Chapter 8
Cellular Uptake Mechanisms and Detection of Nanoparticle Uptake by Advanced Imaging Methods

Kleanthis Fytianos, Fabian Blank and Loretta Müller

Abstract The specific mechanism, of uptake of a nanoparticle by a cell and the subcellular localisation are of great importance regarding the potential effect of the nanomaterial inside the cell. In order to study health risks and the potential of a nanoparticle to be used in biomedical applications, cellular internalization has to be investigated in great detail. This chapter highlights most relevant routes of nanoparticle uptake and includes current approaches for the visualization of particle uptake at the nano-level.

8.1 Introduction

The number of manufactured goods containing nanomaterials has been growing consistently over the last fifteen years [1]. The fundamental interactions of nanoparticles (NPs) with biological interfaces remain incompletely understood [2–6]. This is mainly due to a lack of mechanistic knowledge at the molecular level [2, 7] with regards to nanoparticle uptake by cells in particular. The mode of internalization of NPs into cells will determine intracellular fate and potential effects on the whole organism, including health effects. This is why even for intended therapeutic applications of NPs, possible health concerns need addressing [8–10]. In particular with regards to therapeutic applications, the entire field of nanomedicines has been greatly expanded by the development of a wide range of nanomaterials with a high degree of control over their physical (e.g., size, surface charge, shape, mechanical strength) and chemical attributes. Hence, a better understanding of the physiopathological nature of different diseases and insight into the interaction of nanomaterials with biolog-

K. Fytianos · F. Blank (✉)
Respiratory Medicine, Department of BioMedical Research (DBMR), University of Bern, Murtenstrasse 50, 3008 Bern, Switzerland
e-mail: fabian.blank@dbmr.unibe.ch

L. Müller
University Children's Hospital Basel, Spitalstrasse 33, 4056 Basel, Switzerland

Department of Pediatrics, Inselspital, Bern University Hospital, Freiburgstrasse 18, 3010 Bern, Switzerland

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ical systems at various levels (i.e., systemic, organ, tissue, and cell) are of great importance for further progress towards bench-to-bedside translation [11]. A large number of studies in recent years has aimed at focusing on the molecular interactions involved in the biological actions of NPs [12]. NPs are of similar size to typical cellular organelles and are able to very efficiently enter living cells by exploiting the cellular endocytosis machinery, which may lead to adverse effects and even permanent cell damage [10, 13]. Only specialized cells are capable of phagocytosis, a form of endocytosis in which the cell engulfs larger particles. The most prominent type of phagocytes are macrophages, in general termed as professional phagocytes, since they are the main responsible cell type for clearance of cellular debris and foreign particles in most organs. Other phagocytic cell types of the innate and adaptive immune system include monocytes, neutrophils and dendritic cells. On the other hand, NPs can be internalized by almost all non-phagocytic cells via pinocytosis. It is one of the aims of this chapter to discuss the most important receptor mediated and receptor independent cellular uptake mechanisms for NPs which have been described up to now.

Different uptake mechanisms by cells are determined by the physico-chemical properties of NPs including size [14, 15], shape [14], surface charge [16–18] and surface chemistry [16, 19, 20], which strongly modulate the cellular uptake efficiency. Furthermore, by entering the organism, NPs interact with extracellular biomolecules dissolved in body fluids, including proteins, sugars and lipids forming a specific corona around the particle. The composition of the protein corona (which is discussed in detail in another chapter of this book) depends on diverse physical properties of the NPs like size, shape and surface chemistry and in turn determines the type of interaction with the cells during uptake. In the last two decades, there have been substantial activities to better understand the detailed molecular mechanisms involved in cellular uptake of NPs, often using fluorescence microscopy and other sophisticated biophysical techniques like flow cytometry. This chapter will discuss a number of approaches from recent studies, which were aiming to elucidate the molecular interactions promoting uptake of NPs. There is a number of novel emerging techniques to accurately visualize, monitor, characterize and quantify the uptake of NPs by different cell types. Accurate, well characterized and reliable techniques, to analyse and quantify cellular uptake of NPs is crucial for the design of novel nanocarriers for biomedical applications. A thorough understanding of the involved processes at the molecular level is important for example for the engineering of NPs that do not penetrate cells and evade the phagocytic cells responsible to clear particulates from the systemic circulation like the reticulo-endothelial system, as is the case for NP based contrast agents widely used in medical diagnosis. However, the opposite characteristics are needed for developing NPs designed for selective uptake by specific cells, for example, for targeted drug delivery [12]. The enhanced cellular uptake of NPs at the site of disease is of paramount importance, because targets for many theranostic agents against several disorders (including cancer) are localized in the subcellular compartments [21]. All these facts not only highlight the importance of a better understanding of cellular uptake mechanisms but also have fuelled recent

