Nano Today (2011) 6, 176—185

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/nanotoday

REVIEW

Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies

Tore-Geir Iversen[a,b,*,1], Tore Skotland[a,b,1], Kirsten Sandvig[a,b,c]

[a] *Centre for Cancer Biomedicine, Faculty Division Norwegian Radium Hospital, University of Oslo, Norway*
[b] *Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Montebello, 0310 Oslo, Norway*
[c] *Department of Molecular Biosciences, University of Oslo, 0316 Oslo, Norway*

Received 12 November 2010; received in revised form 31 January 2011; accepted 7 February 2011
Available online 5 March 2011

KEYWORDS
Nanoparticles;
Endocytic mechanisms;
Intracellular transport;
Pharmacological inhibitors;
Toxicity;
Metabolism

Summary During recent years there has been much interest in the use of nanoparticles for *in vitro* studies as well as for delivery of drugs and contrast agents in animals and humans. To this end it is necessary to increase our understanding of how these particles are taken up and transported within the cells, and to which extent they are metabolized and secreted. In this review we discuss the possibilities, challenges and pitfalls of studying endocytic pathways involved in cellular uptake of nanoparticles. Thus, the use of pharmacological inhibitors, expression of mutated proteins, use of siRNAs and colocalization experiments in such studies are critically evaluated. Although the main focus is on cellular uptake, also aspects of intracellular transport, recycling of nanoparticles to the cell exterior, disturbance of cellular functions, and metabolism of nanoparticles are discussed.

Introduction

Nanoparticles have emerged as promising tools both for basic mechanistic studies of cells and animals, as well as for delivery of drugs or other substances *in vitro* and *in vivo* [1—6]. The rate of uptake and intracellular localization of nanoparticles have been studied by many research groups, and several review articles summarizing the published data are available; see e.g. [7—13]. These reviews reveal that it is difficult to draw general conclusions about how to produce particles for optimal cellular uptake, as the rate and mechanism of uptake turns out to be cell-type dependent

Abbreviations: CME, clathrin-mediated endocytosis; CIE, clathrin-independent endocytosis.

* Corresponding author at: Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Montebello, 0310 Oslo, Norway. Tel.: +47 2278 1826; fax: +47 2278 1845.
E-mail address: toregi@rr-research.no (T.-G. Iversen).
[1] These authors contributed equally to this work.

1748-0132/$ — see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.nantod.2011.02.003

and vary between nanoparticles with different size, charge, and other surface properties. There are, however, several reports showing that nanoparticles of 20—50 nm are taken up more rapidly than smaller or larger particles [14—16]. Because particles with a positive charge will bind to the negatively charged cell surface, one would expect positively charged particles to be endocytosed more efficiently than negatively charged particles. In fact, a study in HeLa cells with positively and negatively charged nanoparticles of equal size (80 nm) showed a 2-fold higher uptake of the positively charged particles [17]. In contrast, a higher uptake of negatively charged nanoparticles has been reported in HEK cells [18]. As discussed below, many of the conclusions drawn about cellular uptake of nanoparticles need to be re-evaluated in light of the present knowledge of endocytic mechanisms.

Cell-type specific variation in handling of internalized particles can be expected, and significant differences in intracellular sorting, trafficking and localization of nonconjugated quantum dots (QDs) have been reported in three closely related human prostate cancer cells [19]. It is clear that for delivery of nanoparticles to heterogeneous tumours, differences in cellular uptake and sorting can have significant implications. Importantly, the polyvalent surface of nanoparticles may induce cross-linking of cellular receptors, start signalling processes, induce structural alterations at the cell surface, and interfere with normal cell function [15,20]. Moreover, when studying cellular uptake of nanoparticles one should keep in mind that the rate of endocytosis may also depend on the cell density [21,22].

So far most focus has been on uptake of nanoparticles into non-polarized cells. Importantly, polarized cells can have different endocytic mechanisms on the apical and basolateral pole [23]. Thus, a nonpolarized epithelial cell cannot be expected to correctly reflect the complexity found in epithelial cell layers where clathrin-independent endocytosis (CIE) is selectively regulated at the apical side and caveolae can be found exclusively at the basolateral side [23].

There is still a lot to learn about cellular uptake and intracellular transport of nanoparticles in order to interpret data from *in vitro* studies and to improve the *in vivo* use of the particles. Also, the recent report that caveosomes is an artifact in cells overexpressing caveolin [24] is important for re-interpretation of data already published regarding intracellular localization and degradation of nanoparticles.

In this article we present a summary of the present knowledge of different endocytic mechanisms and we describe how involvement of the various endocytic pathways in uptake of nanoparticles can be studied, including the pitfalls in performing such studies. We also shortly discuss some aspects of intracellular transport of particles, recycling to the cell exterior, metabolism and disturbances of cellular processes caused by nanoparticles.

Endocytic mechanisms

Cells use endocytosis for uptake of nutrients, down-regulation of growth factor receptors and as a master regulator of the signalling circuitry. There are several different types of endocytosis, all based on formation of intracellular vesicles following invagination of the plasma membrane or ruffling giving rise to larger vesicles [25—28]. Phagocytosis (''cell eating'') is used for uptake of large particles such as bacteria, and is the first step in uptake and degradation of particles larger than 0.5 μm. Pinocytosis (''cell drinking'') is used to internalize fluid surrounding the cell, implying that all substances in the fluid phase area of invagination are taken up simultaneously. There are multiple types of endocytic pathways distinguished by specific molecular regulators as shown in Fig. 1. The clathrin-mediated endocytosis (CME) is by far the best studied of these mechanisms and was for a long time believed to be the only endocytic mechanism in addition to phagocytosis and macropinocytosis. However, during the last 20 years several mechanisms of CIE have been described [25,28,29]. These include dynamin-dependent mechanisms (RhoA and caveolin-caveolae/lipid raft dependent) and dynamin-independent mechanisms (Cdc42 dependent and Arf6 dependent). The Cdc42/Arf1 dependent uptake has by some authors also been called the CLIC/GEEC pathway (CLIC, clathrin-independent carrier; GEEC, GPI-AP (glycosyl-phosphatidylinositol-anchored proteins) enriched early endosomal compartment) [28]. Depending on the receptor studied, the so-called ''receptor-mediated endocytosis'' can involve various mechanisms of endocytosis and should therefore not be used synonymously with clathrin-mediated endocytosis as one sometimes can see in the literature. One can expect an increase in complexity not only when it comes to the number of endocytic mechanisms but also regarding their regulation by signalling.

