Letter to the Editor

Uptake of nanoparticles by cells: do you know their number?

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The experimental exposure of organs or cells to nanosized particles (NSPs) may have various intentions, such as proving the efficacy of NSPs as drug carriers or evaluating their toxicological profile. The quantification of cellular NSP uptake as well as the subcellular NSP localization are therefore of general interest. In many studies, so-called 'representative transmission electron micrographs' showing NSPs within cells or cellular compartments are presented. These images are often meant to support or illustrate measurements of cell function and to relate the localization within an organ or cell to the effects of NSPs. In contrast to the accuracy used to characterize the NSPs and their effects, it is astonishing to notice how little attention is paid to the pitfalls of transmission electron microscopy (TEM) and the potential of quantifying the number of NSP within organs, cells or subcellular compartments.

In the September 2011 edition of *Nanomedicine*, Elsaesser and coworkers [1] presented a stereological approach to quantify the total number of NSPs in cultured cells exposed to gold NSPs. The authors applied a fractionator design [2] to estimate the total number of nanoparticles within the cells and a method based on the mean diameter of the cells and cell profile counts from sections [3]. Using TEM they additionally reported the number of particles within subcellular compartments. For those unfamiliar with quantitative morphology a few words on the concepts of stereology should be said. Design-based stereology is a branch of stochastic geometry and provides a portfolio of methods that help to estimate 3D parameters (such as volume, surface area, length and number) from 2D sections without, or at least with minimal, bias [4]. The methods are based on rigorous sampling, do not rely on assumptions about size, shape, distribution or orientation of the structures of interest and provide hard, biological data that can be statistically tested [5]. In the article by Elsaesser *et al.* [1], the fractionator is used to sample a known fraction of the whole experimental unit (here, a cell culture), which is further analyzed. The data obtained from this fraction (i.e., the number of NSPs) are then multiplied by the reciprocal of the sampling fraction to provide the total number of NSPs taken up by the cells of a cell culture. This elegant approach is easy to apply, and once the number and the volume of the cells is known, the mean number of particles per cell can be calculated and related to the cell volume or the organelle volume. For estimation of the number of cells, the authors have made use of a method that requires cells to be convex, thus, it is not free of assumptions. Although in this case the authors have provided two additional, independent measures of cell number and the cells may fulfill the assumption, in general, a design-based stereological approach for estimating the number or volume of cells is preferable. The disector [6] and the (optical) fractionator [2,7] provide unbiased tools to estimate the number, and various local stereological tools (e.g., selector, nucleator, rotator) are available to estimate mean cell volume [8–10].

During the last few years, we and our colleagues have introduced and applied design-based stereological ways to quantify the number of particles within organs or cells and have related the number of particles associated with a compartment to the relative compartment size [11,12]. If particles were randomly distributed within the cells, then the number of particles associated with each compartment would be proportional to the relative compartment volume. Thus, from the observed particle distribution, a second (virtual) distribution can be calculated that would be expected if the particles were randomly distributed. From the observed and the expected distributions a so-called

‘relative deposition index’ is calculated, which allows for statistical testing of three main questions using chi-squared analysis:

- Is the observed distribution of particles random?
- If not, what is the preferential target of the particles?
- Does the spatial distribution differ between two experimental conditions?

These methods helped to identify the alveolar septal interstitium as the preferential target at 1 h and the septal capillaries at 24 h after pulmonary exposure to titanium dioxide NSP [13]. In an *in vitro* study on gold NSPs, we analyzed the uptake and the trafficking of the particles in different subcellular organelles [14]. By means of the relative deposition index, endocytotic vesicles could be determined as the major compartment of intracellular NSP localization and vesicular NSP trafficking over time was observed. No particles were detected in the nucleus, mitochondria, endoplasmic reticulum and golgi apparatus, and the amount of NSPs in the cytosol was negligible compared with the cellular cytosolic volume fraction. In comparison to the study of Elsaesser *et al.* [1], the determination of absolute particle number per cell was performed by multiplying the average intracellular NSP density with the mean cell volume. The intracellular NSP density was estimated by counting the number of NSPs in a systematic random sampled volume of TEM sections. The volume was obtained by multiplying a definite test field area with the TEM section thickness, with the latter being measured by the method of the smallest fold [15]. For estimation of the mean cell volume, a number-weighted sampling procedure was used by application of the rotator on semi-thin TEM sections [10]. This sampling technique has the advantage that it is free of assumptions and can be applied to any cell culture and tissue sample.

In their abstract, Elsaesser *et al.* [1] conclude that their approach “is suitable for any electron dense nanomaterial resolvable by electron microscopy”. To interpret this sentence correctly, it should be mentioned that it may not be trivial to unambiguously identify NSPs within biological samples and that in many cases conventional TEM will not be sufficient [16]. Additional analytical tools such as energy-filtering TEM, energy dispersive x-ray spectroscopy, immunogold labeling or electron tomography may be required. We have recently emphasized this issue by showing that it is hardly possible to distinguish between NSPs and equally sized cellular material or staining precipitations [17]. Therefore, when analyzing NSPs within biological samples it is a prerequisite to ensure that the particles are identified without any doubt. Other nonmicroscopy-related quantitative detection methods, such as inductively coupled plasma mass spectrometry, might be further useful. However, these approaches miss ultrastructural and subcellular information as obtained by TEM and might not exclusively measure intracellular particles. The application of stereology on TEM images combined with further particle characterization therefore offers a unique and accurate technique to quantify NSPs in cells and tissue also at subcellular level.

Conclusion

Design-based stereology is the method of choice for quantifying NSPs within biological specimens. If the particles can be identified unambiguously, there is a wide range of methods to analyze the structure–function relationships of NSP uptake by cells. It is desirable that the use of stereology in NSP research becomes the gold standard for quantitative morphology as it is in other areas of research [18–20].

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