Toxicological assessment of nanoparticle interactions with the pulmonary system

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ABSTRACT

Nanoparticle(NP)-based materials have breakthrough applications in many fields of life, such as in engineering, communications and textiles industries; food and bioenvironmental applications; medicines and cosmetics, etc. Biomedical applications of NPs are very active areas of research with successful translation to pharmaceutical and clinical uses overcoming both pharmaceutical and clinical challenges. Although the attractiveness and enhanced applications of these NPs stem from their exceptional properties at the nanoscale size, i.e. 1–1000 nm, they exhibit completely different physicochemical profiles and, subsequently, toxicological profiles from their parent bulk materials. Hence, the clinical evaluation and toxicological assessment of NPs interactions within biological systems are continuously evolving to ensure their safety at the nanoscale. The pulmonary system is one of the primary routes of exposure to airborne NPs either intentionally, via aerosolized nanomedicines targeting pulmonary pathologies such as cancer or asthma, or unintentionally, via natural NPs and anthropogenic (man-made) NPs. This review presents the state-of-the-art, contemporary challenges, and knowledge gaps in the toxicological assessment of NPs interactions with the pulmonary system. It highlights the main mechanisms of NP toxicity, factors influencing their toxicity, the different toxicological assessment methods and their drawbacks, and the recent NP regulatory guidelines based on literature collected from the research pool of NPs interactions with lung cell lines, *in vivo* inhalation studies, and clinical trials.

Keywords: Pulmonary nanotoxicology ; nanoparticles toxicity ; inhalation of nanoparticles ; pulmonary delivery ; aerosolized drug delivery

1. Introduction

NPs are fueling the development of a novel class of medicines retaining engineered NPs for various theranostic applications such as analytical nano-devices, novel nanotherapeutics, drug delivery and targeting nanocarriers, tissue engineering, clinical, and toxicological applications, and all these applications are under the umbrella of *Nanomedicine*.

Thanks to their nanosize, NPs can easily penetrate the cellular barriers and migrate to the site of action and cross different types of biological barriers (Samaridou and Alonso 2018). In addition, NPs can enhance pharmaceutical properties such as drug stability, dissolution rate, and bioavailability; especially important for poorly soluble and hydrophobic drugs (Merisko-Liversidge and Liversidge 2008). NPs have very versatile capacities to encapsulate different types of molecules; not only drugs, but also macromolecules (Depreter, Pilcer, and Amighi 2013; Tawfeek et al. 2013; Gaggar et al. 2016), biopharmaceuticals (Kunda et al. 2015; Chauhan and Sood 2016), nucleic acids and gene therapeutics (Rudolph et al. 2005; Babincova and Babinec 2006; Kim et al. 2014; Yan et al. 2017). They efficiently

allow for multidrug or combinational therapy targeting (Wang et al. 2016; Costa-Gouveia et al. 2017; Zhang et al. 2017; Yan et al. 2018) achieving synergistic, or multi-targeting, or theranostic applications. Functionalized NPs can deliver the active substance intracellularly (Medina et al. 2009 Sangtani et al. 2018). Furthermore, NPs could be modulated with mechanisms to target only the diseased tissue or cells, either passively, through the enhanced permeability and retention effect (EPR) (increased local blood supply tends to pool the NPs as in case of tumor sites), or actively, through targeting molecules like antibodies (here the tumor cells selectively intake the NPs) (Zimmer 2002; Zamboni et al. 2012). In addition, NPs can be equipped with mechanisms to allow the control of drug release, i.e. sustained or slow release, pulsatile or stimuli-responsive release (Kalaydina et al. 2018; Tan et al. 2018; Zhou, Wang, and Li 2018. The release profile from these carriers can be tuned by enhancing their physicochemical proper-ties. These mechanisms would boost the drug bioavailability at the site of action, bypassing the hepatic metabolism, lowering the off-target systemic side-effects and improving the therapeutic efficacy, patient compliance, and health outcomes. NPs delivered systemically showed better circulatory distribution profiles and less aggregations compared to microparticles (De Jong and Borm 2008; Mansour, Rhee, and Wu 2009; Kalaydina et al. 2018).

NP fabrication based on natural or synthetic materials provide tissue safety and biocompatibility. Recently, biodegradable polymers appeared very attractive for pharmaceutical applications, fueling the development of drug delivery systems due to their biocompatibility, biodegradability, and ease of fabrication and functionalization. More details about the different nanocarriers, their formulation processes and different excipients used to formulate NPs for lung delivery could be found in these sources (Pilcer and Amighi 2010; Vilar, Tulla-Puche, and Albericio 2012; Iyer, Hsia, and Nguyen 2015; Gaul et al. 2018; Chavda 2019). Although nanomedicines are designed to enhance the drug efficacy and reduce its toxicity, potential risks and unique challenges may occur due to the exceptional properties of their engineered nanomaterials. This has lead to the development of a new branch of science, known as *Nanotoxicology,* to understand, determine, and regulate the main factors underlying the toxicological concerns of nanomaterials (Donaldson et al. 2004; Lombardo, Kiselev, and Caccamo 2019).

With the increased application of nanomaterials in various fields of life, human exposure to NPs is raising many concerns. Due to their novel properties, there is a knowledge gap, still to be fulfilled, about their dynamics in the environment and in biological systems, and the safety of exposure in both cases. The pulmonary system is a biological system that is a primary route for NPs exposure, as airborne or aerosolized (particles either liquid or solid that can be suspended in air). The pulmonary system is actively targeted with pharmaceutical NP aerosols to treat either local or systemic pathologies. However, unintentional exposure to NPs in environmental and occupational settings, as well as from NP-based products, has been reported in various research studies and regulatory reports (Steinhäuser and Sayre 2017).

There are many official, and non-official, regulatory bodies to control the industrial and pharmaceutical use of nanomaterials. The challenges here are the lack of standardization specifically addressing the NPs. Details regarding these regulatory bodies can be found in these sources (Abdolahpur Monikh et al. 2018; Coty and Vauthier 2018; Lamon et al. 2019).

NPs testing is commonly achieved via two complementary methods prior to human clinical trials, namely *in vitro* and *in vivo* approaches. The *in vitro* approaches use different cell lines and expose them to a range of NPs concentrations, detecting various endpoints and mechanisms. The *in vitro* approaches provide initial screens, are less ethically problematic, less costly, and offer mechanistic insight to understand the NP interaction with the target cells. Although many researchers argue that the static nature of this exposure lacks the *in vivo* dynamics of NP exposure, many examples of successful correlations have proven that *in vitro* approaches can translate to *in vivo* conditions and, yet, there is capacity for improvement. Promising approaches are being developed and validated to increase the sensitivity and the *in vivo* translation of the *in vitro* models, such as *ex vivo models*, dynamic organoids or miniaturized body-on-chip systems, and *in silico* models (Myatt et al. 2018; Oberdorster and Kuhlbusch 2018; Rothen-Rutishauser, Bourquin, and Petri-Fink 2019).

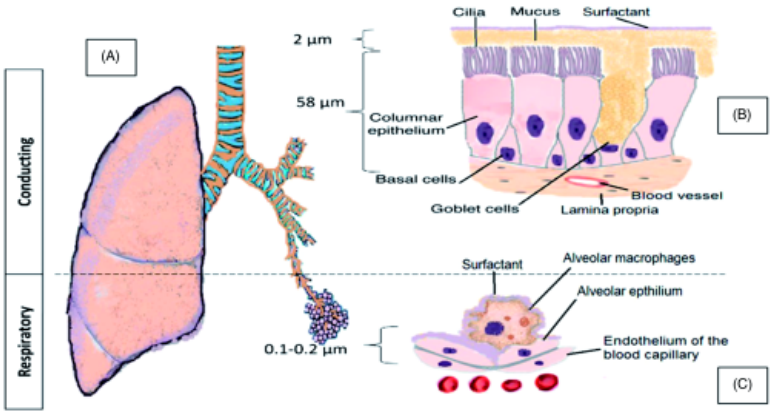
2. Pulmonary route for nanoparticle drug delivery

Respiratory diseases occupy the top four main causes associated with global mortality; chronic obstructive pulmonary disease (COPD), lower respiratory tract infections, lung cancer, and Tuberculosis (WHO). Together they represent the cause of death in one-sixth of global deaths, which is expected to increase to one fifth in the next few years.

Respiratory morbidity accounts for one-tenth of disability-adjusted life-years (DALYs), which is a universal metric used to measure the loss of the productive years in the life of the affected persons. According to the European Lung White Book, (2013), respiratory diseases are responsible for 66% of a million deaths, and more than 6 million cases of hospitalizations accounting for more than forty million days of bed-redden patients each year in Europe (Society 2013). The research pools are actively trying to tackle the respiratory problems with novel approaches, i.e. NP applications, intensifying its abilities for local and systemic drug delivery purposes based on its immense health implications and the unique advantages that can be offered.

The respiratory system is a complex vital organ that has two structurally different regions; conducting (upper) and respiratory (lower) part (Figure 1(A)) (Stocks and Hislop 2002; Osman et al. 2018). The conducting airways start from the mouth/nose, comprising the trachea and extending to approximately 17 branching times until reaching the respiratory bronchioles with progressive narrowing. The lining epithelium is pseudo-stratified ciliated epithelium with tight junctions, with abundant mucous glands, which secret mucous that is responsible for the air filtration, humidity, and acts with the motile cilia to provide mucociliary clearance/escalator (Figure 1(B)). The epithelial thickness is approximately 60 µm lined with a thick mucus layer with a cover layer of a lung surfactant (Kunda et al. 2013). Diseases affecting the conducting airways, i.e. asthma, CF, COPD, impair the respiratory functions by developing pulmonary hypertension and aggravating the bronchoconstriction and congruently, impairing the efficiency of NPs drug delivery to the lungs or the drug absorption. The respiratory airways are distal to the terminal bronchioles consisting of the respiratory bronchioles and alveolar ducts ending in alveolar sacs (18-25 generations). The lining epithelium consists of two main cell types (Figure 1(C)): Alveolar type I, which is the main cell, involved in the alveolar air-blood barrier, and the alveolar cell type II, which is responsible for secreting lung surfactant. It has a plethora of immune cells rich in wandering macrophages that are responsible for macrophage clearance eliminating particle mechanisms (Gordon and Read 2002 Byrne et al. 2015). The lining epithelium is very thin, 0.1-0.2 µm, with a fluid lining thickness of 70 nm. The alveolar epithelium has tight and gap junctions and shows high permeabil-ity. Diseases affecting the respiratory region are very common debilitating conditions, such as infections, tuberculo-sis, emphysema, lung fibrosis, lung cancer, acute distress and pulmonary edema. These conditions might limit the efficiency of NP aerosol delivery.

Figure 1. (A, B, C) Lung structure and epithelial differences. Reprinted with permission from reference (Osman et al. 2018).



2.1. Pharmacological advantages and limitations of drug delivery via the lungs

The pulmonary route for drug delivery provides many advantages and is challenged with some limitations summarized in Table 1. The respiratory part is considered ideal for therapeutic aerosols absorption for many reasons, most saliently, wide surface area, thin lining epithelium, and high permeability. The limitations are the deepest and narrowest areas of the lung, presence of the effective clearance mechanisms, internal humidity, and progressive branching and narrowing impacting the drug away from the target (Rangaraj, Pailla, and Sampathi 2019).

Table 1. Advantages and limitations of the pulmonary drug delivery, and recent strategies to overcome these limitations.

Advantages of the pulmonary route drug administration	Limitations of the pulmonary route	Strategies to overcome the limitations
• The wide surface area of the respiratory part; 80–100 m^2 other sources reported up to 140 m^2, compared with the conducting airway; merely 2–3 m^2, allows for greater contact with the inspired air for gas exchange (Patton 1996)	• The pulmonary airways undergo progressive narrowing that traps the particle away from the deep alveoli.	• NPs size control, the smaller the size is, the deeper the NPs aerosols travel in the lung • NP engineering • Most of the therapeutic aerosols are targeting the lowers respiratory airways
• The respiratory lining epithelium is reduced to submicron thickness (0.2 µm) from approximately 60 in the conducting areas, accompanied with thinning of the fluid lining layer as well (from 8 µm to 70).	• The internal humidity affects the hygroscopic particles favoring their size increase, impaction away from the respiratory areas, and early clearance.	• The internal humidity is being employed to overcome the NPs aerosols impaction in the upper airways using hygroscopic or swellable gel particles.
• Respiratory epithelium is densely vascularized (5 L/min) with fast drug distribution and circulation (Hu, Jiao, et al. 2013).	• The conducting region has very think epithelial lining (60 µm) covered with a thick mucus layer and lung surfactant, representing a challenge for the inhaled particles to penetrate, and aiding in the particle agglomeration favoring their clearance.	• Various absorption and permeation enhancers are employed to overcome the mucous layers, tight junctions and epithelial barrier. • The use of cationic particles that increase the NP-epithelial interaction. • Mucoadhesive versus mucopenetrating functionalization

Advantages of the pulmonary route drug administration	Limitations of the pulmonary route	Strategies to overcome the limitations
• The thin air-blood barrier shows high permeability for small hydrophilic molecules, water and macromolecules suiting the air exchange requirements and aids in NP targeting topical or systemic sites. Its permeability to different drugs is dependent on physicochemical properties, for example, lipophilicity, size, molecular weight (inversely affecting the absorption), etc.	• The epithelial tight junctions prevent drug molecules and NP penetration.	• Tight junction modulators • Permeation enhancers
• It requires lower dose and dose fraction compared to other routes of administration, improving the patient compliance, and lowering dose frequency and decrease the potential side effects (Chandel et al. 2019).	• The effective mucociliary clearance is eliminating inhaled particles representing a further challenge. • The mucous production in healthy individual is 10–20 ml a day and the ciliary velocity is 1–20 mm per min from the peripheral towards the trachea (Rangaraj, Pailla, and Sampathi 2019).	• NP engineering; smaller size, mucou penetrating, absorption enhancers • Hygroscopic particles

