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Review article

Understanding the influence of experimental factors on bio-interactions of nanoparticles: Towards improving correlation between in vitro and in vivo studies

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ABSTRACT

Bionanotechnology has developed rapidly over the past two decades, owing to the extensive and versatile, functionalities and applicability of nanoparticles (NPs). Fifty-one nanomedicines have been approved by FDA since 1995, out of the many NPs based formulations developed to date. The general conformation of NPs consists of a core with ligands coating their surface, that stabilizes them and provides them with added functionalities. The physicochemical properties, especially the surface composition of NPs influence their bio-interactions to a large extent. This review discusses recent studies that help understand the nano-bio interactions of iron oxide and gold NPs with different surface compositions. We discuss the influence of the experimental factors on the outcome of the studies and, thus, the importance of standardization in the field of nanotechnology. Recent studies suggest that with careful selection of experimental parameters, it is possible to improve the positive correlation between in vitro and in vivo studies. This provides a fundamental understanding of the NPs which helps in assessing their potential toxic side effects and may aid in manipulating them further to improve their biocompatibility and biosafety.

1. Introduction

The term nanotechnology was coined by Prof. Norio Taniguchi in 1974 and is defined as the science, engineering and technology conducted at the nanoscale i.e. 1–100 nm. The nanoscale materials generally referred to as nanoparticles (NPs) are highly desirable because of their small size, optical properties, high surface area to volume ratio and their multifunctional nature. Bionanotechnology comprises research at the interface of nanotechnology and biology [2] that has established a niche in biomedical sciences. Liposomes [3–6], peptide-based [7–9] and synthetic polymer-based [10–12], three-dimensional macromolecular assemblies and nanocages [13–15] are examples of hollow/porous core NPs. Solid core NPs may be composed of inorganic metals such as iron oxide, gold, silver, platinum, silicon, quantum dots, titanium dioxide, gadolinium, selenium, copper oxide, zinc oxide or metallic hybrids, or organic carbon nanoparticles. The surfaces of inorganic NPs are generally modified with synthetic or naturally occurring polymers and/or monomers which may be of biological origin such as peptides, proteins, carbohydrates, lipids, DNA, RNA, PNA, aptamers, hybrid bio-synthetic molecules and others. These relatively flexible capping ligands

improve the stability, biocompatibility and functionalize the NPs for various applications or for further modifications. Fig. 1, depicts the various components and configurations of nanoparticle-bioconjugates.

Drugs that have poor pharmacodynamics can be delivered using NPs that may overcome these shortcomings by improving their half-lives, stabilities and bioavailabilities [17]. However, their use is not limited to drug delivery systems (DDS). Their other applications include use as optical imaging agents and analytical probes/biosensors, thus making them suitable theranostics agents [18–21]. Fifty-one nanomedicines have been approved since 1995 by FDA for clinical use with ~77 products in clinical trials as of 2016 [22]. Owing to their potential, nanomaterials are being utilized in the recent fight against SARS-CoV-2 [23,24]. Gold nanoparticles based immunoassays have been developed that enable rapid detection of SARS-CoV-2 infected asymptomatic patients or individuals showing mild symptoms [25,26]. An mRNA vaccine which went into Phase 1 clinical trial in March 2020, codes for the prefusion stabilized spike protein of SARS-Cov-2 and it is encapsulated in lipid nanoparticles which serve as effective delivery agents [27].

The focus of this review is on gold and iron oxide NPs which are the top 2 inorganic NPs in clinical trials (Fig. 1D). Iron oxide NPs are the only metal-containing NPs that have received approval to date for

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Abbreviations

NPs	Nanoparticles
i.v.	Intravenous
i.p.	Intraperitoneal
i.m.	Intramuscular
PEG	polyethylene glycol
PEI	Polyethylimine
AuNPs	gold nanoparticles
FeONPs	iron oxide nanoparticles
SPIONs	super paramagnetic iron oxide nanoparticles
PBS	Phosphate buffered saline
PAMAM	Polyamidoamine dendrimers
MPS	Mononuclear phagocytes system
CMD	carboxymethyl dextran
CvME	Caveloin mediated endocytosis
CME	Clathrin mediated endocytosis
CIE	Clathrin independent endocytosis
DDS	drug delivery system
COVID	corona virus disease
SARS-CoV-2	severe acute respiratory syndrome corona virus 2
PNA	peptide nucleic acid
FDA	Food and Drug administration
MRI	magnetic resonance imaging
PLGA	poly (lactic-co-glycolic acids)
PLA	poly (lactic acid)
PGA	poly (glycolic acid)
PVA	poly (vinyl alcohol)

PMA	poly(myristic acid)
sulfo-LC-SPDP	sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamide)hexanoate
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute
DLS	Dynamic light scattering
TEM	Transmission electron microscopy
FBS	fetal bovine serum
SPR	surface plasmon resonance
PVP	poly(vinylpyrrolidone)
ER	Endoplasmic reticulum
CPP	cell penetrating peptide
TAMs	tumor associated macrophages

Cell lines

HUVECs	human umbilical vein endothelial cells
U937	human monocytes
HeLa	Henrietta Lacks adenocarcinoma
RAW 264.7	mouse macrophages, ascites
MDI-MB-231	human adenocarcinoma breast cancer
bEnd3	mouse brain endothelial
HL-60	human promyelocytic leukemia
J774A.1	mouse macrophages, pleural ascites
HDF	human dermal fibroblasts
SKOV-3	human adenocarcinoma ovary, ascites
A549	human lung carcinoma
B16F10	mouse melanoma
IEC	rat intestinal epithelial cells

clinical use and most of them are MRI contrast agents [22]. Gold nanoparticles (AuNPs) exhibit plasmon resonance which can be followed using UV-Vis spectrophotometric detection assays [28,29], surface-enhanced Raman spectroscopy (SERS) [30] and confocal/luminescence microscopy [31,32]. The magnetic iron oxide nanoparticles (FeONPs), also commonly called superparamagnetic iron oxide nanoparticles (SPIONs) are used as contrast agents for magnetic resonance imaging (MRI) [33,34], for bio-detection such as tracking the implanted stem cells in vitro [35], in binding assays and hyperthermia [36,37] and magnetic field guided drug delivery [21] in cancer treatment. Besides, the electron dense gold and iron NPs are used widely in electron microscopy analyses. Au-Magnetite composites used in (SERS) analyses improve the intracellular signal intensity essential to studying interactions of NPs with biomolecules [30].

Delivery systems must be non-toxic by themselves, should not be cleared quickly from the body and trigger adverse immunological responses. It, therefore, becomes vital to understand their interactions at a molecular level, to determine how suitable they are for delivery and determine the applications for which they are best suited. The review is divided into four sections which discuss the (I) synthesis and functionalizing of NPs, (II) the discrepancies observed between the effects of NPs in vitro and in vivo, followed by a detailed review of (III) in vitro and (IV) in vivo studies of gold and iron oxide NPs, which demonstrate the need to carefully consider experimental factors to improve the correlation between in vitro and in vivo studies. This review also presents recent in vitro and in vivo studies that assess the biosafety/toxicity of NPs and the influence of surface ligands on nano-bio interactions such as uptake and immune response. We will emphasize the importance of standardization in nanotechnology with a focus on the experimental parameters since they have a significant impact on the outcome of studies. Standardization is essential to make valid comparisons between studies and to prevent redundancy in research which help develop the field of nanomedicine [38,39].

2. Synthesis and functionalization of iron oxide and gold nanoparticles with biocompatible ligands

The basic principle of NPs syntheses is to promote nucleation of the monomeric element (e.g. lipids for liposomes and metal ions for inorganic metal NPs), facilitating their assembly in a controlled manner to form stable and well-structured entities with narrow size distributions. Multiple routes and techniques used in NP syntheses have been established that are broadly categorized as chemical, physical and biological. Most chemical and biological methods use facile synthesis techniques that are easily controlled and reproducible, yet low in cost and scalable [40]. Functionalization of NPs has proved essential as they affect stability in the presence of salts and prevent aggregation over time, thereby increasing their shelf-life. They may also have other purposes including-promoting cellular uptake, co-functionalization to promote the delivery of drugs and nucleic acids, use in biochemical assays serving as binding partners, and provide additional functionalities to the delivery system. There are a wide range of biocompatible molecules used to functionalize the NPs for use in nanomedicine which have been divided into 5 major categories in this review (Table 1). The surface composition of NPs is an important factor influencing their overall behavior. Understanding their surface chemistry is therefore essential.

The synthesis method determines how the assembled surface can be further modified with desired molecules for downstream applications. One step syntheses involve the use of functionalizing molecules that serve as both, nucleation and capping agents [41–43]. Widely used Turkevich [41] and Brust-Schiffrin [44] methods for AuNP synthesis contain reducing citrate molecules [42] and hydrophobic thiol ligands such as dodecanethiol [45], respectively, in the synthesis mixture which act as nucleation and capping agents. Magnetic iron oxide nanoparticles are commonly synthesized in the presence of surfactants/synthetic polymers such a dextran [43], poly-vinyl alcohol [46] or as naked iron oxide nanoparticle with cationic ions bound to the surface [47].

