. There are significant differences in the physiologies of laboratory animals when compared with the human body (e.g., blood volume, drug absorption and processing mechanisms, cell surface properties, inhalation behavior in mice and humans) that make the design, conduct, and reporting of in vivo experiments crucial. In addition, each human is unique in physiology and person-to-person variation is expected in how they might respond to nanomedicine. For example, in cancer, genetic variation accounts for the different gene expression within the tumor of patients as well as the individual's immune response, which could explain differences in the performance of nanomedicine, including cancer immunotherapies.[160] Variation in human metabolism, which is affected by age, health, and host genetics, may also play a role in particle disposition and processing. Understanding person-to-person variation, however, could be leveraged for precise NP delivery.[9] The "properties" of the organism including the plasma proteome, gender, strain, and age will be discussed in this section to highlight potential organism-level parameters that can affect NP uptake and hence the efficacy of nanomedicines.

4.1. Plasma Proteome

Unravelling the effect of the biomolecular corona on the interaction of NPs with biological systems has led to a growing number of studies in recent years[125,126,129,161] that complement studies on understanding the influence of material properties on protein corona formation.[162] There are over 10000 proteins in plasma[163] that could potentially interact with NPs, although only a few main protein groups including complement proteins, albumin, apolipoproteins, and glycoproteins have been shown to play major roles in a particle-dependent manner.[126] Recent work by Ju et al. showed that NP interactions with immune cells in human blood are modulated by person-specific biomolecular coronas.[125] No correlation between blood donor, gender, or age was observed, but plasma variance across 23 healthy donors influenced the binding of PEG-coated mesoporous SiO_2 NPs and doxorubicin-encapsulated liposomes to immune cells, particularly monocytes and B cells.

Variance in the protein corona also exists between patients with different diseases, including cancer, diabetes, hemophilia, hypercholesterolemia, as well as healthy and pregnant individuals.[164,165] The therapeutic impact of graphene oxide sheets with biomolecular coronas formed from the plasma of various patients and individuals revealed different uptake in breast cancer cell lines (MCF-7 and MDA-MB-231) as measured indirectly by lysosome induction.[166] Colapicchioni et al. also studied differences in the protein corona from liposomes exposed to the serum of patients with gastric, breast, and pancreatic cancer and found that the enrichment of immunoglobulins was cancer-type dependent, highlighting the potential for exploiting the protein corona for disease diagnosis.[167] The health of the patient can therefore influence the biological identity of the NP, which in turn could affect function and efficacy. This presents opportunities for precision nanomedicine, whereby a patient-derived personalized corona or a single abundant protein coating is formed on particles to improve their therapeutic outcome.[168–170]

4.2. Immune System

The immune system of an organism is a significant barrier in nanomedicine and much effort has been devoted to implementing pre-treatment and particle engineering strategies[171] that allow NPs to evade immune clearance, particularly sequestration by Kupffer macrophages in the liver. A pre-treatment strategy was reported in which non-active NPs (liposomes) were first administered to pre-saturate the Kupffer macrophages to allow better uptake of the subsequently administered therapeutic particles.[172] Transient depletion of Kupffer cells has also been reported, although this might be challenging to achieve clinically.[173] Investigations of targeting and in vivo efficacy of chemotherapeutic NP formulations are often performed on tumor-bearing mice that are immune-deficient (immune system is compromised or absent). It has been demonstrated, however, that the presence of an immune system can affect the therapeutic efficacy of NPs in mice.[174] Soenen and co-workers[174] administered Ag NPs in immune-deficient NOD SCIDγ and immune-competent DBA/2 mice. After 5 days, inflammation, which was used as a measure of therapeutic efficacy, was found in the immune-competent mice but not the immune-deficient mice. Although this result might be due to immune deficiency in NOD SCIDγ, this finding suggests that an intact immune system might be exploited by the immunomodulatory properties of NPs to improve anti-tumor therapy.