Advantages of the pulmonary route drug administration	Limitations of the pulmonary route	Strategies to overcome the limitations
• The high pulmonary bioavailability stems from the epithelial properties and has a significant protease inhibitory activity, no hepatic first pass metabolism, with limited local metabolism, trivial systemic clearance effect, and it also avoids the gut irritation, irritable bowel and food/oral drug interactions. That allows for fast absorption, rapid onset of action, and efficient drug delivery (Patton 1996; Labiris and Dolovich 2003).	• The respiratory airways have macrophages clearance phagocytizing inhaled particles (Nicod 2005, Byrne et al. 2015), that could be beneficial to stimulate the immune response for the vaccination (Chono et al. 2006,Kleinstreuer, Zhang, and Donohue 2008, Rodrigues et al. 2018).	• To overcome the phagocytic clearnaces, various mechanisms can be employed such as optimal size above or below the phagocytic capacity; large porpous particles, hollow particles or trojan particles, swellable or hygroscopic particles, shielding the NPs with PEG or other shielding polumers
• Pulmonary drug administration proved successful in delivering local treatments for respiratory problems as asthma, COPD, lung malignancies, lung infections etc. as well as systemic diseases through delivery of therapeutic molecules as protein/peptide or gene delivery, hormonal therapy or vaccines (Rangaraj, Pailla, and Sampathi 2019).	• Pulmonary system is affected by many diseases that decrease the airflow limiting the efficiency of the aerosol delivery to deep lung.	• Different aerosol delivery devices that can be used to adjust with the lung conditions
	• NP toxicity and Inflammation: NPs should be carefully investigated to be biocompatible and biodegradable materials as to exclude any adverse effects from toxicity or inflammatory effects (Fröhlich and Salar-Behzadi 2014	• Full detailed assessments and NPs characterizations are nanotoxicological evaluated prior to clinical applications

2.2. Aerosol drug delivery of nanoparticles to the lungs

Burgeoning interest has manifested in NP drug delivery to the lungs via aerosols. NP aerosols have superior advantages which include the benefits derived from the advantages of the pulmonary route characteristics: the ability to localize the drug at the site of action with longer retention by preventing its systemic distribution and elimination, achieving a uniform NP distribution and increasing the drug solubility. This is combined with the advantages offered by NP formulation characteristics and can be used for systemic drug delivery resulting in improvement of the clinical outcomes (Takenaka et al. 2001; Hohenegger 2010). The USFDA has approved a liposomal (ARIKAYCE®) amikacin suspension for the treatment of Mycobacterium avium complex (MAC) lung disease via nebulization as the first NP-drug aerosol delivery (Insmed 2019). A list of aerosolized nanomedicines either approved for lung delivery or still in the preclinical or clinical stages is provided in Table 2.

Table 2. Examples of aerosolized nanomedicines to target the lung or systemic targets.

Active drug/molecule	NP carrier	Indication/ disease	Type of study	Ref
Amphotericin B antibiotic, AmBiosome®	Liposome	Lung transplantation infections	Phase III clinical trials. (Rangaraj, Pailla, and Sampathi 2019) NCT00177710	https://clinicaltrials.gov/ct2/show/NCT00177710
Cisplastin antimetastatic agent	Liposomes, sustained release	Metastatic lung cancer	Phase Ib/IIa clinical trails. (Wittgen et al. 2007; Mangal et al. 2017)NCT00102531	https://clinicaltrials.gov/ct2/show/NCT00102531
Ciprofloxacin antibiotic	Pulmaquin™ (FDA) or Apulmiq or Linhaliq™ (EMA)liquid 1;1 mixture of liposomally- encapsulated and free ciprofloxacin. This allows for dual release; immediate and sustained	Lung infections with P. aeruginosa	Phase III clinical trials (NCT02104245)	https://www.drugs.com/history/linhaliq.html
Cyclosporine	Lipososmes	Lung transplant rejection	Phase I/II clinical trails. NCT01650545	https://clinicaltrials.gov/ct2/show/NCT01650545
Interleukin 2 antimetastatic agent	liposomes	Lung metastatsis	Preclinical *in vivo* animal study	(Mangal et al. 2017)
Active targeting EGFR-magnetic NPs.Epidermal growth factor receptor (EGF receptor)	superparamagnetic iron oxide (SPIO) NPs	Stimuli responsive, magnetic hyperthermal tumor ablation in the lungs,on-small cell lung cancer; NSCLC	*In vivo* mice study	(Sadhukha, Wiedmann, and Panyam 2013)
EGFR-targeted cisplastin loaded NPs and non targeted loaded NPs	Gelatin NPs	Cancer lung	Preclinical *in vitro* and *in vivo* lung study	(Tseng et al. 2009)

Active drug/molecule	NP carrier	Indication/ disease	Type of study	Ref
Combined therapy of doxorubicin and cisplatin with two siRNA with active targeting with analog of luteinizing hormone-releasing hormone (LHRH)	Mesoporous Silica NPs	NSCLC	*in vivo* lung study	(Taratula et al. 2011)
Combined therapy of doxorubicin and cisplatin with two siRNA with active targeting with LHRH	Nanostructured lipid NPs	NSCLC	*in vivo* lung study	(Jyoti et al. 2015)
Paclitaxel	PEG_{5000}-DSPEmicelles	Lung cancer	*In vitro* and *in vivo* animal study	(Gill, Nazzal, and Kaddoumi 2011)
Epirubicin	SLNP	Lung cancer	*In vitro/in vivo* studies	(Hu, Jia, and Wending 2010)
9-Nitrocamptothecintopoisomerase I inhibitor	liposomes	Lung cancer, SCLC	Phase II clinical trails. (Verschraegen et al. 2004)NCT00250068	https://clinicaltrials.gov/ct2/show/NCT00250068
Quercetin	PLGA NP coated with magnetic (Fe3O4) NPs	Lung cancer	*In vitro* and *in vivo* studies	(Verma et al. 2013)
Losartan and telmisartan	Polystyrene NPs	Lung cancers	*In vivo* animal study	(Godugu et al. 2013)
Doxorubicin	Albumin NPs with surface adsorbed with apoptotic TRAIL protein (TRAIL/Dox HSA-NP)	Drug resistant lung cancer	*In vitro* and *in vivo* studies	(Choi et al. 2015)
Doxorubicin	56-kDa PEG-PLL dendrimer	Drug resistant lung cancer	*In vivo* animal study	(Kaminskas et al. 2014)
Gene delivery: Akt1 siRNA	PEI NP	Lung Cancer/ Metastases	Preclinical *in vivo* assay	(Ray, Mandal, and Mitra 2015)
Ricin vaccine	Liposomes	Ricin toxicity	Preclinical *in vivo* assay	(Smallshaw, Richardson, and Vitetta 2007)
Pneumococcal surface protein A (PspA)	NPMP	Pneumococcal infection vaccine	Preclinical *in vivo* mouse assay	(Rodrigues et al. 2018)
Indomethacin, ketoprofen	SLNP	Asthma	Preclinical *in vivo* assay	(Mansour, Rhee, and Wu 2009)
Leuprolide	Liposomes	Lung Cancer	Preclinical *in vivo* assay	(Mansour, Rhee, and Wu 2009)
Rifampin, isoniazide, pyrazinamide	PLGA NP/ SLNP	TB	Preclinical *in vivo* assay	(Sung, Pulliam, and Edwards 2007)

Active drug/molecule	NP carrier	Indication/ disease	Type of study	Ref
Tranilast (antiallergic agent)	Nanocystalline powders	Enhanced anti-inflammatory effects in lung in asthma	Preclinical *in vivo* assay	(Onoue et al. 2011)
Lung surfactant proteins	Liposome	AcuteRespiratory Distress Syndrome (ARDS)	On clinic, first approved in Germany and Japan; Alveolfact®, Survanta®, Curosurf®	(Mansour, Rhee, and Wu 2009)
Insulin	PLGA, PEI, Chitosan NP	Diabetes	Preclinical *in vivo* assay	(Hohenegger 2010)
Calcitonin	PLGA-Chitosan NP	Parathyroid diseases	Preclinical *in vivo* assay	(Sung, Pulliam, and Edwards 2007)
Therapeutic peptide	Calcium phosphate NP	Heart failure	Preclinical *in vivo* assay	(Miragoli et al. 2018)

2.2.1. Nanoparticle aerosol generation and deposition into the lungs

Inhalation drug delivery is attained via respirable-sized aerosol particles. The aerosol is described as suspended solid or liquid particles stabilized in a gaseous phase (Moraga-Espinoza, Eshaghian, and Smyth 2018). There are three widely recognized aerosols-generating devices: Nebulizers (Corcoran et al. 2014; Wang, Li, et al. 2017), metered-dose inhalers (MDI) (Moraga-Espinoza, Eshaghian, and Smyth 2018), dry powder inhalers (DPI) (Hoppentocht et al. 2014; De Boer et al. 2017). The mechanism of aerosol generation, advantages and limitations, indications of each device, and the recent developments can be found in the following sources (Dolovich and Dhand 2011; Berlinski 2015; Stein and Thiel 2017; Chandel et al. 2019).

The aerosols are described by their aerodynamic diameter (AD) which is the diameter of a unit density sphere (water droplet) having the same settling velocity in the air to the particle of interest (De Boer et al. 2002). The aerosols that have an inhalable size are of an AD smaller than 10 µm and classified as coarse particles (>2 µm), fine particle fraction (0.1–2 µm), and ultrafine particle fraction (<0.1 µm). The AD range of the pharmaceutical aerosols is between 0.5 and 5 µm (Labiris and Dolovich 2003). A major limitation for NPs lung deposition as a dry powder is their AD is smaller than the optimal size for aerosol deposition; 0.5–5 µm (Bisgaard, O'callaghan, and Smaldone 1999). This means the delivery of NPs as a single/monodisperse NP aerosol to the lungs is almost impossible. Many formulations and delivery strategies are there to overcome this limitation, such as formulating NPs in a bigger carrier as microparticle (Tsapis et al. 2002; Alfagih et al. 2015; Bohr et al. 2015; Mcbride, Price, and Muttil 2017; Wang, Beck-Broichsitter, et al. 2017), delivering NPs aggregates either pure or with excipient carriers such as lactose or L-leucine, or freeze-dried or pre-spray dried in large porous or hollow carriers in DPI, or delivering the NPs as micron-sized agglomerates using nebulization/pMDI that achieves a temporary increase in their AD favoring their lung deposition (Oswald ripening). Recently, effervescent particles technology that involves spray-drying into effervescent excipients has shown better aerosolization and faster release of NPs upon dissolution in aqueous media (Ely et al. 2007, Azarmi et al. 2008, Al-Hallak et al. 2012). NPs drug delivery via DPI is claimed to be better than nebulization/pMDI in being more controlled and stable spherical particles, better loading, and aerosolization performance (Hu, D, et al. 2013; Iyer, Hsia, and Nguyen 2015). The physicochemical properties of aerosol NPs represent an active area in multidisciplinary research as to develop physicochemical characters that are nontoxic, efficient loading and target-delivery vehicle, safe and fit for the purpose, better aerosolization performance, stability, etc (Lewinski, Colvin, and Drezek 2008).

The deposition of inhaled particles into the pulmonary targets is known to be through the following mechanisms either: impaction, gravitational settling, interception, and Brownian diffusion. Cationic charged particles are exposed to another force depositing them into the airways known as electrostatic precipitation. Further details about these mechanisms can be found in the following sources (Gaul et al. 2018 Rangaraj, Pailla, and Sampathi 2019). The NPs aerosol deposition into the lungs is multifactorial dependent process and primarily depends on the physicochemical properties of the NP aerosol, the aerosol generating device; including the inhalation route; oral or nasal, and carrier medium; air (Nebulizers, DPI), or a propellant, and lastly but equally important, patient factors; the underlying pul-

monary pathophysiology including the respiratory volumes, breath-holding, and the severity of the lung disease (Heyder 2004, Stein and Thiel 2017, Rangaraj, Pailla, and Sampathi 2019). These factors and their effects on the deposition are summarized in Table 3.

Table 3. Factors affecting the NPs aerosol deposition in the lungs.