Ligand exchange by direct substitution of surface ligands is one of the

commonly used method for functionalizing NPs [48]. AuNPs form gold-thiol bonds facilitating exchange of smaller ligands such as citrate molecules with larger molecules by direct binding of ligands to NPs via Au-S bond formation (Fig. 1C.) [42,49]. Naked FeONPs have a higher tendency of aggregating due to their magnetic property and are therefore stabilized by suitable surface ligands such as polymers or

cross-linking molecules [40]. These NPs can be further derivatized using ligands as linkers or binding moieties that facilitate electrostatic or covalent binding of molecules. For example, nucleic acids electrostatically bind to cationic surfaces or modified polyethylene glycol (PEG-SH) which covalently binds other thiol containing molecules [19]. Further, NPs can be encapsulated within liposomes which are spherical vesicles

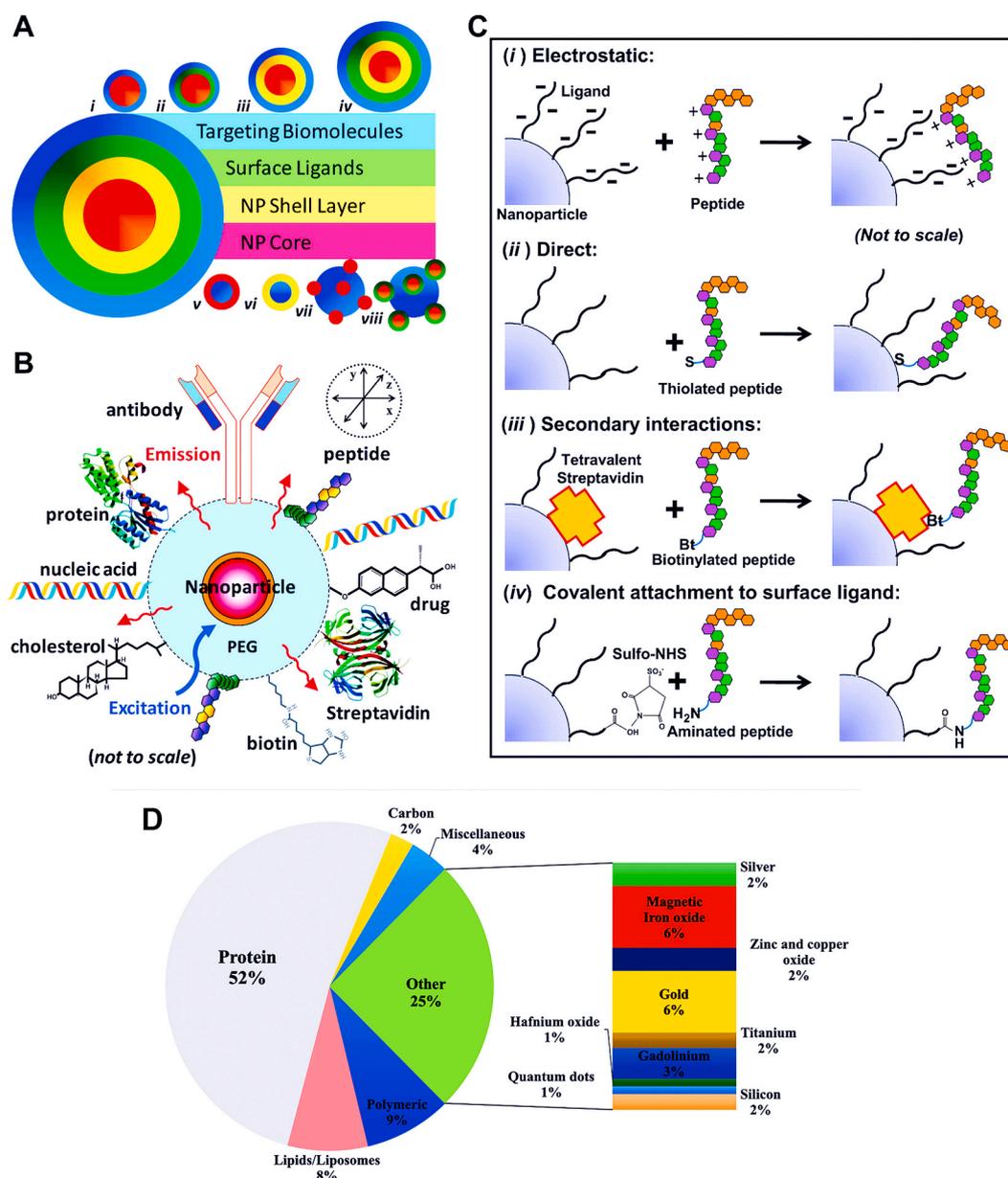


Fig. 1. Nanoparticle (NP) - bioconjugates: (A) A nano-bioconjugate can be composed of varied components of fundamentally different origin. This figure presents these components and their configurations: (i) Biomolecule interacting with NP core, (ii) biomolecule interacting with a NP core via intermediate ligands, (iii) biomolecule interacting with NP shell layer that surrounds the NP core, (iv) biomolecule interacting with NP shell layer/NP core via intermediate ligands, (v) porous NP core containing entrapped biomolecules, (vi) porous or hollow NP core containing entrapped biomolecules surrounded by a NP shell layer, (vii) NP core (or NP core/NP shell structures) particles smaller in size than the much larger biomolecule, (viii) NP core (or NP core/NP shell structures) particles smaller in size than the much larger biomolecule attached via intermediate ligands. (B) A representative NP decorated with multiple functional molecules (e.g., nucleic acids, proteins, drugs, peptides). NPs have great potential since they can provide multiple functions in one active platform. (C) The four general schemes routinely used for the conjugation of peptides to NP materials. These schemes are also representative of the type of interactions involved in the binding of biomolecules in general to NPs. (D) Distribution of types of nanoparticles in clinical trials, explored for use as nanomedicines. Data was obtained in April 2020, from clinicaltrials.gov using the search term 'nanoparticles'. This distribution is representative of active clinical trial studies using nanoparticles as drug delivery systems or imaging agents. Inorganic/metallic NPs in trial have been further categorized based upon their composition.

Adapted with permission from Sapsford, K. E et al. [1] Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques. *Anal Chem* 2011, 83 (12), 4453–88. Copyright (2020) American Chemical Society. Permission for part of the figure obtained from IOP publishing, Aubin-Tam et al. [16]. Structure and function of nanoparticle-protein conjugates. *Biomedical Materials*, 3 (3). © IOP Publishing. Reproduced with permission. All rights reserved.

Table 1
Applications of nanoparticles with different biocompatible surface ligands.

Category of functionalizing molecules	Nanoparticles & Functionalizing molecules	Applications	References
Polymers & carbohydrates	FeONPs + PEG variations (NHS-PEG-SH, PEG-SH)	Magnet guided delivery, MRI, Probes for cancer imaging	Lee [19], Xu [56], Gao [55]
	AuNPs + 2-deoxyglucose	Theranostics	Suvarna [52]
Lipids & Liposomes	AuNPs + Soybean lecithin, cholesterol liposome	Photothermal treatment	Xing [3]
	AgNPs/AuNPs + POPC, POPG, Cholesterol lipid bilayer	SERS probe	Bhowmik [4]
	AuNPs + DC-Chol, DOPE, Chol	siRNA delivery	Kong [5]
	FeONPs + OQLCS polymeric liposomes	MRI	Liao [6]
Peptides	FeONPs + TAT CPP	ROS induced radiation, Dox-delivery	Hauser [63], Morshed [64]
	FeONPs + H625-CPP	MRI, Far red imaging, hyperthermia, drug delivery	Perillo [65]
	AuNPs + Penetratin peptide	Photothermal, NIR	Yin [67]
Proteins & Antibodies	AuNPs, FeONPs + BAPs	CT, confocal imaging, probes for molecular interactions, cell sorting and quantification	Natarajan [49,71]
	AuNPs + albumin variants (BSA, albumin, Nab-paclitaxel)	photothermal chemotherapy, anticancer drug delivery	Nosrati [73], Ruttala [75], Vismara [76]
Nucleic acids	FeONPs + Anti-Her 2, ScFvEGFR	Targeted MRI	Chen [78]
	PEI-siRNA	siRNA delivery	Wang [85]
	AuNPs + Aptamers	LSPR mediated colorimetric detection	Lia [88], Huang [89]
	FeONPs + Aptamers	Detection of biological molecules	Fernandez [90]

POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; DOPE, Dioleoylphosphatidylethanolamine; OQLCS, octadecyl-quaternized lysine modified chitosan; TAT, Transactivator of transcription; CPP, Cell penetrating peptide; ScFvEGFR, short chain variable, anti-EFGR.

composed of amphiphilic lipids, thus imparting added functionalities to both the liposomes and the NPs [3–5].

The review by Sapsford et al. [2] provides details about the types of surface ligands, the functionalizing chemistries and the nano-bio interfaces for different types of NPs. However, this review, does not provide adequate information on the chemistries, syntheses and characterizations of functionalized NPs. We will discuss the functionalization of NPs with biocompatible molecules in the following section.

2.1. Biodegradable polymers and carbohydrates

Synthetic polymers such as poly (glycolic acid) (PGA), poly (lactic acid) (PLA), dextran, poly (lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polyethyleneimine (PEI) and polyvinyl alcohol (PVA) and natural polymers with modified carbohydrate/polysaccharide building blocks (e.g. chitosan) are used widely [50]. Mono- and oligo-saccharides such as glucose, deoxy-D-glucose, rhamnose, maltose, and lactose, are also used as capping ligands for AuNPs and FeONPs [37,51,52]. General

synthesis strategies involve direct synthesis of the nanoparticles in the presence of the polymers [53], co-precipitation of the preformed nanoparticles with polymers in an appropriate solvent [54] and grafting to/ligand stabilization technique that involves coating nanoparticles via functional groups such as thiol and amine groups to gold nanoparticles surface [55].

Biodegradable polymers are widely used as surface coatings, as they are easy to synthesize, widely studied, allow for precise chemical binding of molecules or can be modified with functional groups to bind other molecules using facile chemistries like EDC-NHS [56] and disulfide conjugations [19]. They have been recognized to increase the circulation time of the nanoparticles by preventing opsonization by phagocytes in vivo. Therefore, a wide range of FDA-approved nanoparticles and in vivo devices are coated with one or more of the above-mentioned polymers [57]. A recent report also suggests that PEG-like polymers may not be as inert as currently believed. Their oxidative degradation in vivo can lead to detrimental effects on the cell membrane and affect signal transduction pathways [58]. Therefore, recent emphasis has been on the use of natural or synthetic biocompatible surface coatings which display minimal adverse effects.

2.2. Lipids and liposomes

Lipid amphiphiles comprised of one acyl chain generally form micelles while those with two acyl chains assemble into bilayer-like membrane vesicles called liposomes. Commonly, lipid formulations yield self-assembled structures that are greater than 100 nm. The first liposomal formulation to be approved by the FDA was Doxil in 1995, subsequently 9 additional liposomal formulations with active ingredients (AIs) have been approved [59]. Single chained lipid amphiphiles such as lysophosphatidylcholine and two-chained DOPC, POPC [60], as well as cholesterol and/or their mixtures have been incorporated into liposomes [3], polymeric liposomes [6] (polymer modified lipid components) and to functionalize inorganic core NPs.

