Relationship between Surface Modifications of Nanoparticle and Invasion into Suspension Cells
– The sequel of Nanosafe 2008 –

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Abstract. Nanomaterials have a variety of properties for each material. There is little information available on which kinds of material properties have effects on toxicity and kinetics. This paper presents that a relationship between material properties and hazard data by undertaking a bibliographical survey at first. With respect to cytotoxicity, it probably depends mainly on the particle volume dose and to a certain degree on particle solubility. It can be concluded from these results that there is a relationship between material properties and hazard data. Many activities involving nano risk are occurring all over the world. Secondly, we assayed actually for cellular uptake of three kinds of Quantum dots (15 nm, 5.5×10¹² particles/ml) to demonstrate our result of bibliographical survey. Three different surface modification quantum dots (non-modification, -COOH, -NH₃) were mixed with floating Jurkat cells in each. After thirty minute, we washed these cells three times and detected fluorescence by flow cytometer. Almost all the carboxylate particles invaded a cell, about 60% aminated them also invaded and few non-modification particles were taken up. Nanomaterials are often very broadly categorized and named based upon their basic material composition or product shape. Our results confirm that we have to examine which physical-chemical properties affect some adverse effects for each nanomaterial.

1. Introduction
More than 300 products involving nanotechnology ranging from sunscreens to semiconductors are already on the global market [1]. Supported by government funding, multiple nano-related research projects are being carried out at an unprecedentedly rapid pace. Applications of such research include nano-colloidal platinum solution and nano-sized titanium powder, both wildly used in the cosmetics market. However, many companies have come to withdraw from such research because of the potential adverse effects or uncertain risks [2]. The purpose of this work is to seek out the risk factors of nanomaterials using objective methods and to develop software that can, by revealing the potential risks, support nanotechnology developers. Additionally, by using floating cells and quantum dots, we measured the dose of uptake to these cells and suggested the relationship between surface modification and uptake. Nanomaterials possess various properties of powder engineering: specific surface area, zeta potential, van der Waals forces, crystal type and so on. We attempted to determine which hazards
correspond to which aspects of nanomaterials. It is well known that the diameter or surface area of nanomaterials correlates highly with inflammation response. These results appear reasonable and suit reaction kinetics. In this study, we focused on other parameters and found correlations. We then attempted to predict whether these hazardous nanomaterials could invade or transfer into the human body or not (Figure 1).

Figure 1. This shows the main concept of our survey on nano risk assessment.

Nanomaterials have many kinds of characteristics such as zeta potential, specific surface modification, and diameter (black gear). On the other hand, many kinds of hazard data were shown in past reports such as data relating to inflammation and cancer (gray gear). We predicted which nanomaterial characteristics relate to biomarker values indicating various hazards. Half life and kinetics will depend on the particular features of nanomaterials (light gray gear).

It is well known that a diameter or surface area of nanoparticles correlates highly with an inflammation around alveoli region after inhalation [3-5]. On the other hand, almost all the past research treated various nanomaterials such as titanium dioxide, silver and carbon nanotube in each report. These reports suggest that the relationship between material properties and adverse effect of human health, however, tightly correlates have not been obtained yet. We are trying to develop risk assessment software which can suggest a clear direction of controlling manufacturing processes to support nanotechnology developers from Nanosafe 2008 [6, 7]. By using this software, cell mortality may have relevance to a zeta potential, surface modification and solubility. We focused on surface modification and measured a dose of uptake to floating cells actually.