research into the development of nanocarriers capable of subcellular- and organelle-level targeting, referred to as a completely novel generation of nanomedicines [22].

8.2 Non-ligand Dependent Endocytic Uptake

There are a variety of different biological mechanisms enabling the active uptake of particles into cells. Mostly, it is the specific size of the particles, which defines the mechanisms that are involved. Among the non-ligand dependent endocytosis, we distinguish between the phagocytosis (also called "cell eating") and pinocytosis (also called "cell drinking") (see also Fig. 8.1).

Phagocytosis occurs with particles larger than 0.5 μm. Even tough, many cell types (e.g. epithelial cells, fibroblasts, immune cells) are able to perform phagocytosis, the uptake efficiency of phagocytic cells (macrophages, dendritic cells, neutrophils) is a lot higher and phagocytosis is predominately performed by those cells [24, 25]. Phagocytosis is performed to clean the organism from infectious microorganisms, senescent cells or disabled particles. This process is part of the immune response [26]. Particles, or more precisely, particular ligands on the particles, are either recognized by surface receptors of the phagocytes or by opsonins. Opsonins are small, soluble molecules, such as proteins from the complement system, antibodies, blood serum proteins (e.g. fibronectin, laminin). They recognize particles, bind to their surface and are then recognized by phagocytes [27]. Some of the most important receptors involved in phagocytosis are the following ones: Fc receptors family for immunoglobulins G, the complement receptors, and the α5β1 integrin [28]. The interaction of the particle with the cell surface receptors triggers a signal cascade with the result of particle engulfing: actin assemblies and cell surface extensions are formed to surround the particle. Once completely engulfed, the particle find itself in an intracellular vesicle of approximately 0.5–1 μm, so called phagosomes [25, 29]. Once inside of the cells, phagosomes undergo maturation due to several events of

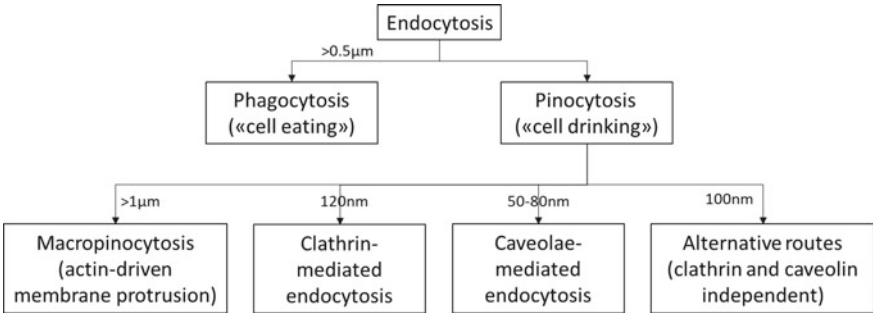

Fig. 8.1 Classification of uptake mechanisms based on size of the up taken particles. Adapted from [23]

fission and fusion with endosomes and lysosomes, resulting in so called phagolysosomes [23]. Particles in the phagolysosomes are degraded and receptors are recycled [23, 30].