Factors affecting NP aerosol deposition	Effect on lung deposition
NP physicochemical characteristics	
Size	• 5–10 µm Aerosols impact in the upper airways • Prone to mucociliary clearance
	• 0.5–5 µm Aerosols sediment in the deeper airways • Prone to mucociliary and macrophages clearance mechanisms
	• Below 0.5 µm Aerosols undergo diffusion in the deepest airways • Less phagocytic uptake, <200 nm are not recognized by macrophages • More cohesive/adhesive agglomerates that difficult to be dispersed
Shape	• Spherical particles are commonly employed as easier fabrication methods. • High aspect ratio/ Fiber-like NPs deposited by interception. This was adapted from pathological example of asbestos.
Charge	• Cationic coated NPs are deposited by the electrostatic interception. • Longer residence or membrane bound time
Density	• NPs deposition in the deepest regions is inversely relate to the particle density. • Hollow particle with lower density and larger size is better deposited than small dense solid particles
Chemistry	• Slight hydrophobic particles increase the absorption but strong hydrophobic will increase the retention time. • Small molecular weight (MWt) hydrophilic particles will be fast absorbed to larger molecules. • High MWt particles are less permeable in the alveolar epithelium
Solubility	• The readily soluble particles will be less prone to clearance than insoluble particles that will be trapped either by the mucociliary or macrophage s clearance • Poor soluble particles with size over 6 µm are cleared faster and most of deposited particles are cleared within 24 hrs post deposition.
Patient factors	

Factors affecting NP aerosol deposition	Effect on lung deposition
Airway geometry and architecture	• The limitations (discussed in Table 1) of the normal lung structure such as airway branching and narrowing, mucous, surfactant, epithelial barriers and clearance mechanisms, person-person variations, age related variations. • In lung pathological conditions that affect the lung normal structure by progressive narrowing, i.e. bronchoconstriction, as in asthma, COPD, chronic bronchitis where the inflamed epithelium with over secretions of mucous, aerosol deposition is altered. The air flow speed is increased with the excessive narrowing, impacting the particles in the upper airways, and the air flow will be shifted to the less affected airways that directs the aerosol away from the diseased sites.
Inhalation pattern	• Oral inhalation has better lung deposition than nasal route.
Airflow velocity	• Fast air flow velocity impacts the particles away from the lower airways • Slower air flow velocity increases the residence time and increases both sedimentation and diffusion deposition.
Air flow pattern	• The air into the lung carries the aerosols; the flow pattern might be either laminar or turbulent. In case of laminar flow, the deposition forces are fluid viscous forces while in turbulent, more inertial forces and Brownian movements of molecules. Further details can be found in (Darquenne 2012) • Inspiratory flow rates are very critical for the aerosl generation and optimum depsotion. Discussed wit the device section below
Humidity	• The humid internal environment can affect the size of hygroscopic NPs and subsequently the mechanism and site of deposition. • Hygroscopic particles can become larger or smaller according to their chemistry • Hygroscopic effect is considered minimal for particles below 0.1 μm • This can be used as a technique to deposit the NPs in the deeper airways by minimizing the upper airways impaction and getting larger and favoring the deposition in the lower airways.
Mucociliary clearance	• Once deposited in the upper airways, the mucociliary clearance works effectively to eliminate the deposited particles to be coughed out or swallowed to the gut. • The mucociliary clearance efficiency is affected in a group of lung diseases as lung fibrosis, CF, or ciliary dyskinesia where the mobility of the cilia is impaired and the mucous is very thick leading to longer particle retention that might predispose to local toxicity.
Macrophages clearance	• NPs deposited in the alveolar epithelium will be subjected to clearance by the alveolar macrophages • The alveolar sac can contain up to 6 wandering macrophages continuously acting to eliminate any foreign materials • Soluble particles will fast evade the elimination by absorption and translocation to the epithelium • Particles slow low solubility or designed for controlled sustained release have longer residence time and more prone to macrophages uptake and subsequent clearance to mucociliary region, lymphatic and RES or slowly degrading. • Particles size of 1.5–3 μm are commonly engulfed by macrophages.

Factors affecting NP aerosol deposition	Effect on lung deposition
Target site	• If the NPs aerosols are deposited away from the target site, it lowers the efficacy of the treatment. Prior understanding of the disease and the disease site to target the delivery is a must. For example, in case of asthma, it would be wisely to target a NP steroid aerosol depositing that should be uniform all over the lung tissue to target all the inflammatory cells in the respiratory tree. • Another example, in case of infection, the site of where the infection happening is predetermined, i.e. in the upper or lower airways, affecting the epithelium or localizing in the lumen.
The aerosol generating device factors	
Nebulizers	• Commonly used device in uncooperative patient • FPF ranging from 60–80% • Deliver mixes of drugs in one shot • Effective in lower inspiratory flow 6–8 l per min • ~ 10% dose is deposited, various patient training techniques as to breathe deeply and breath-holding can increase the deposition to 17% • Main concerns are the negative effect on the formulation structure integrity, the carrier or the drug or macromolecules might get damaged due to the nebuliation forces. New generations of nebulizers such as vibrating mesh technologies, that deliver more uniform particles that can increase the deep lung deposition and maintain the formulation integrity are being developed and used. Further details can be found in (Chandel et al. 2019).
MDI	• Commonly portable device and used by COPD, asthma patients. • FPF can be up to 70% • ~20% of the dose deposited with minimal inspiratory flow rate is necessary ~20 l/min. • The formulation integrity is achieved with producing inhalable aerosols and using a propellent. • The main issue that might render the treatment unsuccessful in some patient is the lack of coordination. • Not suitable for delivery of high doses and long-term storage as loss of stability or degradation might happen. • New MDI have been developed allowing less patient effort to actuate, such as Autohaler® and Easybreath®; shown better lung deposition and less inspiratory volumes required (Chandel et al. 2019).

Factors affecting NP aerosol deposition	Effect on lung deposition
DPI	• DPIs have superior advantages over the other devices based on more stable powder formulation • DPIs are breath-actuated minimizing the lack of coordination • DPIs might have similar deposition rate to the MDIs. • Inspiratory flow, humidity and temperature have great effect on the DPI aerosol performance • An inspiratory flow rate of at least 30 l/min is necessary for aerosol generation. • Difficult use in debilitating conditions, elderly, and children • Newer generations and further details are in the following source (Chandel et al. 2019).

2.2.2. Challenges of formulating nanoparticle aerosol for lung delivery

NPs aerosol delivery is still not well established and faces many challenges. Apart from the toxicological potentials of the nano-specific components, the aerosolized formulations may contain other stabilizers or emulsifiers that were used either during the fabrication process or in the final product that must be considered as part of their safety. The aerosol deposition is not a straightforward process and needs extensive optimization of the formulation. The diseased lung condition that might add more difficulty in achieving the aimed deposition capacity. The loading efficiency and the stability of the loaded active agent must be worthwhile. Translation from benchtop to the clinics is not easy with challenges to be considered, i.e. the dose- and dosage-form defining problems (Scherließ and Etschmann 2018, the shelf-life, the scalability, the ease and safety of the use, and the last but not the least is the cost (Iyer, Hsia, and Nguyen 2015).

3. Unintentional pulmonary exposure to nanoparticles

Unintentional exposure to NPs covers any exposure to NPs not intentionally planned such as NPs emitting from natural sources (such as volcanos, soot and fire ashes). Unintentional exposure to anthropogenic NPs can occur in occupational settings (during manufacturing processes using raw NPs materials or generating them as secondary by-products), and recently increased in-market consumer products that are based on NPs. Table 4 provides a non-inclusive list of examples of NPs that can be found as airborne from occupational settings and/or from consumer products (Zhang et al. 2015, Foss Hansen et al. 2016, Kuhlbusch, Wijnhoven, and Haase 2018). This exposure can happen via different routes of entry, such as the skin and oral route, but inhalation exposure to the airborne NPs is very concerning due to the vulnerability and inevitable nature of inhalation with the inherent ability of NPs to be easily suspended in air (Steinhäuser and Sayre 2017).

Table 4. Examples of NPs that can be found airborne in occupational settings and/or from consumer based products.

NPs	Occupational and/or consumer exposure
Ag	Textiles, Electronics, food packaging, medical devices, antimicrobial and disinfectant sprays, refrigerators and air humidifiers
Iron NPs (Fe_2O_3, Fe_3O_4)	Automotor, cosmetic, electronic and medical products, refrigerators and air humidifiers
TiO_2	Electronic devices, cosmetic industry, water and cleaning products, solar cell industry, antimicrobial and disinfectant sprays
Gold (Au NP)	Medical products, electronic industry, fuel and lubricants, food and beverages,

NPs	Occupational and/or consumer exposure
Nanocrystals and quantum dots (cadmiumSelenide, cadmium sulfide, leadSulfide)	Solar cells, electronics, semiconductors and dye and medical uses
ZnO	Cosmetics, paints and varnish, packaging and personal care products, paints, cleaning products, sprays
Silica NPs (SiO_2)	Paints, coatings and food packaging. antimicrobial and disinfectant sprays
Aluminium oxides (Al_2O_3), hydroxides ($Al(OH)_3$), oxo-hydroxides (AlO(OH))	Grinding tools, automotor, polishing, electronic, plastic, dyes industries
Nanoclays	Cosmetics and personal care, packaging, water managements, flame retardants, paints
Ceramics	Paints, personal care products, filtration systems, coatings
Cerium oxide (CeO_2)	Coatings, paints, automotor and fuel cell industry
Carbon NPs: C60, C70, Nanotubes, Carbon Black, Graphene NPs	Electronic devices, catalysts industries, water managements and filtrations, sensors, automotor and sports, clothing and packing, sensors, plastics, cosmetics, refrigerators and air humidifiers

There are countless challenges and issues when it comes to assessing unintentional exposure to NPs. The huge variations of the same chemical structure of NPs, i.e. different sizes, shapes, different functionalization, and impurities, manifest a massive burden to evaluate the safety of each NP on a single basis. Knowledge gaps still exist regarding NP behavior in the environment from their release site to their deposition into an organism; their interactions with other ecological elements; their biodegradation or bioaccumulation; the amount or the internal dose that can be deposited in an organism; and to what extent by which route of exposure the dose accrues. This knowledge will help to determine if their environmental concentration is decreasing or increasing over time, set the measures to control it, identify the post-exposure ecological and biological effects, and determine if the interaction will render them less or more toxic (Kuhlbusch, Wijnhoven, and Haase 2018). The literature has many studies presenting the increased risk of pulmonary diseases, and other cardiovascular problems, in those living in very air-polluted cities and near motorways, and relating these problems to diesel exhaust particles, fibers, fire ashes and soot, as well as their constituent particle types: coarse, fine, and ultrafine particles. More studies are needed to uncover the toxicity of the ultrafine particle fraction. The major difficulties in the real-time assessment of exposure at the environmental setting are due to the lack of adequate technologies that can count the particles, assess their size fractions, and determine their nature and probable sources from direct air sampling (Nowack 2017). Most of the current sampling is achieved either at the site of release of these particles, for example, from industrial drainage, or at the site of settling of these particles, for example samples from soil or water that are miles away from the production site. Many technological hurdles are still to be overcome to assess the environmental journey of NPs either from natural or anthropogenic sources to build realistic quantitative exposure risk assessments and firm conclusions (Nowack 2017; Jantunen et al. 2018).

The assessment of NPs exposure at the occupational settings is still developing to address the emission of NPs, set measures to control their release into the environment and to develop the protective equipment for the workers. Many studies have suggested the relationship between the releases of particulate matter at the workplace and long-term human diseases. One of the earliest studies draws attention to workplace exposure to NPs where seven women developed serious lung issues after long-term occupational exposure to NPs and MPs of polyacrylate, silica, and silicates. These particles were found in their tissue samples and Bronchoalveolar lavage (BAL) but no firm conclusion of causation against the NP fractions was found (Kuhlbusch, Wijnhoven, and Haase 2018; Forest et al. 2019). Asbestosis and silicosis are well-known lung pathologies caused by occupational exposure to inhaled dust and particles of bioresistant materials as asbestos, silica, and other minerals. There are well-established measures to do air monitoring during the occupational setting but these still lack the assessment of the ultrafine fractions that probably have more reactive and larger surface areas, as well as larger numbers of these particles. Air monitoring has overlooked consideration of an individual's susceptibility (i.e. any concurrent breathing problems, breathing patterns, or genetic or general predisposition), or their relative personal exposure (i.e. how long the exposure, frequency of that exposure, what particles, pure or mixed with impurities, soluble or bioresistant, their physicochemical properties) (Abdolahpur Monikh

et al. 2018). The calls for co-implementing bio-monitoring is urgently needed where measuring the internal dose can reflect the deposited dose and assess the individual clinical picture. Some research studies are proposing approaches of monitoring the load of NPs in patients' samples and their clinical conditions and propose extracting these particles to be used for *in vitro* experiments to assess their toxicity; what is called *in vivo* to *in vitro* testing (Forest et al. 2019). Other studies call for assessing different particles collected from different environmental or occupational settings and then studying their safety. This creates unrealistic economical, technical, ethical and time-scale burdens to achieve, hence the calls for grouping NPs to allow for read-across of their safety and the urgent need to enhance the reliability and relevance of the *in vitro* testing, abiotic functional assays, and *in silico* models (Abdolahpur Monikh et al. 2018; Basei et al. 2019; Lamon et al. 2019).

With the increasing number of products that contain NPs (either embedded in a solid matrix where aerosolization is very rarely expected, or in a suspended form where aerosolization can occur), much less is known about how NPs can be released into the environment, and other associated ecological and biological effects. Most of the consumer exposures, for example, spray products containing NPS, are based on modeling methods that measure the exposure on simulated environments but still suffer from the same issues of the occupational exposure, i.e. neglecting the frequency of the exposure, the individual susceptibility, and the different NPs interactions prior to deposition that might aggravate the harmful effects (Nowack 2017, Steinhäuser and Sayre 2017, Kuhlbusch, Wijnhoven, and Haase 2018, Basei et al. 2019). More studies are urgently needed to study the release of NPs from occupational settings and consumer products into the environment, and to assess the short-term and long-term exposure risks. When this knowledge is available, this will help set accurate quantitative risk-exposure estimates and set regulatory measures to protect humans and the environment when handling, using, and disposing of NP-containing materials.

