The influence of biomolecular corona on nanomaterial-cell interactions and nanotoxicity

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Abstract

This paper is based on the premise that nanomaterials in bio-fluid would adsorb proteins and other biomolecules onto the surface in complex biological environments and therefore nanomaterial-cell interactions would be influenced by biomolecular corona. The formation of nanomaterial-corona complex and their impacts on nanomaterial-cell interactions are briefly described. Various studies on nanomaterial-cell interaction with and/or without biomolecular corona are discussed with an emphasis on effects of the biomolecular corona. The importance of standardized nanocytotoxicity assessment methods is stressed.

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I. Introduction

Nanomaterials are distinguished for their unique properties, such as atomic or molecular size scale and extremely high surface-to-volume ratio. It is believed that, with the similar size scale to

that of biomolecules, nanomaterials can penetrate some biological barriers, enter inner and interact with cells. With the development of medical applications of nanomaterial, the concerns of nanotoxicity grow. To make use of nanoparticles in biomedicine, we need to understand the impact of nanoparticles on human health comprehensively. The importance of nanoparticle–protein interactions cannot be stressed enough.

Studies on nanosafety involving different levels from molecule, cell to organism, this paper is going to focus on the cellular level. At this level, many of earlier studies focused on the cellular uptake of nanoparticle and the outcomes of exposing cells to nanoparticles, regardless of interactions of nanoparticles with actual biological environment. First we need to clarify what nanomaterials could undergo in complex biological environments.

The formation of bimolecular corona

When nanomaterials enter complex biological fluid, such as blood plasma or interstitial fluid, with the high surface energy, they would adsorb biomolecules onto their surfaces to stabilize themselves. It is logical and reasonable. It took many years and much effort of predecessors to find this fact and to evoke the scientific communities to realize and accept it. The concept of "nanoparticle-corona" has been widely accepted since it was first introduced in 2007 [1]. Adsorbing biomolecules leads to changes in surface properties of nanomaterials. It will definitely influence their interactions with cells. Understanding the formation of nanomaterial-corona complexes is of significance to understand nanomaterial-cell interactions in biofluids.

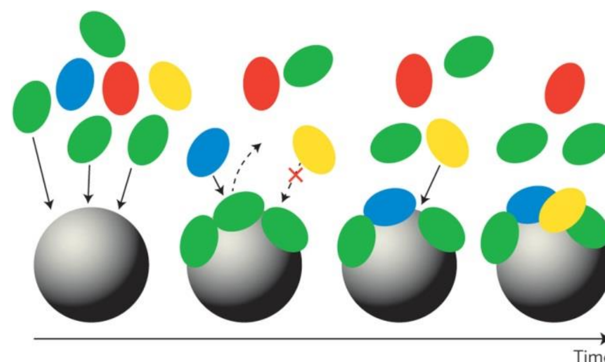

Figure 1 Formation and evolvement of the biomolecular corona. Reprint from [2].

The composition of the corona is not static but evolving with time and environment. As shown in Fig. 1, at the beginning, an initial corona forms from those biomolecules (in green) that first reach the surface. The initial composition is typically made up of those highly abundant proteins. Then

molecules with higher affinity (in blue) arrive and replace those of low affinity. A third molecule (in yellow), with a low affinity for the bare surface, now adsorbs on the nanoparticle surface as a consequence of favorable interactions with the adsorbed biomolecules (in green and blue). An unfavorable biomolecule (in red), does not adsorb at all. The formation of biomolecular corona is very fast (within 0.5min). Actually, after the formation, the composition keeps relatively stable and the number of protein could change a lot [3]. The structure of corona could be divided into two layers. The inner one is called hard corona, with relatively fixed composition, binding tightly to the nanomaterial surface; the outer one is referred to as soft corona, with lower affinity to the nanomaterial, exchanging with the environment rapidly.