Pinocytosis occurs in almost all cell types and is characterized by the uptake of fluid, which may—by coincidence—also contain particles. This process can be divided into four modes: Macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and alternative routes (see also Fig. 8.1) [31]. Macropinocytosis is the process of the nonspecific uptake of large amounts of external fluid via the uptake of large vesicles (0.5–10 μm) known as macropinosomes [32]. As particles may be localized close to the cell membrane, they may end up in one of those macropinosomes and thus, are taken up by the cells [23]. This mode of uptake is typical for the uptake of viruses [33], bacteria [34], and apoptotic cell fragments [35], and thus contributes to the antigen presentation in the major histocompatibility complex II [36, 37]. Macropinocytosis is an actin-driven process, which needs stimulation: growth factors (e.g. epidermal growth factor or platelet derived growth factor) interact with receptor tyrosine kinase [33], what activates the signal cascade to initiate actin polymerization to form membrane protrusions [38]. These protrusions fuse with the membrane generating uncoated endocytic vesicles of the size of about 1 μm. The shape of the macropinosomes is irregular. The uptake capacity by this mode is very high, but rather unspecific [39]. The clathrin-mediated endocytosis is the "classical" uptake mechanism, as it is known for a long time and very well characterized [40]. It is a complex pathway, which is initiated at the apical membrane of polarized cells (e.g. endothelial or epithelial cells) [31, 41, 42]. The whole process starts by the induction of a curvature in the membrane leading to the formation of vesicles [11]. Various proteins are involved in this induction, e.g. epsin [43], endophilin [44], clathrin assembly lymphoid myeloid leukemia protein [45]. The neck of a newly formed invagination is surrounded by a ring of the GTPase dynamin. The activity of the GTPase dynamin is responsible for the release of a vesicle from the plasma membrane [46, 47]. Once the vesicle is released, it can be targeted to more mature endosomes, lysosomes or multivesicular bodies, or it is recycled to the plasma membrane surface [23]. Usually, particles of sizes between 120 and 150 nm can be internalized by clathrin-mediated endocytosis [14, 48], however, also particles of 200 nm have been reported to enter the cells via this pathway [15]. The uptake route for non-targeted NPs depends on the particle characteristics (e.g. particle size, shape, surface charge) and also on the cell type supposed to take up the particle [23]. The detailed mechanism of the particle internalization is not completely understood. However, the efficient uptake of particles of about 100 nm in diameter via clathrin-mediated endocytosis, speaks for the main uptake route [23]. Particles of a diameter between 50 and 80 nm are predominantly uptaken via caveolin-mediated endocytosis. The uptake of particles via this process can be mostly observed on the basolateral side of endothelial cells [31, 49, 50]. The process starts with the building of caveolae (small, stable membrane-associated structures with flask-like shape) [51, 52], which are coated with caveolin-1 [53]. They have been shown to interact with pathogens, e.g. SV40 virus [54] cholera toxin subunit B or shiga toxin [55]. It is unclear what happens to NPs, once they are inside the caveolae, which have

been internalized by the cell. For a long time, it was thought that particles are localized inside so-called caveosomes (special type of caveolar endosomes), which are further transported to the Golgi apparatus. The pH inside the caveosomes is neutral and thus, there is no degradation as in lysosomes [11]. However, more recently, the caveosomes were shown to be artifacts of caveolin overexpression [23, 56], and routes of the intracellular trafficking of NPs are controversially discussed again [23]. This route is of importance for small NPs, as NPs between 20 and 40 nm are a lot faster taken up by this route than NPs of 100 nm [50, 57].

As discussed above, recent studies have highlighted a number of different non-ligand dependent mechanisms which are of great importance for the internalisation of NPs by cells. However, there are endocytic routes for NPs which do not fit the so far described categories. Most of them are clathrin- and caveolin-independent and are summarized as "alternative routes" [23]. Those routes have first been described for the entry of cell surface proteins and bacterial toxins. It has also been proposed to be involved in other cellular mechanisms, such as membrane repair, polarization, spreading and intracellular signaling [58]. Depending on the subtype of pathways, various proteins are involved, especially actin and actin-associated proteins [59]. Additionally, NPs can also enter a cell via passive diffusion [60, 61].