In a major part of the literature on cellular uptake of nanoparticles the discussion is restricted to clathrin-mediated and caveolae-mediated endocytosis in addition to phagocytosis and macropinocytosis. However, the con-

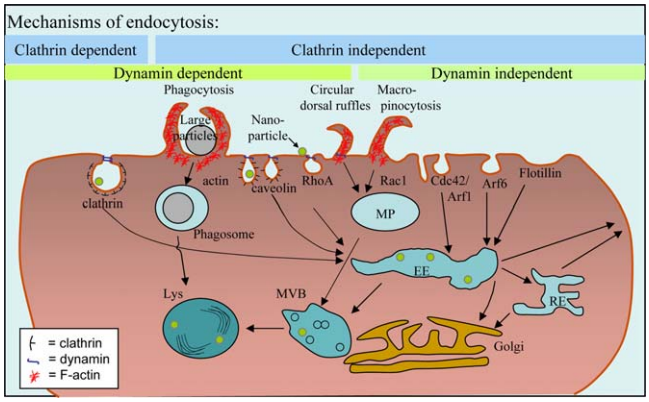

Figure 1 Model of endocytic mechanisms and intracellular transport. Nanoparticles (green dots) and other substances taken up by endocytosis are enclosed within the early endosomes (EE), phagosomes or macropinosomes (MP). These vesicles with particles then mature down the degradative pathway and become multivesicular bodies/late endosomes (MVB) which fuse with lysosomes (Lys). Alternatively, the nanoparticles may be transported back to the cell surface either directly from EE or through the recycling endosomes (RE). The pH drops gradually from the cell surface to lysosomes where pH is 4.0—5.5. The lysosomes contain proteases and other enzymes that degrade most biological substances.

Table 1 Toolbox of pharmacological inhibitors used to study endocytosis.

Agent	Effect	Mechanism affected	Pitfalls
Amiloride (or its derivative EIPA or HOE-694)	Blocks macropinocytosis by lowering submembraneous pH (cytosolic pH close to the membrane) and preventing Rac1 and Cdc42 signalling [87,88].	Macropinocytosis	
Chlorpromazine	Inhibitor of Rho GTPase	CME [89]	Not efficient in all cell lines
Cytochalasin D	Inhibits actin polymerization and may thus lead to actin filament disassembly [44]	Macropinocytosis and may affect several endocytic mechanisms	Not necessarily efficient in adherent cells [44], except for macropinocytosis
Latrunculin A	Sequesters actin monomers, blocks actin polymerization and may thus lead to actin filament disassembly [44]	As for cytochalasin D	As for cytochalasin D
Jasplakinolide	Stabilizes actin and promotes actin assembly [44]	Macropinocytosis [44]	
Dynasore	Inhibitor of dynamin function	Several mechanisms; see Fig. 1.	
Methyl-β-cyclodextrin	Cholesterol depletion by extracting cholesterol	Macropinocytosis and both CME and CIE giving rise to small vesicles	Should be checked for possible leakage of K^+ (more sensitive than protein leakage)
Filipin	Interacts with cholesterol	A number of clathrin-independent and cholesterol-dependent mechanisms (see text)	Unstable and toxic
Nystatin	Interacts with cholesterol	As for filipin	Toxic
Lovostatin	Lowering of cholesterol content by inhibiting cholesterol synthesis	See pitfalls	Uncertain if agents inhibiting cholesterol synthesis are sufficient to inhibit endocytosis
Genistein	Inhibitor of several tyrosine kinases	Inhibits caveolae pinching [43]. Used as a caveolae inhibitor, but not specific for this process (see text)	Affects several processes
Phosphoinositide 3-kinase inhibitors (e.g. wortmannin, LY94002)	Inhibit phosphatidylinositol 3-kinase	Macropinocytosis and compensatory RhoA mediated endocytosis [90]	

clusions in many of these studies reveal limited knowledge about endocytic mechanisms. Typically, it is claimed that caveolae-mediated endocytosis is involved if the uptake is reduced by cholesterol-depleting agents. However, such agents will also affect several other endocytic mechanisms (reviewed in Ref. [27]). Another argument is that the particles are taken up by caveolae because they colocalize with cholera toxin. Again, the argument is not necessarily valid as cholera toxin is taken up by several endocytic mechanisms [30,31]. Caveolae are characterized by electron microscopy as small (50—80 nm), flask-shaped invaginations of the plasma membrane coated by caveolin-1. To our knowledge, no studies have unambiguously demonstrated the existence of larger caveolae able to accommodate uptake of nanoparticles larger than 100 nm. Today we know that caveolae are mostly quite stable cell-associated structures with a number of functions that do not involve vesicle formation [29,32]. Thus, the presence of a ligand in a certain structure such as the caveolae, does not necessarily imply that the ligand is internalized at any significant rate from this structure.

Caveolae-mediated endocytosis was originally described as the way of entry of SV40 viruses into cells [33]. Later the same group reported SV40 to be taken up even more efficiently by clathrin- and caveolin-1 independent endocytosis [34]. Recently, they concluded that the caveosome itself is an artifact in cells over-expressing different constructs of caveolin-1 and that the term caveosome no longer should be used [24]. Thus, articles describing uptake of nanoparticles into caveosomes should be reviewed in light of this new knowledge. This is especially important in light of caveolae-mediated endocytosis of nanoparticles (into caveosomes)

being regarded as a route away from lysosomal degradation of the particles. The caveolae that do pinch off are considered to fuse with normal acidified endosomes being able to transfer material to lysosomes [24,35]. The involvement of caveolae in transcytosis across endothelial cells is well described, but does not necessarily imply that a similar mechanism is involved in uptake into the cell interior of other cell types [36].

Most studies so far have been focusing on uptake of nanoparticles into non-polarized cells. Polarized epithelial cells have been described to have different endocytic properties on the apical and basolateral side [23], and one would expect that also in endothelial cells the two poles will differ. To our knowledge there are only a few publications describing uptake of nanoparticles on the apical side of polarized cells (see e.g. [37]), and no publications showing endocytosis from the basolateral side or clearly demonstrating transcytosis of nanoparticles over endothelial cells *in vitro*. Thus, there is an obvious need to study endocytosis and transcytosis of nanoparticles in polarized cells. In conclusion, the discussion above illustrates that more studies of endocytosis of nanoparticles are required, as well as a critical discussion both about the best way to perform such studies and how to interpret already published data.