Effects of nanomaterial-corona complexes

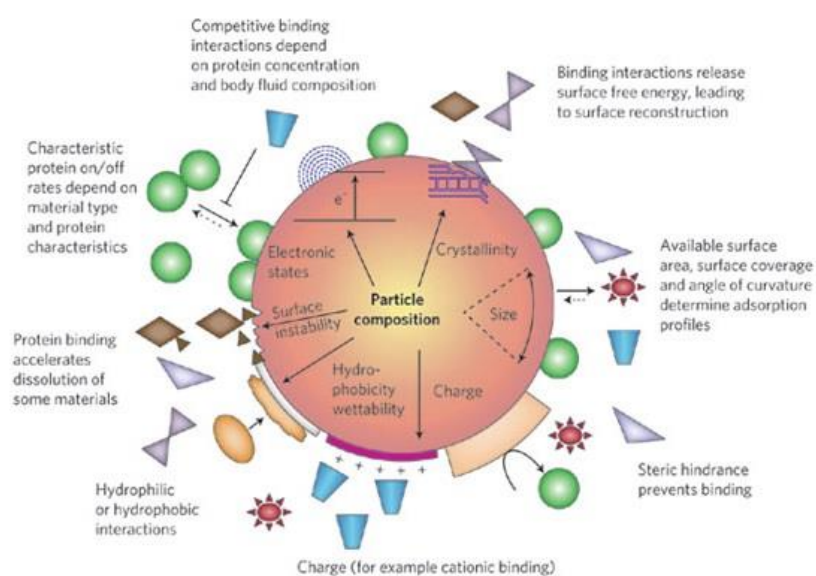

Figure 2 Effects of protein corona surrounding a nanoparticle (NP). Reprint from [4]

The effects of nanomaterial-corona complexed on nanomaterial-cell interactions are displayed in several aspects, as indicated in Fig. 2. : firstly, biomolecular coronas change the morphology of nanomaterials, for example curvature, which could have influence on nanomaterial-cell interaction. Secondly, biomolecular coronas provide nanomaterials new biological identity. The original biological identity of the pristine nanomaterial could be shadowed by the biomolecular corona when it forms. Biomolecules binding to the surface of nanomaterials may have specific interaction with cell membrane receptors, increasing the efficiency of cellular uptake of nanomaterials; they can also change the surface charge, imposing repulsive electrostatic interactions, lowering the cellular uptake of nanomaterials instead. Thirdly, the binding of biomolecules releases surface free

energy of nanomaterials, reducing the possibility of interactions significantly. Many earlier studies are based on the assumption that nanoparticles can keep its “clean” surface in biofluids before meeting with cells, which is normally not the case. Actually, before the adhesion to the cell membrane, nanomaterials have met with biomolecules in the biofluid. It means that there is no pristine surface. Nanomaterial is not necessarily to attach to cell membranes owing to their high surface free energy.

In the absence of biomolecular coronas, nanomaterial-cell interactions are only between cell and nanomaterial itself. In the presence biomolecular corona, the situation is much more complicate. There are not only interactions between the nanomaterial and cell membrane, but also other multiple interactions, i.e. interactions of biomolecules and nanomaterials, interactions of biomolecules in the environment and that have adsorb, or interactions between nanomaterial-corona complexes and cell membranes.

This paper will focus on the different results and conclusions with and without the biomolecular corona. It should be admitted that this field is not fully understood. There are no universal criteria to assess the nanomaterial-cell interactions and nanocytotoxicity they bring. This makes it a tough task to get a reliable comparison between different literatures. Therefore, the importance of standardized nanocytotoxicity assessment methods is emphasized and many factors are taken into account.