4.3. Strain

With regards to rodent models, differences in strains (e.g., mice variants) have been shown to result in different NP uptake. C57BL/6 and BALB/c mice are common strains used for biodistribution and pharmacokinetics studies. However, variance in organ distribution and general response to NPs in the two strains has been observed.[175] Jones et al. investigated particle (300 nm cylindrical PEG hydrogel NPs) clearance in C57BL/6 and BALB/c mice to ascertain the role of immune status in NP clearance.[175a] C57BL/6 and BALB/c mice are prone to Type 1 immune responses and Type 2 immune responses -, respectively. NP uptake

by BALB/c and C57BL/6 was shown to be different and it could be associated with the higher uptake of particles by granulocytes in peripheral blood and spleen of BALB/c than C57BL/6 (**Figure 8**). Yokel et al. studied 4 nm CeO_2 NPs in both strains. BALB/c mice were generally found to be more responsive to the intraperitoneal-injected NPs.[175b] Tumor blood flow has also been shown to differ between mouse strains, which might have implications in NP-mediated photodynamic therapy and other vascular-related therapies.[176]

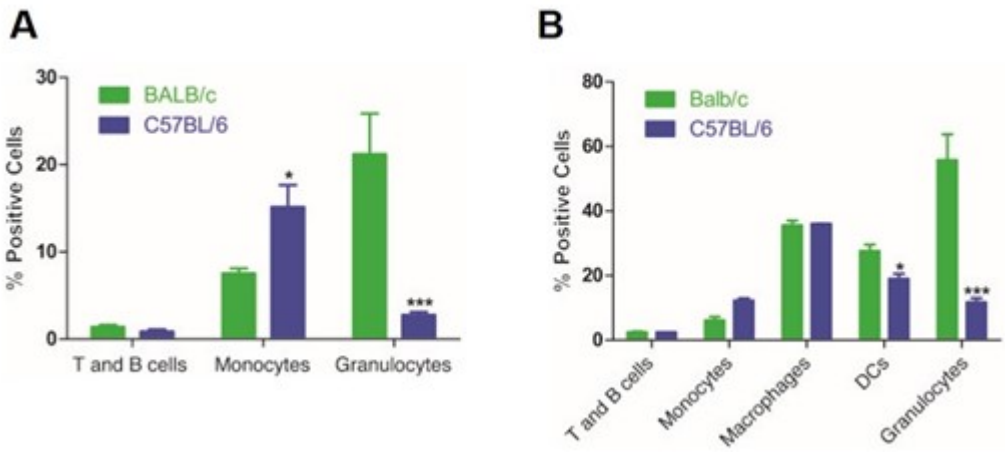

Figure 8. Effect of mouse strain on 300 nm PEG NP clearance. Flow cytometry analysis showing the uptake of fluorescently labeled NPs by immune cells from (A) peripheral blood and (B) spleen of Balb/c and C57BL/6 mice. (A) In blood, higher uptake of particles by granulocytes in BALB/c mice ($P < 0.002$, unpaired 2-tailed *t*-test) and significantly higher uptake by monocytes in C57BL/6 mice ($n = 4$) ($P < 0.05$, unpaired 2-tailed *t*-test). (B) In the spleen, slightly higher uptake of particles by DCs ($P < 0.05$, unpaired 2-tailed *t*-test) and granulocytes ($P < 0.003$, unpaired 2-tailed *t*-test) in BALB/c mice. Reproduced with permission.[175a] Copyright 2013, American Society for Clinical Investigation.

4.4. Gender

In female mice, Schroeder and co-workers reported a twofold increase in NP accumulation in the ovaries of ovulating female mice compared with nonovulating mice.[177] This led to increased ovarian toxicity and reduced fertility when compared with the results obtained following administration of the free drug. In an ovarian cancer model, NP accumulation in the ovaries during ovulation improved the therapeutic outcome. However, in a breast cancer model, the higher accumulation in the ovaries rather than the tumor reduced therapeutic

efficacy. This finding suggests that the ovulation state of females should be considered in the design of female-specific nanomedicines.[177]

