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The Impact of the Physiochemical Properties of Manufactured Nanoparticles on *In vitro* and *In vivo* Evaluation of Particle Toxicity

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Abstract

Recently, many *in vitro* studies evaluating the effects of nanoparticles on cellular physiology have been reported. In *in vitro* systems, the nano-objects induce not only primary effects but also confounding (artificial) effects. Investigations into the physiological and pathological effects induced in cells by *in vitro* exposure to nano-objects may be confounded by the specific physical and chemical properties of the objects. For example, protein adsorption from the culture media to the surfaces of nano-objects can essentially starve the cells. In addition, certain nanoparticles can release metal ions into cell culture or bioassay reagents. The protein adsorption and metal ion release by the nano-objects can interfere with ELISA and LDH assays, producing inaccurate results. Moreover, unstable or non-homogenous suspensions of nano-objects can result in imprecise *in vitro* evaluations of nano-objects. For accurate *in vitro* testing of nanoparticles, we should consider the effects of these three important properties of nanosuspensions: protein adsorption, metal ion release, and suspension stability.

Keywords: Nano-object; Nanosuspension; Adsorption; Metal ion; Suspension stability

Introduction

A nano-object is defined as an object with one or more external dimensions being nanoscale (1-100 nm). A nano-object includes three kinds of materials, a nanoplate, a nanofiber, and a nanoparticle (Figure.1). Nano-objects, particularly metal oxides, metal nanoparticles, and nanocarbons, have various industrial and medical applications. As the field of nanotechnology shifts from scientific study to engineering, the application focus shifts from the laboratory to the market. In the past ten years, various studies describing the biological effects of nano-objects have been reported. Evaluations of the biological activity of manufactured nanoparticles have been performed both in cell culture (*in vitro*) and in animal models (*in vivo*). *In vitro* testing is used to understand the toxic mechanisms of nanoparticles and as pre-screening for the *in vivo* tests. Thus, *in vitro* testing plays an important role in the evaluation and understanding of nanotoxicity. Although the *in vivo* tests are essential for determining the total toxic effect of the particles, such as establishing a no observable adverse effect level (NOAEL) for exposure, the *in vivo* system is complex. In order to understand the biological mechanisms of nanoparticle activity, simplified *in vitro* systems are simple essential. In addition, compared with *in vitro* systems, *in vivo* modeling is expensive and time-consuming. Thus, pre-screening of test materials by *in vitro* testing is beneficial. With regard to nanoparticle-induced inflammation and oxidative stress, the *in vitro* data correspond to the results of the *in vivo* tests [1,2]. However, some evaluations, such as that of the carcinogenicity of nanoparticles, are problematic *in vitro*.

As describes above, *in vitro* testing is essential to evaluate the biological activity of nanoparticles, however, it has been recently suggested that the physical and chemical features of nanoparticles can affect the results of *in vitro* experiments [3,4]. For example, results of *in vitro* tests are sometimes influenced by the physical properties of the nanoparticle suspension [5-8]. Therefore, characterization of the suspension is important for accurate interpretation of data produced by *in vitro* testing. Without properly characterization of the test suspension, the results of nanotoxicity test may be misinterpreted. In some cases, we can observe "confounding" effects caused by the specific physical and chemical properties of the nanoparticle. The confounding effect is an artificial effect in many cases. These confounding effects are specific to *in vitro* experimentation, because they either do not occur or have minimal effect *in vivo*. Importantly, nanoparticle-induced effects observed *in vitro* must be confirmed *in vivo*. If the effect is observed only *in vitro*, it is possibly not toxicologically relevant. Therefore, investigators should distinguish between the primary effects and confounding effects of nanoparticles when evaluating biological

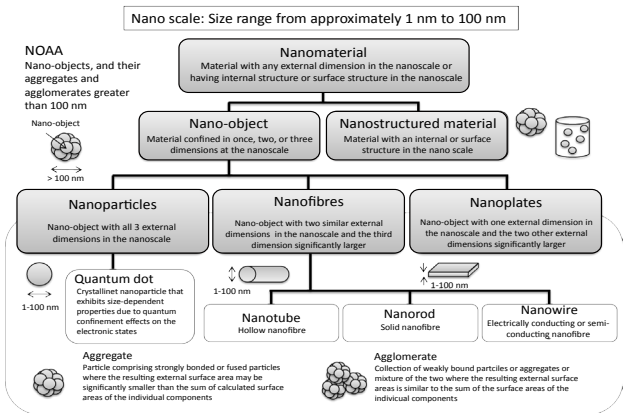

Figure 1: Definition of materials related to nanotechnology. The definitions are based on ISO/TS 27687:2008 [39] and reference [40].