A reverse phase evaporation method that involves exchanging the existing surface ligands with lipids in an organic solvent followed by transfer to an aqueous solvent, is commonly used for lipid membrane assembly on NPs. This technique has been employed in the synthesis of hybrid lipid bilayer coatings on NPs where inner and outer layer have different compositions [6,60]. Another common technique involves adsorption of liposomes [4], on the NPs where the charged head moieties interact with the surface and encapsulate the NPs within liposomes [5]. However, lipid membranes often have low stability in solution due to fusion, leading to increases in the particle size [61]. This can be remedied by increasing the surface charges that promote repulsion between particles or by incorporating spacers such as PEG that sterically hinder particle association. These methods improve colloidal stability.

2.3. Peptides and amino acids

There are innumerable synthetic and naturally occurring peptides composed of different permutations and combinations of amino acids that have been used to coat NPs. In addition to the 20 amino acids encoded by the universal genetic code, other commercially available unique amino acids have been utilized. Depending on the chosen amino acids different functional and structural properties have been observed. With their chemically addressable functional groups, they are easily modified or adducted and they also allow for stoichiometric control of attached targeting/therapeutic molecules for in vivo delivery. Ligand-exchange, direct binding and assembly and covalent binding to functional moieties are the synthesis approaches commonly utilized to functionalize NPs with peptides (Fig. 1C.). Although amines bind to the gold surfaces, the strength of Au–N (~4 kJ/mol) bond is much weaker than the Au–thiol bond (137 kJ/mol) that is commonly used to bind cysteine containing peptides under appropriate conditions [49,55,62].

Cell penetrating peptides like HIV-1 derived TAT peptide [63]

promote the uptake of molecules or complexes that cannot penetrate the cell membrane efficiently by themselves. They are therefore used to co-functionalize the surface of nanoparticles and are widely explored for delivery of nanoparticles in radiation therapy [63], chemotherapy [64], cancer theranostics [65] and other targeted therapies [66,67]. Another class of peptides called self-assembling peptides generally consist of amphiphiles that can assemble on the surface of the nanoparticles to form a membrane called self-assembling monolayers (SAMs) similar to lipids. A library of the self-assembling peptides developed by Chee et al. have phosphate groups that promote binding with iron oxide nanoparticles [68], and have been demonstrated to form stable monolayers on their surface. The self-assembling branched amphiphilic peptides (BAPs) [69,70] form water-filled, bilayer delimited cationic vesicles called BAPCs, like liposomes, and have also been conjugated to AuNPs and FeONPs [49]. These NPs with the peptide bilayer mimic the vesicles and also possess the inherent properties of iron oxide and gold NPs such as electron dense core, magnetism and plasmon resonance, thus making them useful probes for imaging and to study their nano-bio interactions [71].

2.4. Antibodies and proteins

Specific proteins can be used to functionalize NPs for targeted delivery or to serve as binding partners in assays. Abraxane® is an FDA-approved chemotherapeutic drug that consists of nanoparticle albumin bound (nab)-paclitaxel. Albumin is an abundant serum protein used as surface coating for NPs as it improves bioavailability, has low immunogenicity and good biocompatibility [72,73]. Nab-paclitaxel and its variations comprise a major percentage of the protein based nanomedicines in clinical trials (Fig. 1D). This success has fostered the use of albumin as a surface coating for additional NPs delivery systems [74–76]. Antibodies/immunoglobulins are widely used due to their high specificity in detecting and binding to specific antigens and have been successfully employed for disease treatments as antibody drug conjugates (ADCs), four of which are commercially available [77]. Since protein structure defines function, any structural alterations due to temperature transitions or pH, limit the chemistries available for attachment to NPs. General strategies for binding antibodies and proteins to inorganic surfaces therefore include covalent binding to a modified surface [78–80] or by physical adsorption promoted by electrostatic interactions [79]. The orientation of the antibody is more important for its functioning than its coverage on the surface and hence orienting covalent binding strategies are more widely employed [79].

Finetti et al. [80] used “click” chemistry to immobilize anti CD-63 and anti-rabbit-IgG on the surface of AuNPs. Thus, using the benefits of click chemistry, antibodies immobilized NPs can be produced for a wide range of applications. Antibody immobilized AuNPs are also widely used in immunostaining for analysis using electron microscopy, and plasmon resonance mediated confocal imaging [31,81,82]. Antibodies tagged with fluorescence molecule on AuNPs allows for dual imaging, reducing cost and time.

2.5. Nucleic acids/aptamers

NPs are commonly coated with nucleic acids such as DNA, dsRNA, ssRNA, siRNA, mRNA, and microRNA, as they facilitate the delivery of the nucleic acids into cells or for use in binding assays. DNA grafted polymers such as poly (acrylic acid) embedded DNA are also used for functionalizing nanoparticles as they facilitate polyvalent DNA nanostructure formation [83]. A common strategy for functionalizing NPs with nucleic acids is to utilize the electrostatic interactions between the negatively charged nucleic acids and cationic NPs which mediates their adsorption to NPs [84,85]. This does not require extensive modification of the nucleic acids [86]. Recently nucleic acids have also been identified as templates that control and facilitate inorganic NPs synthesis [87]. Aptamers that bind with high affinity and specificity to proteins and

peptides are commonly conjugated to AuNPs and FeONPs for detection of molecules using colorimetric binding assays [88,89] and magnetic isolation [90], respectively.

Table 1 summarizes the varied biomedical applications of FeONPs and AuNPs with the different biocompatible ligands discussed here.

3. Correlation and discrepancies between in vitro and in vivo studies

In vitro studies are often indicators of potential outcomes in animal studies and provide mechanistic information at the cellular level. They allow researchers to explore the effect of different doses, chemicals at relatively lower cost and reduced time. They also allow for probing the underlying mechanisms leading to toxicity, immunogenicity, metabolic changes and analyzing gene expression profiles. These cell culture studies reduce the number and cost of animals required to statistically assess the effect of NPs [91]. NPs on the other hand encounter a very complex environment in vivo which cannot be mimicked accurately in vitro. And therefore, there are obvious discrepancies due to these inherent differences between in vitro and in vivo environments. Khlebtsov et al. [92] have examined the lack of correlation between in vitro and in vivo behavior of NPs. They emphasize on the need for systematization of data obtained from various studies on NPs, to gain a fundamental understanding of factors affecting their bio-interactions.

The inconsistencies observed between their effects in vitro and in vivo is also due to differences in experimental factors [93–97]. For example, one basic consideration is to use the cell lines/primary cell types for in vitro studies that belong to the same species that is being investigated in vivo. Surprisingly, this is overlooked often [93,94,96]. Zhang et al. [98] observed inconsistencies where PEG-AuNPs were cleared quickly from circulation in mice even though in vitro studies demonstrated their reduced uptake by RAW 264.7 mouse macrophages. Review of literature available suggests that there are two main reasons for the discrepancies observed – (i) lack of fundamental understanding of the effect of NP’s properties on biointeractions and (ii) lack of standardization/differences in experimental parameters [92]. Therefore, one must take caution before extrapolating in vitro results to the NPs behavior in vivo. This review focusses on understanding the impact of the experimental factors to aid in setting standards for assessing NPs and thus, help in improving the positive correlation between in vitro and in vivo studies as well as to make fair comparison between studies.

There are hundreds of reports on the toxicity of NPs in vitro ‘or’ in vivo but very few recent studies have compared their effects in vitro ‘and’ in vivo. Table 2 summarizes the studies belonging to latter group.

4. Nano-bio interactions in vitro

Recent reviews by Foroozandeh et al. [100] and Behzadi et al. [101] discussed the effect of nanoparticle physicochemical properties such as size, shape, surface composition on their uptake and intracellular trafficking. Unfortunately, few articles discuss the effect of experimental parameters on cellular uptake. In the following section the importance of carefully selecting cell lines, determining effect of dosage, time and media type in understanding NP interactions will be discussed. We also review recent studies that explore cellular uptake routes, immune responses and toxicity induced by AuNPs and FeONPs with different surface compositions.

4.1. Influence of various experimental parameters

4.1.1. Cells and culturing techniques

Cell lines used to study NPs are commonly selected based on availability; they should be chosen based on the applications of NPs and the expected in vivo exposure [102]. Several studies have shown that nanoparticle uptake and toxicity profiles vary between cell lines, cell sub-types and to some extent between species [102–106]. The uptake of

Table 2

Summary of studies on effects of NPs in vitro 'and' in vivo.

NPs	Surface coating	Cells	Animal model & strain	Route of administration	Conclusion	Ref
AuNPs	Citrate	HepG2 HT29	Wistar rats	Intraperitoneal (i. p.)	No effect was observed on cytokines secretion & other serum contents in vivo but damage to genetic material by smaller NPs, observed in vitro.	Lopez-Chaves [93]
IONPs	PEI & PEG	RAW264.7 SKOV-3	BALB/c	Intravenous (i.v.)	PEI-IONPs >> toxic to cells in vitro & ↑↑ toxicity in vivo causing death of mice at specific dosage Size & surface functionalization have a huge impact on the cellular uptake, toxicity, tissue distribution & clearance in vivo	Feng [99]
AuNPs & Nanorods	Polyallylamine hydrochloride (PAH)	C3H fibroblasts HTC	Wistar rats	Oral	Some adverse effect on liver cells in vitro & oxidative stress and inflammation in vivo. No major adverse effects observed in vivo	Bernardi [96]
AuNPs	PEG	RAW264.7	Swiss mice	i.v.	Dramatically lower uptake of NPs in vitro but fast clearance of NPs in vivo. No correlation between in vitro and in vivo data	Zhang [98]

NPs is also dependent on cell-specific functions [107].