The way disease can impact males and females, and hence susceptibility to NP treatment, should also be considered. Bharadwaj et al. found gender-dependent permeability in the BBB in traumatic brain injury (TBI) mouse model.[178] At 24 h post-administration, NP (PEGylated polystyrene) accumulation was greater in TBI female mice than in male mice, suggesting that the pharmacokinetics and pharmacodynamics of nanomedicine delivery to the brain after TBI may differ between female and male subjects, with the potential effects of hormonal variance warranting further studies.[178]

Gender differences were also observed in AgNP- and AuNP-induced toxicity in rodents.[179–181] For instance, Gender-related differences were found in how NPs affected specific organs, particularly the liver and kidneys. Kim et al. found a twofold increase in the accumulation of AgNPs in the female kidneys when compared with the male kidneys.[179]

4.5. Age

The development of the coronavirus disease 2019 (COVID-19) vaccines has highlighted the need to assess potential vaccines and therapeutics in different age groups, and indeed clinical trials often involve age-related studies of therapeutic efficacy and safety.[182,183] Age-related studies involving NPs have largely focused on toxicity studies of NPs, including zinc oxide, silver, copper, aluminum, and titanium dioxide (**Figure 9**).[184–186] Older animals have generally been shown to be more susceptible to the adverse effects of nanomaterials than younger animals.[187] A study of Fe NP distribution in mice showed higher distribution of the NPs in the spleen, liver, lung, kidney, and brain of elderly (14–16 months) mice versus young mice (3–4 months) (Figure 9).[188] These studies highlight that physiological, immunological, and genetic changes in animals or humans as a result of old age or ageing may impact NP uptake and accumulation, which may be exploited or avoided depending on the NP function.