II. Nanomaterial-cell interactions and nanotoxicity

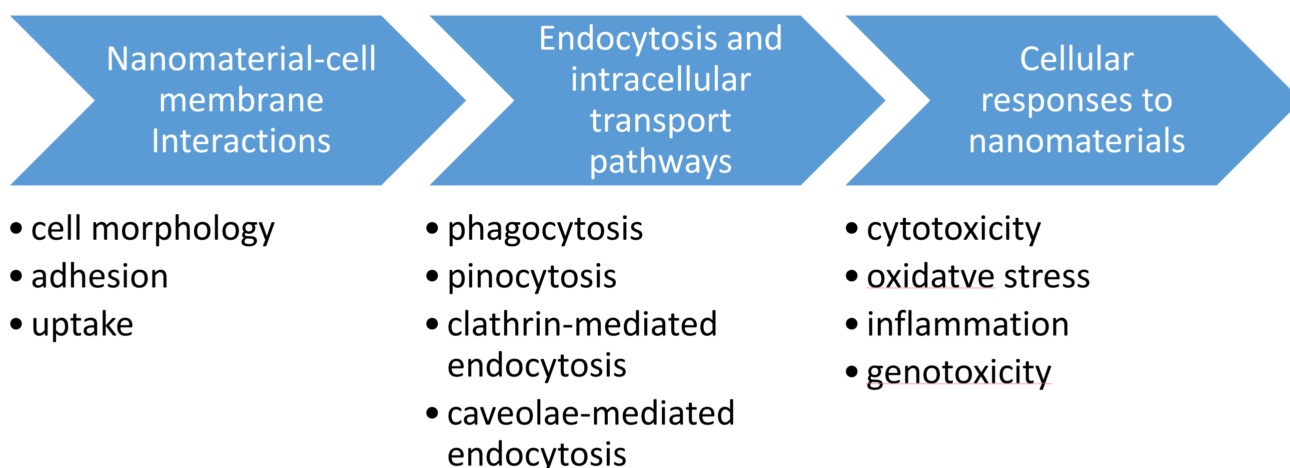

Figure 3 Prominent methods that are used to study the impact of nanomaterials on cells.

As shown in Fig. 3, current researches mainly focus on these three processes of nanomaterial-cell interactions in published papers. To monitoring the interactions on the cell membrane, no single method is enough. For cell morphology, confocal microscopy combined with electron microscopy is widely used. For cellular uptake, the commonly used method is flow cytometry. The internalization process is quiet rapid; to avoid that, studies on cellular adhesion and uptake usually need a condition under the temperature of $4^{o}C$. By a low temperature, cellular internalization is inhibited, making it possible to study the cellular adhesion to nanomaterials. The mechanism of cellular internalization is not clear yet. In order to investigate the possible toxicity of nanomaterials, in vitro toxicity tests are usually used, including cytotoxicity (viability), oxidative stress, inflammatory reactions, and genotoxicity (DNA damage) etc. But the sensitivity of those assays varies. Therefore the nontoxic concentrations are of inconsistency in literatures.

Most studied nanomaterials include metal (e.g. Au [5-7] and Ag [8]), metal oxide (e.g. superparamagnetic iron oxide nanoparticles (SPION) [9-11] and TiO_2[12,13]), polymer (e.g. polystyrene (PS)[14-17]), carbon (e.g. carbon nanotubes[17-20] and graphene[21]) and others like silicon oxide[22]. Because the main approaches for nanomaterials to human body are exposure via skin, inhalation and sequent ingestion, commonly used cell models are mainly cell lines of skin, lung, and gut. Besides, lipid bilayer is another model that is widely used.

This part will cover some typical nanomaterials mentioned above and present different results in the presence and absence of biomolecular coronas. It needs to be declared that it is difficult to make comparison among published works. One of the main reasons is the difference of nanomaterials and cell models for disparate researches. What’s more, different researches vary in conditions and evaluation methods, and even the concentrations of nanomaterials are expressed in different units (nM or g/ml). These variances make comparison of experimental results even tougher. As many researchers have proposed recently, in order to get reliable, comparable and reproducible data, it is of utmost importance to establish standards for in vitro tests, in terms of cell lines choice, sample preparation procedures, cytotoxicity test assays, for example.

The purpose of this paper is to present a broad overview of currently available in vitro data on nanomaterial-cell interactions studies (summarized in the Appendix) and to reevaluate some results from experiments without considering the concept of the protein corona. For some early studies on nanomaterial-cell interaction, although the concept of protein corona was not taken into account, the experimental design involved the condition for the formation of it. To be specific, adding extra

serum in cell culture media for cell and nanomaterials may facilitate the development of nanomaterial-protein corona. We should pay more attention when evaluating these results.

Au

Gold nanomaterials, especially Au NPs, are widely used in biological and medical application such as biosensor, imaging and DNA detection. The advantages of gold NPs are presented in numerous papers. Firstly, they are available in a wide range of size and surface functionalities. Secondly, they are easy to image and detected with electron microscopy even at low concentrations in complex matrices, which allows label-free nanomaterials tracking in cells. Thirdly, they have high chemical stability in biological environments and low toxicity to cells. As a consequence of these advantages, gold NPs seem to be standard materials for researches on nanomaterial-cell interactions. Listed in table1 are some studies on nanomaterial-cell interactions with gold nanoparticles.