4. Nanotoxicology and the regulatory bodies of nano applications

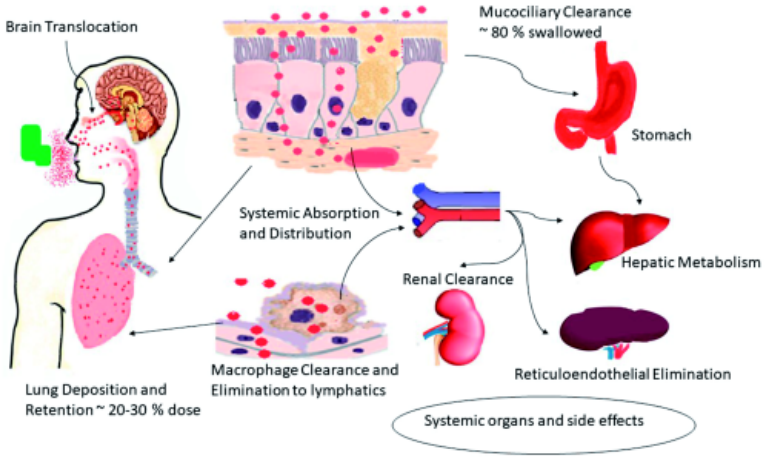
Nanotoxicology was enabled in 2004 as a new category of the classical toxicology to address the challenges with NPs assessments (Donaldson et al. 2004). It focuses on uncovering the mechanistic relationship between NPs physicochemical characteristics and the consequential biological effect. Moreover, it scopes optimizing the experimental conditions for *in vitro* and *in vivo* evaluation to detect possible NPs interference with different assays, and to provide the safety assessment data for this nanomaterial, and their applications (Joris et al. 2013). The novelty and complexity of NPs and their biological responses had raised issues regarding; which is the critical parameter in their toxicity, i.e. what is the NP dose mass, number, physical or chemical characteristic, route-dependent toxicity or a combination of all (Buzea, Pacheco, and Robbie 2007). The Dearth of standardized assays and pre-set regulations have made it difficult to compare the available literature regarding their safety. Over two decades, the scientific community has undergone extensive debates and yet failed to come to a common ground about the NPs metrics, definitions, classifications, characterizations, safety/toxicity characteristics, toxicity endpoints, target endpoints, occupational/environmental exposure limits, and standardized assessment methods. Many government and non-government bodies are working closely to address the regulatory challenges with efforts to bridge the international harmonization and standardizations. For example, nano-based products for consumer use are mostly under regulation by many organizations within USA; as USNCL, FDA and EPA. These organizations have a set of protocols to evaluate the safety of these products prior to their marketing, starting from benchtop development, manufacturing, occupational, environmental, and biological risk assessments and clinical trials. Similarly, in Europe, organizations such as EUNCL, EMA, and others are providing standards and protocols to regulate nano-based products. They provide a set of protocols to assess the safety of nanomedicines (Sainz et al. 2015; Accomasso, Cristallini, and Giachino 2018; Siegrist et al. 2018). In the UK, organizations like DEFRA, FSA, DH, ISO, OECD, REACH are controlling the handling, use, and assessment of nanomaterials (Rasmussen et al. 2019). Detailed and recommended methods for physicochemical characterizations of NPs, for occupational, environmental, and consumer exposure modeling; and for *in vitro/in vivo* methods of nanotoxicological testing, agreed by a variety of regulatory bodies to assess different nanomaterials, can be found in these sources (Sainz et al. 2015; Drasler et al. 2017; Nowack 2017; Steinhäuser and Sayre 2017; Abdolahpur Monikh et al. 2018; Kuhlbusch, Wijnhoven, and Haase 2018; Lamon et al. 2019).

4.1. Inhaled nanoparticle ADME kinetics and toxicity

Upon pulmonary administration of NPs aerosols, the smaller the size of the NP aerosol, the deeper they can travel into the lung. Once deposited, NPs interact with a group of biological and cellular barriers starting from the surfactant layer, mucus, epithelial cells, vascular endothelium, and interacting with different molecular and cellular structures in

the biological environments prior to exerting a drug response (Figure 2). NPs have to be absorbed and transported into their targets faster than the rate of their clearance and degradation. NPs will be up taken by the alveolar epithelium where they can be transported through the paracytosis or transcytosis pathways (Forest, Vergnon, and Pourchez 2017). Locally delivered NPs, results in prolonged lung retention, which is aimed to achieve site-targeting, increase the duration of action, lower the dosing frequency, and minimal off-target side effects. On the other hand, the longer lung retention might cause local NPs toxicity, and the possibility of NPs escape through the thin alveolar epithelium to systemic circulation producing off-target side effects (Bourquin et al. 2018). For example, in an *in vivo* study involving female Wistar strain WU rat, Ag NPs of 50 nm size and coated with polyvinyl pyrrolidine (PVP) (previously tested *in vitro* against alveolar macrophages and were more cytotoxic as determined by LDH release than NPs of 200 nm) were administered via intratracheal instillation in a single exposure of a dose from 0, 37.5, 75, 150, 300, 600 (*w/v*) µg per rat lung as a shot of 500 µl isotonic saline solution, and compared to600 µg dose of Ag NP-200 nm. At day 3 and 21 days post-exposure, BALF was collected, with histological evaluation of the lung tissues, liver, kidneys, RES (spleen, and lymph nodes) for subsequent Ag quantification and imaging by ICP-MS and microscopy. Ag NP 50 and 200 nm at 600 µg were associated with severe pulmonary inflammation with dead cells, ruptured macrophages, and detached epithelial cells denoting the high toxicity of the Ag NPs, so the dose was limited to 300 µg. The 200 nm size showed more prominent inflammatory signs than the 50 nm and the study was continued with the 50 nm NPs. The BALF was evaluated for the high level of cytokines and other inflammatory mediators, where Ag NP-50 nm doses from 75–150 µg showed a reversible inflammation, 300 µg had caused progressive inflammation with infiltrating neutrophils, cell proliferation and DNA damage with single and double-strand damage. Significant accumulation of Ag NPs in the distant RES organs was associated with doses above 75 µg, with focal pools of Ag NP partly attributed to the wondering macrophages, other localizations were found in the kidney proximal tubules, denoting the ability of the NPs to be retained locally and migrate systematically causing target and off-target side effects. A drawback in this study, is the lack of Ag NPs quantification of the lung tissue load (Wiemann et al. 2017).

Figure 2. Pharmacokinetic of inhaled NPs and their potential toxicity.



Systemic NPs delivery via inhalation, NPs traverse the whole respiratory membrane to the blood where they will be carried and delivered to the systemic targets. NP systemic translocation was reported to be size dependent process but no solid threshold is clear, for example, it is ten times higher for NPs of 20 nm than 80 nm Iridium, or forty times higher for 1.4 nm than 18 nm Au NPs (Rinaldo et al. 2015). NPs can be retained at the site of action by EPR effect as in case of tumors or been actively targeted by certain antibodies to migrate to certain types of cells. Some NPs travel through the olfactory epithelium to the brain (Samaridou and Alonso 2018).

NP metabolism and clearance have shown many variations. With the use of lung delivery for both systemic and local delivery, the low metabolizing activity will increase the pulmonary retention of NPs and prolong their action (Olsson et al. 2011). This could be advantageous, where the lung acts as a stable reservoir of the NPs for sustained delivery and clearance. Up till now, the full lung metabolizing capacity is not fully recognized with little information known about any enzyme induction or inhibition apart from smoking; a well-known lung metabolizing inducer (Kroon 2007), the lung enzymatic inhibition could increase the potential clinical side effects and toxicity of locally

delivered NPs (Olsson et al. 2011). Particle elimination and NPs elimination from the lung can be attributed to their dissolution/solubility in the interstitial fluid or physically eliminated by mucociliary or macrophages clearance (Rinaldo et al. 2015).

The biodegradable NPs products will eventually be cleared by the renal system. The renal clearance is limited to NP or NP products size lower than 8 nm (Longmire, Choyke, and Kobayashi 2008). The physical clearance of NPs could be achieved either with the fast mucociliary in the conducting zone, or the slow macrophages clearances in the respiratory zone. The NPs swallowed into the stomach, might undergo fecal clearance or reabsorption to the systemic circulation. Furthermore, the macrophages might entrap these NPs resulting in an inflammatory reaction upon exceeding the macrophages ability to digest. Macrophages have less-efficient clearing for NPs with a size below 70 nm, moreso, rod or fiber-like NP are very challenging for phagocytosis resulting in frustrated macrophages (Bakand and Hayes 2016). NPs can be drained to the local lymphatics and lymph nodes either delivered by loaded macrophages or sole NPs drainage to lymphatics. From lymphatics, NPs can travel to the systemic circulations and end up in the reticuloendothelial system (RES) (liver, spleen, bone, and others) where further chemical digestion or physical retention will occur causing off-target side effects. It is unknown, how much of the inhaled NPs is retained in the lungs or escape to the systemic circulation, or the duration within the lung retention (Zhao et al. 2019). Although the enhanced properties of NPs stem from their nanosize and their physicochemical properties but it still requires optimization of the NP and its formulation to suit the planned route of entry as evidenced in many studies (Donahue, Acar, and Wilhelm 2019). Kreyling et al. had published a series of NPs administration studies via intratracheal instillation, oral, and IV injection for the same type of NP in rats where differences in the bioavailability, distribution, clearance and elimination were noticed from 1 hr up to 28 days (Kreyling, Holzwarth, Haberl, Kozempel, Hirn, et al. 2017; Kreyling, Holzwarth, Haberl, Kozempel, Wenk, et al. 2017; Kreyling, Holzwarth, Schleh, et al. 2017) indicating the unsuitability of adopting the kinetics for NPs from one route to be applied to another route of administration. Another study had shown the lung retention of gadolinium NPs administered as contrast agent was much better and tumor-limited with no lung inflammation induced by local lung delivery compared with the same particles injected through IV route indicating targeting the lung by local delivery shows superior results from systemic lung-targeting (Bianchi et al. 2014; Dufort et al. 2015). *In vivo* animal study in Sprague-Dawley male rats were exposed to TiO2 NPs anatase ~20 nm, 15 mg/m^3 during 6 hrs inhalation using nose-only inhalation chambers. Rats were euthanized at 0, 3, 6, 12, 24, 48, 72 hrs, 7 and 14 days. Quantification of the NPs retention by ICP-MS in the lungs, blood, heart, brain and olfactory tissues, lymphatic, liver, pancreas, salivary glands, spleen, lymph nodes, thymus, feces and urine were done at the time intervals. Lung slices were collected for TEM analysis after 6 hrs exposure. Oxidative damage were detected by the malondialdehyde (MDA; marker for lipid peroxidation) using thiobarbituric acid reacting substances (TBARS) assay from the blood levels and tissue levels at 1, 3, 7 and 14 days intervals. In the Lungs, the max peak NPs load was achieved 48 hrs after the exposure, with a slow progressive decrease over 14 days, denoting that the NPs were retained/impacted with slow translocation to the lung tissues. In the blood, the max peak was 12 hrs post-exposure and high levels were detected in lymph nodes and other internal organs, denoting the systemic translocation. The total amount translocated to blood and lymphatic was low compared to the retained amount on the lungs. MDA was significantly high in the lungs, blood and main tissues (liver, spleen, kidneys) 24 hrs after exposure with fast decline over 14 days. Feces and urine contained large amounts of the eliminated NPs and this can be explained by the mucociliray clearance to the GIT, liver drainage, blood translocation to the kidneys. Olfactory bulb and brain contained high levels of NPs but there was no firm conclusion if the NPs had been translocated through the olfactory bulb and nerves to the brain, or simply was directed from the systemic circulation. From this study, it was concluded that lung translocation to the blood was the main source for NPs available for the systemic distribution. Highest levels of NPs were in lungs followed by liver and then kidneys during max peaks and over 14 days. Feces primarily and urine secondarily were main elimination routes for the NPs denoting the effectiveness of mucociliary clearance (Pujalte et al. 2017). Another animal study demonstrated the systemic distribution and accumulation after inhaling Gold NPs and off-target initiating inflammation (De Matteis 2017).

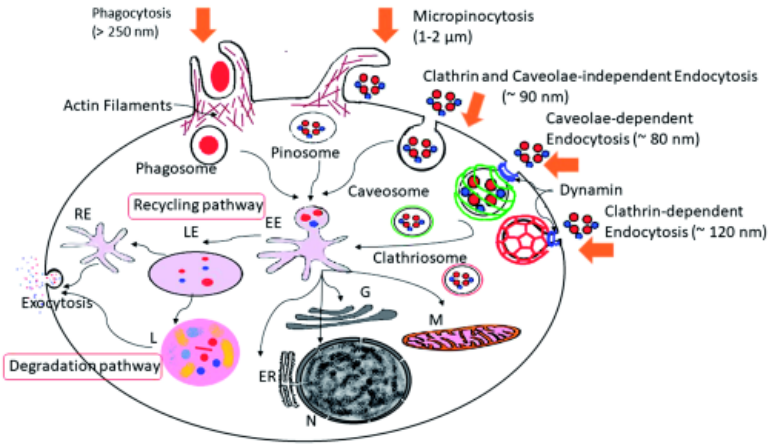
The NP kinetic variations from one route of administration to another, i.e. inhalation versus parenteral and oral routes. The NPs physicochemical and formulation properties designed to suit a specific route, are not going to be suitable for another route because of the differences in biological barriers at the site of entry with differences in the structural, physiologic and chemical environments. Another strong factor is the protein corona, upon contact of NPs with the fluid environment of the barrier site, a layer of proteins and other molecules of that fluid medium will wrap the NPs giving what is called a new identity for the NPs that plays in the safety and efficacy of the treatment (Moore

et al. 2015). This may lead to NPs aggregates and more prone to phagocytic uptake, or the physicochemical properties may change implicating changes in their kinetic behavior either limiting their effect or intensifying their toxicity (Bello and Warheit 2017). Further details about the protein corona and its effects on NP activity can be found in these sources (Monopoli et al. 2011; Vilanova et al. 2016; Ânia et al. 2018; Nierenberg, Khaled, and Flores 2018; Cao et al. 2019). A stealth/shielding technique for NPs are currently designed to limit the effect of protein corona (Vij et al. 2010). Therefore, a single NPs safety scenario concluded by one route might not guarantee the safety for another route of administration. This means each route of entry must be tested separately. A nanotoxicological evaluation on a single NP case should be assessed covering the nanocarrier physicochemical properties, nanocarrier-drug formulation factors, with *in vitro/in vivo* evaluation prior to clinical trials. Recently applying *in silico* models after *in vitro* studies was successful to predict the *in vivo* kinetics of gold aerosolized NPs (Bachler et al. 2015; Donahue, Acar, and Wilhelm 2019). A growing concern which is not yet much explored is the ability of NPs to cross the placental barrier and causing developmental fetal problems.

