CHAPTER FIVE

LIPID MEMBRANES AS TOOLS in NANOToXICITY STUDIES

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Abstract

The uniqueness of nanoparticles/nanomaterials requires a new experimental
methodology for nanotoxicity studies to complement the conventional techniques
of traditional toxicology. Manufactured nanoparticles are potentially capable of
inducing defects in lipid membranes such as physical disruptions, formation of
holes, thinned regions, etc. The effects of nanoparticles on cell membranes are
one of the key issues we are concerned about. Many methods may be employed
to directly or indirectly explore this issue. Our aim is to show and discuss
possibilities where nanoparticle–cell membrane or nanoparticle–vesicle interac-
tions can be demonstrated. The aim of such studies is twofold. First, they help to
characterize the membrane disruption potential of nanoparticles as their intrinsic
property and second, they provide evidence to link the biological activity of
nanoparticles to their toxic potential. The effects of nanoparticles were observed
on lipid vesicles which are simplified biological membranes. Interactions between
nanoparticles (C60) and lipid vesicles (POPC) were demonstrated. Nanoparticles
caused differences in size distribution of the population of vesicles incubated with
nanoparticles when compared to the control population of vesicles.

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Advances in Planar Lipid Bilayers and Liposomes, Volume 10 © 2009 Elsevier Inc.
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We also present and discuss interactions of nanoparticles with cell membrane in \textit{in vivo} model system. Model invertebrate organism was orally exposed to a suspension of nanoparticles (nanosized TiO$_2$, nanosized ZnO, and C$_{60}$). After the exposure, destabilization of digestive gland cell membrane was assessed by acridine orange and ethidium bromide (AO/EB) differential fluorescent staining.

Results of studies on unintentional interactions of nanoparticles with biological membranes could benefit to the safe use of nanoparticles that are already available in medical, pharmaceutical, or food application. Understanding the nanoparticle-induced defects in biological membranes is among the major challenges of nanotoxicology. In the future, research on nanoparticle–membrane interactions needs to go toward understanding the mechanism of interaction which could lead to less hazardous nanotechnologies.

1. \textbf{Introduction}

Nanoparticles differ substantially from bulk materials having the same composition. Novel properties distinguishing nanoparticles from the bulk material typically become apparent at critical particle lengths below 100 nm. Particles of this size have numerous potential technological applications [1] but maybe hazardous as a result of a variety of interactions with biological systems possibly leading to harmful effects. Evaluating the potential hazards related to exposures to nanoscale materials and its products has become an emerging area in toxicology and health risk assessment.

Particle surface area and particle number determinations have been postulated to play significant roles in the development of nanoparticle-related effects. As the particle size is reduced, the proportion of atoms found at the surface is enhanced relative to the proportion inside its volume. This results in nanoscale particles, which are likely to be more reactive, thus generating more effective catalysts from an applications standpoint. However, from a health implications perspective, reactive groups on a particle surface are likely to modify the biological (potentially toxicological) effects. Therefore, changes in surface chemistry forming the “shell” on a (core) nanoparticle-type may be important and relevant for their biological potential. In addition, surface coatings can be utilized to alter surface properties of nanoparticles to prevent aggregation or agglomeration with different particle-types, and/or can serve to “passivate” the particle-type to mitigate the effects. It is interesting to note that surface coatings, functioning to reduce aggregation and to facilitate particle dispersion, enhance the efficacy of the particle-type in its \textit{designed application}, but may also accelerate translocation of the nanoparticle throughout the body [2, 3].

Recent studies indicate that the toxicity of some nanoparticulates may be related, in large part, to the surface reactivity of the particles, indicating
that the particle surface–cellular interactions may take precedence over the core particle or particle size/surface area per se in influencing the development of (cyto)toxic responses [4]. It was shown that nanoparticles can strongly interact with cell membranes, either adsorbing onto it or compromising the membrane integrity to result in the formation of holes, membrane thinning and lipid peroxidation [5–9]. Recent papers report in vivo and in vitro [7] effects of nanoparticles on membrane stability and pore forming. The term hole or pore can refer to a wide range of structural changes that could lead to enhanced permeability ranging from the formation of an actual hole in the membrane to more subtle changes in content of the membrane leading to enhanced diffusion. Different mechanisms of permeabilization of cell membrane by nanoparticles have been proposed [10]. Membrane permeability could arise from a reduction in density of the plasma membrane. In this case, a hole or pore corresponds to a region of reduced material (lipid, protein, cholesterol, etc.). Furthermore, it is well-known that the phase transition of membrane lipids from the liquid crystalline state to the gel is followed by the ion permeability increase [11, 12]. To what extent nanoparticles affect phase transition of membrane lipids and subsequently increased ion permeability is of high importance in all bio–nano interaction studies.

