Reply to "Comment on `Short Ligands Affect Modes of QD Uptake and Elimination in Human Cells'"

■ In a recent review, Iversen *et al.* described the challenges and pitfalls associated with experimental studies of the cellular uptake and trafficking of nanoparticles.[1] The authors reiterate these points[2] in relation to the work of Al-Hajaj *et al.*, who recently published an *ACS Nano* article devoted to the uptake and elimination of quantum dots (QDs) by human cells. Al-Hajaj *et al.* used several complementary methods coupled with pertinent control experiments in order to show the complexities in nanoparticle entry into and exit from the cells.[3] The gist of their article is that nanoparticles with identical cores but different short ligands can enter into and exit from the cell by different pathways.[3] The QDs employed were carefully characterized using a wide range of physicochemical and spectroscopic tools, including asymmetrical flow-field flow fractionation (AF-4), an analytical technique ideally suited to the detection and quantification of nanoparticle aggregates. The study by Al-Hajaj *et al.* was intended to stimulate further research in this area by exploring new approaches that complement the traditional pharmacological, genetic, physical, and analytical methods.

We support the view that several different methods must be used to indicate, suggest, or provide evidence for the route of cellular entry of nanoparticles and of their exit from cells.[1,2] Skotland's group stresses that controls are absolutely essential to validate internalization studies. We strongly support this point of view. Listed below are experiments carried out in our laboratory prior to submission of this work to *ACS Nano*. These measurements were carried out using techniques previously published by us and others. Detailed descriptions were not explicitly included in the article by Al-Hajaj *et al.* for the sake of clarity and conciseness.

(1) Confocal microscopy studies were performed with the four types of QDs. Studies with two QD samples are presented in the article by Al-Hajaj *et al.*[3] Confocal microscopy data were generated from z-stacks.

(2) Confocal micrographs with or without z-stacks or electron micrographs (EM) provide semiquantitative data and must be complemented with other techniques, such as FACS analysis and spectrofluorometry. These two approaches are important *per se* and are not merely complementary to imaging techniques since they involve extensive washing of the cells in order to remove particles loosely bound to the cell surface. The washes include an acid treatment, which is not suitable for confocal microscopy with collection of z-stacks since acid-inflicted cell damage may occur during the long data acquisition times required.

(3) Experiments with inhibitors were performed with the four QD samples. Data collected with two QD samples are given in the article;[3] similar data are obtained with other QDs. Interestingly, THA did not reduce the adsorption of the QDs to the cell surface, as suggested by Skotland *et al.*; if anything, an increased adsorption and/or invagination on some cell surfaces *with reduced* intracellular QD content was noticed. The mechanism of THA-promoted extracellular aggregation is currently unknown (dotted line in Figure 1).[3]

(4) In order to provide sufficient EM information on the spatial distribution of nanoparticles, three-dimensional (3D) EM reconstructions are required. Almost all EM studies published so far in different areas of biology provide only two-dimensional images of random sections. Three-dimensional EM reconstructions are impractical, given the time required to collect the necessary data from enough cells to satisfy statistical requirements. Two-dimensional EM images are illustrative and informative, but they do not provide quantitative answers. One would like to have access to dynamic 3D data with a resolution similar to that of EM. A few laboratories around the world are currently exploring new EM approaches that allow for dynamic cell studies without fixation. Resolution of current confocal microscopes is indeed limited. Co-labeling experiments are currently the best one can do, using commercial dyes to label specific organelles and appropriate software packages (*e.g.*, Imaris) for quantification. We have done this in the past, and we provide a detailed protocol for how to assess the extent of colocalization from multiple z-stacks.[4] In the Al-Hajaj *et al.* study, we used similar approaches. It would have been repetitious to describe them in detail again.

(5) Experiments with and without inhibitors need to be performed in order to show possible effects on adsorption to the cell. We have done many experiments using inhibitors to assess if their presence affects QD emission and/or aggregation. Examples are given in the article by Al-Hajaj *et al.* Additional examples were provided to the reviewers. New data that demonstrate the advantages of AF-4 for the analysis of aggregates will be part of a follow-up manuscript. The effects of inhibitors on nanoparticle association are indeed an interesting area of research, particularly relevant to *in vivo* situations where drugs are coadministered in experimental animals and in humans. At the cellular level, atomic force microscopy (AFM) should be employed in conjunction with AF-4.

In summary, the main findings of Al-Hajaj *et al.* are (1) QDs with the same core carrying on their surface small, chemically distinct ligands behave differently when placed in contact with a given cell type and are taken up to different extents; (2) multiple routes, rather than one way of entry, are at play; (3) asymmetrical flow-field flow fractionation data are essential to provide information about the state of nanoparticle aggregation; (4) multiple complementary methods must be used in studying nanoparticles' entry into and elimination from the cell; and (5) there is a need to further explore the fate of nanoparticles when cells are exposed to different pharmacological agents, particularly those in clinical practice, because

they can affect the cellular trafficking of nanoparticles. Further studies using traditional and new methodologies will give us a better understanding of the journey of nanoparticles "from the outside in and the inside out". We are only beginning to appreciate the complexity of these processes. As long as one is aware of the intricacies of this fascinating area of research and points out new possible contributors and approaches to be employed, progress in the understanding of the biology of nanoparticle journeys will ensue, leading to effective new therapeutic applications.

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