Methods used to study endocytosis of nanoparticles

Pharmacological inhibitors

Pharmacological inhibitors are often used to investigate which endocytic mechanism is responsible for cellular uptake of nanoparticles. This approach is far too often based on the assumption that these inhibitors have specific effects on a given endocytic mechanism, but as discussed below and summarized in Table 1, this is normally not the case.

Cholesterol depletion with methyl-β-cyclodextrin (mβCD) and perturbation of the cholesterol function by addition of the cholesterol-binding drugs filipin or nystatin are among the most popular tools for studying endocytic mechanisms involved in uptake of nanoparticles. However, cholesterol is not only important for the caveolae-mediated uptake (as mentioned above), but also for other CIE mechanisms such as macropinocytosis [25,27,38]. Furthermore, clathrin-dependent uptake also requires cholesterol and will be inhibited upon treatment with mβCD [39,40]. Thus, depletion of cholesterol can clearly not be used to identify one endocytic mechanism. Moreover, when using mβCD for cholesterol depletion in cellular uptake studies, it is important to check that the treatment does not destroy the membrane, e.g. by inducing ion leakage through the membrane [39]. In some cases protein leakage is measured, but for instance K^+-leakage with subsequent inhibition of protein synthesis [39] can be expected to occur even at a lower extent of membrane permeabilization. Also an increased entry of Ca^{2+} through the membrane will have a number of effects other than those caused by a change in cholesterol content. For instance, a cytosolic Ca^{2+}-binding protein such as calmodulin can upon increased levels of Ca^{2+} activate various kinases and phosphatases [41]. Also, enzymes like Ca-dependent phospholipase A2 will be activated upon influx of Ca^{2+}, change the membrane composition and through release of arachidonic acid and formation of metabolites have a number of downstream effects [42].

Genistein is an inhibitor of several tyrosine kinases and genistein has been reported to inhibit SV40 induced vesicle formation from caveolae [43]. However, this does not imply that genistein can be used as a selective inhibitor of caveolae function. Genistein will for instance inhibit uptake via clathrin-coated pits of receptors that need tyrosine phosphorylation for accumulation in clathrin-coated pits (e.g. the EGF receptor), and genistein is reported to inhibit F-actin recruitment to clathrin-coated pits and internalization via clathrin-mediated uptake [18]. It should be noted that the inhibitory effect on endocytosis of the three agents cytochalasin D, latrunculin A, and jasplakinolide, all known to perturb intracellular actin dynamics (Table 1), have been described to depend on both the cell line used and the experimental protocol [44]. Recently, the efficacy of endocytosis inhibitors such as genistein, mβCD and potassium depletion on the uptake of transferrin and lactosylceramide were reported to be highly cell line dependent [45].

Methods such as potassium depletion of cells to remove clathrin from the membrane [46] or incubation of cells with hypertonic sucrose [47] were originally used (more than 20 years ago) to block clathrin-dependent endocytosis and to demonstrate the existence of different uptake mechanisms. However, these are unspecific methods with side-effects on cellular physiology. For instance, potassium depletion leads to inhibition of protein and DNA synthesis [48], induces changes in ion fluxes and internal pH, and causes depolarization of cells and reduction in cell volume [49]. Incubation of cells with hypertonic medium, for instance by the addition of 0.45 M sucrose, were in leukocytes found to block receptor-mediated endocytosis but not fluid phase endocytosis. However, high sucrose can also affect other types of endocytosis than the clathrin-dependent, since not only receptor-mediated uptake but also fluid phase uptake was completely blocked in fibroblasts [50].

Thus, these old methods should preferably not be used today when a number of other methods exist that more specifically affect a given process. When addressing the mechanisms of endocytosis, it is warranted to combine different methods since several chemical compounds and methods originally considered being specific later turn out to have additional effects.

Cells expressing mutated proteins and the use of siRNA

Although expression of mutated proteins has advantages compared to the old methods described above, the use of such mutated proteins can have side effects and be less specific than originally believed (see Table 2 and the discussion below). Expression of a mutated protein may result in a higher concentration than that of the normal endogenous protein and thus give rise to lower-affinity interactions not observed in cells lacking such mutated proteins. One should keep in mind that siRNA treatment often performed for 2—5 days, may result in unwanted cellular changes and give observations not relevant for the target protein. More-

Table 2 Examples of endogenous proteins knocked-down or used in mutation experiments when studying endocytosis.

Protein	Properties	Mechanism affected
AP180 and other adaptor proteins	Necessary for nucleating clathrin-coated pits on the plasma membrane	CME
Arf6	Small GTP-binding protein	Arf6-mediated CIE [91] and macropinocytosis [88]
Caveolins	Caveolins can stabilize receptors in caveolae and at the plasma membrane outside caveolae [92]	Caveolae mediated endocytosis. May also regulates other endocytic mechanisms; internalization of autocrine mobility factor is enhanced in cells with less caveolin-1 [92]
Cavins	Scaffolding of caveolae [93]	Discussed in Ref. [93]
Cdc42	A Rho-family GTPase	Cdc42/Arf1 mediated endocytosis [94], phagocytosis [95] and macropinocytosis [88]
Endophilin	Necessary in biogenesis of clathrin coated vesicles	CME
Dynamin	A large GTPase important for membrane scission	Several endocytic mechanisms (see Fig. 1)
Eps 15	Housekeeping component of the CME machinery [96]	CME
Phosphoinositide 3-kinase	Generating PIs necessary for recruitment of factors involved in endocytosis	Several mechanisms, e.g. macropinocytosis [88]
Rac1	Regulation of actin cytoskeleton	Regulator of RhoA mediated endocytosis [97] and macropinocytosis [38]
Rho-A	Regulation of actin cytoskeleton	RhoA mediated endocytosis [98]

over, recent results from our own lab demonstrate that even transfection agents used in connection with siRNA treatment can cause cellular stress reactions (unpublished data). It is also important to be aware of that down-regulation of a factor involved in one endocytic mechanism may lead to up-regulation of other endocytic pathways, and it is therefore a challenge to quantify how the different endocytic mechanisms contribute to uptake in untreated cells [51]. As most methods described above for elucidating the endocytic mechanisms involved are not specific for any of the endocytic mechanisms, the best way to perform such studies is to combine the use of different inhibitors, mutated proteins and siRNAs. It should be clear from the discussion above, that one has to be very careful when interpreting data from such studies, and we urge nanoparticle scientists to collaborate with experts in endocytosis when trying to elucidate the endocytic mechanisms involved.