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outcomes. Table 1 summarizes the primary and confounding effects of nanoparticles on *in vitro* and *in vivo* tests and the biological relevance. In this review, we examine the necessary factors for accurate *in vitro* testing of nanoparticles. We focus on three important properties of nanoparticles: (1) adsorptive properties, (2) release of metal ions, and (3) stability of the suspension.

Adsorption Properties of Nanoparticles

Most important confounding influence nanoparticles may have on an *in vitro* system is surface adsorption. Compared with micro-scale particles, nanoparticles have large surface areas and thus, nanoparticles adsorb proteins and salts from the culture medium [9]. In many cases, fetal bovine serum (FBS) and/or horse serum is added to the medium as a supply of essential nutrients and growth factors. When nanoparticles are added to a serum-supplemented medium, serum proteins immediately adsorb to the nanoparticles and form a protein layer on the surface, which called a "protein corona." [10]. The protein corona is involved in the adhesion of the nanoparticles to the cellular surface [11,12]. A major protein present in serum is serum albumin. Bovine serum albumin (BSA) shows negative zeta potential at neutral pH [13,14]. Nanoparticles with a positive zeta potential adsorb more BSA than do nanoparticles with a negative zeta potential. The zeta potential of nanoparticle in the culture medium is dependent upon the adsorbed proteins. Since the nanoparticles adsorb BSA present in culture medium rather than water, the zeta potential of the nanoparticles is negative. In many cases, it is approximately -10 to -30 mV [15-17] (Table 2). Zeta potential influences the cellular uptake of nanoparticles indirectly via protein adsorption [18], and whether or not particles adsorb large amounts of protein is an important *in vitro* property of the nanoparticles. However, nanoparticles can also adsorb proteins *in vivo*. Biological fluids such as saliva, pulmonary surfactant, and skin moisture are largely made up of proteins and inhaled or administered nanoparticles may adsorb these proteins. Therefore, protein adsorption and formation of the protein corona occurs not only *in vitro* but also *in vivo*, and thus contribute to the biological effects of nanoparticle. On the other hand, protein adsorption by nanoparticles can also cause confounding effects *in vitro*.

Adsorption of medium components onto the nanoparticles leads to the depletion of media nutrients and starvation of the cells. In addition, nanoparticles adsorb salts such as calcium from the medium [8,9]. Calcium starvation causes changes in cellular morphology change and growth inhibition [3]. The lipids present in serum may also be adsorbed by the nanoparticles; however, the influence of lipid adsorption on cellular physiology is still unclear. The cellular starvation associated with nanoparticle exposure is an artificial effect specific to the closed experimental *in vitro* systems. Nanoparticle-induced cellular starvation does not occur *in vivo*, because nutrients are continually replenished. Therefore, the biological effects of nanoparticles can be overestimated using cell culture systems in which do not account for this starvation affect. In addition, nanoparticles can adsorb secreted cellular proteins such as cytokines and lactate dehydrogenase (LDH). The levels of these secreted proteins are frequently used as markers of inflammation and cytotoxicity. Nanoparticle exposure can induce the secretion of inflammatory cytokines by cells or physically injure cellular membranes, resulting in LDH release into the surrounding medium. Thus, experimental methods that rely upon cytokine or LDH release as measurements of nanoparticle-induced toxicity may significantly underestimate particle effects [3,6]. Therefore, *in vitro* evaluation of nanoparticles should carefully account for these confounding effects.

Influential factor	Effect for experimental systems	*in vitro* test	*in vivo* test	
Adsorption ability	Adsorption of molecules to particles	Yes	Yes	primary effect
	Influence of adsorption effect on experimental result. (Starvation effect, adsorption of marker molecules, etc.)	Yes	No	confounding effects (artificial)
Metal ion release	Metal ion release from nanoparticles	Yes	Yes	primary effect
	Accumulation of metal ions in experimental system	Yes	No	confounding effects (artificial)
	Influence to experimental reaction such as ELISA	Yes	No	confounding effects (artificial)
Stability of nanosuspension	Formation of secondary particles, aggregates and/or agglomerates	Yes	Yes	primary effect
	Accumulation of the large secondary particles onto cells by gravitational settling	Yes	No	confounding effects (artificial)

Table 1: Important factors of nanotoxicology and these effects on experimental systems.