8.3 Receptor-Mediated Cellular Internalization and Nanoparticles for Targeted Uptake

The study and characterization of expression and overexpression of specific receptors on the surface of target disease cells has proven to be essential for the research and development of novel nanomedicines. This type of approach helps to improve cellular uptake of biomedical NPs in therapeutic and/or diagnostic procedures, while side-effects and off-side toxicity can be reduced as much as possible [11]. Well-characterized examples of receptors known for active disease cell targeting are folate receptor (FR), transferrin receptor (TfR), epidermal growth factor receptors (EGFR), G-protein coupled receptor (GPCR), low density lipoprotein receptor (LDLR) and lectins [62]. Receptor-mediated internalization of biomedical NPs has been investigated for various cargos such as vaccines, drugs, DNA, and RNA. For efficient cell targeting and internalization, different high affinity ligands have been conjugated to the surface of NPs. The major types of targeting ligands include peptides, high-affinity small molecules, aptamers (i.e. oligonucleotides or peptides) and antibodies. Recent developments using ligand-conjugated NPs for biomedical applications are described in this subchapter, with specific focus on FR, TfR, EGFR, prostate-specific membrane antigen (PSMA), integrin and neonatal Fc-receptor (FcRn). For a very comprehensive overview of targeted uptake, please consult the review written by Yameen et al. [11]. We have included a synthesis of this work in the following text.

8.3.1 Folate Receptor (FR) Targeting

FR is a glycoprotein of 38-44kDA molecular weight. There are two isoforms, FR-α and FR-β. While FR-α is expressed on some normal epithelial cells (e.g. kidney) [63] and on many epithelial cancer cells (e.g. ovarian, renal, brain) [64], FR-β has been shown to be present on activated macrophages [65] and on hematopoietic malignancies [66]. As folic acid binds with high affinity to the FR. This allows creating folate-conjugated nanocarriers which are selectively taken up by cells expressing FR [67]. This approach involving FR-β could be used to target chronic inflammatory diseases (e.g. diabetes type 2 or arteriosclerosis). The benefit of folate-conjugated NPs, liposomes, oligonucleotides, and chemotherapeutics has been shown in many in vivo and in vitro studies [68]. E.g. chemotherapeutic drugs loaded into folate-functionalized NPs showed higher antitumor activity compared to non-targeted NP therapeutics in an in vivo ovarian peritoneal metastasis model [69]. Folic acid is a small molecule, which has advantages compared to the use of peptides or antibodies as targeting ligands. It is highly stable in acidic or basic media and at high temperatures, there is no risk of toxicity or immune reaction as it is a vitamin, and the chemical modification and a potential scaling-up for clinical applications is easily doable [11].

8.3.2 Transferrin Receptor (TfR) Targeting

TfR is a cell membrane glycoprotein. It is present as a homodimer of two identical subunits located transmembranely. The main functions are the mediation of the cellular uptake of iron from plasma glycoproteins (e.g. transferrin) and the regulation of cell growth [70, 71]. While TfR is most probably expressed on all cells, the expression level varies: it is highly expressed in immature erythroid cells, placental tissue, and rapidly dividing cells. The fact that it is about 100 times higher expressed on cancer cells compared to normal cells [72], makes it to one of the most attractive targets for the cancer therapy with receptor-mediated endocytosis using drug NPs. Transferrin has been successfully used as ligand for TfR-mediated intracellular delivery of nanotherapeutics, as there are TfR-targeted nanomedicines in various stages of clinical trials [73].