4.2. Nanoparticle interaction at cellular and molecular levels

NPs ability to overcome the biological barrier and interact with the cell is a physicochemical-dependent process. Each NP delivery system should be characterized and studied on a single case scenario. The aim of NPs as drug delivery carriers is to deliver the cargo to certain cells, or more specifically, to certain subcellular locations, to exert a response. Cellular uptake can be mediated by various mechanisms; passive or carrier-mediated or endocytosis. For NP-mediated drug delivery, nanocarriers are of high molecular weight and mostly subjected to vesicular transport or endocytosis (Wang et al. 2012; Jameson et al. 2019). Endocytosis pathways involve many processes; pinocytosis processes and phagocytosis. Pinocytosis is the cellular uptake of small-sized particles and fluids and includes micropinocytosis, clathrin-, and caveolin-dependant and independent mechanisms (Figure 3). While the phagocytosis is the uptake of larger debris, particles, and bacteria and is only carried by professional immune cells such as macrophages and neutrophils. Further details of these different mechanisms can be reviewed in these sources (Aderem and Underhill 1999; Conner and Schmid 2003; Elkin, Lakoduk, and Schmid 2016; Donahue, Acar, and Wilhelm 2019; Jameson et al. 2019). NP uptake mechanisms are physicochemical dependent processes and cell-type dependent. Post uptake NPs vesicles will be fused with the early endosome (EE) where a low pH digestive activity can take place degrading the NPs. The lysosomal digestion carries many potentials of intracellular and targeting delivery of pH-sensitive NPs. This is of benefit in the case of tumors where their extracellular environment has low pH (Dominska and Dykxhoorn 2010). Lysosomal/endosomal escape is a challenge for the subcellular NP targeting to cell organelles (Li, Cheng, et al. 2014), i.e. nucleus (Li et al. 2017, Pan, Liu, and Shi 2018) and mitochondria (Salnikov et al. 2007; Chen et al. 2016; Eftekhari 2018). Endosomes have complex machinery that allows for NP vesicular sorting, digestion and degradation, and waste-exocytosis and recycling as well as initiating cellular death in case of toxic NP overload. Full endosomal maturation cycle and their role in NP degradation can be reviewed in these sources (Scott, Vacca, and Gruenberg 2014; Elkin, Lakoduk, and Schmid 2016; Rothen-Rutishauser, Bourquin, and Petri-Fink 2019). Some NPs can be found without vesicles in the cytoplasm (Bourquin et al. 2018). NPs may translocate to cytoplasm, mitochondria, nucleus or other cellular organelles and molecules, and may evoke a cytotoxic response (Jiang et al. 2010; Shi et al. 2011; Donahue, Acar, and Wilhelm 2019).

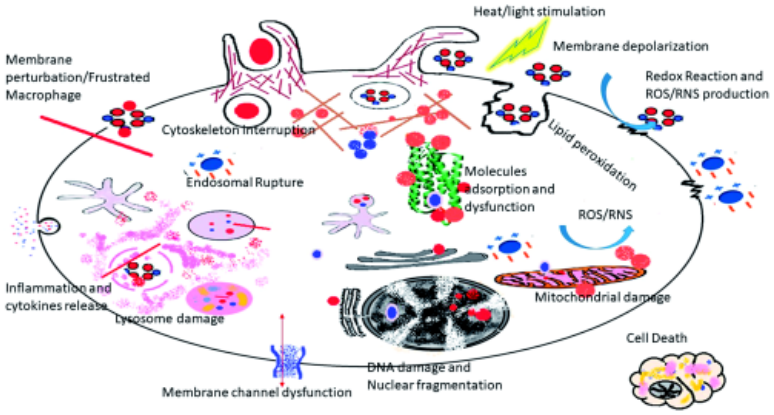
Figure 3. Main uptake mechanism of NPs and the endocytic pathways. EE: Early Endosome-low pH; LE: Late endosome-low pH; ER: Endoplasmic reticulum; L: Lysosome-very acidic; N: Nucleus; M: Mitochondria; G: Golgi; RE: Recycle endosome.



4.3. Proposed mechanisms of NPs toxicity

NP toxicity is still one of the hot topic areas due to the novelty of their materials that render the NP with unique physicochemical properties. These exceptional properties are not only tremendously critical for their efficacy but also for their toxicity with partly or complete dose-independent. Cellular injury might vary from trivial reversible injuries recovered by the efficient cellular repair mechanisms to severe or irreversible injuries inducing cell death or long term adverse effects (Donahue, Acar, and Wilhelm 2019). Various intersecting toxic mechanisms were reported upon exposure to various types of NPs, or even to the same chemical structure NP with variable physiochemical properties (Figure 4). Up till now, there is no agreed solid background as to which is the single and the most critical parameter for NP toxicity, for example, is it the size only, or the chemical composition, or the mass? Unlike the same bulk counterparts, the mass dose is not such critical for the toxicity (apart from being overtly overdosed that would generate toxicity anyway) and the full identity of physicochemical properties of the NPs is critical. As a result, a full thorough NP characterization is a must prior to their testing and drawing conclusions. In this literature review, the authors focused on NPs toxicity that was based on different types of NPs, such as organic NPs, i.e. polymeric natural or synthetic that are commonly used for drug delivery to the lungs, carbon-based such as CNTs and carbon black that are common in environmental and occupational setting inhalation, and metallic NPs such as Ag, Au, Zn, Silica, TiO_2 NPs that have more risks of occupational and consumers risk from inhalation. Although most of the *in vitro* research pool is based on lung cell lines exposure to suspended NPs and that is due to the expensive nature and the extreme difficulty to achieve aerosolization exposure to *in vitro* cell lines. The value of the initial *in vitro* step is establishing the relationship between the NP physicochemical identity to biological or toxicological responses, uncovering the underlying mechanisms, a possible high throughput screening, developing *in silico* predictive modeling, and reducing the animal load (Xia et al. 2016). The *in vivo* based experiments are inhalation exposure but with varied routes of delivery, i.e. oral- or nasal-delivery, whole body exposure, intratracheal instillation or cannulation. These studies will provide solid frameworks to understand the toxicity of NPs aerosols on the lung due to the novelty of the aerosolized NPs.

Figure 4. NP cellular uptake and interactions with different mechanisms of cytotoxicity.



4.3.1. Cell membrane disruption

Membrane disruption due to NPs interactions could be mediated via various mechanisms (Donahue, Acar, and Wilhelm 2019; Farnoud and Nazemidashtarjandi 2019). Positively-charged NPs interact with the negatively charged cell membrane altering, depolarizing or damaging the membrane i.e. thinning, pore formations and erosions (Yeh et al. 2013; Jameson et al. 2019). The negative charge NPs could also affect the membrane potential through the electrostatic interactions with the lipids causing lipid leakage (Mu et al. 2014). Surface chemistry is another important factor, where a lipid-bilayer damage and RBCs hemolysis was attributed to the presence of surface Silanol groups with amorphous silica NP and not with mesoporous Silica NPs (Slowing et al. 2009). ROS production and subsequent lipid peroxidation is another membrane damaging mechanism where it could be prevented by the use of antioxidants (Sayes et al. 2005). Inhibition or stimulation of the membrane ion channels upon NPs interaction with subsequent loss of control over the cellular ionic pool can lead to membrane damage. Membrane depolarization and loss of control of Ca^{2+} influx with further proliferation inhibition were dependants on surface charge, with positive NPs having an influence in both malignant and nonmalignant human cells (Arvizo et al. 2010). Blocking of K^{+} channels is another example of membrane induced damage (Chhowalla et al. 2005), for example, impurities of yttrium within Carbon NPs were found to inhibit the K^{+} channels (Jakubek et al. 2009). Cytoskeleton alteration is another mechanism where NPs can functionally block the F-actin and α- or β-tubulins, which are the major functional proteins. This cytoskeleton plays an important role in preserving the cellular shape, motility, adhesion, transport, and cellular division and proliferation. PEGylated NPs were found to reduce this cytoskeleton affect (Tarantola et al. 2009). Polystyrene NPs showed inverse correlation between the size and the membrane interaction *in vitro* cell membrane model (Peetla, Stine, and Labhasetwar 2009; Accomasso et al. 2016). Fiber SWCNT NPs showed an increase in the ROS production due to physical needle-like effect disrupting the cell membrane and other subcellular structures, for example entangling the internal actin cytoskeleton, centrosome structures, nucleus, and DNA (Saifi, Khan, and Godugu 2018).

4.3.2. Oxidative stress

When ROS production exceeds the cellular ability to inhibit, this induces what is called oxidative stress where free radicals will react with the cellular components, proteins, membranes inducing cellular dysfunction, inflammation, lipid peroxidation, mitochondrial shutdown, molecular and DNA damage, and eventually cell death, either through the apoptosis if its less severe insult or through necrosis with severe toxic insults (Mu et al. 2014, Donahue, Acar, and Wilhelm 2019, Evans et al. 2019). Furthermore, almost all types of NPs had shown toxicity that is linked to the production of ROS and this could be determined by measuring free radicals and GSH levels (Anttila et al. 1997). NPs ability to induce the ROS is dependent on its physicochemical properties. The metallic NPs enhance the ROS production by catalyzing reactions like Fenton's or Heiber-Weiss with free radicals production. Park et al. showed the reduction of ROS production after addition of antioxidant N-acetylcysteine with Ag NPs (Saifi, Khan, and Godugu 2018). ZnO NPs triggered cytotoxicity in primary pulmonary cell line BEAS-2B, via ROS, Ca^{2+} spill, mitochondrial membrane disruption that was observed as well in A549 cells (Shin, Song, and Um 2015). ZnO NPs were associated with ROS and acute inflammation *in vivo* lung study and was claimed to be due to the NPs dissolution or its ability to shed

Zn ions that induced the oxidative stress and inflammation. Carbon NPs such as carbon black NPs or CNTs were able to induce ROS in *in vitro* cell-free media and to cultured cells. Organic compound redox-cycling reactions are responsible for production of free radical anionic compounds such as polycyclic aromatic hydrocarbons (PAHs). ROS could be due direct insult to the mitochondrial membranes or processes with inability to energize the cell (Xia et al. 2016).

4.3.3. Cell organelles damage and mitochondrial shutdown

NPs ability to interact with the cellular components and induce membrane damage and ROS will suggest potential toxicity on the mitochondria. Direct NP-mitochondrial interaction was noticed causing lipid membrane damage and leakage in which 3 nm gold NPs were localized into the mitochondrial membranes causing its dysfunction and leakage (Salnikov et al. 2007). Lysosomal damage either by chemical sponge theory or physical needle like shaped NPs rupturing the lysosome and releasing Ca^{2+} from the endoplasmic reticulum with eventual loss of control of mitochondrial permeability. In addition, lysosomal rupture and enzymatic spillage might activate apoptosis pathways and other carbon-based and fullerenes are reported to cause mitochondrial damage. The mitochondrial damage with loss of energy production and antioxidant properties will results in high ROS, nuclear and DNA damage, and membrane disruption (Saifi, Khan, and Godugu 2018, Donahue, Acar, and Wilhelm 2019).

4.3.4. DNA damage and mutagenicity

This could be direct NP genotoxic effect or indirect effect through the induction of inflammation, inflammatory mediators or ROS, molecular and organelle damage (Doak and Dusinska 2017[Singh et al. 2017). Due to the high surface energy of the NPs, DNA as any other biological molecule is subjected to surface adsorption. This bind-ing will induce both conformational and functional deformity of these biomolecules. Carbon based NPs were found to induce DNA-double stranded to cleave under light or with the presence of copper ions (Mu et al. 2014). Gold NPs were found to induce conformational changes as relaxation of the DNA strand coils, cleavage of the double strands (Railsback et al. 2012) and functional changes as inhibition of the transcription (Mcintosh et al. 2001). TiO_2 NPs caused oxidative stress and production of free radicals that resulted in subsequent DNA damage (Donaldson, Beswick, and Gilmour 1996). TiO_2 NPs showed DNA damage by causing breaks and other oxidative DNA dysfunction with impairing the repair mechanisms (Saifi, Khan, and Godugu 2018). Cationic PLL coated silica NPs were found to cause DNA transcription inhibition that was size dependent (strong irreversible DNA adsorption with size 40 nm NPs that was reversible with the smaller size 10 nm NPs) (Zinchenko, Luckel, and Yoshikawa 2007). Long-term studies are required to investigate NPs-genotoxicity with the development of cancer. Some QDs NPs were reported to cause DNA fragmentation (Mu et al. 2014).