Although immortalized cell lines are easier to maintain, readily accessible and widely studied, they differ from cells in vivo due to repeated in vitro manipulations and the initial immortalization itself [102]. Joris et al. [102] observed that mouse and human neural stem cells clearly showed a more pronounced effect of exposure to FeONPs in terms of toxicity, mitochondrial activity, calcium homeostasis and ROS generation in comparison to neural progenitor and cancer cell lines. The phenotype expressed by cell lines and the processing of NPs differed dramatically from primary cells of similar origin. Therefore, studies in cell lines cannot be considered a final endpoint. No one cell line will emerge as a universal one but, by testing nano-safety in multiple cell types, one increases the power of prediction for in vitro utility and safety.

The reported cell lines have been classified according to cell culture methods- as traditional or non-traditional. Non-traditional cell culturing methods such as 3D cell cultures [103], transwell membrane set-up [108] and sandwich cultured cells [91] are being actively used to better mimic in vivo conditions [109]. Larger NPs which are usually heavier, have a propensity to sediment over time and in a 2D cell culture set up this influences the uptake of NPs to a great extent [103,107,108]. Bancos et al. [108] used a transwell membrane set up for the RAW 264.7 cell line, to study the uptake of 10 nm citrate capped AuNPs and compare it with their uptake in a 2D cell culture and in cell suspension. There was an obvious effect of sedimentation of NPs on the cellular uptake. The cells in the transwell set-up which encountered NPs suspended in media only, incorporated the least number of AuNPs compared to their 2D counterpart and cells in suspension which internalized the highest number of NPs by 24 h. Besides, a transwell set-up facilitates co-culturing of multiple cell types simultaneously, to evaluate the effect of NPs treatment on the crosstalk between the cell types or to study transcytosis [110–112].

Three-D cell cultures that make use of a scaffold increase the surface area of exposure, while only ~50% area is available in a 2D cell culture. The MD1-MB231 breast cancer cells in 2D culture, in comparison to their 3D counterpart, had increased viability and showed a lesser change in the cytoplasmic actin network that plays a major role in intracellular processes [103]. Thus, the toxicity of the NPs could be underestimated by testing their effect in just 2D cell cultures and immortalized cell lines. In vivo, NPs and drugs have a tendency to accumulate in the liver generally, which clears foreign materials and thus, the liver is an important tissue to consider for studying NPs. The sandwich hepatocyte culture model uses primary hepatocytes, grown between two layers of collagen that keeps them competent and polarized with functional bile networks and helps to assess the hepatotoxicity of drugs and NPs accurately [113]. While 3-D cultures mimic the in vivo environment more closely not all labs have transitioned to this approach. Traditional 2D cultures still predominate in the current literature.

4.1.2. Media composition and protein corona

Cell culture media composition varies depending upon the requirements of each cell line. Examples of two commonly used cell culture media are Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI), which vary from the human and mouse plasma in glucose and ion concentrations [114]. Sodium, calcium, bicarbonate, chloride, sulfate and glucose are at significantly higher concentrations in DMEM in comparison to RPMI. Media composition plays a crucial role since the NPs interact with and bind the various medium components including fetal bovine serum (FBS) proteins that help to maintain normal growth and proliferation of cells. The formation of this surface coating, called the biocorona, is dependent upon the physicochemical properties of the NPs and affects their uptake. The protein components of the biocorona have been widely explored but other components such as lipids, nucleotides and ions, are poorly characterized [115]. Biodistribution of NPs in vivo is also affected by the biocorona. With the different compositions of serum in vivo and cell culture medium, there will be differences observed in the uptake of NPs [97,116].

Maiorano et al. [104] studied the biophysical characteristics of 15 nm–80 nm citrate-AuNPs in DMEM and RPMI supplemented with 10% FBS. AuNPs in RPMI showed an abrupt increase in diameter, due to formation of protein corona in 1 h of incubation after which it remained constant up to 100 h. AuNPs in DMEM showed a gradual increase in diameter to 200 nm and then plateaued since the protein corona volume was independent of AuNP size, unlike NPs in RPMI. RPMI increased the interparticle interactions while AuNPs in DMEM were more stable due to the large protein corona which reduces interparticle interactions. Gunnarsson et al. [28] observed a similar effect where AuNPs pre-exposed to protein poor medium had a higher tendency to aggregate than in protein rich medium. Interestingly, 15 nm AuNPs exerted more adverse effects on cells in RPMI in comparison to DMEM. Hence, while designing and implementing studies, we should consider the choice of cell culture media which is crucial [117].

Another non-trivial factor to be considered is the method by which NPs are administered as documented by Moore et al. [107] When poly (vinylpyrrolidone) (PVP) coated AuNPs were administered as a concentrated bolus directly to J774A.1 mouse macrophages, the protein corona formation was 2-fold higher than AuNPs pre-mixed with media. The macrophages also phagocytosed more AuNPs administered as a concentrated dose in comparison to the pre-mixed AuNPs. This study emphasizes how a minor detail such as the initial administration of NPs can affect the outcome of the study. Thus, to be able to compare studies between research groups, we should consider every minor detail and develop a robust analytical method. Due to a lack of standardized/universal methods of testing NPs, it is difficult to compare and obtain a better understanding of NPs bio-interactions.

4.1.3. Dosage and time

The effect of NP concentration and incubation times on cells is difficult to determine based on the many published protocols. Most studies used NPs in the nM to μM range [28] and tested their effect using a single dosage over 24–72 h. Time can be a limiting factor since cells overgrow and lose viability over time. Cells in vivo encounter NPs not as a single high dose but at diluted concentrations for a longer period of time. This can lead to higher cumulative doses. FeONPs bound anticancer agents are being explored to achieve selective accumulation of these agents in tumor which might require them to be administered in multiple doses. Thus, it involves repeated exposure over long periods to the NPs. For example, chemotherapeutic agents like doxorubicin are administered at 60–75 mg/m² dose at regular intervals of 21 days [21, 33–35,91]. Hence, more studies are needed to assess the effect of repeated exposure to NPs at prescribed intervals [91].

Gokduman et al. [91] studied the effect of single or cumulative dose of varying concentrations of SPIONs on hepatocytes over a period of 7 days. Although no significant difference was observed between LD₅₀ for the single or cumulative doses, loss of hepatocyte functions was observed after 48 h in the cumulative treatment. Similarly, Lotsch et al. [118] used 0.1 nM of AuNPs to mimic unintended environmental exposure and assessed the effects of acute versus chronic exposure (up to 2 weeks) to AuNPs on human dermal fibroblasts (HDF) at a genetic level. Proliferation and viability of HDF cells remained unaffected over 14 days but acute exposure to PEG-AuNPs nanorods induced a measurable difference in the gene expression, while the cells developed an adaptive response to the chronic exposure. Exposure levels can vary greatly between clinical applications and environmental exposures. Thus, the type of dosage (acute vs chronic), the level and time of exposure to NPs should not set arbitrarily but by a selective process, keeping the future applications of NPs under consideration, to make a fair and relevant comparison between in vitro and in vivo studies.

Xu et al. [119] conducted a hierarchical cluster analysis of a library

of 21 gold nanoparticles with different physicochemical properties. Unlike experimental studies which demonstrated a strong correlation between individual physicochemical properties and biological effect, the correlation analysis suggested that no such conclusion could be drawn. Rather the crosstalk between various physicochemical factors governs the bio-interactions of NPs. Thus, different experimental factors (Fig. 2.) and a combination of NPs physicochemical properties appear to play a significant role.

4.2. Influence of NPs with different surface compositions on cellular interactions

4.2.1. Toxicity of NPs and their effect on reactive oxygen species (ROS) generation

Cell viability tests are widely used to assess the toxicity of NPs. This typically involves a single dose of NPs followed by short-term evaluations of viability. Whereas, in vivo studies focus on studying the systemic effects and accumulation of NPs. Therefore, there is an apparent disconnect between most in vitro and in vivo studies [120]. Reactive oxygen species produced by cells in response to NPs is a potent early marker for nanoparticle toxicity [94,98,114,115]. Transition metals such as iron (Fe^{+2}) in FeONPs can generate ROS by reacting with hydrogen peroxide (H_2O_2) to form hydroxyl free radical ($\text{OH}\bullet$) through the Fenton reaction [94,116,117]. These can disrupt the mitochondrial activity, cause damage to DNA and lead to lipid peroxidation which destabilizes the cell membrane making it more susceptible to oxidation and may lead to rearrangement of lipid rafts, thus, affecting cell signaling [116–118]. Organs such as the brain can be affected significantly due to their high lipid composition, reduced available antioxidants and high oxygen tensions than other tissues [119]. Oxidative stress exerted by NPs may be inevitable in some cases and can be ameliorated by the naturally occurring antioxidants [94] or by supplementation with antioxidants such as thymoquinone to reduce these effects [121].

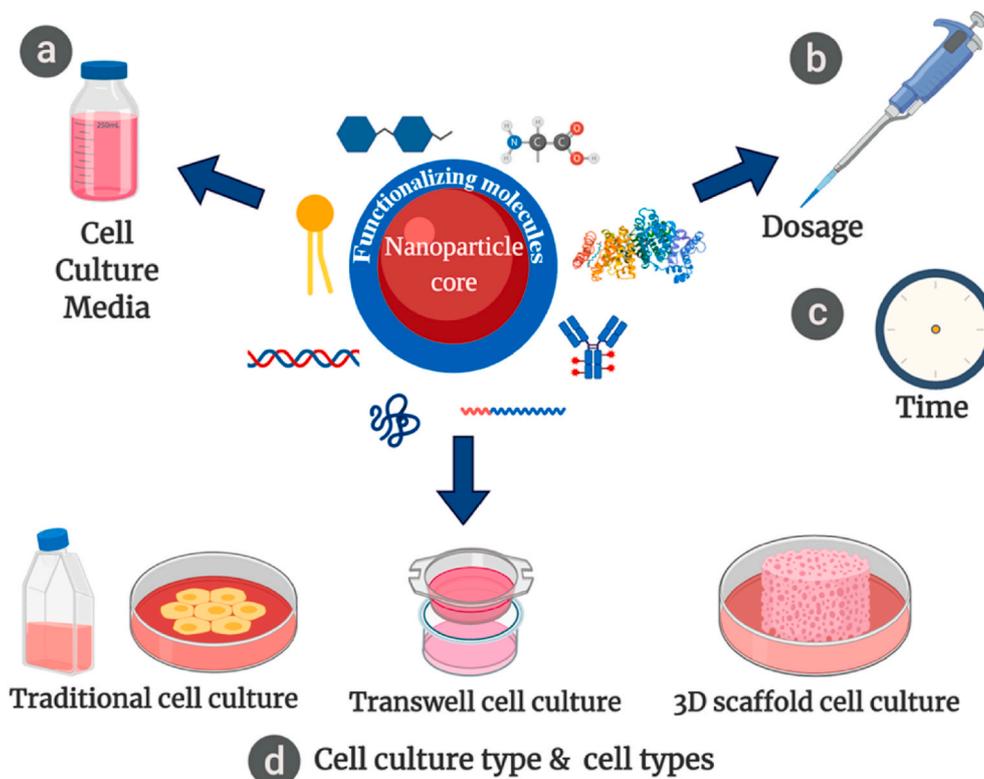


Fig. 2. Experimental factors impacting in vitro studies –(a) Cell culture medium determines the biocorona formation around NPs. (b) Dosage, exposure type and (c) time should be chosen based on intended applications and potential exposure levels. (d) Cell culture methods, cell types and cell origin are the other factors that must be carefully selected to accurately determine the net effect of NPs.