8.3.3 Epidermal Growth Factor Receptor (EGFR) Targeting

EGFR is a member of the ErbB tyrosine kinase family and can stimulate tumor growth, invasion, and metastasis. It is overexpressed in various tumors, e.g. colorectal, brain, breast or ovarian [74–76]. Following the binding of ligands to EGFRs, the EGFRs homo- or heterodimerize with other members of the ErbB receptor

family and other cell-surface tyrosine kinases [73]. Monoclonal antibodies as well as small molecules have been used as EGFR-targeting ligands: epidermal growth factor, transforming growth factor-α, heparin binding EGF-like growth factor, epigen, betacelluin, and epiregulin [77]. Nanocarriers for drugs using EGFR to deliver drugs to specific cells are widely used and already reached clinical trial phase I [73]. One example for the use of EGFR for targeted anticancer therapy are cisplatin-encapsulated gelatin NPs with EGF. Those NPs improved the in vitro targeting ability and anticancer effects in A549 cells (high EGFR expression) compared to HFL1 cells (low EGFR expression). Additionally, the effect was shown in a tumor-bearing mouse model (with high EGFR expression) [78].

8.3.4 Prostate-Specific Membrane Antigen (PSMA) Targeting

PSMA is a type 2 integral membrane glycoprotein overexpressed on the surface of prostate carcinomas and of the neovasculature of many solid tumors [79, 80]. This makes PSMA a valid cancer target for therapies. One example is BIND-014, which has reached a Phase II clinical trial. It is a docetaxel-loaded, polymer-based nanomedicine with a small-molecule ligand (S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid, ACUPA) against PSMA [81].

8.3.5 Integrin Targeting

Integrins modulate interaction between endothelial cells and the extracellular matrix and are—via this interaction—involved in many vital cellular functions, such as adhesion, migration, invasion, stress responses, proliferation, differentiation, survival and apoptosis. They belong to a family of heterodimer transmembrane receptors [62]. The receptor αvβ3 is overexpressed in tumor-related endothelial cells during angiogenesis compared to endothelial cells in normal tissues [82–85]. Cyclic-RDG peptides were identified as promising targeting ligands for this receptor [62, 86, 87]. Recently, Pt(IV) prodrug-loaded PLGA-PEG NPs functionalized with cyclic-RGD were shown to be more efficacious compared to cisplatin in a non-particulate administration mode in a orthotopic human breast cancer xenocraft model. Additionally, it was better tolerated than the cisplatin [88].

8.3.6 Neonatal Fc-Receptor (FcRn) Targeting

FcRn is highly expressed in the neonatal intestine and in the apical region of epithelial cells in the small intestine of adults [11, 89]. This receptor has been used to test the uptake of nanomedicine delivered orally by Pridgen et al. [90]. PLA-PEG polymer

nanoparticle were functionalized on the surface with polyclonal IgG Fc fragments. Those NPs were shown to be transported transepithelially in vitro and in vivo. In mice, the particles were found in the lamina propria and also in other organs, showing the ability to enter the systemic circulation. The adsorption was 11.5 times higher for the targeted NPs compared to nontargeted ones. As it is a challenge for the nanomedical application to find ways to overcome the intestinal epithelial cell barrier [91], these findings using the FcRn receptor are of high interest for the future development. The oral administration route is of more convenience for patients, especially if frequent administration is required [92].

Recent studies have highlighted the importance of specific receptors for targeting by biomedical nanocarriers. A good knowledge of receptor mediated mechanisms of internalisation offers novel opportunities for treatments which are more efficient and show less side-effects due to precise targeting, in particular, for the treatment of cancer.