4.3.5. Inflammation

Upon macrophage recognition and phagocytosis, inflammatory mediators are released which initiates an inflammatory response. This is a physio-pathological reaction which in excess or persistence of inflammation could predispose to autoimmune disease, long term diseases, and cancer (Donahue, Acar, and Wilhelm 2019). For the lung, the inflammatory potential of NPs designed for drug delivery should be excluded. The size (below or larger than the alveolar macrophages) will bypass the macrophages recognition and phagocytosis. NPs agglomerates are another problem during drug delivery of NPs to the lungs, as they increase the size of the particle favoring their impaction away for the alveoli or favoring their clearance. When the macrophages clearance is overwhelmed by NP size, shape, chemistry, particle number and agglomerates or surface group with prolonged pulmonary retention due to slow or incomplete degradation and metabolism, inflammatory response will be evoked. Due to their nanosize, NPs showed longer retention time and less efficient lung clearance in *in vivo* inhalation study (Bakand, Hayes, and Dechsakulthorn 2012). It has been known from the pathogenesis of lung silicosis that long fibers were trapped longer due to defective clearance with subsequent inflammatory induction. Another *in vivo* study showed the lungs had overburden and prolonged retention of long fibers (4-12 nm) TiO_2 NPs compared with the spherical NPs (Porter et al. 2013). ZnO NPs of 50 nm size were investigated against air-blood barrier of co-cultured continuous lung NCI-H441 and the vascular HPMEC-ST16R in transwells, where increased levels of the proinflammatory mediators, IL-6 and IL-8, were detected (Shin, Song, and Um 2015; De Matteis 2017). Hence, multiple parameters of NPs physicochemical characteristics play a role in inflammation induction (Braakhuis et al. 2014). Furthermore, Amorphous silica NPs induced inflammation and cell death with generation of ROS (Fu et al. 2014).

A new potential mechanism involved in the inflammation cascade is the NLRP3 inflammasome activation with rising IL-1β. Certain pathogens, toxins or particles might induce this mechanism with a rise of the IL-1β through acti-

vation of caspase 1 and initiation of inflammatory induced apoptosis or known as pyroptosis. Several studies have shown NPs can induce the NLRP3 activation, for example, some liposomes and polymer-based NPs. This is beneficial in case of vaccines delivery. A detailed source for this mechanism and its assessment can be found in these following sources (Sharma et al. 2018; Shirasuna, Karasawa, and Takahashi 2019).

4.4. Cell death mechanisms

Cell death is the final fate after any irreversible cytotoxic or stress insult and can be either programed cell death known as apoptosis, or unprogramed cell death known as necrosis. Very recently, the Nomenclature Committee on Cell Death had adopted new considerations to replace the old cell death classification to the new adopted and emerging mechanisms proved by many studies that have discovered variable mechanisms of the programed cell death such as apoptosis, autophagy, ferroptosis, pyroptosis, necroptosis, paraptosis, lysosomal-induced, autoimmune-induced, and many others. These processes have different fundamental mechanisms with different underlying biological and histological picture of the cell death (Mohammadinejad et al. 2019; Tang et al. 2019). The uncontrolled cell death (known as necrosis) is commonly a passive accidental cell death due to energy failure or simply bursting in response to severe acute insult. Detailed information for these mechanisms is found in these sources (Rothen-Rutishauser, Bourquin, and Petri-Fink 2019; Tang et al. 2019). NPs can induce complex death mechanism with many intersecting activation of different death pathways with a physicochemical dependent manner (Mohammadinejad et al. 2019). NPs can trigger many intrinsic and extrinsic pathways and induce apoptosis, autophagy, lysosomal-induced cell death, for example, in some metallic NPs such Nickel oxides, silicon dioxide, TiO_2, and SWCNTS. These NPs-induced cell death mechanisms involve caspases activation, ROS production, mitochondrial shutdown, lysosomal damage (De Stefano, Carnuccio, and Maiuri 2012). Although some inconsistencies are found in the literature regarding the necrosis where some researchers considered the loss of cell or the decreased viability as necrosis. There are many studies that claim different cytotoxicity's with different cell death mechanisms after exposure to TiO_2 NPs but lack the specifications of NPs crystal structure. TiO_2 with different nanosizes and crystal structure (rutile and anatase) had induced cell death; anatase TiO_2 NPs induced necrosis whereas the rutile TiO_2 NPs induced apoptosis and was concentration dependent.

4.5. NPs physicochemical-dependant toxicity determinants

4.5.1. Nanoparticle size, surface area, and aspect ratio

NPs have intrinsic properties that play a role in their beneficial advantages as well as their toxicity (Figure 5 and Table 5). Due to their nano-size range, they have large surface area-to-volume ratio, which explains their high reactivity and strong adsorption properties. Moreover, the high surface energy tends to facilitate the NP-cellular or biomolecular interaction, and NP-NP interactions. This cellular interaction might be in a beneficial way. On the contrary, it may increase the production of ROS and/or RNS. This is due to high surface area catalysis (Liu et al. 2010). Oberdörster et al demonstrated in pulmonary *in vivo* study that smaller size NPs showed more persistent lung inflammation with prolonged pulmonary retention and interstitial translocation, and impaired macrophage clearance than larger size NP of the same crystal structure (Oberdorster, Ferin, and Lehnert 1994). NPs with size 1.4 nm showed more cytotoxicity than 15 nm size of the same NP structure by inducing more ROS and mitochondrial damage (Pan et al. 2009). Carbon black NPs with larger surface area (270 m^2/g) had more cytotoxicity on rat lungs than larger particles with smaller surface area (22 m^2/g) (Nikula et al. 1995; Driscoll et al. 1996). Silica NPs with size of 1.2 and 22 nm showed passive transport compared with the larger NPs (greater than 22 nm) that transported through membranous vesicles in *in vitro* study (Roiter et al. 2009). A size range of Ag NPs was assessed after inhalation in rats, NPs with the smaller size (18, 34 nm) showed higher cytotoxicity than larger NPs (60, 160 nm) after 24 hrs exposure. The cytotoxicity was measured by the amount of leaked LDH that showed to be size and surface area-dependent rather than dose-dependent damage (Braakhuis et al. 2016). The size of NPs is suitable for many uptake and endocytosis mechanisms with larger particles around 100 nm or more are endocytosed via clathrin-mediated while the size below 80 nm is mediated by caveolae-dependent mechanism. However, there are many examples of larger or smaller NPs uptaken by various endocytic mechanisms (Shin, Song, and Um 2015).

Figure. 5. Nanoparticle physiochemical properties.

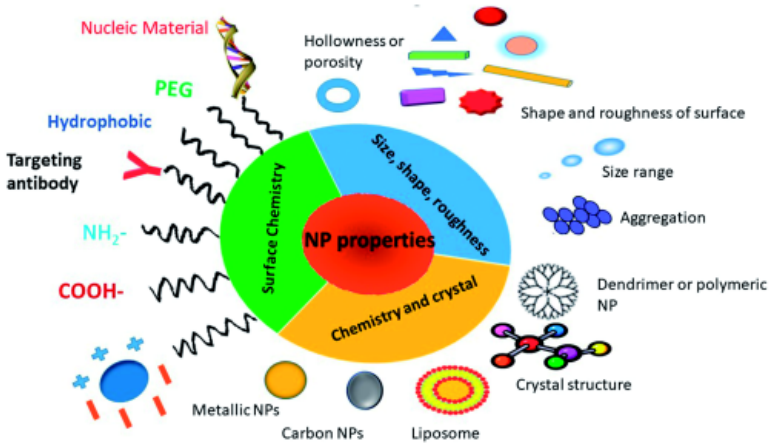


Table 5. NPs properties and possible cytotoxic effects.

NP physicochemical properties	Physicochemical dependant cytotoxicity
NP Size	Cellular penetration and Crossing tissue barriersCellular injury and membrane damageEscape Defense mechanisms and longer retentionTranslocation to other organsInflammationSubcellular localization and organelle impairmentDNA damage
NP agglomeration	Cellular injury by interrupting cellular processes or overloading its capacity.
Surface area	Increased reactivity and toxicityIncrease ROS production
NP shape	Rod or fiber-like more toxic than rounded shaped NPInflammatory induction
NP charge	Positive NP: more cellular interaction and cytotoxicityNegative NP: might increase or decrease cellular uptake, prone to macrophages clearance.
Chemical structure, composition, and purity	Increased toxicityMembrane depolarizationROS generation Mitochondrial damage Inflammation and immune modulation Cellular injury and metabolic impairmentDNA damage Transporting/adsorbing contaminants and toxins
Insolubility/biodurability (bioaccumulation)	Bioresistant inside living systems such as lungs.Persistent Inflammation Long term effects-Cancer

CNTS are considered to have a high aspect ratio, which means that one of the NP dimension is very long, i.e. fiber or rod shaped structure. CNTS have many applications in both industrial and pharmaceutical drug delivery applications. CNTS have shown the pro-fibrogenic properties with NLRP3 activation with IL-1β, TGF-β and growth factors PDGF-AA involved in inflammation, fibrosis, and cell death. Other high aspect ratios of NPs were investigated in vitro, such as CeO_2 NPs (with >200 nm length and aspect ratio >22 nm), showed more inflammation and cytotoxicity compared to the spherical shaped (Xia et al. 2016). Small aspect ratio 6-10 nm showed cell membrane perforation and organelle damage and leakage of lysosomal contents with the stack bundle or needle shaped perforation (Lin et al. 2014).

4.5.2. Nanoparticle surface properties

NPs with a specific charge, surface coatings or modifications are intentionally designed to achieve some pharmaceutical or therapeutic targets such as increasing the stability of the formulation, increasing the efficiency of protein adsorption with cationic NPs, reducing the immune system recognition, prolonging the circulatory half-life (Mu et al. 2014). This surface charge is measured by determining their Zeta potential. The surface charge is designed using different surface coatings or modifications, i.e. positively charged NP (coated with amine groups such as 1,2-Dioleoyl-3-trimethylammoniumpropane (DOTAP), chitosan), or negatively charged NP (coated with acidic groups such as

Polyvinyl alcohol (PVA), poloxamer188 (PF68) or neutral NP charge (near the neutral or slightly negative charged NPs) (Mura et al. 2011). The NP charge plays a major determinant role for their cellular uptake and interactions. Positively charged NPs exert higher cellular uptake, internalization and biomolecular interactions more than negatively and neutrally charged NP and subsequently a higher cytotoxic effect (Bhattacharjee et al. 2010). Their cytotoxicity has been associated due to membrane depolarization, ROS production or mitochondrial damage (Schaeublin et al. 2011). Quartz NPs with surface negatively charged coating showed less particle uptake and inhibition of the oxidative DNA damage in A549 cells compared with the non-coated quartz NPs (Schins et al. 2002). Negatively charged liposomes showed longer blood circulation in comparison to the neutral NP, with the positively charged showed faster clearance and higher toxicity (Bourquin et al. 2018). Similarly, positive charged polymeric dendrimers PAMAM/G4 showed more toxicity compared to the anionic NPs on zebrafish and mouse embryos (Gatoo et al. 2014). Another study had shown the effect of surface coating and the resultant charge; liposomes loaded with elcatonin peptide for inhalation systemic delivery were coated with chitosan oligosaccharides (CH–OS) and polyvinyl alcohol (PVA, anionic emulsifier with hydrophobic anchor) were tested *in vitro* against A549 and *in vivo* rat lung to show enhanced electrostatic interaction with the cell line for the former and reduced for the later. However in *vivo*, both showed longer residence time but CH–OS liposome had interacted with the membrane causing membrane damage and opening formation with faster peptide absorption. Furthermore, the PVA-liposomes showed a more sustained release profile (Murata et al. 2012).

Ag NPs of different sizes and surface coatings were tested in a series of in *vitro* and in *vivo* experiments (Silva et al. 2015). Ag NPs of size 20 nm and 110 nm and coated with either citrate (C20, C110) or polyvinylpyrrolidone (PVP) (P20, P110) were tested *in vitro* regarding their shedding of Ag + ions in media, and the cytotoxicity responses against human bronchial epithelial (BEAS-2B) and murine macrophage cell (RAW 264.7). The findings showed faster dissolution of NP 20 nm size with Ag + ions productions with more cytotoxicity and oxidative stress induction than NP 110 nm. *In vivo* comparisons have confirmed the findings where C20, C110, P20, P110 were administered intratracheal single-dose exposure to a serial dose (0, 0.1, 0.5, 1 mg/kg body weight (BW) in male Sprague Dawley rats. At day 1, 7, 21 postexposure, BALF, lung tissues were collected. All different sized particles had induced significant acute inflammatory polymorphs infiltrations which were clearly manifested in the BALF on days 1 and 7 and persistent to 21 days for higher doses of 0.5 and 1 mg/kg BW. At higher doses, the lungs showed patchy centriacinar infiltration of inflammatory cells such as neutrophils, monocytes and macrophages, necrotic cells and cellular debris that gradually resolved by day 21, but higher doses of NP110 showed persistent cellular infiltrations and debris until day 21. BALF showed higher levels of LDH, protein content, neutrophil count, and inflammatory cytokines. This indicated the smaller size NPs had faster dissolution than larger particles. Another *in vivo* study compared the above male Sprague Dawley (SD) rats exposure to Brown-Norway (BN, asthma and allergic lung model) rats to same single exposure to Ag NPs C20, P20, at a dose of 0.1 mg/kg BW (Seiffert et al. 2015). Both had developed inflammatory lung response, but the BN rats showed eosinophilic allergic and neutrophilic type of inflammation with higher protein content and oxidative stress markers while SD rats showed more neutrophilic inflammation with high levels of cytokines. The response was severe for the C20 than P20, suggesting that the PVP had a role in neutralizing or reacting with the Ag NP. These studies illustrated the effect of NP size, surface area, coating, and animal species on the NP cytotoxicity.