Until recently, the biophysical behavior of lipid membranes was not of fundamental importance in toxicological studies. This is rapidly changing with the emergence of nanoparticles and questions regarding their safety. Concerns about cell plasma membrane disruption resulting in toxic effects are also paramount in the minds of nanoparticle designers focused on nanoparticle applications.

A convenient system for the study of the effect of various substances on membranes is artificial phospholipid membranes, which can be readily obtained by forming phospholipid vesicles in water solution. As the phospholipid bilayer being the basic constituent of the cell membrane, it is believed that the cell membranes and phospholipid membranes share some important features. Phospholipid vesicles are suitable since the composition of the membrane bilayer can be controlled to some extent. As artificial membranes are much less heterogeneous than cell membranes, it is easier to focus on a particular mechanism of interest. Also, giant phospholipid vesicles are large enough to be clearly observed live under the phase contrast optical microscope, so some processes such as shape transformations can be directly followed. Vesicles can be made of different lipid compositions while the surrounding solution can be manipulated by adding substances and changing the temperature.

It was previously found that some substances can have strong effect on giant phospholipid vesicles, so it would be possible that we would observe such features also due to the presence of nanoparticles. To show examples of such effects on the giant phospholipid vesicles, Fig. 1 shows that upon
addition of a certain plasma protein, which also interacts with phospholipids on the microtitter plate (apolipoprotein H), causes adhesion between vesicles (A). Vesicles were created by electroformation in saccharose solution and rinsed with equiosmolar glucose solution. Since the intact membrane is impermeable to sugar molecules, the sugar composition inside (saccharose) differed from the composition outside (saccharose and glucose) and the inside of the vesicle is darker than the surrounding solution (A, black arrow). After addition of apolipoprotein H, the membrane of some vesicles became permeable to sugar so the inside and the outside solutions mixed (A, white arrow). Furthermore, it can be seen in Fig. 1B that lateral segregation of membrane material was also observed (B). Bar = 10 μm. Adapted from Ref. [13].

Vesicles can adhere to each other due to the mediating effect of the surrounding solution. Figure 2 shows a population of giant phospholipid vesicles before (A) and after (B), the addition of the low-molecular-weight heparin dissolved in blood plasma into the suspension of vesicles. A strong effect of the mediated interaction can be observed.

Constituents of the outer solution can affect the shape of the entire vesicle. Figure 3 shows a pronounced transformation of shape of a vesicle with a long protrusion after the addition of the phosphate buffer saline to the outer solution. Although the membrane seems to stay intact, it can be seen that the shape of the protrusion changes from a tube-like to a bead-like. The shape changes can take place due to electrostatic properties of the membrane–solution interface, due to entropic effects and also due to difference of the osmotic pressure between the outer and the inner solution. The vesicles shown in Figs. 1–3 were created by electroformation method as described in Refs. [13, 14] and are mostly (over 90%) unilamellar [15].

As mentioned above, nanoparticles have also a potential to interact with biological membranes, so similar effects on vesicles could be expected also
due to the presence of nanoparticles. Giant phospholipid vesicles appeared to be an ideal system to study these interactions. An example is presented below. In addition, an in vivo system is presented too which can complete and upgrade the results obtained in in vitro system with lipid vesicles. The combination of in vivo and in vitro methods could significantly improve the relevance of results in nanotoxicology.

2. Effects of Engineered Nanoparticles on Lipid Membranes

2.1. Vesicle Shape Transformation Study

To a large extent, morphology of biological membranes is due to the ability of the molecules within them to move laterally along the membranes. Freely suspended membranes form closed vesicles which exhibit a variety of different shapes and shape transformations.
Phospholipid vesicles (also called liposomes) are under investigation both as models for biological membranes and as carriers for various bioactive agents such as drugs, diagnostic and genetic materials, and vaccines [16–19]. An essential parameter that describes the quality of liposome suspensions is the mean size, respectively the size distribution. A rigorous control of vesicle size and lamellarity within the vesicle population is stringent for achieving the desired behavior and thus the performance of vesicles for these purposes. In most laboratories routine liposome size analysis is carried out by photon correlation spectroscopy (PCS) using commercial instruments. This technique gives a measure for the mean size of the vesicles. Although PCS allows in principle the determination of particle size distributions, the reproducibility and reliability of the method for calculation is insufficient. Quantitative determination of the liposome size distribution, thus, is still difficult [20]. Although a number of powerful approaches like electron microscopy, ultracentrifugation, analytical size exclusion chromatography, and field-flow fractionation have been suggested, none of these approaches has found widespread use due to various limitations.