8.3.7 Methods of 3D Fluorescence Microscopy and Flow Cytometry to Determine and Characterize Cellular Uptake of Nanoparticles

Over the last decade, a wide range of different types of NPs have been developed, especially for the biomedical sector (i.e. nanomedicine and targeted drug delivery). Biomedical NPs can be suitably designed in order to reach the site of interest (i.e. lungs, tumours) and interact with specific cell types. Initially, NPs will interact with the cells of the specific site by either attaching to their membranes or be internalized. Cellular uptake is one of the most important factors to be investigated since it can determine the overall effect of the particles to the cells. Their effect could be drug release to a tumour site or immunogenic peptide delivery that can ultimately lead to effects like tumour size decrease or modulation of immune cells respectively. Fluorescently labelled NPs are widely used in pre-clinical applications at in vitro, in vivo and ex vivo settings. Imaging, bio-distribution and clearance can provide solid information for early development studies and thus dictate the direction of future experiments and strategies [93]. Alongside novel nanoparticle designs, technological advancements in the field of biomedical imaging (i.e. Stochastic Optical Reconstruction Microscopy—STORM) and scientific software (i.e. sophisticated image processing) can also help in the further development of the field, understanding of uptake mechanisms and ultimately lead to new scientific discoveries. Complementary to that, a portable device that can detect fluorescently labelled NPs as well as bacteria and viruses [94] has been reported. This device is compatible with smartphones, since it can be used as an extension that is attached to the camera of a smartphone. It utilizes an opto-mechanical system that contains a 450 nm diode. Such a device has potential to be used for quick quality control of NPs as well as for field applications [94]. The aim of this part of that chapter is to discuss about

some important nanoparticle types that are used in cellular uptake studies and some common and novel detection techniques (mainly based on fluorescence) that are used for their detection and cellular uptake measurements.

8.3.8 Quantum Dots and Inorganic Nanoparticles

Quantum dots have a size of 2–10 nm. Due to their very high surface to volume ratio, narrow excitation wavelengths (where according to their size they show a unique color) and electrical properties, they are widely used in semi-conductor and electronics industry and for fluorescence imaging. For the biomedical field they can be used for imaging and labelling purposes (i.e. cells or tissues). If used at concentrations of less than 100 μg/mL they do not interfere with cellular viability and cytotoxicity [95, 96]. Importantly, it has been shown that streptavidin-conjugated quantum dots can be used for cancer diagnosis. Since they can be utilized as contrast agents for biomedical imaging, they have been used in Magnetic Resonance Imaging (MRI), intra-vital in vivo imaging, flow cytometry and other bio-analytical assays. It is expected that in the future further developments will be established and their applications range will increase [97]. Bagalkot et al. [98] and Fontes et al. [99] highlight the importance of quantum dots in biomedical research, especially in the field of cancer imaging. They describe how quantum dots can specifically monitor drug release on target cancer cells, in real time. This is mainly attributed to their highly modifiable surface, electron density and photo-stability, which allows prolonged imaging times.

Gold NPs are one of the most studied types of inorganic nanomaterials. Due to their optical properties and evidence of low cytotoxicity and high affinity with biomolecules (i.e. peptides and nucleic acids) they are considered to be one of the most promising agents for imaging, drug delivery and diagnostic applications. Besides transmission electron microscopy, a novel and effective method to visualize uptake of gold NPs in cells and tissue is hyper-spectral imaging. The only system currently available for hyper-spectral imaging is the Cytoviva system (www.cytoviva.com). This instrument makes use of the unique spectrum of gold NPs, which enables accurate detection. Images are analyzed pixel-wise and the gold NPs can be mapped within the tissue or cells, due to their distinct UV-Vis spectrum. A recent example of the use of hyper-spectral imaging of gold NPs is given by England and colleagues [100]. However, the standard technique to measure gold and other inorganic NPs in cells remains transmission electron microscopy [101].