Localization of intracellular nanoparticles

Most studies of the cellular uptake and transport of nanoparticles have been performed using fluorescently labelled nanoparticles and confocal microscopy. The limited resolution of this technique sometimes makes it difficult to conclude whether the nanoparticles are taken up into the cell or just bound to the cell surface. Electron microscopy after serial sectioning or staining of the plasma membrane with ruthenium red is essential to be sure if true vesicles are observed or if they are just invaginations still connected to the cell surface (Fig. 2C). Measuring the colocalization with various markers of intracellular organelles (Table 3) can be helpful, but it should be kept in mind that apparent colocalization may be obtained from structures in close proximity without real colocalization in the same organelle; see [52] for an example of how an apparent colocalization turns out to be an artifact due to low pixel resolution. False colocalization in a cell can easily be obtained if one

Table 3 Markers commonly used to identify cellular structures.[a]

Markers	Compartment localized
Caveolin	Caveolae
EAA1	Early endosomes
ESCRTs	Late endosomes
GM130 or giantin	Cis-Golgi or cis/mid-Golgi cisternae
LAMP-1	Lysosomes
LDL	Clathrin-coated pits
LysoTracker	Lysosomes and late endosomes (pH < 5.2)
Rab 7	Late endosomes
Rab 11	Recycling compartment
TGN46	Trans-Golgi
Transferrin	Clathrin-coated pits; early and recycling endosomes

[a] *Warning*: Some of these markers may move to other compartments if nanoparticles disturb normal intracellular transport.

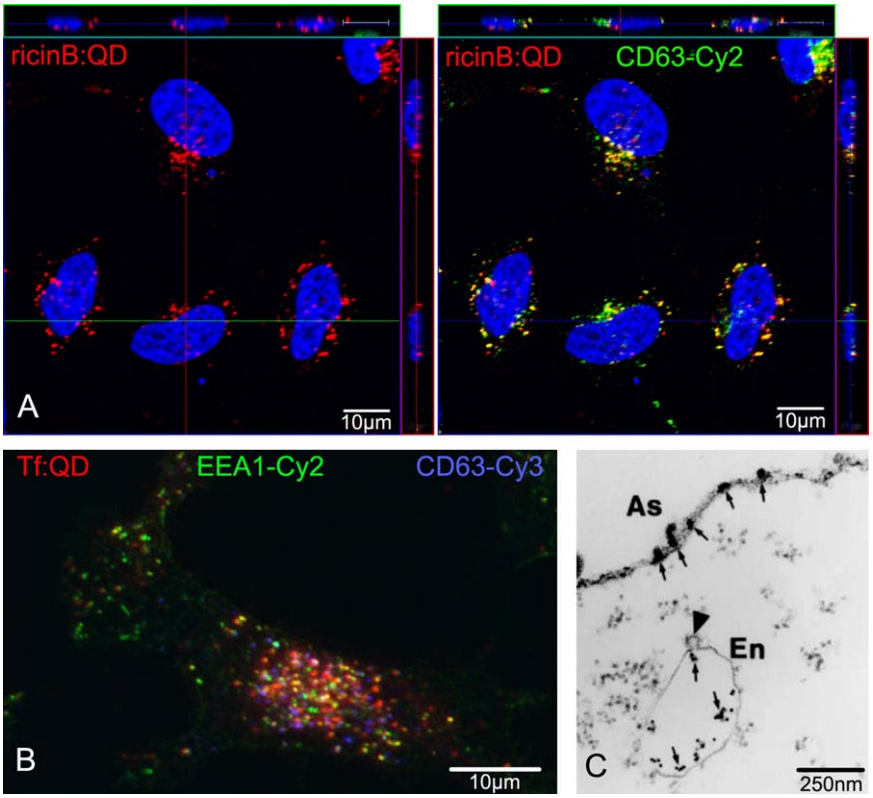

Figure 2 Cellular uptake and intracellular localization of nanoparticles in cells. Panel A shows ricinB:QD and panel B shows transferrin (Tf):QD bioconjugates in HeLa cells. The QDs were bound to the cell surface at 4 °C, and then internalized by the cells for 3 h at 37 °C. The cells were fixed and prepared for immunofluorescence microscopy imaging by labelling with antibodies against the early endosomal marker EEA1 and the lysosomal marker CD63, followed by the corresponding secondary antibody—fluorophore conjugates. The images show the QD bioconjugates in red, and the intracellular markers as indicated in the images. Yellow or pink color in the merged image indicates colocalization: Yellow = red + green, pink = red + blue. The images display partial colocalization of ricinB:QDs with CD63, and of Tf:QDs with EEA1 and CD63. The side-view (z-stack) of cells with ricinB:QDs also demonstrate their intracellular perinuclear localization (nuclear Hoechst staining in blue). Panel C shows an electron microscopy image demonstrating uptake of 10 nm gold nanoparticles within an endosome (En) of MDCK II cells (a section of Fig. 7C in Ref. [86]). The cells were treated with ruthenium red to stain the apical surface membrane (As), thus showing that the particles are in endosomes and not in an invagination connected to the cell surface.

of the fluorescent markers displays large patches or continuous areas of fluorescence that inevitably will overlap with fluorescent spots of the other marker. Furthermore, utilizing fluorescence microscopy and flow cytometry as analytical tools to elucidate cellular uptake of nanoparticles in cells has also been difficult due to electrostatic interactions between nanoparticles and the plasma membrane. Confocal microscopy is easy to use, but because of the limited resolution one should to a larger extent use electron microscopical techniques both to identify the intracellular localization of nanoparticles and to look for possible formation of aggregates within endosomes/lysosomes with low pH (aggregate formation might give rise to a malfunction of the endosomal/lysosomal system in a given cell). One should also keep in mind that the size of the intracellular vesicles containing the endocytosed nanoparticles may give important information regarding the uptake mechanism, as one should expect to see larger vesicles following uptake by macropinocytosis after short time of uptake.