The protein corona rendered positive polymeric and some metal NPs a negative charge with a loss of the membrane depolarizing effect abolishing the hemolytic effect (Cho et al. 2014). It was previously mentioned, the protein corona might limit the effect and/or increase the NP toxicity by masking the charge, increasing the aggregation, enhancing the macrophages uptake and initiating inflammation and immune response.

Shielding the NPs is a mechanism where the NPs are covered with hydrophilic polymers, commonly PEG, to limit the protein adsorption. PEGylated NPs for drug delivery show more stable and longer shelf-life formulations. *In vitro* PEGylated NPs showed lower ROS productions and reduced cytotoxicity than non-PEGylated NPs (Bhattacharjee et al. 2013). PEG NPs showed *in vivo* enhanced bioavailability, reduced immune recognition, longer circulatory half-life and reduced renal clearance (Vij et al. 2010). Another study showed the size and the density of PEG coating had increased the circulatory life of micelles in mice (Wang et al. 2015). Quartz NPs with the surface negatively charged by coating showed less particle uptake and inhibition of the oxidative DNA damage in A549 cells compared with the non-coated quartz NPs (Schins et al. 2002). Negatively charged liposomes showed longer blood circulation in comparison to the neutral liposomes, with the positively charged liposomes showing faster clearance and higher toxicity

(Bourquin et al. 2018). Silver NPs of 15 nm size was coated with Polysaccharides (PS) and hydrocarbon (HC). PS and HC showed different agglomeration pattern and morphology when dispersed into the alveolar lining and lysosomal fluids. The PS coated silver NPs showed de-coating after dispersion (Braydich-Stolle et al. 2014). Three types of amorphous Silica NPs (unfunctionalized, amine or positively coated, carboxylate or negatively coated) were tested *in vitro* against A549 in presence and absence of bovine lung surfactant Alveofact. The presence of lung surfactant increased the toxicity of unfunctionalized and amine functionalized silica NP whilst no change was observed for the carboxylated silica NP and was associated with an increase in the IL-8 production irrespective to the presence or absence of the surfactant. This was explained by the reactivity of the silanol (Si–O–H) group formed with the addition of lung surfactant (Kasper et al. 2015).

Mucoadhesive or penetrative coatings have major influences on the aerosol deposition, retention, and toxicity. Schneider et al. studied the effect of surface coating with mucoadhesive properties (MAP) or mucopenetrating properties (MPP) using polystyrene NPs in *ex vivo* models and *in vivo* mouse models. MAP NPs showed increased entrapment within the mucous layer, aggregation and subsequent clearance, regardless of their size. MPP NPs up to a size of 300 nm (after this, entrapment by the mucous mesh networks) showed better uniform lung distribution and enhanced retention. The NPs were loaded with dexamethasone and studied again in *vivo* concluding the superior effects of the MPP NPs over the MAP NPs and the free drug in reducing acute inflammatory mouse lung model (Schneider et al. 2017). Surface hydrophobicity is another critical factor where it determines the pace of NPs crossing to the underlying epithelial layers and affect the efficiency of the surfactant layer (Hu, Jiao, et al. 2013). *In vitro* study had evaluated the interaction of the NPs with the protein content of the lung surfactant layer where magnetite NPs were functionalized by either hydrophilic starch or hydrophobic phosphatidylcholine, both of negative charge. The hydrophobic component of the used lung surfactant (SP-A) was adsorbing to the lipophilic NP, whereas the hydrophilic (SP-D) surfactant proteins were adsorbing to the hydrophilic NPs. Herein was observed an increase in the macrophages uptake of the hydrophobic NPs evidencing the protein corona role in the NP clearance (Ruge et al. 2012). Hydrophobicity modification by coating Au NPs with peptides/or amino acids can dramatically limit or enhance the cellular uptake (Mu et al. 2014).

MWCNTs had showed increasing the fibrogenic nature with the increase in their hydrophobicity. Other rare earth oxides (REOs) that might be involved in environmental lung exposure were shown to be unstable in the acidic or lysosomal media undergoing dissolution and releasing toxic ions that increase their toxicity. REOs and their ions react with the membrane phosphate groups destabilizing the cell membrane, organelle damage, leaking of lysosomal enzymes and mitochondrial shut down, oxidative stress, NLRP3 inflammasome activation, pyroptosis, and induce autophagy cell death (Li, Ji, et al. 2014, Xia et al. 2016).

The amount of protein participating in NP corona formation with the lung fluid was investigated by Raesch and his group using swine lavage lung surfactant. Magnetite NPs was functionalized with three different coatings (phosphatidylcholine; (lipophilic NP), PEG5000; Hydrophilic PEG NP), and PLGA coating (PLGA NP). The lipid adsorption to NPs was the smallest to PEG NP and the highest to lipid NP. The proteins were more than 300 types identified on the surface of the NPs, with selectivity of SP-A and SP-D adsorption for lipophilic to hydrophilic NPs. Further details and the limitation of the study could be found in this source (Raesch et al. 2015).

4.5.3. NP morphology

The shape of the NPs is another cytotoxic factor affecting the membrane vesicle formation, cellular uptake and digestion, circulatory retention and distribution. NP shape can vary from rounded or oval spheres, ellipsoids, wires or rods, cubes, sheets, cylinders and many others. Generally, the spherical NPs are considered to be safer and faster to be endocytosed than rod- or fiber-like NPs. Spherical NPs can be deposited in the lung via different mechanism such as impaction, settling or diffusion, but the longer aspect or fiber-like NPs are deposited by interception. Pal et al. investigated the shape-dependent cellular interactions for silver NPs where the cellular uptake of spherical NPs showed faster internalization compared to rod NPs (Vij et al. 2010). The fiber-like NPs cause macrophages frustration with increase in the inflammation potential and carcinogenicity, i.e, asbestos. TiO_2 fiber NPs with 15 mm length had higher toxicity compared to shorter 5 mm fibers with more persistent lung inflammation in mice. The SWCNT with a rod-like shape showed higher pulmonary cytotoxic potential compared to spherical (Gatoo et al. 2014). Needle-shaped NPs caused endothelial cell membrane disruption compared to spherical NPs. Non-spherical polymeric NPs showed reduced cell internalization (Mu et al. 2014). SWCNTS were more effective in calcium channel blocking than spherical fullerenes. Hydroxyapatite NPs with different shapes (sphere, needle, plate, rod) were tested against BEAS-2B

cells, showing plate and needle shapes were more toxic than the others and this was related to their direct contact cell damage. Graphene nanosheets were investigated and found to cause direct cell membrane damage due to its adsorption ability that was reduced with increasing serum level concentration in the media. This confirmed the role of protein coronas in reducing the toxicity (Zhao et al. 2013). Recent studies compared the same NPs with different shapes and the resultant cytotoxicity and showed nanowires were more toxic compared to the others. Rare shapes such as filamentous-, worm-, needle-, disc, or ring shaped are gaining popularity for drug delivery applications and may become the next generation drug delivery carriers, though at present, the traditional spherical NPs seems the safe delivery carriers for lung and most of the delivery applications (Truong et al. 2015)

4.5.4. Nanoparticle chemistry, crystal structure, composition, and dissolution

The chemistry of the NPs has cytotoxic implications; affecting the rate of cellular uptake, internalization, ability to produce oxidative stress and cell death (Xia et al. 2006). Different chemistry (Xia et al. 2008) and crystal structure (Braydich-Stolle et al. 2009); show different potential in cytotoxicity, ROS production, and inflammation (Mu et al. 2014). For example, TiO_2 (a common sunscreen NPs) rutile and anatase are allotropes of the same chemical composition but different crystal structures. Rutile NPs showed more toxicity evaluated by ROS production, membrane depolarization, DNA damage than the anatase NPs. The crystal structure affects the aggregation properties where *in vitro* study showed anatase NPs soft and smaller aggregates were more internalized than hard bigger size rutile NPs aggregates (Gatoo et al. 2014). Impurities or any leftover solvents after the NPs manufacturing might play a role in their cytotoxicity (Forest, Vergnon, and Pourchez 2017). The chemical properties that affect the particle solubility and biodegradability should be considered to achieve biodegradable and avoid bio-resistant/bioaccumulating NPs. To what extent, the bio-resistant particles could affect the lungs is still currently a gap in knowledge where chronic studies might help to clear (Jeong et al. 2011). The NPs dissolution ability influences their toxicity. NPs are either easily soluble/degradable or poorly soluble. The poorly soluble or more persistent NPs after the inhalation might predispose to increase the inflammation and lung toxicity. Dissolved NPs can easily escape to the blood and spread to internal organs and cleared by the kidneys. The more persistent NPs will be retained and cleared by mucociliary and macrophages clearance to be distributed later to RES organs, liver drainage to GIT and cleared by feces. International Commission on Radiological Protection (ICRP) classified the NPs into three classes depending on their ability of dissolution: Soluble material with retention half time <10 days, partly soluble within 10 to 100 days, and poorly soluble NPs over 100 days. Other factors depends on dissolution especially with the metallic NPs are the ability to release toxic ions, for example, Ag, Zn, etc (Xia et al. 2016).

4.5.5. Nanoparticle aggregation

The aggregation of NPs is another property that depends upon other NPs characteristics and the dispersing media. It needs to be considered to understand the poor correlation between different toxicity studies, i.e. inhalation or instillation or *in vitro* studies. The aggregation of NPs could play a double effect; it could increase the NPs uptake as larger amounts and more particles will sediment on the cellular surface (Limbach et al. 2005), or it could reduce the cellular uptake if the aggregates are bigger than cellular size to permit the uptake (Drescher et al. 2011). *In vivo* it may play an effective role in reducing their toxicity due to easier macrophages clearance (Takenaka et al. 2001). The aggregates might increase the retention of NPs either in the lung or RES exerting increase in the inflammation and fibrosis (Gatoo et al. 2014).

4.5.6. Repeated dose exposure and bioaccumulation

The repeated lung exposure might happen in both environmental and occupational settings where small doses deposited and might accumulate inducing chronic effects. Some studies tried to mimic chronic exposure by repeated short-term exposure and showed persistent cell damage. Animal studies with doses near the occupational values showed variable degree of lung damage. The bioaccumulation for NPs is not aimed therapeutically (Pietroiusti et al. 2018).

4.5.7. Other physicochemical properties

NP elasticity, porosity, chirality, bandgap, electrical, optical and magnetic properties (Han et al. 2019; Vallabani, Singh, and Karakoti 2019) among many other factors that will have an influence on the design of NPs, loading, efficiency of the carrier, cytotoxicity, delivery method, the desired application, and therapeutic performances. Due to the

novelty of NPs, there is limited literature discussing the cytotoxicity for lung delivery based on these factors, however, the rapidly evolving field will uncover their potentials and hazards.

4.5.8. The dose-dependent toxicity

From the previous knowledge, it can be concluded that the mass dose of the NPs is not alone responsible for the toxicity as the conventional parent bulk molecules where the interpretation of the physicochemical factors induced toxicity should be considered.

In vitro and *in vivo* nanotoxicology studies have shown conflicting results regarding the dose-dependent NP toxicity. The nanotoxicology and inflammation studies are usually conducted with large NP doses, and any material at a high dose could be toxic for the living organisms. Therefore, discrimination between test conditions should be conducted within the range of the aimed therapeutic doses. However, a large piece of information regarding the long-term NP exposure or the bioaccumulation is still unknown (Takenaka et al. 2001). Detailed thorough physicochemical NP characterization and systematic nanotoxicology evaluation of their properties are prerequisites prior to their applications.

5. Nanotoxicology assessment methods

For nanotoxicological evaluation, NPs biocompatibility must be confirmed by two methods; *in vitro* then progress into the *in vivo* methods (Table 6) prior to clinical trials. There are different methods that are increasingly getting more popular in the research communities, i.e. ex vivo, and in silico methods. Prior to NP testing, thorough physicochemical characterization of NPs is a must (Donaldson et al. 2009, Wu, Zhang, and Watanabe 2011). This allows better understanding, easy comparability of different studies, and correlating the NP physicochemical characters with their toxicity or biological profiles that helps better optimization for nano-medical applications (Sayes and Warheit 2009).

Table 6. Summary of advantages and limitations of the different assessment methods.