Recently, Zupanc et al [21] studied the shape transformations of lipid vesicles by machine learning methods taking advantage of a possibility of direct observation of the vesicles within a population comparing to the above methods which are based on indirect measurements. They developed automated image segmentation and analyses for assessing population differences among vesicles incubated in different media.

In the study conducted by Zupanc et al. [21], differences in shapes between a population of nanoparticle-incubated vesicles and a control population were sought. Although the two populations did not show differences at a first sight (Figs. 4A, B and 5), subtle changes in size and shape could be revealed by quantitative analysis of the images. The authors discuss the imaging technologies which generate a wealth of images but require also quantitative image analysis as a prerequisite to turning qualitative data into quantitative values. Such quantitative data are expected to

Figure 4 (A, B) Lipid vesicles incubated without (A) or with nanoparticles C_{60} (B). There are no differences in the shape transformation that could be observed by eye.
open the way toward a detailed view of interactions between nanoparticles and biological membranes. The method based on computer aided analysis of lipid vesicles shape transformations holds many promises for future investigation of morphological characteristics of lipid vesicles.

We believe that the giant phospholipid vesicles are a promising system for the study of complex interactions of nanoparticles with biological membranes. A significant advantage of the system is that nanoparticles–giant phospholipid vesicles interactions could be studied under highly controlled conditions.

2.2. Membrane Destabilization as a Base for In Vivo Studies on Effect of Nanoparticles on Cell Membranes

Different mechanisms of permeabilization of cell membrane by nanoparticles have been proposed [10]. For example, membrane permeability could arise from a reduction in density of the plasma membrane. In this case, a hole or pore corresponds to a region of reduced material (lipid, protein, cholesterol, etc.). Another possibility for the hole or pore formation involves a change in plasma membrane content. For example, the formation of dendrimer/lipid vesicles could create a localized region that was lipid poor and protein and cholesterol rich. In addition, nanoparticles can provoke oxidative stress which in turn leads to lipid peroxidation and cell membrane permeabilization [22, 23].

Studies on juvenile largemouth bass brain cells confirmed that fullerenes do in fact induce oxidative stress [24]. Another possible mechanism

![Diagram](image.png)
of nanoparticle membrane destabilization involves direct oxidation of the lipid membranes. For example, in the case of nanosized TiO\textsubscript{2}, efficient destruction of bacteria has been ascribed to ultra-structural alterations of membranes, especially when irradiated with visible light [25]. In the case of nanosized ZnO, external generation of hydrogen peroxide has been considered to be one of the primary factors of antibacterial activity [26]. In the case of C\textsubscript{60} [27], it has been suggested that induction of lipid peroxidation by C\textsubscript{60} can result from direct physical contact with biological membranes.

An appealing method for assessing cell membrane permeabilization employs acridine orange/ethidium bromide (AO/EB) staining and is widely conducted in \textit{in vitro} studies with different types of cells. Recently, Valant \textit{et al.} [28] modified this method to be applicable for cell membrane stability assessment of entire organ where a model animal is exposed \textit{in vivo}.

The AO/EB assay is based on the assumption that changes in cell membrane integrity result in differences in permeability of cells to AO and EB dyes. Different permeability by the two dyes result in differentially stained nuclei as follows. Acridine orange is taken up by cells with membranes that are intact or destabilized, and in the cell, emits green fluorescence, as a result of its intercalation into double-stranded nucleic acids. Ethidium bromide, on the other hand, is taken up only by cells with destabilized cell membranes, and it emits orange fluorescence, after intercalation into DNA [29]. The assay has been applied to a variety of medical [30], pharmacological [31], biotechnological [32], and cell biology [33] studies.