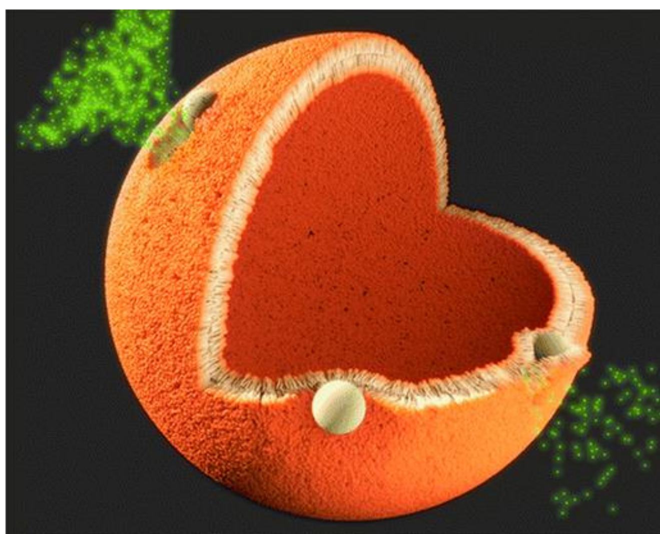

Figure 4 The diagram for liposome leakage assay. Reprint from [5]

The choice of cell might be the first factor we need to consider for standardized nanotoxicology tests. Lipid bilayers are commonly used models as mimics for cell membranes. Babak Y. Moghadam et al. [5] used lipid bilayer vesicles (liposome) as cell membrane model to investigate the effects of nanomaterials with different surface functionality by dye-leakage assay (Fig. 4). Liposome leakage assay is based on fluorescence intensity measurements. Disruption of liposomes with self-quenched fluorescent dye loaded leads to the leakage of fluorescent dye and thus the increase in fluorescence. 10 nm Au NPs were coated with poly(diallyldimethylammonium chloride)(DAD, positively charged), tannic acid (TAN, negatively charged) and poly(vinylpyrrolidone)(PVP,

negatively charged). They compared the liposome leakage results for Au DAD, Au TAN, Au PVP, melittin (a membrane lytic peptide), and a control sample with only liposomes present and found that Au NPs with both positive and negative surface charge induce leakage. Positively charged particles resulted in faster and greater leakage than negatively charged ones. Negatively charged particles had minimal effects. Particles with identical surface functionality and different core materials of Au and $TiO_{2,}$ the leakage results are similar. These findings suggest that surface functionality, rather than particle core composition, plays a key role in the interactions between NPs and lipid bilayers.

The results do show the effects surface charges in the interaction of NPs and cell membranes. However, there are several pitfalls presenting. Firstly, lipid bilayers are simple mimics for cell membranes. In addition to the lipid bilayer, the cell membrane also contains a number of various proteins which can play a role in specific NP-cell interactions and NPs internalization. Secondly, a single assay of the liposome leakage assay cannot demonstrate details of interactions. As they mentioned the mechanism of the leakage is no clear and further researches are needed; it can be caused by particles passing through the membrane, holes left on the membrane after passing, or other reasons. Thirdly, there is no NP-corona complex in this study. The environment is oversimplified as a buffer solution at pH 7.4. Liposomes are negatively charged. The interactions of NPs and liposome are dominated by electrostatic interactions. In view of the complexity of real biological environment, the situation could be different. Since the high surface energy of these NPs, they tend to adsorb molecules onto their surfaces in the environment. Positively charged particles are likely to adsorb negatively charged molecules and then positively charged species may be adsorbed. The net result could be a decrease of the amount of positive charge or conversion of positively charged particles to negative charged particles. Similar effects apply to negatively charged particles. So we cannot reach to a conclusion that the NPs will cause cellular membranes disruption leading to cytotoxicity based on these observations at this point.

Different literature varies in assays for cytotoxicity assessment, impeding reliable comparison. The test assay is one of the crucial factors for standardized nanotoxicology tests. Stefaan J. Soenen et al.[6] proposed the importance of using multiple assays for cell–NP interaction studies. They investigated the effects of poly(methacrylic acid)(PMA)-coated diameter Au NPs on three cell types of C17.2 neural progenitor cells, human umbilical vein endothelial cells (HUVEC), and PC12 rat pheochromocytoma cells, evaluating the cell viability, physiology, morphology and cell functionality. PMA itself would not elicit cytotoxic effects at the concentration range of their experiments,