Together with cholera toxin subunit B, the glycosphingolipid lactosylceramide has also been used as a fluorescent marker for caveolae and lipid raft dependent uptake. However, one should be cautious when using lactosylceramide as a marker since it has been shown that exogenously added glycosphingolipids can cause formation of glycosphingolipid-enriched lipid domains at the plasma membrane and increase the ability of caveolae to pinch off [53,54]. Interestingly, addition of glycosphingolipids was reported to induce internalization of integrins via caveolae-like structures [53].

As shown in Fig. 1, one can expect nanoparticles taken up by cells to be found mainly within endosomes or lysosomes. These organelles have low pH, and lysosomes contain proteases and other enzymes that degrade a variety of biological substances. Most studies report nanoparticles to be found within cellular structures that are likely to be endosomes or lysosomes, and in some studies this has been addressed in more detail. Thus, cationic polystyrene nanospheres were mainly observed within LAMP-1 positive organelles [55], i.e. lysosomes, and different QDs colocalized with markers for endosomes and lysosomes [56], such as EAA1 (marker for early endosomes), LysoTracker (marker

for acidic organelles/late endosomes) and CD63 (marker for lysosomes); see Fig. 2A and B.

Nanoparticles of varying size and composition may be taken up by various endocytic mechanisms ending up in different intracellular trafficking pathways. Anionic polymeric particles of 43 nm in hydrodynamic diameter were reported to be internalized mainly via clathrin-dependent endocytosis and directed to the degradative pathway, whereas nanoparticles of 24 nm were taken up via a cholesterol-independent, non-clathrin- and non-caveolae-dependent pathway with no routing into the degradative pathway of HeLa and HUVECs [57]. Likewise, a significant fraction of ricinB-QDs (30 nm) and transferrin-QDs (40 nm) was reported to be routed into non-lysosomal vesicles of HeLa, HEp-2 and SW480 cells [56,58]. It is not surprising that the particles themselves can affect the routing (see below) and therefore probably also the composition of the organelle in which they are found. Thus, the mechanism of sorting and routing of nanoparticles into these poorly characterized endosomal entities awaits further investigations.

Delivery of nanoparticles to the cytosol

Delivery of nanoparticles into cells *in vitro* by using electroporation or microinjection is outside the main scope of the present discussion; for a review see [11]. The possibility to deliver substances directly into the cytosol by using positively charged ''cell-penetrating peptides'' has been an issue of much discussion for many years. Several authors have reported or discussed the option of coupling such peptides to nanoparticles [11,59,60]. Although different groups have come to different conclusions, most nanoparticles coupled to cell penetrating peptides seem to end up in endosomes [61], whereas microinjection results in a more homogenous distribution throughout the cytosol [60]. Nativo et al. [62] reported uptake of gold nanoparticles (16 nm) modified by positively charged cell-penetrating peptides. Although some of the particles were found in the cytosol, the fraction ending up in endosomes was always close to 100% (Mathias Brust, personal communication). Recently, it was claimed that a slow, but efficient endosomal release was obtained with QDs (8—10 nm) functionalized with an amphiphatic palmitoylated peptide [63]. Interestingly, the endocytosed peptide-QDs accumulated within endosomes that were labelled with transferrin, a characteristic marker of early endosomes and the recycling pathway. Thus, one might wonder whether these small peptide-QDs are sorted into the recycling pathway rather than following the degradative pathway to lysosomes, and if some of the particles in the image may be bound to the cell surface following slow recycling.

Some very interesting data were published by Verma et al. [64] (see also editorial comments [65]), who stated that particles made with striations of alternating anionic and hydrophobic groups crossed the plasma membrane without being endocytosed, whereas particles coated with the same molecules in a random order did not. These data are very surprising and one would like to understand the molecular mechanism of such a process. Thus, it will be very interesting to see the follow-up studies of these particles.

Nanoparticles that absorb protons in response to the acidification of endosomes can be used to disrupt these vesicles via the ''proton sponge'' effect (swelling and increased osmotic pressure) and thus deliver small molecules and proteins to cytosol [66]. Nanoparticles can also be delivered to the cytosol from endosomes by the use of photochemical internalization; this technique includes the use of substances (e.g. porphyrins) that are taken up in the endosomal membrane and after light treatment generate free radicals that destroy the endosomes [67,68].

Disturbances of intracellular transport and other cellular processes induced by nanoparticles

Several review articles about the cellular toxicity of nanoparticles are available [69—74], thus we will not discuss this issue in any detail. However, we would like to point out that several of the methods used to detect cellular toxicity require rather large effects on the cells, e.g. the trypan blue used to detect dead cells or the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) used to assess mitochondrial activity by conversion of MTT into formazan. Many of these studies are also performed at too few time points. The toxic effects of nanoparticles should be measured at time points when the cells contain intact particles as well as partly degraded and completely degraded particles. It is in fact not a trivial matter to describe the metabolism of nanoparticles and thus to select time points for studying toxic effects. We have described approaches for investigating metabolism of nanoparticles in more detail in a recent review article [5]. In our opinion it is essential to study the potential disturbances of intracellular trafficking and signalling, e.g. down-regulation of receptors for growth factors and lack of recycling of membrane and receptors. When using nanoparticles to study cell function it should be kept in mind that polyvalent binding sites at their surfaces may induce cross-linking of cell surface proteins, and thereby interfere with normal biological processes [15,20]. The polyvalent binding sites may also result in an increased binding affinity (avidity) to cell surface receptors. Thus, multivalent interactions due to binding of ligands to nanoparticles have been reported to increase the avidity by 4 orders of magnitude [75]. A large change in affinity may of course affect the cellular response to a given ligand and could affect processes such as low pH-induced dissociation of ligand from receptors in endosomes. Since large particles may be unable to enter the recycling pathway (see below) this would lead to cellular accumulation of particles.

The extent to which nanoparticles are able to distort the normal intracellular trafficking was recently studied by measuring the uptake and intracellular transport in three different cell lines using QDs (hydrodynamic diameter of 30 nm) coupled to three proteins that bind to different cell receptors [56,58]. The proteins studied were transferrin, the plant toxin ricin (binding to galactose residues on the cell surface) and Shiga toxin (binding to the glycosphingolipid Gb3). Interestingly, the intracellular transport was changed following uptake and accumulation of these QDs, thus demonstrating that QDs may have significant effects

on cell function. Recently, different shaped mesoporous silica particles were reported to influence cell functions such as migration and the cytoskeleton organization in different ways [76].