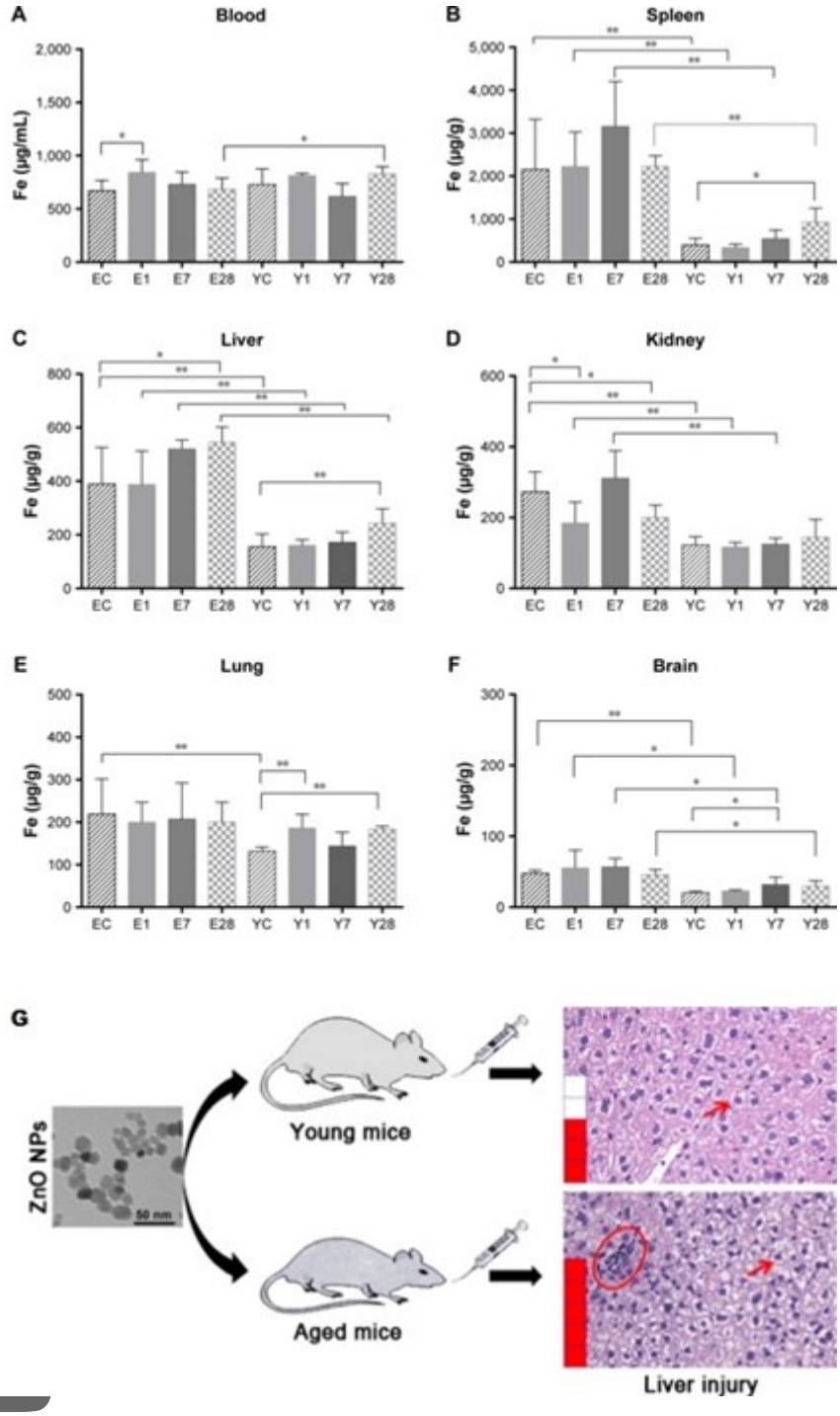

Figure 9. (A–F) Biodistribution of Fe-based magnetic NPs at 0 ("C"), 1, 7, and 28 days after intraperitoneal injection in elderly ("E"; 14–16 months) and young ("Y"; 3–4 months) Swiss mice. Reproduced with permission.[188] Copyright 2019 Dove Medical Press Limited. (G) Age-related hepatoxicity of orally administered ZnO NPs in young versus aging C57BL/6J mice. Reproduced with permission.[184] Copyright 2016, Elsevier B.V.

5. Conclusions and Perspectives

The physicochemical properties of NPs are known to impact their interactions with cells and biological systems. Advances in materials science, and particle engineering and characterization have facilitated studies into how NP properties can be tailored to modulate bio–nano interactions. However, understanding the biological factors that govern the desired application is also essential in the studies of NP uptake in biological systems and the design of effective nanomedicines. At the single cell level, the cell type, cell surface properties and uptake mechanisms, cell proliferation state, including cell cycle, and cell culture conditions are some of the major parameters that can influence NP uptake. In more complex 3D models, the tumor size and model type can also influence uptake. At the whole organism level, the immune competence of the model, gender, strain, age, and blood proteome are also key considerations. Discussion on the methods used to characterize NP uptake is beyond the scope of this review but it is acknowledged that the different methods used make it challenging to compare different NP uptake studies, leading to calls to standardize the way bio–nano interactions, including NP uptake, are studied and reported.[189–191] Several recent studies have aimed at quantifying experimental and biological factors that govern bio–nano interactions, including cell–particle interactions,[192a,192b] receptor binding,[192b] tumor targeting,[7] pharmacokinetics,[192c] and NP dosage.[192d] Nevertheless, it is evident that beyond tailoring NPs to improve their efficacy in humans, more consideration of the physiological state of the site of disease from the organism level down to intracellular properties in target cells is needed (**Figure 10**). As a starting point, particle design for nanomedicines might refer to the following reasoning/guideline:

1. Which site in the body is affected by the disease? What are the biological barriers (e.g., immune response) that must be overcome to reach the target site? This could influence administration route, and particle size and surface (e.g., personalized biomolecular corona, PEGylation).

2. At the target site, how will the NP reach the target cell? Would it need to permeate through the ECM and biological fluids, including mucus? What are the immune defenses at the target site? Particle size, mechanical properties (e.g., elasticity), and surface properties may play a role.
3. At the cell interface, what mechanisms or pathways are available for cell internalization? Are there cell barriers that could influence uptake? This could necessitate surface modification strategies to improve uptake (e.g., targeting ligands, cell-penetrating peptides, fusogenic materials) in addition to particle size, shape, and elasticity considerations.
4. What and where is the intracellular target, if any? What are the intracellular barriers to drug release and effective drug function (e.g., endosomal escape, degradative enzymes)? Particle composition that enables drug release is important, as well as any signaling molecules attached to the drug, if necessary, to allow trafficking to the intracellular target site.