according to previous reports. Their findings show that the cellular uptake of PMA-coated Au NPs is concentration-dependent. This is in line with previous works by other research groups. They examined effects of Au NPs on cell viability, cytoskeleton, spreading, homeostasis, proliferation, functionality, stem cell differentiation, reactive oxygen species (ROS) induction and focal adhesions and associated signaling. By these multiply methods, they finally defined a noncytotoxic concentration of 10 nM based on cell functionality assessment, which is 1/10 of that obtained by commonly used assays (100 nM). To explain the low noncytotoxic concentration, there is another possibility. In this work, they did not take corona-NP complexes into account; in the description of experimental methods in this paper, they did not mention incubation of NPs in serum (FBS, for example) or other conditions that could provide proteins for corona-NP complexed forming. After internalization into cell, protein corona may not play an important role in the interaction between cell and NPs; the core component itself could interact with the components in cells. Based on this assumption, the formation of NP-corona complexes will not lower the noncytotoxic concentration. On the other hand, on the assumption that protein corona could impede direct interactions of NPs and cell membranes and thus cellular adhesion and uptake efficiency of NPs decrease, the noncytotoxic concentration will definitely be reduced with NP-corona complexes forming.

A multiparametric study could be necessary considering the cytotoxic effects are cell-specific. For particular cell lines, one of those parameters is the determining factor for the noncytotoxic concentration. With different assays, disparate noncytotoxic concentrations can be obtained. Attention should be paid to build a standardized assessment method.

For standardized tests, NP-corona complexes should be considered. When considering the formation of NP-corona complex, new problems arise. There are a number of studies focusing on the formation kinetics and composition of protein corona under different conditions. These issues are not well understood. Gabriele Maiorano et al.[7] demonstrated that cell culture media where NP-corona complexes have effects on cellular response. They investigate two commonly used cell culture media (i.e. Dulbecco Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI)). With same NPs and cells, they found that NP-corona complexes formed in DMEM are less abundant in cells, showing lower cytotoxicity. It proves that the internalization and cytotoxic effects are influenced by the formation of NP-corona complexes.

Similar effects are demonstrated in other nanomaterials.

PS

PS NPs are generally considered to be non-toxic and biocompatible and widely used in biomedical applications, for example, drug delivery systems design. However, Gretchen J. Mahler et al.[23] reported that when exposed to high dose of PS NPs, the iron transportation of intestinal cells increased due to disruption of the cell membrane. That suggests PS NPs could potentially harm cells when interacting with them. We need further exploration of PS NP-cell interactions. On the other hand, PS NPs can be easily synthesized in a wide range of sizes with distinct surface functionalities and thus they are proper model particles to study the effects of the particle characteristics on various conditions.

Anna Lesniak et al.[14] used PS NPs to study the effect of protein corona on NPs adhesion to the cell membrane and found that the adsorption of NPs to cell membrane reduced with the formation of protein corona. They performed experiment with carboxylate modified PS NPs of 100nm on both A459 cell and POPC lipid bilayers and got similar results. As discussed in the previous part, PS NPs shows the similar effect of the presence of a protein corona in cellular adhesion and internalization as Au NPs. Since the release of the free energy of the NP surface, the nonspecific interactions between NPs and cells are reduced, as a result, the adhesion to the cell membrane of NP–protein complexes strongly decreases compared to bare NPs. The lowered adhesion then results in a lower uptake levels. The reduced uptake levels in turn affect the cytotoxicity.

It was reported that the presence of protein corona can reduce the cytotoxicity of gold and nanotubes[19]. However, the mechanism of toxicity reduction is unknown. The first possible mechanism can be simple a lower uptake level as mentioned above. The second possibility can be that the protein or other biomolecular adsorb onto the surface play a role. The influence of the presence of protein corona on the aggregation of NPs can also be a reason. In the preparation of NP, in order to stabilize NPs in suspension, NPs are normally functionalized by different charged group. After the formation of protein corona, the electrostatic interaction can be neutralized. The NPs could be destabilized by the protein corona. Citrate-coated SPION was observed to aggregate and resulted in higher cytotoxicity in the presence of protein corona [9]. This effect can probably play a role in other way around for different nanomaterial, coating and cells.

Understanding the effect of corona on the cellular adhesion/binding is of significant to drug targeting. With corona forming in bio-environment, the targeting would be lost to some degree. Two possibilities present. One is that the targeting is covered by corona, losing targeting. The other

is that corona giving new acceptor pair, resulting in new targeting. All in all, it is a phenomenon we cannot get rid of, so the wiser way is to make use of it. Since the biological identity of biomolecular corona can cover that of NPs, we can design the targeting function on the composition of corona. It needs further exploration and tests.