In the modified AO/EB assay, the digestive glands (hepatopancreas) of a well-known group of terrestrial invertebrates, terrestrial isopods (\textit{Porcellio scaber}, Isopoda, Crustacea) were taken as a model test system (Fig. 6A–F). Animals were orally exposed to a suspension of nanoparticles (nanosized TiO\textsubscript{2}, nanosized ZnO, and C\textsubscript{60}). Digestive gland cells came in direct contact with nanoparticles. The deferential staining showed that all tested nanoparticles, that is, nanosized TiO\textsubscript{2}, fullerenes (C\textsubscript{60}), and nanosized ZnO (Fig. 7), have cell membrane destabilization potential (Fig. 8A and B). Among them, C\textsubscript{60} is the most biologically potent. Differently pretreated particles differ in their biological activity as well. Sonicated nanoparticles are more biologically aggressive than nonsonicated nanoparticles. As expected, bulk material failed to cause any membrane destabilization.

The AO/EB assay is applicable for ranging chemicals and nanoparticles according to their cell membrane destabilization potential. The advantage of the modified AO/EB method is its applicability to \textit{in vivo} study under a realistic exposure scenario, which is via mouth to the digestive gland epithelium.
Figure 6  (Continued)
3. Lipid Membranes in Nanotoxicity Studies

The lack of metrology for nanotoxicological evaluation contributes much of the confusion in the current exposure/risk assessment framework, causes uncertainty in the prediction of toxicity of nanoparticulate material and adds to the challenge of bio–nano interaction research. The lack of assessment technology is a critical issue for regulators and investors, agencies who fund the research or industries that expect to profit from nanotechnology.

At the moment, the use of existing toxicity tests for chemicals is recom- mended also for assessing the hazards of nanoparticles. A major reason for that is the familiarity and interpretability of these tests. However, in the future new tests are need which would include specific characteristics of nanoparticles.

On the basis of present knowledge, it appears that lipid vesicles fulfill many of requirements to be used in assessment of biological potential of nanoparticles in vitro. They are simple models for biological membranes and provide highly controllable and repeatable experimental conditions. In addition, such in vitro system is cost effective mean for toxicological and pharmaceutical studies.
Figure 7  Scanning electron micrograph shows nanosized ZnO which membrane destabilization potential was tested in in vivo test system with P. scaber. The suspension of nanoparticles was orally applied and came in direct contact with the surface of gland epithelium cells (see Fig. 6E and F). After application digestive gland cells are stained with AO/EB (see Fig. 8A and B). (photo taken by M. Bele, National Institute of Chemistry, Ljubljana, Slovenia)

Figure 8  Micrographs of hepatopancreatic tissue of P. scaber taken by fluorescent microscope. (A) A control, untreated animal. No nuclei are stained with EB; (B) severely affected cell membranes. Cells with destabilized membranes have nuclei stained with EB (gray-white). (photos taken by J. Valant, Department of Biology, Biotechnical faculty, University of Ljubljana, Ljubljana, Slovenia)
In the future, studies on lipid vesicles could provide basic understanding of nanoparticle–membrane interactions and, second, the information on biological reactivity of nanoparticles could be used as an additional biological characteristic of nanoparticles apart from their physicochemical properties.

It is very important that biological effects of nanoparticles be tested also under realistic *in vivo* conditions because nanoparticles can be subject to a variety of alterations before they interact with cells. This could not be predicted in *in vitro* cell culture tests.

*In vivo* test with *P. scaber*, which is presented here, assures realistic exposure conditions which could reveal specific and unique interactions of the nanostructures with biological components inside the organism.

The same as in toxicity testing with chemicals most probably also in testing effects of nanoparticles a multilevel approach will be proposed. Such approach could involve more levels of testing that utilize both *in vitro* and *in vivo* methods. Perhaps, it will be visible that tier 1 evaluates nanoparticle–membrane interactions in a simplified *in vitro* system. Positive results at this level can be used either to direct further testing or just to rank–order the nanoparticles in terms of their membrane–destabilization potency.

### 4. Conclusions

A lot of knowledge already exists on interactions between nanoparticles and lipid membranes. This knowledge might significantly contribute to emerging field of nanotoxicology and support determination of safe doses of nanoparticles for humans and environment.

We presented here two different approaches for studying nanoparticle–membrane interactions. The application of machine-learning approach in studying shape transformations of vesicles illustrates the potential of computational imaging in understanding of the dynamics of nanoparticle–vesicle interactions. The other AO/EB staining allows assessing the nanoparticle–cell membrane interactions under realistic *in vivo* conditions.

Nanoparticles will have several beneficial applications in the future, but they should be applied at safe doses. However, at the same time, they have the potential to act as dangerous toxic compounds. This behavior is not surprising. The natural nanoparticles, oligonucleotides, and proteins, as well as more complex functional nanoparticles such as viruses have always presented humanity with a bipolar, Janus face.

### REFERENCES