Feng et al. [99] observed that cationic PEI coated FeONPs were endocytosed in high numbers compared to PEG-FeONPs and were more toxic to cells as they dramatically reduced cell viability in a concentration dependent manner. Increased ROS generation that disrupted the cell cycle by arresting cells in G2-phase cell cycle, led to apoptosis. Genotoxicity induced by the PEI-FeONPs was observed to be an indirect effect and not due to direct interaction with the DNA [99]. In contrast 60 nm 'naked' FeONPs intercalated with DNA base pairs in primary lymphocytes and generated high levels of ROS that reduced the cell viability [121]. Micronucleus formation and chromosomal abnormalities were observed in rats, further suggesting the naked FeONPs were genotoxic. Therefore, surface composition can play an important role in preventing excessive cellular damage by influencing the subcellular localization and intracellular processing [122,123].

Different cell types have varied antioxidizing abilities. For instance, macrophages have higher resistance to ROS and thus, high ROS levels do not significantly affect their viability [99]. FeONPs and AuNPs might interfere with colorimetric cell viability assays, due to their strong absorbance, light scattering or plasmon resonance in the visible light range [94,124]. This leads to under or over estimating the viability of cells if suitable controls are not in place. Hence, NPs may seemingly be non-toxic based on the cell viability and ROS analysis but may cause changes to cellular and molecular responses such as but not limited to impaired calcium homeostasis, perturbed mitochondrial activity, morphological changes affecting the functioning of intracellular

pathways, disruption of protein-protein interactions, ER stress induced by unfolded protein response, and differences in genomic profiles which are not detectable by these conventional assays [102,118]. A better analysis beyond just toxic and non-toxic is essential to describe redox effects triggered by NPs. Gokduman et al. [91] also hypothesized based on the ROS generation profile that instead of using absolute values, one should consider the time period at which there are logarithmic increases in ROS, since it will be more sensitive for early detection of NPs induced cytotoxicity. Table 3 summarizes the cellular toxicity induced by NPs with identical or different surface chemistries and how they may differ based on the experimental factors.

The introduction of most types of foreign species warrants a response from a biological system. It is essential that one ascertain the overall effect of NPs, before concluding they are safe to use. Standardization of detection techniques and measuring harmful nano-bio interactions is absolutely essential for the advancement of bionanotechnology [38, 118]. Recognizing this need, the International standards organization (ISO) established a committee in 2005 for standardization in nanotechnology [125]. The FDA also set up a nanotechnology task force to identify approaches to ensure safe use of nanomaterials [126]. Therefore, a continued collaborative effort will hopefully help in advancing nanotechnology in the treatment of diseases.

4.2.2. Cellular uptake mechanism and pathways of NPs

Cells may use an active, energy dependent endocytic pathway or

Table 3
Effect of nanoparticles on cellular toxicity and viability.

NP	Surface coating	Cell line	Media	Dosage	Time of exposure	Changes observed leading to cellular toxicity	Reference
6 nm SPIONs	Citrate	HL60 in suspension MDA-MB231 (2D) MDA-MB231 (3D) bEND 3	RPMI	0–150 µg/mL	24 h	<ul style="list-style-type: none"> Cells in 2D culture less affected than cells in 3D ↑ Surface area of exposure = ↑ toxicity 	Milla et al. [103]
15 nm, 40 nm, 80 nm AuNPs	Citrate	Hela U937	DMEM & RPMI	1 pM - 1 nM	48 h & 96 h	<ul style="list-style-type: none"> Viability of HeLa cells ↓ than U937 AuNPs in RPMI more toxic to cells than AuNPs in DMEM 	Maiorano et al. [104]
10 nm AuNPs	citrate	HUVECs	DMEM	0–64 µg/mL	24 h	<ul style="list-style-type: none"> ↓ cell viability at 5% serum in comparison to 10% serum ↑ uptake in low serum media 	Gunduz et al. [120]
AuNPs	Nanospheres with Citrate & PAA Nanorods with PAA & PEG	Human dermal fibroblasts	DMEM	0.1 nM Acute vs chronic exposure	24 h (Acute) 3, 7, 14 days (Chronic)	<ul style="list-style-type: none"> AuNPs were generally non-toxic Uptake: PAA rods > PAA spheres > citrate spheres ~ PEG rods Gene expression changes observed ↑ in nonchronic exposure Cells developed adaptive response to chronic exposure EG rods ↑ gene expression changes 	Falagan-Lotsch et al. [118]
~4 nm AuNPs ~4 nm SPIONs	polymer PMA	hNSC mNSC ReNcell C17.2 LA-N-2 Neuro-2A	DMEM	0–150 nM	24 h	<ul style="list-style-type: none"> AuNPs were more toxic than FeONPs to all cells Primary stem cells more susceptible to FeONPs induced toxicity Human cells morphology was affected more than mouse cells in general 	Joris et al. [102]
10 nm SPIONs	Proprietary ligands	Primary Rat hepatocytes	DMEM	0–400 µg/mL One high dose vs cumulative dose	24 h & 48 h	<ul style="list-style-type: none"> Cell viability ↓ with ↑ concentrations in single dose vs cumulative treatment Cumulative dosage more deleterious to hepatocyte functioning and metabolic competency ll treatments induced ROS production 	Gokduman et al. [91]
10 nm & 30 nm FeONPs	PEG & PEI	RAW 264.7 SKOV	DMEM	0–400 µg/mL	1, 2, 4, 16 h	<ul style="list-style-type: none"> PEI FeONPs more toxic than PEG FeONPs PEI FeONPs ↑ ROS, Apoptosis PEG FeONPs induced autophagy 	Feng et al. [99]
60 nm FEONPs	Naked	Rat Primary lymphocytes	RPMI	0–800 µg/mL	24 h	<ul style="list-style-type: none"> ↓ cell viability, ↑ ROS, ↑ Genotoxicity 	Ansari et al. [121]
~16 nm FeONPs	DHCA	Human Bone derived mesenchymal stem cells	MEM	0-1000 Fe µg/mL	1, 4, 24 h	<ul style="list-style-type: none"> Moderate effect on cell viability ↑ ROS 	Hachani et al [94].

energy independent passive diffusion to internalize NPs. Table 4 summarizes the uptake pathways used by nanoparticles with different surface chemistries in various cell types. Endocytosis is broadly classified as – Clathrin mediated endocytosis (CME), caveolae mediated endocytosis (CvME), macropinocytosis and clathrin and caveolae independent endocytosis. Phagocytosis is a type of endocytic pathway which is only employed by immune cells such as macrophages, neutrophils and dendritic cells [127].

Cargo is transported intracellularly in endocytic vesicles formed by cell membrane invaginations. Endocytic vesicles can be classified based on the protein markers on the vesicle membrane associated with the endocytic pathway, further influencing the cargo's intracellular sorting (Fig. 4). CME and macropinocytosis promote the fusion of endocytic vesicles with the highly acidic lysosomes (~pH 5) that can cause degradation of the functionalizing ligands and NPs themselves. While the cargo transported in the caveosomes, enter the Golgi and endoplasmic reticulum, bypassing the lysosomes. CvME also favor transcytosis like in the case of Nab-paclitaxel [127–130]. Some oncology and viral medications such as trastuzumab emtansine (T-DMI) [131] and chloroquine [132], respectively, target the endocytosis pathways. Hence, studying the mechanism of uptake is important for the fundamental understanding of nano-bio interactions and drug delivery.

Chemical inhibitors of endocytosis are commonly used to study the endocytic uptake pathways. Some inhibitors may have a generalized inhibitory effect while some are relatively more specific. Methyl- β -cyclodextrin although commonly used as an inhibitor of CvME, it can also inhibit cholesterol dependent clathrin and caveolin independent pathways [133]. Similarly, dynasore may inhibit dynamin independent endocytic pathways as well [134]. Therefore, the chemical inhibitors should be selected wisely and the results should be interpreted appropriately. siRNA mediated knockdown of proteins, essential to specific endocytic routes on the other hand is less ambiguous than chemical inhibitors [135]. In some cases other endocytic pathways may be upregulated to compensate for inhibition of one pathway. Although the net uptake of NPs may seemingly be unaffected, one should not discount changes in the uptake mechanism [71,135,136].

Endocytosis of NPs is time dependent [137]. He et al. [138] observed that although the uptake of cationic CALRRRRRRRR (R8) peptide functionalized AuNPs was slower in comparison to the hydrophobic CALNNPFVYLI (PFV) peptide coated AuNPs, in the initial 1 h, their net uptake was higher at the end of 12 h of incubation. IEC-18 epithelial cells also seemed to use different endocytosis pathways to internalize peptide bilayer coated FeONPs in a time dependent manner [71]. The surface composition plays a crucial role since they may also help in endosomal escape as observed for highly cationic NPs [70,139].