Nanoparticle	Primary particle size (nm)	Zeta potential (mV)		Reference
		Water	DMEM, 10% FBS	
TiO_2	11	-8	-10	[25]
ZnO	13	-15	-5	[25]
CeO_2	8	+15	-10	[25]
Nanodiamond	Unknown	-51.66	-23.7	[41]
Nanodiamond	Unknown	+42.39	-11.29	[41]
Nanodiamond	Unknown	+45.58	-11.93	[41]

Table 2: Zeta potential of nanoparticles in water and culture medium.

Metal ion Release

In some cases, nanoparticles are more soluble than micro-scale particles. It has been reported that ZnO, CuO NiO, Cr_2O_3, and MgO nanoparticles can release their metal ions into the culture medium [15,19]. Silver nanoparticles also show cytotoxicity by Ag^+ release [20]. Many of these metal ions, such as Zn^{2+}, Cu^{2+}, Ni^{2+} and Ag^+, have cytotoxic activity. The "soluble" nanoparticles show not only extracellular metal ion release but also intracellular metal ion release after cellular uptake [21]. Extracellular metal ions have the same cytotoxicity mechanisms as soluble metal compounds such as $ZnCl_2$. The uptake of extracellular metals is regulated by specific membrane ion channels, and therefore, metal ions do not easily traverse cellular membranes [22]. For example, extracellular Ni^{2+} influx into cells is blocked by calcium channels [23]. On the other hand, phagocytosis of nanoparticles results in the release of the metal ions inside the cell. Consequently, intracellular metal ion concentrations increase, and various cytotoxic mechanisms are induced, such as enhanced oxidative stress and enzyme dysfunction [2,24]. Thus, nanoparticles behave as both particles and metals in the cell.

Additionally, dissolution rate of nanoparticles is also important. The dissolution rate is different in the kind of the dispersant. In many cases, nanoparticles more soluble in the medium than the water. For instance, ZnO nanoparticles showed different solubility by the medium and water [25]. There is a report that the solubility of silver nanoparticles depended on the concentration of Cl- [26]. Solubility of nanoparticles in the biological fluids such as saliva, the pulmonary surfactants, and

the sweats might be also different. And surface property of the nano-object affect to a biodurability [27]. The dissolve rate is important for not only the cytotoxicity but also biodurability.

The cytotoxic activity of nanoparticles depends upon which metal is released. Previous studies report that ZnO and CuO nanoparticles release Zn^{2+} and Cu^{2+}, respectively, into the culture medium, where they induce severe cellular oxidative stress [2,15,28,29]. On the other hand, although MgO and $CaCO_3$ nanoparticles also release Mg^{2+} and Ca^{2+}, the ions are relatively benign [15,30]. In addition, "insoluble" nanoparticles such as TiO_2 and CeO_2 produce only minor biological effects compared to those of "soluble" nanoparticles [15]. The activities of metal ions released from nanoparticles are also observed *in vivo*. In particular, the pulmonary toxicity of "soluble" nanoparticles such as ZnO and NiO is more severe than that of "insoluble" nanoparticles such as TiO_2 [31].

Metals released from nanoparticles are responsible for not only the primary physiological effects but also confounding effects on *in vitro* systems. For example, some metal ions affect LDH activity. The LDH assay is one of the most widely used methods for ascertaining cytotoxicity. Damage to the cellular membrane induced by toxic agents such as nanoparticles results in leakage of the intracellular enzyme LDH into the surrounding medium. In many LDH assay kits, leaked LDH is determined based on its enzymatic activity. However, certain metals such as Zn^{2+} and Cu^{2+} can inhibit LDH activity, and thus, cellular viability is overestimated. In addition, high concentrations of Zn^{2+} can interfere with ELISA reactions [32]. The release of metals from nanoparticles *in vivo* into body fluids such as saliva, pulmonary surfactant, and blood may decrease over time. However, a measurement of the released metal concentration *in vivo* is difficult to obtain, and very little is known about the kinetics of metal release *in vivo*. In contrast, an, *in vitro* system is closed system. The released metals accumulate and the metal concentration in the system increases with time. Therefore, the metal ion concentration differs between *in vivo* and *in vitro* systems. As such, an understanding of the metal ion concentration in the nano-object suspension is important for the evaluation of biological activity.