CNTs

Carbon nanotubes are categorized as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Since the discovery of carbon nanotubes in 1991, CNTs attracted interest in many fields, including biomedical application such as biosensors, biomolecular recognition devices and molecular transporters. The toxicity studies of CNTs began in 2001; the results, however, are highly contradictory. CNTs with intrinsic properties, such as surface charge, shape, length and layer numbers, exhibit different toxicity features. In addition, external factors, like impurities, surface charge, modification and the formation of protein corona, also influence CNTs toxicity significantly.

CNTs are one-dimensional fiber-like nanomaterials. Normally, they are out of nanoscale at one dimension at least, which make they harmful when interaction with phagocytic cells. When CNTs have a length which exceeds 20 μm, they are too large for phagocytic cells to internalize them. In these circumstances, phagocytic cells will attach and eject lysosomal contents onto the surface, especially if the surface has been opsonized. This is referred to as "frustrated phagocytosis". It is the cause of tissue injury in many different autoimmune diseases. Frustrated phagocytosis can be observed in response to MWCNTs as shown in Fig. 5.

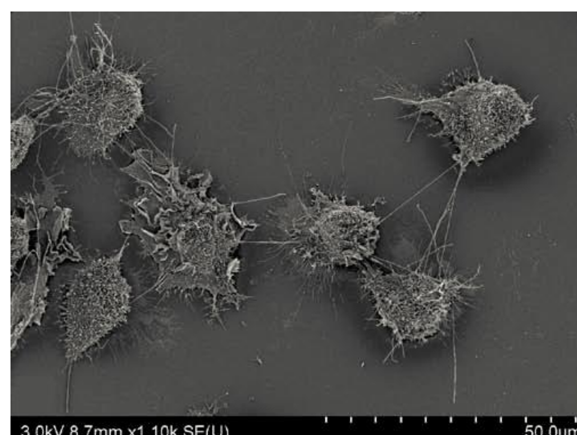

Figure 5 SEM image of J774.A1 cells undergoing frustrated phagocytosis induced by MWCNTs. Reprint from [17]

Maria Davoren et al. [18] evaluated the in vitro toxicity of SWCNTs on human A549 lung cells. Stock suspensions of SWCNTs were prepared in serum containing (5%) and serum-free (0%) medium. After exposure 1.56–800 µg/ml SWCNTs to cultured A549 cells for 24 h, cellular morphology and toxicity were detected (detected by alamar blue (AB), neutral red (NR), coomassie blue (CB), MTT assays and measurement of Adenylate Kinase (AK) and Interleukin-8 (IL-8)). Exposure to SWCNTs resulted in ultrastructural and morphological changes in cultured human A549 lung cells. They did not observe SWCNTs internalization in the A549 cells. Besides, SWCNTs were found to interfere with a number of the dyes used in the cytotoxicity assessment. Therefore, further understanding of these interactions is needed to pick proper assessment methods. As shown in Fig. 6, among the multiple cytotoxicity assays used, the AB assay was the most sensitive. In the presence of serum, lower SWCNTs toxicity was observed. They concluded SWCNT to have low acute toxicity for 24 h exposure.