Different cell types may use different endocytic pathways for the uptake of the same NPs [140] and a single cell type may use multiple pathways for the uptake of NPs [71]. Srijampa et al. [141] identified that monocytes and macrophages generally studied for their phagocytic response may also use other endocytosis pathways alongside phagocytosis for NPs uptake. B. End endothelial cells internalized more of the negatively charged FeONPs in comparison to epithelial cells, using CvME, which was enhanced in the endothelial cells since they overexpressed the caveolin-1 protein [142]. R8-AuNPs used energy independent direct translocation alongside CME and macropinocytosis to enter tumor cells [138].

PEG is commonly used to improve the circulation time of NPs but it also prevents their cellular uptake to a large extent. The hydration shell of PEG prevents opsonization of NPs thereby preventing direct interaction with model liposomes used to determine interactions between membrane lipid components and surface ligands. CPP-PEG capped AuNPs on the other hand interacted efficiently with the lipid bilayer inserting themselves into the membrane [138]. The initial interactions or binding of the functionalizing molecules to the cell surface dictate the subsequent internalization events [143]. The NPs may be recognized by receptors which recruit proteins like clathrin, actin, dynamin that direct encapsulation in vesicles for internalization. For instance, glucose coated AuNPs developed to actively target aggressive head and neck tumors for computed tomography (CT) imaging, were endocytosed within 3 min of incubation by tumor cell lines which expressed high levels of GLUT-1 transporter, using CME [130].

Table 4
Endocytosis of nanoparticles.

NP	Surface coating	Cells	Inhibition mediated by	Uptake route	Reference
AuNPs	Glucose	A431 A549 PC3 B16F10 LNCaP 3T3	Chemical inhibitors	GLUT-1 transporter mediated, CME & CvME Lower uptake in GLUT-1 low cell lines via diffusion	Dreifuss et al. [130]
AuNPs	PEG-SH - Cell penetrating peptides (CPP)	A549 B16F10	Chemical inhibitors	CME & Macropinocytosis	He et al. [138]
AuNPs	PEI-PEG + pDNA	HeLa	Chemical inhibitors	CME	Li et al. [144]
AuNPs	His-PIMA-PEG-OCH ₃ /NH ₂ + SVS-1 antimicrobial peptide	HeLa CHO	Temperature	Energy independent, non-endocytic route	Kapur et al. [145]
FeONPs	Siloxane with free –COO groups	Primary human lung cells b.End MDCK	Chemical inhibitors & Temperature	CvME	Sun et al. [142]
SPIONs	silane/silica with free –COO groups	HeLa	siRNA silencing	CvME & CDC42 mediated fluid phase endocytosis	Bohmer et al. [135]
SPIONs	PEG + Folic Acid + Fluorophores	HeLa MCF-7 MDA-MB435	Chemical inhibitors	CME	Vannier et al. [146]
SPIONs	Sienna + (Trademarked)	A549 MDA HeLa THP-1	siRNA silencing	CME & ↓ CvME CvME & Macropinocytosis	Guggenheim et al. [140]
AuNPs	citrate	HUVECs	Chemical inhibitors	CME, Macropinocytosis & Phagocytosis	Gunduz et al. [120]
SPIONs	Branched amphiphilic peptide bilayer coated magnetic nanobeads (BAPc-MNBs)	IEC-18	Chemical inhibitors	Macropinocytosis CvME, CME, Macropinocytosis (1 h) Clathrin caveolae independent endocytosis (4 h)	Natarajan et al. [71]

4.2.3. Immune responses to NPs

NPs can elicit an immune response by interfering and interacting with intracellular signaling pathways directly or indirectly via the reactive oxygen and nitrogen species produced. For example, the transition metals on the surface of NPs or in SPIONs generate ROS as described previously, which triggers a pro-inflammatory response [147, 148]. Cytokines and chemokines are used as indicators of an immune response since they are immune cell secretions or they are secreted by other cells to attract immune cells, in response to invading pathogens or foreign substances [149]. NPs may also cause changes in the conformation or cause unfolding of proteins binding them and trigger the immune system to react to the altered-self molecules [150].

NPs with the same core composition and size but different surface coatings can elicit different immune responses [151]. Anionic hydrophilic ligand coated AuNPs did not affect LPS stimulated J774A.2 and RAW 264.7 macrophages, while hydrophobic ligands and tetraethylene glycol coated AuNPs elicited an anti-inflammatory response. PEI-SPIONs interacted with the TLR4 receptor on macrophages and activated them, while negatively charged dextran and DMSA coated SPIONs had negligible effects. This indicates that surface charge may have influenced the observed immune response. [152]. Table 5 summarizes studies on the immunogenicity of NPs with varied surface compositions.

The immunogenicity of the NPs may also be used advantageously, as in tumor therapy and vaccine development. [110,153,154] FDA-approved Ferumoxytol is an iron supplement used commonly to treat chronic kidney disease and the formulation contains SPIONs coated with polyglucose sorbitol carboxymethyl ether. Zanganeh et al. [110] demonstrated that cancer cells treated with Ferumoxytol attracted M1 macrophages. An mRNA transcriptome analysis confirmed M1 related TNF- α and CD86 overexpression along with reduction in anti-inflammatory M2 related CD206 and IL-10 expression. The pro-inflammatory response was beneficial as they prevented tumor growth by inducing tumor cell cytotoxicity, mediated by an increase in caspase-3 activity in cancer cells and a 16-fold increase in hydroxyl (\cdot OH) radicals. [110].

AuNPs have been explored as adjuvants by Dykman et al. [154] and Niikura et al. [153] The average antibody titers in response to BSA and the bacterial CpG antigens increased substantially when they were coupled to 15 and 50 nm AuNPs, in combination or individually. [154] Niikura et al. also used AuNPs as adjuvants and coated them with West Nile virus envelope (WNVE) protein. They observed increased anti-WNVE titers and inflammatory cytokine production by bone-marrow derived dendritic cells, when treated with WNVE-AuNPs. Thus, NPs are good immune potentiators and may serve as two-in-one adjuvant and vaccine delivery platforms. All in all, understanding the effect of NPs on immune response can also help us determine their

potential applications and how to counter their effects.

5. Nano – biointeractions in vivo

The NPs bio-interactions in the complex in vivo environment are dependent on their physicochemical properties, contributing to their translocation to the different organs and tissues and ultimate clearance. [97,156] Therefore, it is vital to discern the relationship between the NPs and the interactions with endogenous molecules that influence their biodistribution. In this section, the effects are discussed relative to different administration routes on tissue distribution, their systemic toxicity profiles and the immune responses generated in vivo. The in vivo studies reviewed here are limited to animals of the *Mus* and *Rattus* genus.

5.1. Effect of route of administration on biodistribution of NPs

NPs can be administered via different routes, namely-intravenous (i. v.), intramuscular (i.m.), transdermal (across the skin), subcutaneous (under the skin), intradermal (into skin), epicutaneous (on the skin), intratumoral, intraperitoneal (i.p.), intracerebral and oral delivery. The route of administration has an obvious role to play on the tissue distribution which is generally chosen based on the end application of NPs. However, i. v. injections are used more commonly since they can provide a near instantaneous response and is suitable for delivery of materials that cannot be absorbed efficiently or that can undergo proteolytic or pH disruption. Another major advantage of i. v. injections is the increased bioavailability of drugs. [157] The animal model selected for a particular study may influence the administration route. [158] Intramuscular delivery in mice is generally not recommended as their muscles are small, making it difficult to get reproducible results. [159] The genetic background of animals will also show variations in NPs interactions due to differences in their response to foreign molecules. The C57BL/6 and the BALB/c mice, for example, fundamentally exhibit different immune responses that could affect their adaptive immunity. C57Bl/6 and BALB/c are prototypical, Th1 and Th2 type mouse strains, respectively, and therefore can have an altered response to NPs. [160]

When NPs are administered, they have to cross various hurdles before they reach the target. They have to overcome primary defense barriers such as the gastrointestinal, circulation barriers and skin barriers depending upon the route of administration. [161] A significant percentage of administered NPs reach the tissue but they have other hurdles to overcome, such as being sequestered by resident macrophages of the mononuclear phagocytic system (MPS) like Kupffer cells in liver, macrophages in the marginal zone or the red-pulp region of the spleen and alveolar macrophages in lungs. [99,156,162–164] Larger

Table 5
Immunogenicity of nanoparticles.

NP	Surface coating	Cells/Cell line	Changes in secreted cytokines, chemokines & iNOS	Type of overall response	References
FeONPs	Polyglucose sorbitol carboxymethyl ether	RAW 264.7	TNF- α \uparrow , IL-10 \downarrow , iNOS \uparrow	Pro-inflammatory, M1 macrophages polarization	Zanganeh et al. [110]
FeONPs	Ovalbumin	RAW 264.7 DC2.4	TNF- \uparrow , IL-6 \uparrow , IFN- γ \uparrow	Pro-inflammatory, NPs behave as adjuvants	Zhao et al. [155]
SPIONs	PEI- stabilized with: Zonyl-FSA Surfactant Pluronic-F127 surfactant	SVEC	TNF- α \uparrow , IL-6 \uparrow , IL-23 \uparrow , CCL1 \uparrow , CCL4 \uparrow , CCL5 \uparrow , TGF- β \downarrow , iNOS \uparrow	Pro-inflammatory, reduced cell migration	Mulens-Arias et al. [152]
		HUVECs	CCL2 \uparrow , CCL5 \uparrow , CXCL12 \uparrow , IL-23 A & TNF- α levels unchanged, slight \uparrow TGF- β 1 & VEGFA	Pro-inflammatory, reduced angiogenesis	
		THP-1	IL-1 β \uparrow , IL-6 \uparrow , TNF- α \uparrow , CCL2 \uparrow , IL-12 \downarrow	Pro-inflammatory & altered M2 macrophages function	
AuNPs	Hydrophilic zwitterionic polymer (ZDiMe)	J774.2 & RAW 264.7	TNF- α unchanged	Neutral response	Moyano et al. [151]
	Hydrophobic zwitterionic polymer (ZDiPen)		TNF- α \downarrow	Anti-inflammatory	
	Tetraethylene glycol modified		TNF- α \downarrow	Anti-inflammatory	

NPs are typically metabolized in the liver and secreted into bile for excretion, while smaller NPs may be filtered out through the kidneys. [165–167] PEI and PEG coated SPIONs when injected i. v. into SKOV-3 tumor bearing mice were cleared from circulation within 24 h of injection and accumulated primarily in liver, spleen and tumor with trace amounts found in lungs, heart and kidneys. PEI-FeONPs accumulated the least in tumors but had increased uptake in the kidneys, suggesting faster clearance from the body. [99] Faster clearance from circulation causes reduced accumulation in the tumor, as also noted by Bailly et al. [168].