2. Methods

2.1. Bibliographical survey
To better estimate the potential risk of nanomaterials, correlations were investigated between material properties and various biomarkers indicating adverse effects on humans. Nanomaterials have a variety of properties such as solubility, iso-electric point, crystal shape, BET specific surface area and so on. I and Professor Yamaguchi at the University of Tokyo predicted a relationship between material properties and hazard data by undertaking a bibliographical survey. For a viability analysis of various types of particles, many papers were selected. The selected papers consisted of experiments involving viability assays performed in vitro on nano and micro particles of different chemical species. Defining a research domain via a set of queries is not a rudimentary task [8]. We use the following as queries to define the relationship between material parameters and nano risks to human health:
We collected citation data on publications from the Science Citation Index (SCI) and the Social Sciences Citation Index (SSCI), compiled by the Institute for Scientific Information (ISI), because SCI and SSCI are two of the best sources for citation data. We also used the Web of Science, which is a Web-based user interface of ISI’s citation databases. A total of 17,480 papers were retrieved. The data includes all ISI records, i.e. articles, letters, reviews, editorials, meeting abstracts, and so on. Some of these papers may be not relevant to nano risk because we collected data simply by the query described above. Therefore, we focused on the maximum connected component, which currently comprises 6,475 papers and accounts for 37.0%. In other words, we regarded papers not citing other papers in the component as sidetrack in the mainstream of nano risk research field and ignored them. The retrieved data were converted into a non-weighted, non-directed network. Subsequently, the network was divided into clusters using the topological clustering method [9,10]. Traditionally, co-citation has been used to analyze a citation network. However, because co-citation is accompanied by a time lag to create a link, and analysis of intercitation is more relevant in the similarity of pairs of documents than co-citation [11], we used intercitation as a link. The clustering algorithm is based on modularity $Q$, which is defined as follows [9,10]:

$$Q = \sum_{s=1}^{N_m} \left( \frac{l_s}{l} - \left( \frac{d_s}{2l} \right)^2 \right)$$

(2)

where $N_m$ is the number of clusters, $l_s$ is the number of links between nodes in cluster $s$, and $d_s$ is the sum of the degrees of the nodes in cluster $s$. In other words, $Q$ is the fraction of links that fall within clusters, minus the expected value of the same quantity if the links fall at random without regard for the clustered structure. Since a high value of $Q$ represents a good division, we stopped joining when $\Delta Q$ became minus. A good partition of a network into clusters means there are many within-cluster links and minimal between-cluster links.

After clustering the network, we characterized each cluster by the titles and abstracts of papers that are frequently cited by the other papers in the same cluster. It does not mean that all papers in the cluster study the same topics as covered in these frequently cited papers. In fact, each paper studies its own topics, and each paper has its own unique focus. However, as a first approach, it is reasonable to treat these inter-cited papers as a cluster to investigate the brief structure of a research domain and to consider the frequently cited papers in the cluster as representative of the same. The clustered network is visualized by using a large graph layout (LGL) [12], which is based on a spring layout algorithm where links play the role of spring connecting nodes. Thanks to such layout, papers that cite each other and form a group can be located in closer proximity.

2.2. Dose-mortality analysis in vitro – size effect –

From the experimental results reported in the papers, dose and viability data sets were singled out and scatter diagrams were made with dose as the horizontal axis and “% death” as the vertical axis. “% death” means the mortality rate of cells exposed in vitro to nano/sub-micro/micro particles versus control cells calculated using the formulation: 100 (％of cells) - % of still viable cells. On the other hand, the kinds of unit measurements used for dosages were varied by using 4 unit measurement types. “μg/ml” is a unit measurement for particle weight over cell culture media volume. “cm²/ml” is, in one way, a type of unit measurement for particle surface area compared by BET measurement over cell culture media volume, and, in another way, a measurement of particle surface area calculated using a particle’s minor and major axes. When a particle has a spherical shape, calculated surface area ($m^2/g$)
should be $6/pD$ where $\rho$ (g/cm$^3$) is density and D (µm) is diameter. If a particle’s form is fibrous, calculated surface area should be nearly $4/pD$ supposed $D<<$Length. “No. of particles” is a unit for particle count over cell culture media volume. In addition, in the scatter diagrams, each plot had different marks depending on size: one type of key for particles with a primary diameter less than 120 nm and another type of key for those above 400 nm. Generally “nano-sized” is said to be 1-100 nm, but in this paper the collected particle data were for particles of 1-120 nm and of 400-10 000 nm, so the separation value was determined as 130 nm.