Cellular excretion and degradation of nanoparticles

There are a few articles describing exocytosis of nanoparticles (meaning recycling of the particles to the cell exterior) [14,16,77]. They all conclude that exocytosis of the particles is much slower than endocytosis. Whereas the rate of endocytosis seems to be fastest for particles of 20—50 nm, the rate of exocytosis decreases with increasing particle size [14,16]. Chithrani and Chan [14] reported that the fraction of endocytosed nanoparticles varied for different cell lines and that the percent of cellular nanoparticles being exocytosed within 1 h in HeLa cells was approximately 35, 10 and 5% for nanoparticles of 14, 50 and 74 nm, respectively. An open question is also what happens to nanoparticles released from cells that have been killed, e.g. by targeted nanoparticles containing drugs.

How stable are nanoparticles following cellular uptake? This will obviously differ between various nanoparticles. Several groups have focused their research on biodegradable particles that are expected to be metabolized within endosomes/lysosomes, and reviews are available about such particles made of polylactic acid and similar polymers [3], albumin particles or liposomes [78,79]. In the following, we discuss the stability of metal-based nanoparticles.

The QDs seem to be very stable both *in vitro* and *in vivo*. Thus, fluorescence of CdSe-based QDs (emitting at 605 nm) was reported to be retained in human mesenchymal stem cells for the whole observation period of 52 days, whereas smaller QDs with the same chemical composition (emitting at 525 nm; approximately half the size of the 605 nm particles) gave a rapid decrease of fluorescence within 2—7 days after uptake [80]. A recent study of fluorescent QDs revealed that they were observed two years after injection in mice, although a range of significantly blue-shifted emission peaks with increased band widths clearly demonstrated some metabolism [81]. These data show that metabolism of such particles may be a challenge for *in vivo* use. It should be kept in mind that although fluorescence of nanoparticles may give useful information about the stability of QDs, fluorescence cannot be used to obtain good quantitative data for metabolism of the particles as the fluorescence of partly degraded particles is unknown (discussed in Ref. [5]).

Iron oxide particles have been used as contrast agents for 20 years and have been shown to be safe after intravenous injection [82,83]. The degradation of such particles has been studied in rats [84], and they have even been shown to be solubilised in the absence of any enzymes at a pH similar to that found in endosomes and lysosomes [85]. The metabolism of iron oxide particles is therefore most likely taken care of by lysosomes, i.e. the intracellular system for degradation of ferritin, the iron-storage protein with a core of iron-oxide mineral within its cavity. As discussed in a recent review article [5] there is very little information available about metabolism of non-iron oxide metal-based nanoparticles.

Summary

We have summarized different methods that can be used to investigate cellular uptake of nanoparticles and the pitfalls in such studies. The complexity, the combination of advanced chemistry and cell biology, makes it important that future research on nanoparticles is performed as a close collaboration between scientists with different backgrounds. This is important to prevent misleading/wrong interpretations and thus aid in bringing nanoparticles faster into clinical use.

References

[1] T. Jamieson, R. Bakhshi, D. Petrova, R. Pocock, M. Imani, A.M. Seifalian, Biomaterials 28 (2007) 4717—4732.
[2] X. Michalet, F.F. Pinaud, L.A. Bentolila, J.M. Tsay, S. Doose, J.J. Li, G. Sundaresan, A.M. Wu, S.S. Gambhir, S. Weiss, Science 307 (2005) 538—544.
[3] J.K. Vasir, V. Labhasetwar, Adv. Drug Deliv. Rev. 59 (2007) 718—728.
[4] S.S. Agasti, S. Rana, M.H. Park, C.K. Kim, C.C. You, V.M. Rotello, Adv. Drug Deliv. Rev. 62 (2010) 316—328.
[5] T. Skotland, T.G. Iversen, K. Sandvig, Nanomedicine 6 (2010) 730—737.
[6] N.M. Zaki, N. Tirelli, Expert Opin. Drug Deliv. 7 (2010) 895—913.
[7] D. Maysinger, J. Lovric, A. Eisenberg, R. Savic, Eur. J. Pharm. Biopharm. 65 (2007) 270—281.
[8] V. Mailander, K. Landfester, Biomacromolecules 10 (2009) 2379—2400.
[9] A. Verma, F. Stellacci, Small 6 (2010) 12—21.
[10] H. Hillaireau, P. Couvreur, Cell. Mol. Life Sci. 66 (2009) 2873—2896.
[11] J.B. Delehanty, H. Mattoussi, I.L. Medintz, Anal. Bioanal. Chem. 393 (2009) 1091—1105.
[12] W.A. Hild, M. Breunig, A. Goepferich, Eur. J. Pharm. Biopharm. 68 (2008) 153—168.
[13] G. Sahay, D.Y. Alakhova, A.V. Kabanov, J. Control Release (2010).
[14] B.D. Chithrani, W.C. Chan, Nano Lett. 7 (2007) 1542—1550.
[15] W. Jiang, B.Y. Kim, J.T. Rutka, W.C. Chan, Nat. Nanotechnol. 3 (2008) 145—150.
[16] H. Jin, D.A. Heller, R. Sharma, M.S. Strano, ACS Nano 3 (2009) 149—158.
[17] O. Harush-Frenkel, N. Debotton, S. Benita, Y. Altschuler, Biochem. Biophys. Res. Commun. 353 (2007) 26—32.
[18] L.W. Zhang, N.A. Monteiro-Riviere, Toxicol. Sci. 110 (2009) 138—155.
[19] S. Barua, K. Rege, Small 5 (2009) 370—376.
[20] G. Gur, Y. Yarden, Nat. Biotechnol. 22 (2004) 169—170.
[21] J. Kaplan, Nature 263 (1976) 596—597.
[22] B. Snijder, R. Sacher, P. Ramo, E.M. Damm, P. Liberali, L. Pelkmans, Nature 461 (2009) 520—523.
[23] K. Sandvig, B. van Deurs, Gene Ther. 12 (2005) 865—872.
[24] A. Hayer, M. Stoeber, D. Ritz, S. Engel, H.H. Meyer, A. Helenius, J. Cell Biol. 191 (2010) 615—629.
[25] K. Sandvig, M.L. Torgersen, H.A. Raa, B. van Deurs, Histochem. Cell Biol. 129 (2008) 267—276.
[26] M. Wieffer, T. Maritzen, V. Haucke, Cell 137 (2009) 382—383.
[27] G.J. Doherty, H.T. McMahon, Annu. Rev. Biochem. 78 (2009) 857—902.
[28] S. Kumari, S. Mg, S. Mayor, Cell Res. 20 (2010) 256—275.
[29] M.T. Howes, S. Mayor, R.G. Parton, Curr. Opin. Cell Biol. 22 (2010) 519—527.
[30] M.L. Torgersen, G. Skretting, B. van Deurs, K. Sandvig, J. Cell Sci. 114 (2001) 3737—3747.