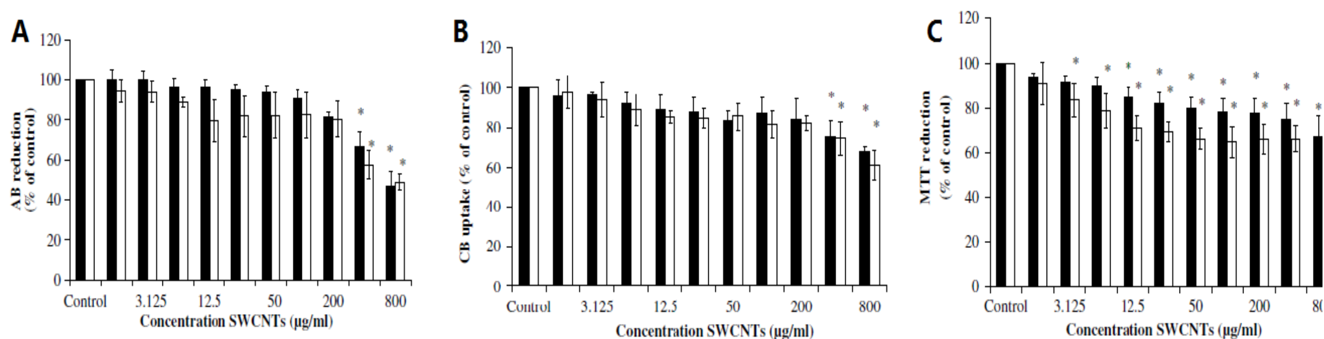

Figure 6 Cytotoxicity of SWCNT to A549 cells after 24 h exposure determined by (A) AB assay, (B) CB assay and (C) MTT assay. Exposures were conducted in media containing 5% serum (■), and serum free media (□). Adapted from [18].

Cuicui Ge et al. [19] also observed the effects of protein corona in reducing SWCNTs' cytotoxicity. After 6 h and 12 h of 30 µg/mL SWCNTs with and without protein-coating, cell viability was detected with cellular cytotoxicity assays. The cell viability was much lower in the absence of proteins, as shown in Fig. 7.

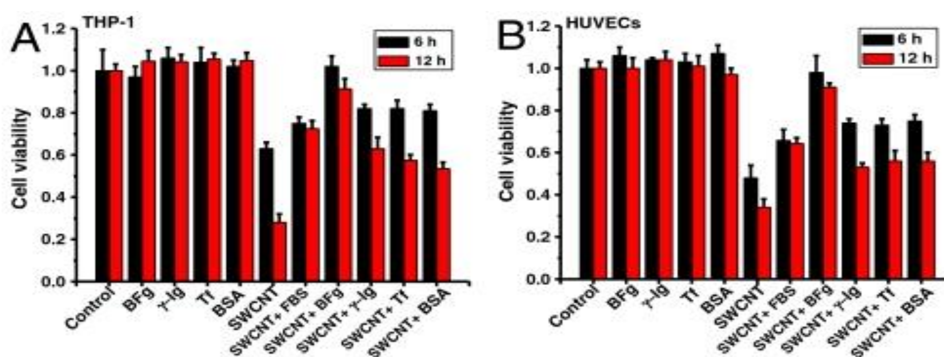

Figure 7 Proliferation and viability of cells in the presence of SWCNTs with/without protein coatings. The differential cytotoxicity of THP-1 (*A*) and HUVEC cells (*B*) in 30 µg/mL SWCNTs with/without protein coatings after treatment for 6 and 12 h. Reprint from [3]

The influence of protein corona on CNTs cytotoxicity indicates that in standardizing assessment test, the formation of nanomaterial-corona complexed must be taken into account. In summary, factors that will significantly influence experimental results on studying nanomaterial-cell interactions include:

1. Intrinsic factors: core material composition, size, shape, surface charge, surface functionalization, cell lines, etc.
2. Extrinsic factors: the presence and composition of biomolecular corona, dose, incubation duration, exposure duration, etc.
3. Others: assessment assays, etc.

III. Conclusions

Based on a number of studies, it is proven that when nanomaterials enter complex biological environment, biomolecules adsorb to their surface, forming nanomaterial-corona complexes. Nanomaterial-cell interactions are intermediated by the biomolecular corona. Reduced adhesion to cells is commonly observed in the presence of biomolecular corona. Cytotoxicity is influenced by the new properties that biomolecular corona brings to nanomaterials.

Although much progress have made in recent years in the field of NP-cell interactions and nanosafety, data on nanocytotoxicity with nanoparticle-corona complexes are limited. Several important problems regarding nanocytotoxicity need to be addressed.

Firstly, the mechanism of the toxicity caused by nanomaterials need to be clarified. A traditional view is that the nanocytotoxicity is caused by the unique size scale of nanomaterials. It is commonly

observed that smaller the particle, higher the cytotoxicity. However, the composition of the core materials also plays a role. Secondly, lack of standardized assessment method impedes comparison between different researches. As shown in the appendix, different studies in this field are comparable regarding to materials, cells, dose, and other conditions. Third, advanced techniques for studying on NP-cell interactions are needed. Many factors in this field are unknown. To make use of nanotechnology in biomedical applications, solving the problems of nanosafety is necessary.