2.3. Uptake measurement using floating cell

Three different surface modification quantum dots were mixed with floating cells in each. One quantum dot was just coated with polyethyleneglycol (non-modification; plane) and others were modified with a carboxyl (-COOH) or amino (-NH$_3$) group on polymer layer. The median diameter was 15 nm and the number concentration was $5.5\times10^{12}$ particles/ml. Jurkat floating cells increased at confluence were washed by PBS(-) and were blended with each quantum dot solution. After thirty minutes, we washed these cells three times by PBS(-) solution and detected fluorescence of quantum dot by FACS calibur (Becton Dickinson). Lower there graphs indicate the uptake percentage of each particle in Jurkat cells. X-axis is the number of cells and Y-axis is the height of fluorescent plus; FL2-H (BP 585/42 nm). These procedures were shown in Figure 2.

3. Results

Eleven Papers [13-23] discussing in vitro experiments concerning the viability of cells except macrophages were selected from our search. These papers checked the endpoint of cell viability after 3 days of exposure. For papers in which no BET surface areas described, calculated surface areas were substituted for BET surface areas (If a particle has spherical shape, calculated surface areas (m$^2$/g) should be $6/\rho D$ where $\rho$ (g/cm$^3$) is density and D (µm) is diameter. If it is fibrous in form, calculated surface areas should be nearly $4/\rho D$ assuming $D<<$Length.). The collected cell viability data were used to make scatter diagrams. Figure 3-(a) shows a conventional diagram whose horizontal axis is particle dose (µg/ml) and whose vertical axis is % death (100% - viability (%)). Its keys were differentiated in particle diameter between 0-120 nm and 400-10 000 nm. Plots of 0-120 nm and 400-10 000 nm overlapped significantly so that the effect of size on cell viability is likely to be indicated by particle weight or particle volume (since particle weight and volume show similar values). Figure 3-(b) indicates a diagram whose horizontal axis is the particle dose of BET surface areas (cm$^2$/ml). There seems to be a split between the 0-120 nm group and the 400-10 000 nm groups. Figure 3-(c) shows a diagram whose horizontal axis is the particle dose of calculated surface areas (cm$^2$/ml). Here BET
surface areas of amorphous silica were converted to the smaller value of calculated surface areas, but
the total trend of scattering plots was not altered significantly. Figure 3-(d) indicates a diagram whose
horizontal axis is the particle dose of the number of particles (cm$^2$/ml). Two groups were split in two
more widely and the size effect for cell viability is not likely to be the number of particles.

Figure 3. Relationship between the ratio of cell mortality and particle dose in a culture medium.

Particle dose was written in weight concentration (a). BET specific surface area was measured in
eleven selected articles. The x-axis of (b) is it and (c) is calculated surface area. Particle number was
calculated by weight concentration (d). There was a highly significant difference between the
0-120 nm group and the 400-1000 nm group in the ratio of cell mortality (Wilcoxon, two-tails,
$p < 0.01$).

The collected cell viability data are all shown in Figure 4 with each type of mark representing a
particle species. Figure 4-(a), (b) indicated that a different crystal type gave a different dose-response
curve if these materials were the same. Of course, each material or each oxidation number is presented
in a different effect level to cell mortality as shown in Figure 4-(c), (d). Additionally, a regression line
$Y = a + b \ln(x)$ was drawn on each particle species, and EC50 (the concentration of $\mu$g/ml that reduced
cell proliferation to 50% of unexposed controls) was evaluated. Seen as Figure 5, an x-y scatter
diagram was developed, with the x-axis particle solubility (g/l) and the y-axis EC50 calculated as
described previously. As can be seen, a possible correlation exists: the greater the solubility, the
smaller the EC50 (meaning greater cytotoxicity). At the same time, the plots for anatase TiO$_2$ as well
as Fe, Fe$_2$O$_3$ and ZrO$_2$ contain results that do not clearly support such a correlation. There may be
other, more important factors affecting their cytotoxicity, or the data volume may have simply been
insufficient.
Figure 4. Relationship between the ratio of cell mortality and particle weight in a culture medium.

The ratio of cell mortality indicated a high level of linearity as the weight of the particle is high in each substance. Each substance showed a certain gradient. Toxicity will probably vary depending upon the crystal type of titanium (a).