[31] H. Shogomori, A.H. Futerman, J. Biol. Chem. 276 (2001) 9182—9188.
[32] A.M. Hommelgaard, K. Roepstorff, F. Vilhardt, M.L. Torgersen, K. Sandvig, B. van Deurs, Traffic 6 (2005) 720—724.
[33] L. Pelkmans, J. Kartenbeck, A. Helenius, Nat. Cell Biol. 3 (2001) 473—483.
[34] E.M. Damm, L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, A. Helenius, J. Cell Biol. 168 (2005) 477—488.
[35] G. Scita, P.P. Di Fiore, Nature 463 (2010) 464—473.
[36] B. van Deurs, K. Roepstorff, A.M. Hommelgaard, K. Sandvig, Trends Cell Biol. 13 (2003) 92—100.
[37] O. Harush-Frenkel, E. Rozentur, S. Benita, Y. Altschuler, Biomacromolecules 9 (2008) 435—443.
[38] S. Grimmer, B. van Deurs, K. Sandvig, J. Cell Sci. 115 (2002) 2953—2962.
[39] S.K. Rodal, G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, K. Sandvig, Mol. Biol. Cell 10 (1999) 961—974.
[40] P. van Kerkhof, M. Sachse, J. Klumperman, G.J. Strous, J. Biol. Chem. 276 (2001) 3778—3784.
[41] A. Ishida, N. Sueyoshi, Y. Shigeri, I. Kameshita, Br. J. Pharmacol. 154 (2008) 729—740.
[42] D.B. van Rossum, R.L. Patterson, Cell Calcium 45 (2009) 535—545.
[43] L. Pelkmans, D. Puntener, A. Helenius, Science 296 (2002) 535—539.
[44] L.M. Fujimoto, R. Roth, J.E. Heuser, S.L. Schmid, Traffic 1 (2000) 161—171.
[45] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, Mol. Ther. 18 (2010) 561—569.
[46] J.M. Larkin, M.S. Brown, J.L. Goldstein, R.G. Anderson, Cell 33 (1983) 273—285.
[47] G. Daukas, S.H. Zigmond, J. Cell Biol. 101 (1985) 1673—1679.
[48] M.L. Ledbetter, M. Lubin, Exp. Cell Res. 105 (1977) 223—236.
[49] I.H. Madshus, T.I. Tonnessen, S. Olsnes, K. Sandvig, J. Cell Physiol. 131 (1987) 6—13.
[50] J.E. Heuser, R.G. Anderson, J. Cell Biol. 108 (1989) 389—400.
[51] H. Damke, T. Baba, A.M. van der Bliek, S.L. Schmid, J. Cell Biol. 131 (1995) 69—80.
[52] N. Balasubramanian, D.W. Scott, J.D. Castle, J.E. Casanova, M.A. Schwartz, Nat. Cell Biol. 9 (2007) 1381—1391.
[53] D.K. Sharma, J.C. Brown, Z. Cheng, E.L. Holicky, D.L. Marks, R.E. Pagano, Cancer Res. 65 (2005) 8233—8241.
[54] Z.J. Cheng, R.D. Singh, D.L. Marks, R.E. Pagano, Mol. Membr. Biol. 23 (2006) 101—110.
[55] T. Xia, M. Kovochich, M. Liong, J.I. Zink, A.E. Nel, ACS Nano 2 (2008) 85—96.
[56] T.G. Iversen, N. Frerker, K. Sandvig, Prog. Biomed. Opt. Imaging (2009), 71890T-9.
[57] S.K. Lai, K. Hida, S.T. Man, C. Chen, C. Machamer, T.A. Schroer, J. Hanes, Biomaterials 28 (2007) 2876—2884.
[58] C. Tekle, B. Deurs, K. Sandvig, T.G. Iversen, Nano Lett. 8 (2008) 1858—1865.
[59] C.C. Berry, Nanomedicine 3 (2008) 357—365.
[60] I.L. Medintz, T. Pons, J.B. Delehanty, K. Susumu, F.M. Brunel, P.E. Dawson, H. Mattoussi, Bioconjug. Chem. 19 (2008) 1785—1795.
[61] G. Ruan, A. Agrawal, A.I. Marcus, S. Nie, J. Am. Chem. Soc. 129 (2007) 14759—14766.
[62] P. Nativo, I.A. Prior, M. Brust, ACS Nano 2 (2008) 1639—1644.
[63] J.B. Delehanty, C.E. Bradburne, K. Boeneman, K. Susumu, D. Farrell, B.C. Mei, J.B. Blanco-Canosa, G. Dawson, P.E. Dawson, H. Mattoussi, I.L. Medintz, Integr. Biol. (Camb.) 2 (2010) 265—277.
[64] A. Verma, O. Uzun, Y. Hu, Y. Hu, H.S. Han, N. Watson, S. Chen, D.J. Irvine, F. Stellacci, Nat. Mater. 7 (2008) 588—595.
[65] T. Xia, L. Rome, A. Nel, Nat. Mater. 7 (2008) 519—520.
[66] Y. Hu, T. Litwin, A.R. Nagaraja, B. Kwong, J. Katz, N. Watson, D.J. Irvine, Nano Lett. 7 (2007) 3056—3064.
[67] K. Berg, M. Folini, L. Prasmickaite, P.K. Selbo, A. Bonsted, B.O. Engesaeter, N. Zaffaroni, A. Weyergang, A. Dietze, G.M. Maelandsmo, E. Wagner, O.J. Norum, A. Hogset, Curr. Pharm. Biotechnol. 8 (2007) 362—372.
[68] N. Nishiyama, Y. Morimoto, W.D. Jang, K. Kataoka, Adv. Drug Deliv. Rev. 61 (2009) 327—338.
[69] A. Nel, T. Xia, L. Madler, N. Li, Science 311 (2006) 622—627.
[70] S.J. Klaine, P.J. Alvarez, G.E. Batley, T.F. Fernandes, R.D. Handy, D.Y. Lyon, S. Mahendra, M.J. McLaughlin, J.R. Lead, Environ. Toxicol. Chem. 27 (2008) 1825—1851.
[71] T. Xia, N. Li, A.E. Nel, Annu. Rev. Public Health 30 (2009) 137—150.
[72] B.A. Rzigalinski, J.S. Strobl, Toxicol. Appl. Pharmacol. 238 (2009) 280—288.
[73] C.F. Jones, D.W. Grainger, Adv. Drug Deliv. Rev. 61 (2009) 438—456.
[74] N. Lewinski, V. Colvin, R. Drezek, Small 4 (2008) 26—49.
[75] C. Tassa, J.L. Duffner, T.A. Lewis, R. Weissleder, S.L. Schreiber, A.N. Koehler, S.Y. Shaw, Bioconjug. Chem. 21 (2010) 14—19.
[76] X. Huang, X. Teng, D. Chen, F. Tang, J. He, Biomaterials 31 (2010) 438—448.
[77] P.V. Asharani, M.P. Hande, S. Valiyaveettil, BMC Cell Biol. 10 (2009) 65.
[78] V.P. Torchilin, Adv. Drug Deliv. Rev. 58 (2006) 1532—1555.
[79] E. Igarashi, Toxicol. Appl. Pharmacol. 229 (2008) 121—134.
[80] O. Seleverstov, O. Zabirnyk, M. Zscharnack, L. Bulavina, M. Nowicki, J.M. Heinrich, M. Yezhelyev, F. Emmrich, R. O'Regan, A. Bader, Nano Lett. 6 (2006) 2826—2832.
[81] J.A. Fitzpatrick, S.K. Andreko, L.A. Ernst, A.S. Waggoner, B. Ballou, M.P. Bruchez, Nano Lett. 9 (2009) 2736—2741.
[82] C. Corot, P. Robert, J.M. Idee, M. Port, Adv. Drug Deliv. Rev. 58 (2006) 1471—1504.
[83] Y.X. Wang, S.M. Hussain, G.P. Krestin, Eur. Radiol. 11 (2001) 2319—2331.
[84] K.C. Briley-Saebo, L.O. Johansson, S.O. Hustvedt, A.G. Haldorsen, A. Bjornerud, Z.A. Fayad, H.K. Ahlstrom, Invest. Radiol. 41 (2006) 560—571.
[85] T. Skotland, P.C. Sontum, I. Oulie, J. Pharm. Biomed. Anal. 28 (2002) 323—329.
[86] O. Garred, S.K. Rodal, D.B. van, K. Sandvig, Traffic 2 (2001) 26—36.
[87] M. Koivusalo, C. Welch, H. Hayashi, C.C. Scott, M. Kim, T. Alexander, N. Touret, K.M. Hahn, S. Grinstein, J. Cell Biol. 188 (2010) 547—563.
[88] M.C. Kerr, R.D. Teasdale, Traffic 10 (2009) 364—371.
[89] L.H. Wang, K.G. Rothberg, R.G. Anderson, J. Cell Biol. 123 (1993) 1107—1117.
[90] P. Khandelwal, W.G. Ruiz, G. Apodaca, EMBO J. 29 (2010) 1961—1975.
[91] H. Radhakrishna, J.G. Donaldson, J. Cell Biol. 139 (1997) 49—61.
[92] P. Lajoie, I.R. Nabi, Int. Rev. Cell Mol. Biol. 282 (2010) 135—163.
[93] C.G. Hansen, B.J. Nichols, Trends Cell Biol. 20 (2010) 177—186.
[94] S. Sabharanjak, P. Sharma, R.G. Parton, S. Mayor, Dev. Cell 2 (2002) 411—423.
[95] H. Park, D. Cox, Mol. Biol. Cell 20 (2009) 4500—4508.
[96] A. Benmerah, M. Bayrou, N. Cerf-Bensussan, A. Dautry-Varsat, J. Cell Sci. 112 (Pt 9) (1999) 1303—1311.
[97] A. Grassart, V. Meas-Yedid, A. Dufour, J.C. Olivo-Marin, A. Dautry-Varsat, N. Sauvonnet, Traffic 11 (2010) 1079—1091.
[98] C. Lamaze, A. Dujeancourt, T. Baba, C.G. Lo, A. Benmerah, A. Dautry-Varsat, Mol. Cell 7 (2001) 661—671.