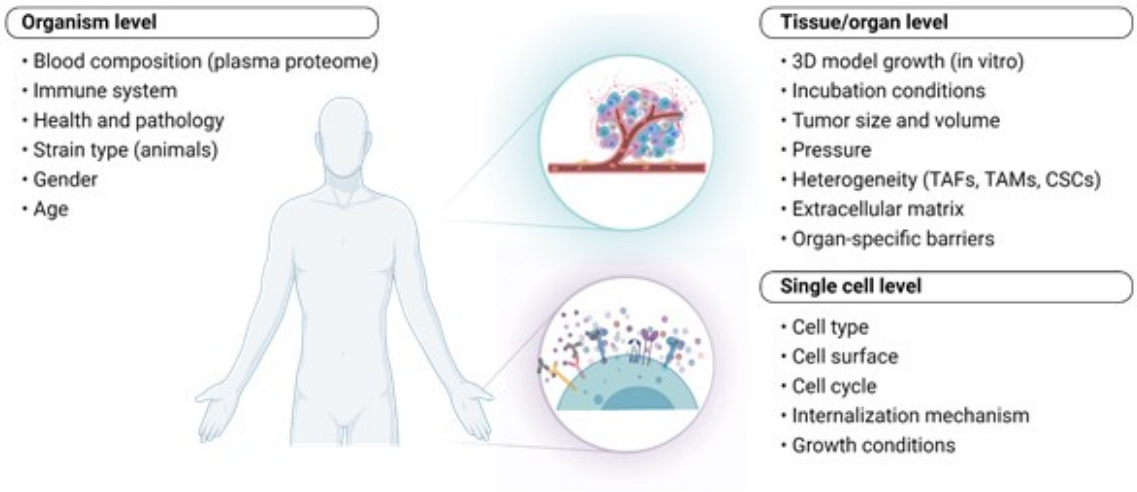

Figure 10. Biological factors at the single cell, tissue/organ, and organism level that influence NP uptake. These factors must guide NP design to improve the outcomes of nanomedicine. Created with BioRender.com.

It is clear from the above reasoning/guideline that biological factors are key to guiding particle design in nanomedicine. Further investigation in the influence of disease physiology and biological parameters will broaden our understanding of bio–nano interactions and guide the design of nanomedicines with improved efficacy that can potentially be translated to the clinic. However, it should be acknowledged that the underlying biological factors and barriers have been difficult to identify, and mechanistic studies have been limited by the models and methods available to investigators. It is therefore also important to apply interdisciplinary methods to investigate new approaches or methods that help us identify these barriers and understand how they influence bio–nano interactions. This includes, at the single cell level, identifying genes and proteins that govern the cellular uptake pathways of NPs and identifying the relevant proteins in the protein corona that interact with cell surface receptors. Emerging chemical biology tools could be leveraged to monitor NPs inside live cells and allow the study of endocytosis of NPs in vivo. Moreover, as the field matures, basic bio–nano studies should go beyond phenomenological conclusions and delve deeper into mechanistic experiments while being complemented by unbiased discovery studies that make use of high-throughput screens to generate new hypotheses.

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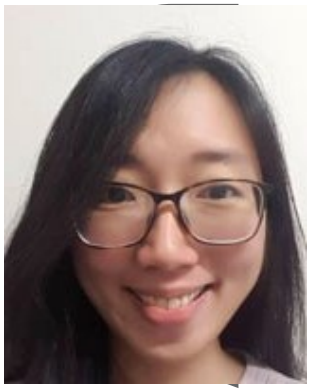

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Studies of bio–nano interactions have largely focused on the influence of the physicochemical properties of materials on their interactions with biological systems. This review focuses on the "bio" in bio–nano interactions and highlights the impact of biological factors at the cellular, tissue, and whole organism level on nanomaterial uptake and efficacy.

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A Focus on "Bio" in Bio–Nanoscience: The Impact of Biological Factors on Nanomaterial Interactions