Figure 5. Relationship between solubility and EC50.

EC50 was determined by the concentration of μg/ml that reduced cell proliferation to 50% of unexposed controls.

To use a Jurkat cell which floats in the medium, we measured the dose of invasion to this cell after 30 minutes. This cell is incubated in medium and FBS generally. However, some reports showed that nanoparticles adducted to the protein floating in an alveolus surfactant. Three kind of quantum dots (plane, -COOH, -NH\textsubscript{3}) mixed with only PBS(-) or PBS(-) plus FBS solution. Right column graphs in Figure 6 indicate the uptake percentage of each particle in Jurkat cells. X-axis is the number of cells and Y-axis is the height of fluorescent puls, FL2-H (BP 585/42 nm). Center is the result of flow
cytometry after blended cell and particle with PBS(-) and right is with PBS(-) and 2% FBS. Few non-modification quantum dots were taken up to cells (0.19%). On the other hand, 99.3% carboxylated particles and 62.9% aminated them were invade to cells after thirty minutes shown in Figure 6.

Figure 6. Uptake of 15 nm quantum dots in Jurkat cells.

Top is FSC-H/SSC-H figure of PBS solution only. Left column shows a percentage of cell uptake and center and right columns show FL2-H/SSC-H figure. Center column is treated cell and quantum dot in a PBS(-) solution and right column is treated them in a PBS(-) with 2% FBS. Polymer coated non-modification quantum dot was mixed with Jurkat cells and measured before staining (the 1st line)
and after staining (the 2\textsuperscript{nd} line). Modified quantum dot of carboxyl and amino base showed in the 3\textsuperscript{rd} and 4\textsuperscript{th} lines.

4. Discussion
To study the effects of particle size and particle species on nanomaterial risk to health, dose-mortality analyses in vitro were performed for this paper. Our results suggest that the effect of size on cytotoxicity is due to particle weight or volume and that another cytotoxic factor is particle solubility. The effect of particle size on cytotoxicity has been argued in many papers. Nano and micro alumina and titania particles have been tested using viability assays, and viable osteoblast density has not always been smaller in nano particle cultures than micro particle cultures when particle weight dose (µg/ml) has been equivalent [14]. This trend is also seen in Figure 2 of our results showing data of additional particle species and more plots. Another paper argued that cytotoxicity does not depend on size or chemical species but, decisively, on total volume [11]. The authors introduced 5 species of ceramic particles into cell culture and measured the dose-response curve with varying doses using three criteria: particle number, particle surface area, and particle volume. Their results were quite similar to our results in terms of splitting formation of plots on scatter diagrams. On the other hand, the cytotoxic effect of aggregated nanomaterials was investigated with little correlation found between BET specific surface area and the cytotoxicity index [15]. For this paper, only BET specific surface area was chosen for determination as to whether it is a strong factor for cytotoxicity, but a number of other parameters, such as solubility, may also require study. Based upon our analysis and examination of already-published papers, the chief factor with respect to cytotoxicity appears to be the particle volume dose with a subordinate but still significant additional factor possibly being particle solubility. A possible mechanism could be that particle volume obstructs intra-cellular adherence or cell-extracellular matrix interaction so that the cell proliferation rate slows depending on particle volume. Under such conditions, a using equivalent particle dose for a given surface area does not work because smaller particles are more susceptible to being pushed away. In addition, dissolved materials in culture media may affect cellular osmotic systems, or larger surface areas of dissolving particles may increase particle volume and thereby obstruct various kinds of adherence or interactions.

From the cell culture experiment, nano particles (15 nm) can invade suspension cells in functional-dependent manners. Especially, almost all the carboxyl base modified quantum dots were taken up to cells in a short time. The similar results were reported [24] from a few years ago. A cell surface has a negative charge and a carboxylated quantum dot also has the same charge. These cells and particles act repulsively by Coulomb’s force. A drug is designed to an anionic generally because of negative surface charge. Our result and some past reports are conflicting about the surface charge. In this experiment, we are not able to distinguish an uptake and adhesion between cell and particle. We will try to reveal these two problems by Nanosafe 2012.

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