Tore-Geir Iversen is a senior researcher and Project Leader at the Centre for Cancer Biomedicine, The Norwegian Radium Hospital in Oslo, Norway. He earned his Ph.D. at the Norwegian University of Science and Technology (NTNU), Trondheim in 1995, at that time studying microbial genetics. He joined the group of professor Sandvig in 1997, then studying endocytosis and intracellular transport of different protein toxins. In 2006 he turned his focus into studying how nanoparticles are endocytosed and transported in cells. His group was the first to demonstrate that accumulation of nanoparticles within endosomes could induce changes in the normal intracellular transport of the cell. Current research interests also include more applied biological studies about nanoparticles and the criteria required for their clinical use in therapy and imaging.

Tore Skotland is a guest researcher at The Centre for Cancer Biomedicine, The Norwegian Radium Hospital in Oslo, Norway. He received his Ph.D. in biochemistry from the University of Bergen, Norway in 1980. After 11 years in basic research (protein chemistry and enzymology) he moved to pharmaceutical R&D in 1983 where he stayed for 26 years within the same company Nycomed/Amersham/GE Healthcare, i.e. one of the world leading companies in developing contrast agents for medical imaging. The last 20 years he was heading the work to characterize the metabolism and excretion of all types of contrast agents in the company (water soluble as well as particle based) for CT, MRI, SPECT, PET and optical imaging. He is co-author of approximately 75 publications.

Kirsten Sandvig is a group leader at The Centre for Cancer Biomedicine, The Norwegian Radium Hospital and professor at The University of Oslo in Norway. She received her Ph.D. in biochemistry at the University of Oslo in 1979. Professor Sandvig has for many years contributed significantly to our present knowledge about endocytosis and intracellular transport with approximately 250 publications in the field. She was the first to describe recycling of endocytosed material to the cell exterior, the first to describe retrograde transport from endosomes to ER, and also among the first to describe the existence of clathrin independent endocytosis. She has throughout her carrier used different types of protein toxins in her studies, which has resulted in numerous prizes for her scientific contributions.