Meta-analysis conducted demonstrated that only 0.7% of total administered NPs reach tumor. Removal of Kupffer cells increased the uptake of NPs to only 2% from 0.7% in tumors. Therefore, although sequestration by macrophages in the liver affects the bioavailability of NPs, one needs to look at other organs and their effect on delivery of NPs to disease sites such as tumor. [164] Thus, sequestration of NPs can affect their ability to deliver therapeutics to target cells. A research group at the FDA was interested in understanding the effect of repeated doses of 10 nm AuNPs on the MPS and their importance in clearance of the NPs. [162] They hypothesized that chronic exposure to NPs would lead to saturation of the MPS system and, thus, lead to unforeseen toxicity or changes. However, 8 weeks of chronic exposure to 10 mg/kg AuNPs in BALB/c mice did not establish a steady state in the MPS i.e. they were not saturated. AuNPs accumulated the most in the liver, followed by spleen, causing tissue discoloration. The carcass showed high amounts of AuNPs due to possible accumulation in lymph nodes.

There is a complex interplay between size [169], charge [156], functionalizing molecules [97] and composition of the metallic core [170] that affects the tissue distribution and indirectly their use as imaging agents. [169] Sharma et al. [156] observed that cationic FeONPs accumulated mainly in lungs while the same size anionic FeONPs functionalized with carboxymethyl dextran, accumulated in the spleen and liver. The ligand density on NPs also have an effect as discerned by Xue et al. [171] Intravenously injected 15 and 22 nm FeONPs with 2 kDa or 5 kDa PEG (i.e. different densities of PEG coating) showed obvious differences in tissue distribution. Although the lower MW PEGs covering NPs were cleared faster from blood, they persisted the longest in liver and spleen.

There has been an increase in studies exploring intradermal delivery using microneedles as it is minimally invasive. [172] Dur et al. [173] delivered proinsulin peptide using glucose, mannose and GSH functionalized AuNPs to generate immune tolerance and prevent or delay onset of type 1 diabetes. They used intradermal delivery with the aim of delivering them to antigen presenting Langerhans cells in the skin, thus, generating an appropriate response from T-cells. The 5 nm AuNPs were distributed through the reticular dermis to the basement membrane zone and in keratinocytes, Langerhans cells and dermal cells, within 4 h but the colloidal 50 nm AuNPs were retained in the dermis. Repeated subcutaneous injections of similarly sized (~13 nm) iron oxide–zinc core-shell NPs delivering tumor antigens into dendritic cells, led to the accumulation of the NPs at the injection site and not in other tissues. Thus, the NPs were not effectively distributed and therefore required further modifications to prevent accumulation at the injection site. [174] Hence, we see that the interplay between various factors including the administration method affect the successful delivery of the NPs and the outcome of NPs mediated drug delivery.

5.2. Systemic toxicity and immune response to NPs

In vitro studies can guide one in explaining the effects of NPs in vivo. For instance, ROS and RNS generated in response to NPs activates the cells and induces secretion of cytokines/chemokines (Fig. 4). This leads further to the infiltration of immune cells, which may cause tissue necrosis or induce apoptosis of cells causing organ damage. Thus, the immune response to the NPs can lead to a cascade of events that induces toxicity. In vivo, toxicity is determined by assessing ultrastructural

changes in the tissues (Fig. 3), comparing cytokine levels and other molecular markers in serum and analyzing blood cell counts (hematology) [171], which may be direct or indirect indicators of tissue damage and systemic toxicity.

Sharma et al. [156] observed infiltration of cells in the lungs of PEG-PEI FeONPs treated mice. Sub-chronic exposure to AuNPs caused edema in alveolar septa of lungs, enlargement of kidney corpuscles, infiltration of Kupffer cells in liver sinusoids and mild hyperplasia in spleen. [175] Mast cells infiltration in organs in response to coated and uncoated SPIONs, alongside macrophages was reported for the first time by Sabareeswaran et al. [163] Mast cells are actively involved in the secretion of active molecules that induce inflammation, necrosis and even allergic reactions. Hematoxylin and eosin (H&E) staining and TEM/SEM analysis are commonly used and allow visualization of tissue sections. The former provides information on morphological and ultra-structural changes, accumulation of NPs in specific types of cells in tissues while the sub-cellular localization and changes are generally assessed using the latter technique. [93] NPs induced cellular toxicity may lead to changes in cell surface area and morphology (shape) due to disruption of actin, increased lipid droplets in cytoplasm, granular cytoplasm and disrupted endosomal membrane. [93,156,163] (Fig. 3) Sequestration of NPs by antigen presenting cells such as macrophages although limits their biodistribution, they may have an added advantage in applications such as vaccine delivery. Uptake of NPs by antigen presenting cells in major immune potentiating sites such as lymph nodes and spleen can induce an enhanced immune response to the antigens. [176] Therefore, certain NPs can be developed for specific applications, but only with a fundamental understanding of the nano-bio interactions.

NPs that get past the MPS are internalized by other specialized cells in the tissues such as hepatocytes of epithelial origin and they may also accumulate in the extracellular/interstitial spaces in tissues. [156] Damage to the hepatic tissue is marked by an increase in serum alanine amino transferase (ALT) enzyme, while elevated aspartate amino transferase (AST) can be indicative of both cardiac function impairment and liver damage. [177] Other commonly used pharmacological indicators of implied toxicity are weight loss and serum levels of creatinine, bilirubin, blood urea nitrogen (BUN) and lactate dehydrogenase (LDH). PEG coated FeONPs caused increased metabolic stress in the liver leading to increase in serum ALT and AST. [171] Five nm PBS stabilized AuNPs injected i. v. caused increase in ALT and AST levels in BALB/c mice [178] while 20 nm dextran coated AuNPs did not affect ALT and AST levels 14 days post injection. [168] PEI-FeONPs at 5 and 2.5 mg/kg doses were highly toxic to mice leading to death, but a dose of 1.5 mg/kg was well tolerated. [99] Sharma et al. [156] observed a similar effect of PEG-PEI FeONPs where 2 mg/kg dose was highly toxic to mice. Thus, NPs with different physicochemical properties exhibit different levels of toxicity and have varied effects in vivo. Table 6 summarizes the bio-distribution and systemic effects of NPs with different surface compositions.

6. Conclusion

Nanoparticles developed over the past two decades are used widely in biological applications. Therefore, it has become essential to study the nano-bio interactions and understand the impact of exposure to nanoparticles. If designed optimally, in vitro studies can give a wealth of information that can be well correlated to the in vivo effects of nanoparticles, which can further help improve the success of nanomedicines. Thus, there is an increasing need for standardization in the field of bionanotechnology. Besides, understanding the limitations of the characterization techniques and cellular assays for nanomaterial assessment, identifying supplementary techniques and assays for verifying NPs bio-interactions and reasoning the use of specific experimental parameters will help achieve the goal of replacement, reduction and refinement. Most successful nanomedicines that have been approved for commercial

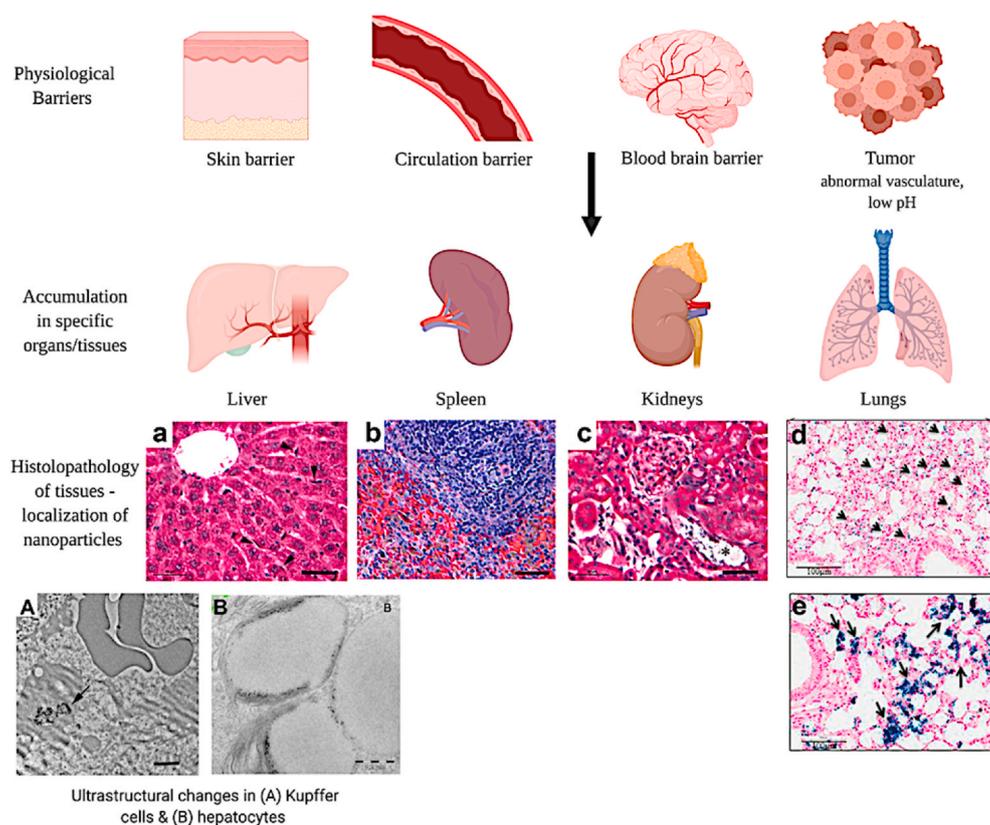


Fig. 3. Localization of NPs in tissues and the physiological barriers to their uptake – Biodistribution of NPs in organs/tissues is influenced by their ability to cross the various physiological barriers. Sequestration by macrophages of the mononuclear phagocytic system (MPS) causes faster clearance of NPs from circulation and they accumulate mainly in organs such as liver and spleen. Histopathological & electron microscopy analyses provide information on the localization of NPs within tissues i.e. accumulation of NPs in specific cells, extracellular or in interstitial spaces as well as sub-cellular localization, morphological changes and damage to tissues and cells. H & E staining of NPs treated - (a) Liver showing FeONPs localized in Kupffer cells, (b) Spleen with increased macrophages in the red-pulp area, (c) Kidneys showing slight degeneration of tubular epithelial cells cytoplasm, (d) and (e) Lungs with NPs localized in the alveolar epithelium and interstitial spaces, respectively. Electron micrographs show accumulation of NPs in the (A) cytoplasm of Kupffer cells and (B) lipid droplets of the hepatocytes.