Stability of the Nanosuspension

Nanoparticles form aggregates and/or agglomerates in suspension. In particular, they form larger aggregates/agglomerates in suspension fluids with a high salt concentration such as cell culture medium. The nanoparticle aggregates, in turn, can form secondary particles consisting of a complex between the aggregate and adsorbed proteins [3]. In the unstable suspension, a gravitational settling of the large secondary particles occurs. In cell culture testing, precipitation of the particles results in a non-homogenous exposure and alterations in the effective concentration of particles that the cells receive. Furthermore, while secondary particles in a stable suspension enter cells via diffusion, in an unstable suspension, the secondary particles reach the cells by diffusion and/or gravitational settling [33,34].

Particle size can also influence the biological effects of nanoparticles. Nanoparticle suspensions include secondary particles of various sizes and the size of the secondary particle that reaches any given cell might be different depending on the exposure time. For example, secondary particles of TiO_2 nanoparticles in the range of 90-130 nm had minimal effects on cell viability and induction of oxidative stress [35]. Thus, although the effects of secondary particle size on cellular physiology is still unclear, this association of secondary particle size with a biological effect is a primary effect of stable suspensions of nanoparticles. On the other hand, if large secondary particles accumulate on cells by gravitational settling, the effective cellular exposure concentration of the nanoparticles is difficult to determine and will not reflect the suspension concentration. Furthermore, large amounts of accumulated particles on cellular surfaces may alter the biological effects of the particles, which will differ from the effects produced by stable suspension. Therefore, an understanding of the stability of the nano-object suspension is important for the evaluation of biological activity (reviewed in reference [4]).

Other Factors of Note

Other physiochemical properties of nanoparticles should be taken into consideration when evaluating biological effects *in vitro* vs. *in vivo*. The pH of the nano-object suspension is important for cellular metabolism and growth. Although commonly used culture medium such as MEM, DMEM and RPMI 1640 have pH buffering ability, the release of large amounts of metal ions from nanoparticles can leads to pH changes in the medium. These pH changes alter cellular physiology to a greater extent in the closed *in vitro* system than *in vivo*. On the other hand, the pH of the medium also changes with cellular metabolism, and the culture medium becomes more acidic over time. Although the biological consequences of pH changes are minimal, understanding how pH affects nanoparticle-induced toxicity is important.

The potential endotoxin contamination of nanoparticle preparations should be also noted. Contamination of endotoxin affects the expression of inflammatory markers such as IL-1 and IL-8. Adsorbed endotoxin on the surface of nanoparticles confounds experimental results. Moreover, nanoparticles interfere with the limulus amoebocyte lysate (LAL) test used to detect endotoxin [7,36]. These observations should be taken into consideration when analyzing nanoparticle-induced cytokine expression *in vitro*.

Finally, the concept of "cell vision" needs to be considered [37]. The cellular responses induced by nanoparticles such as induction of oxidative stress, apoptosis and cell membrane injury are similar regardless of the cell type. However, the strength and specificity of these responses differ by cell type. For example, macrophages produce proinflammatory cytokines such as IL-1β and TNF-α. The cytotoxic effect of these cytokines on nerve cells is more pronounced than for epithelial cells [38]. Thus, the most suitable cell type should be selected for experimentation.

Conclusion

The purpose of *in vitro* testing in toxicology is different from that of *in vivo* examinations. The *in vitro* test is not simply an alternative evaluation tool to an *in vivo* examination. One of the important purposes of *in vitro* testing is to understand the mechanisms of the biological activities of nanoparticles. We can obtain holistic information on the biological consequences of nanoparticle exposure from *in vivo* tests. For example, evaluation of carcinogenicity of a nanoparticle only by *in vitro* testing is insufficient. For the effective evaluation of the biological responses to nanoparticles, it is important to understand the advantages and the limitations of *in vivo* and *in vitro* tests. Sometimes, the nanoparticles induce confounding effects *in vitro*. For accurate evaluation of cellular responses to nanoparticles, an understanding of the properties of nanoparticles-medium suspensions is important. At a minimum, the measurement of three properties is necessary: (1) protein adsorption, (2) metal ion release, and (3) suspension stability.

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