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Appendix

nanomaterial	Cell(s)	Size	Coating	Dose and duration	Corona	assay	results	Ref.
Au	Lipid bilayer	~10nm	DAD(+), TAN(-), PVP(-)	0.005 mg/mL to 0.1 mg/ml for 6 h (steady state)	×	Liposome leakage assays	Leakage is detected at ENM concentrations down to 30 ppb. Liposome leakage is time dependent and increases with ENM number density.	[5]
	C17.2, HUVECs, PC12	4 nm	PMA(-)	5, 10, 20, 50 and 100nM for 2,6,8,12 and 24 h	×	LDH	Similar concentration dependent cellular uptake for all three cell types with different efficiency. Higher NP concentrations (200 nM) reduce cell viability.	[6]
	HeLa and U937	15, 40 and 80 nm	Citrate(-)	500 pM for 48h and 96h	√	LDH, *Apoptosis/necrosis* ,TUNEL, WST-8 assays	The cellular uptake of AuNPs in RPMI is higher than in DMEM, overall exerting higher cytotoxic effects.	[7]
SPION	Human lymphoblastoid cells	8.3 nm	Citrate and PAA	1-10 mM for 24h	√	TEM, Flow cytometry	Devoid of protein corona, citrate-coated particles were shown to aggregate, whereas PAA coated particles exhibit an outstanding stability. PAA coated NPs exhibit a reduced adsorption and/or uptake toward the cells compared to citrate coated particles.	[9]
	L929	4 nm		100,200 ,400 mM24, 48, and 72 h.	√	MTT	After exposure to coated SPION, cell viability demonstrated to be higher compared with uncoated SPION.	[10]

	Human Alveolar Epithelial Cell	9.3-10.4 nm	$-NH_3^+$ $-COO^-$	100, 1000, and 2000 µg/mL. for 24h	√	Trypan Blue Dye Exclusion, PB, MTS,ROS	Proliferation reduction is dose dependent and highest for bare SPIONs. Negatively charged SPIONs were the most biocompatible.	[11]
TiO_2	Syrian hamster embryo (SHE) cells	≤20 and >200 nm	-	10.5, 1.0, 5.0, and 10.0µg/mL for 12, 24, 48, 66, and 72 h	×	MN assay	Cells treated with ultrafine particles(20 nm) had increased micronuclei number and resulted in apoptosis while particles of 200 nm did not induce the change of micronuclei.	[12]
	macrophage cell line (J774.2)	29 and 250 nm	-	0.0975, 0.195 0.39 0.7 mg/mm2 (Particle dose)	×	MTT	Compared with particles of 250nm, ultrafine paricles (29 nm) impair alveolar macrophage phagocytosis more and slowed the clearance.	[13]
PS	A549 and Lipid Bilayers	40 and 100 nm	Carboxylate(-)	100 µg/mL for	both	QCM-D	The presence od protein corona strongly reduces nanoparticle adhesion and nanoparticle uptake efficiency.	[14]
	ISO-HAS1and Lonza	~110nm	amine (+)carboxylate(-)	100 µg/mL for 0.5 min, 2 min, 5 min, 15 min, 30 min, 60 min, 120 min, 240 min and 480 min.	√	Haemocompatibility assays.	Rapid corona formation (<0.5 minutes) is found to affect haemolysis, thrombocyte activation, nanoparticle uptake and endothelial cell death at an early exposure time.	[15]
CNTs	A549 cells	L5–15 µm, D20–60 nm; L1–2 µm, D60–100 nm; L1–2 µm, d <10 nm	-	cytotoxicity :24 h exposure to 5, 50,100, 500 µg/ml DNA damage: 3 h with 50 µg/ml CNTs	×	Cytotoxic Assay(methylene blue assay), comet assay, DNA damage	long and thick MWCNTs induce the strongest DNA damage and increase the total cell number in abdominal lavage fluid	[20]

	THP-1 and HUVES	outer diameter <2 nm and 5 to approximately 30 μm in bundle length	-	30 μg/mL for 6 h and 12 h	√	CCK-8 assay	the competitive bindings of blood proteins on the SWCNT surface can greatly alter their cellular interaction pathways and result in much reduced cytotoxicity for these protein-coated SWCNTs	[19]
	A549	-	-	3.125, 12.5, 50, 200, 800 μg/ml for 24h	both	AB, NR and CB assays, MTT	In the presence of serum, lower SWCNTs toxicity was observed.	[18]