Adapted and reprinted (a), (b), (c) from *Nanomedicine-Nanotechnology Biology and Medicine*. 12 (6), Sabareeswaran, A.; Ansar, E.; Varma, P.; Mohanan, P.; Kumary, T., Effect of surface-modified superparamagnetic iron oxide nanoparticles (SPIONS) on mast cell infiltration: An acute in vivo study, with permission from Elsevier. © (2016) (d), (e) *Scientific Reports*, Sharma, A.; Cornejo, C.; Mihalic, J.; Geyh, A.; Bordelon, D.; Korangath, P.; Westphal, F.; Gruettner, C.; Ivkov, R., Physical characterization and in vivo organ distribution of coated iron oxide nanoparticles. © (2018) Springer Nature. (A) & (B) . *Nanomedicine-Nanotechnology Biology and Medicine*, 14 (1), Lopez-Chaves, C.; Soto-Alvaredo, J.; Montes-Bayon, M.; Bettmer, J.; Llopis, J.; Sanchez-Gonzalez, C., Gold nanoparticles: Distribution, bioaccumulation and toxicity. In vitro and in vivo studies, with permission from Elsevier. © (2018).

use are relatively simple, well-described systems that have been widely studied. Given the volume of information available on different types of NPs with different physicochemical properties, a concerted effort by researchers such as depositing information on and creating a database of NPs will enable the analysis of the complex data using various computational tools available. Deciphering the complex nano-bio interactions and understanding the crosstalk between various biophysicochemical properties of nanoparticles is essential for the growth of nanobiotechnology.

6.1. Definitions

1. Nanotechnology – The strictest definition of nanotechnology by the National Nanotechnology Initiative (NNI) and Environmental Protection Agency (EPA) refers to structures roughly in the 1 nm–100 nm size regime in at least one dimension. Despite this definition, nanotechnology refers to sub-micron i.e. up to hundreds of nanometer sized assemblies synthesized by bottom-up or top-down approaches [180].

2. Nanoparticles - Nanoparticles refers to nanomaterials in the sub-micron range for the purpose of this review.
3. Nanomedicine - Therapeutic or imaging agents that use nanoparticles to control the biodistribution, enhance the efficacy, or otherwise reduce toxicity of an active agent/drug or biologic [22].
4. Ligands and functionalizing molecules – Molecules of different chemical and biological origin used to cap/coat nanoparticles for improving their stability or providing added functionalities.
5. LD₅₀ – Median lethal dose which measures the dose that kills half the members of a population over a specified period of time.
6. Nano-bio interactions – The interaction between nanoscale entities and biological systems/molecules.
7. Non-traditional cell culture techniques – Cell culture techniques that do not use the conventional two-dimensional adherent cell culture or non-adherent, cells in suspension method, are defined in this review as non-traditional cell culture techniques.
8. Standardization – Establishing and implementing a set of standards based upon general consensus by authorities with expertise in the field.

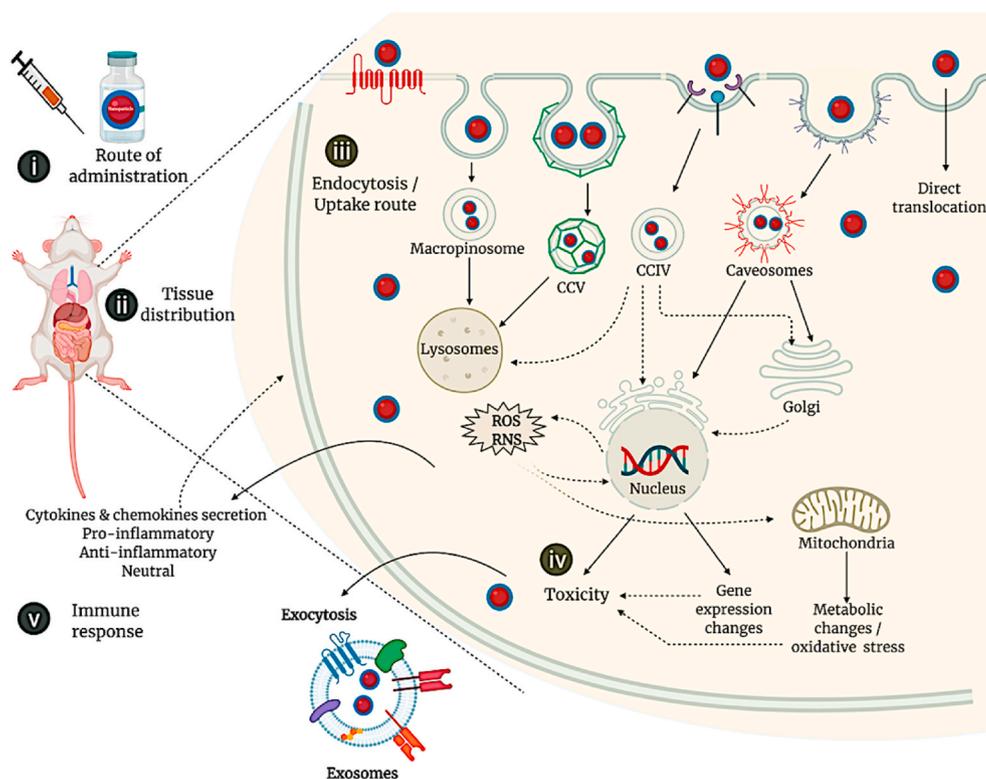


Fig. 4. Bio-interactions of nanoparticles – Nanoparticles can be administered via different routes (i) which shapes their tissue distribution (ii). The cellular interactions of the NPs ultimately affect their fate in vivo. The physicochemical properties of the NPs impact the uptake mechanism (iii) which consecutively determines their intracellular fate. The NPs are sorted into different compartments based on the endocytic route. The NPs can affect the gene expression by directly interacting with the DNA or indirectly due the reactive oxygen species (ROS) generated. This may also lead to metabolic changes. Reactive nitrate species (RNS) produced mainly by immune cells such as macrophages and neutrophils along with the ROS are considered to be indicators of cellular activation. Altogether, the intracellular changes may cause cellular toxicity (iv) and cause an immune response by inducing changes in cytokine and chemokines secretion (v). NPs can also be exocytosed in vesicles called exosomes which may be inherently targeted to different tissues. Therefore, a cascade of events determines the bioavailability, clearance, toxicity profile and thus, the net effect of NPs. CCV-Clathrin coated vesicles; CCIV- Clathrin caveolin independent vesicles.

Table 6
Biodistribution, systemic toxicity and immune response to nanoparticles in vivo.

NPs	Surface coating	Animal model & strain	Route	Biodistribution	Toxicity and/or immune response		Ref
					Biochemical	Histopathological changes	
FeONPs	PEG & PEI	BALB/c	i.v.	Liver, spleen > lungs, heart, kidney	• ALT↑ (PEG), LDH ↑ (PEI)	• ↑ macrophages in hepatic portal area	Feng [99]
FeONPs	PEG-PEI, Carboxymethyl-dextran	Nude mice	i.p.	Spleen, liver (CM-dextran), Lungs (PEG-PEI)	• Death at 2 mg/kg PEG-PEI FeONPs dosage	• PEG-PEI in lung epithelial cells & interstitial spaces but not in macrophages	Sharma [156]
FeONPs	PAMAM dendrimer	BALB/c	i.p.	kidney, liver, lungs > tumor	• ↑ BUN, hyperglycemia, • ↑LDH, ↑ Bilirubin	• Edema & loss of cytoplasm in liver • Apoptosis in cardiac tissue	Salimi [179]
AuNPs	PBS stabilized (proprietary)	BALB/c	i.v.	liver, spleen > lung, kidney > brain, heart	• Slight ↑ ALT, AST	• Slight liver hemorrhage	Xia [178]
AuNPs	Citrate	BALB/c	i.v.	Liver > spleen > lung > sternum > kidney > skin, heart, uterus, muscle, blood, brain		• Discolored spleen, liver due to increased accumulation	Weaver [162]
AuNPs	Dextran	nude mice	i.v.	Liver > spleen >> tumor, lung, heart, brain	• No apparent acute/chronic toxicity	• Most NPs in Kupffer cells • No hepatological changes or inflammation in kidney, spleen, heart	Bailey [168]
AuNPs	Citrate	Wistar rats	i.p.	Spleen, liver, kidney, intestines	• ↑ lipid peroxidation & carbonylation • No changes in TNF-α, IL-1β, IL-6 and IL-10	• ↑ lipid droplets in hepatocytes	Lopez-Chaves [93]

9. Liposomes – Amphiphilic bi-tailed lipid molecules like diacylglycerols self-assemble to form bilayer membrane delimited spherical vesicles, generally referred to as liposomes.
10. Micelles – Amphiphilic molecules may aggregate in water to form spherical vesicles called micelles.
11. Click chemistry – Group of reactions that are quick, simple, easy to purify, versatile and provide high yields is referred to as click chemistry.
12. Biocorona – Layer/s or coating of biological molecules such as proteins, lipids, nucleic acids, nucleotides and such formed on the surface of nanoparticles is referred to as the biocorona.

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Author contributions

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