Assessment method	Advantages	Limitaions
• *In vitro*	• Initial faster screen • High throughput screen • Easy to perform • Easy laboratory control • Easier dosing • Immortal continuous cell lines • Mechanistic and toxicity studies • Permeability and uptake studies • Non-animal alternative	• Not physiological representative • Lack of multicellular interactions • Difficult to translate *in vivo* • Short term exposure • Lack of standardizations • Difficult to compare between different studies • Variations between primary and immortalized cell lines
• *Ex vivo*	• Fast screen • Relatively controlled and easy dosing • Better multicellular and organ response • Mechanistic and toxicity study	• Lack of biodistribution data • Difficult to maintain and handling • Short term exposure

Assessment method	Advantages	Limitaions
• *In vivo*	• Whole body exposure • Biodistribution data • Single or repeated exposure • Acute or chronic toxicity • Short term and long-term studies	• Training and handling • Expensive and technically demanding • Animal discomfort and cruelty • Labor demanding • Interspecies variability • Sometimes poor human translation
• *In silico*	• Predictive ability for mechanistic and toxicity • No animal cruelty • Computer-based studies	• The availability of enough information that enables the study • Lack of experiments standardizations makes it difficult to compare different results

5.1. *In vitro* methods

Cell-based toxicological screening is the first step prior to *in vivo* toxicological evaluation. They are commonly known as cell culture or tissue culture methods. They provide an initial rapid, less costly, and non-animal assessment of NPs. These assessments are very desirable both ethically and economically with better control over the test conditions, allowing wide range of concentrations, single or multiple parameters, and types of NPs to be tested (Frohlich and Salar-Behzadi 2014; Warheit 2018). The most common endpoints are the cellular uptake, internalization and intercellular transport, membrane potential, mitochondrial function, effects on the cytokines or chemokines and cell signaling, oxidative stress (ROS or RNS), cell death and apoptosis, gene regulation and toxicity. The goal of these methods is to achieve a cell-NP interaction response that discriminates between the NPs compatibility versus toxicity (Lewinski, Colvin, and Drezek 2008). These methods commonly employ an enzyme-linked assay with final read-out by fluorometric or spectrometric detectors. Other methods are through special stains and dyes and evaluated under microscopy and flow cytometry. The cells are usually exposed to NP dispersions within a variable range of time (few hours up to 2 or 3 days) then processing the cells for the endpoint results (Wu, Zhang, and Watanabe 2011, Joris et al. 2013). For example, a group of tests including the Alamar Blue, Tetrazolium, Neutral Red, Trypan blue-based assays that are used to detect general toxicity of NPs, LDH assay to detect membrane potential, DCF Fluorescence assay, lipid peroxidation or Glutathione assay to detect the oxidative stress potential. Inflammatory response can be assessed using ELISA kits screening a variety of cytokines and chemokines. Genotoxicity assessed by using micronucleus, COMET, and chromosomal aberrations tests (Jones and Grainger 2009; Drasler et al. 2017; Savage, Hilt, and Dziubla 2019).

The choice of the cell is usually dependant on the aimed target tissue, the route of administration, and the intended application. Types of cells used are either primary or secondary cell lines. The secondary cell lines are a genetically-transformed immortal cell line, hence called continuous or cancerous cell line. They are more widely used and commercially available from many vendors, i.e. American Tissue Type Culture Collection (ATCC). They provide easier experimental handling and maintenance, faster growth and shorter experiment time. The primary cell lines are a harvest of a target tissue on special plates with nutritional media. They are more challenging in isolation, differentiation and phenotyping, maintenance, and special experimental and ethical requirements. As a result, their use is very expensive and onerous testing but more closely representative for the *in vivo* counterparts. The 2 D monoculture is a single and classic cell culture model and commonly lacks the multiple cell interactions and cross communications. The co-cultures of different cell lines for example, lung epithelial cells with macrophages could provide more accurate results by the resemblance of the different cell mixture within the lung tissue. 3 D models have closer representation of a mixture of different cell types within the tissue and provide the cell-cell interaction and communication with

enhanced *in vivo* correlated results (Hughes et al. 2007, Jones and Grainger 2009). 3 D co-culture models known as spheroids or organoids are grown in a 3 D scaffold made of inert materials as collagen, matrigel®, or others, supplemented with stem cells or tissue cell mixtures and stimulated by different stimuli to differentiate the cells into lung structures. ''Organ on chip'' or miniaturized lung and Microfluidic systems are another 3 D co-culture models where the lung organoid is subjected to mechanical and physical factors mimicking the biological environment, i.e. biological-air interface. The advantages of these miniaturized lungs are the very controllable nature, reliable, more physiologically relevant, and simulating biological environment with the ability to screen many drugs prior to *in vivo* (Iyer, Hsia, and Nguyen 2015).

5.2. High-throughput screening methods

Recent advances in cell-based assays allow for toxicity and/or efficacy screening of multiple nanomaterials at multiple concentrations with multiple cell lines, simultaneously. This expansion of experimental design is practically enabled through the miniaturization and multiplexing of the experimental apparatus and method by utilization of either ultra-small 384-well cell culture plates or nanodrop sample chambers on a chip. The nanodrop assay setup allows for different assays with suitable detection features (e.g. fluorescence, and luminescence) to be performed in a fraction of the volume without the cell-activation or photometric effects of the culture plate since the cell culture is performed in a self-contained drop. However, since cells are typically microns in size, nanodrops do not necessarily capture cells themselves, only fluidic cellular exudate for assay and analysis. By assaying numerous material types/functionalization's and material concentrations on numerous cell types, all in parallel, complex interactions between materials and cells may be ascertained through complex data analysis that correlates phenotypes with multi-well plates, cell culture, detection schemes, and recognition schemes (Lemaire et al. 2007, Jan et al. 2008, Jones and Grainger 2009, Savage, Hilt, and Dziubla 2019).

5.3. The main shortcomings for in vitro cell models in evaluating the inhaled NPs

Besides the inherent limitations of *in vitro* cell lines (Table 6), the exposure to NPs is assessed under submerged conditions which is well-proven for many chemicals but when it comes to assess airborne NPs, it is unrealistic and not comparable to the *in vivo* air liquid interface (ALI) exposure. Moreover, the NPs physicochemical properties can be altered when dispersed in biological media prior to testing, limiting the translation to the *in vivo* setting. Hence, ALI exposure systems are being developed and described in many studies (mostly in-house development) to enhance their reliability, relevance, reproducibility, and predictability. Different mechanisms of spray/aerosolization are being developed to generate NPs droplets that can be deposited on the cell layers by similar deposition mechanisms as in the real inhalation exposure, i.e. impaction, gravitational settling, electrostatic deposition, diffusion.Furthermore, the dosing and the dosimetry definition with poor translation from *in vitro* mass or number concentration into *in vivo* surface area needs to be considered and applied. For assessing the lung/or inhalation exposure, the biological response is a strong cross communication between the different types of cells within the lung (that could be up to 40 different cell types) indicating the need of co-culture models under ALI. This should not underestimate the value of the *in vitro* cell lines as a fast screening, inexpensive, and mechanistic approach, but more enhancement is needed. Further details regarding ALI considerations, challenges and improvement can be found in the following sources (Paur et al. 2011; Lacroix et al. 2018; Upadhyay and Palmberg 2018; Ihalainen et al. 2019).

5.4. *Ex vivo* methods

These methods employ viable animal tissues or whole organs while their structure maintained under simulated natural conditions. Either these tissues or organs obtained from humans, e.g. *in vitro* skin absorption test (OECD TG 428), or animals sacrificed for any other cause excluding the need for the experiment, e.g. slaughterhouse (isolated Chicken Eye test; OECD TG 438) (Rasmussen et al. 2019). *Ex vivo* models evaluate NP uptake, distribution, and toxicokinetics. They provide conditions that are realistic and mimic the *in vivo* response, and more control over experimental conditions. For example, *ex vivo* lung slices from either human or rat or mouse are used to assess the respiratory system for various types of NPs to detect cytotoxicity, oxidative stress, genotoxicity, alteration of protein expression, or inflammation (Rasmussen et al. 2019). The use of *ex vivo* studies is new and some variations are there preventing their standardizations (Wick et al. 2015). Moreover, the inability to relate the appropriate dose to the *in vivo* studies remains a challenge. Despite their novelty, these promising studies can provide very complex and realistic information about the NPs uptake, transport and mechanism of action.

5.5. *In vivo* methods

Inevitably, the *in vivo* studies will follow the *in vitro* experiments to validate a range of realistic NPs doses on animals. *In vivo* animal studies provide more consistent clues for uncovering the NPs kinetics, and understanding of the biological exposure-response, dose-range, and toxicity to support the subsequent human clinical trials. Some concerns arise when testing the NPs *in vivo*, first, the dose is difficult to translate from *in vitro* doses to the *in vivo* animals where it is not uncommon for *in vitro* experiments to have very high toxic dose ranges (Oberdorster and Kuhlbusch 2018). The complexity of NPs that can alter the kinetic behavior according the different route of administration requires a specific administration model for each route. The main disadvantages, the issues of interspecies variability; where the lung of mice, or rats is much smaller and less complex compared to human lung, the difficulty in real time bioanalytical assessments of NPs *in vivo* (Saifi, Khan, and Godugu 2018; Zhao et al. 2019).

Registration, Evaluation and Administration of Chemicals (REACH) regulations were adopted for NPs evaluation even though they are different from bulk chemicals. "When assessing nanotoxicity *in vivo*, the following aspects ought to be evaluated according to REACH guidelines: acute, subchronic and chronic toxicity, skin and eye irritation or corrosion and skin sensitization, genotoxicity, reproductive toxicity, carcinogenicity and the NPs toxicokinetics (Joris et al. 2013). Full documentation of the experiment from animal behavior, food and water intake, samples of blood, urine and histopathological specimens of target and other organs, with the toxicity endpoints must be collected. Further tissue organ slices can be used for *in vitro* experiments to get more detailed and representative assays (*ex vivo*) (Joris et al. 2013).

5.6. *In silico* methods

The use of computer-based programs that use huge piece of available data to predict the NPs toxicity. Quantitative structure-activity relationships (QSAR) are rapidly evolving approaches that need developing for the exceptional characteristics of NPs that are different from their conventional chemical counterparts (Myatt et al. 2018). The aim of these models is to provide very accurate models with pre-set standard of parameters that predict and link the NPs based on their physicochemical characteristics and their potential bioactivity, behavior, and toxicity (Nel et al. 2013). For example, QSAR models have been developed on physicochemical characters (Wagner 2018 or pharmaco-kinetic models based on *in vitro* and/or *in vivo* data (Rodrigues et al. 2018), environmental behavior models (Lin et al. 2018), e.g. mode of transport and fate. A study by Gupta et al. had successfully predicted the *in vitro* cytotoxici-ty of five different NPs based on their physicochemical characters (Yan et al. 2017). *In silico* models are yet to be validated for the NPs models even though they are validated by the OECD guidance for conventional chemical testing (Suzuki et al. 2017; Qiu, Clement, and Haynes 2018; Rasmussen et al. 2019). Successful QSAR models are in need for data availability of accurate and consistent grouping of NPs based on physicochemical characters, mechanisms of action, exposure scenarios, standardized *in vitro* and *in vivo* testing conditions. This is a big challenge as of the NPs novelty and changeability. Furthermore, the availability of these models remains another challenge, as not much is known about how to build up these models and what parameters needed. Sharing and reporting data have mutual benefit to close the gaps in NP knowledge (Joris et al. 2013; Raies and Bajic 2016; WHO 2018).

6. Conclusion

The fast-growing field of nanomaterials and its wide variety of applications in various fields of life requires urgent needs of developing methods to efficiently assess their safety prior to marketing. With the increasing research evaluating the safety of the NPs, more understanding of the potential risks associated with their physicochemical properties is emerging. The more detailed physicochemical characterization of NPs prior to their testing, the better understanding, easier comparability and conclusiveness the results are.

However, endless efforts from different regulatory bodies are there to set standards and protocols for NPs testing and inhalation modeling exposures to help minimize any potential risks that might occur when any type of NPs are employed, there are still many technical challenges as well as more need to implement and harmonize international standardizations. Nanotoxicity assessments are currently evaluated *in vitro* prior to *in vivo* animal study. *In vitro* experiments are achieved under submerged conditions and highlight single-cell type-dependent response that lack the whole body or whole organ cross communication response. Newer methods such as 3 D models, co-cultures, lung on chip microfluidics, and testing under ALI provide more physiologically relevant models. *In vivo* methods suffer from

animal specificity when translated to humans in addition to their ethical issues and cost problems. Most of the nanotoxicology studies are performed under high doses that might not be realistic and more relevant doses and concentrations are required. Other models are being developed to enhance the relevance and reliability of the nanotoxicology assessments such as *ex vivo* and *in silico* models that have shown many successful examples of correlation to *in vivo* results.

Unfortunately, the scientific community had shown many discrepancies in their NPs experimentations and hence urgent needs to harmonize standards and protocols for NPs testing. These protocols will ensure single profile of assays that each NPs can be tested on and ensure more general acceptance of conclusive results.

NP pulmonary drug delivery has a very promising potential as noninvasive route that can deliver the drug locally or systemically. Yet there are challenges, optimizing NPs formulation for lung delivery, full understanding of NP aerosol fate and behavior after lung deposition, systemic behavior, and physicochemical properties and their effects on the cellular interactions, NP retention, and biocompatibility. There are many studies evidenced that the dose and physicochemical factors as size, shape, chemistry, etc, all play roles in NPs cytotoxicity. Various mechanisms could be intersected for the same NPs cytotoxicity such as ROS, membrane depolarization, DNA damage, inflammation, cell death. Assessment of unintentional lung exposure to NPs in different environmental and occupational settings as well as from consumer products are still challenged with many technical and economic problems that require development of standards and protocols to assess their safety and set measures to control their environmental release and public exposure. Many studies are needed to close the knowledge gap regarding the release from occupational settings and consumer products into the environment to build realistic quantitative exposure-risk assessments for NPs safety.

Disclosure statement

The authors declare they have no conflict of interest.

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