8.3.9 Fluorescently Labelled (Nano)Particles

Polymer based NPs and microparticles are generally regarded as safe for use for biological studies. Apart for very low records for cell viability and cytotoxicity, they

possess several competitive advantages compared to other NP types. For example, due to their chemical properties they can bind with many fluorescent molecules and thus become easier visible under fluorescent microscopes. Their chemical synthesis is also a straight-forward procedure. Additively, they are biodegradable and biocompatible, thus there are no concerns regarding bio-accumulation [102]. For all the above mentioned reasons, polymer based NPs have become a standard tool for cellular uptake, detection and imaging studies, mainly on a research scale [103]. In a recent review, Wolfbeis gives a very comprehensive overview of the nanomaterials that are mainly used in fluorescence-based imaging. For example, polymer-based particles, lipid-based particles and carbon nanomaterials among others. Bio-conjugation techniques like covalent and non-covalent binding of the fluorescent molecules to the nanomaterial surface are also reviewed.

8.3.10 Laser Scanning Confocal Microscopy and Live Cell Imaging

Understanding the interactions of internalized particles with endocytic vesicles is of crucial importance in order to determine mechanism of actions for therapeutic purposes. This can be done via Live Cell Imaging or Structured Illumination [104]. Widely used cell lines or primary cells can be used as cellular models to study such interactions with fluorescently labelled particles. These models allow concentration and time dependent studies in order to examine the uptake and accumulation of particles in cells [101, 12].

Complementary to that, working with realistic nanoparticle concentrations is important in terms of biomedical applications like vaccine development. For example, gold NPs were exposed to immune cells at concentrations ranging from 10 to 100 μg/mL in vitro, since different concentrations might result in different functional and immunological responses [105]. Especially for cellular internalization and co-localization studies, confocal microscopy is the most common and suitable technique to use since it allows: improved resolution compared to wide-field illumination techniques because the confocal aperture can be closed down to eliminate higher orders of the diffraction pattern. Furthermore, confocal systems allow z-stack projections with improved z-resolution that can prove if particles are internalized. Complementary to that, 3D rendering of the acquired images as well as further image processing (i.e. adjusting the transparency of specific channels) is possible with the use of scientific software (e.g. Fiji or IMARIS). A vivid example of what is currently possible in 3D rendering was recently shown by Lehmann et al. [106] who visualized fluorescent magnetic hybrid NPs after uptake by human blood monocyte derived macrophages in vitro with sub-cellular localization inside lysosomes and not entering mitochondria (Fig. 8.2). More specifically, Blom et al. [107] identified that virosome and liposome uptake by bone marrow derived dendritic cells is done via clathrin-mediated endocytosis as well as phagocytosis. Confocal microscopy was

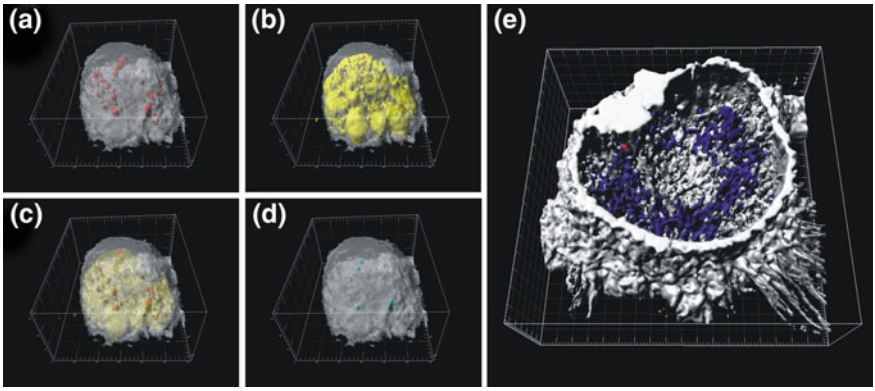

Fig. 8.2 Visualization of fluorescent magnetic hybrid NPs in macrophages with Laser scanning microscopy and 3D rendering. **a** Visualization of NPs (red) inside macrophages (light gray) shown in a 3D reconstruction. **a**–**d** Localization and co-localization (turquoise) of the particles with lysosomes (yellow) are shown. **c** By making the cell-body channel (light gray) transparent as well as the lysosome channels, the particles colocalized with the lysosomes become visible. **d** The colocalization of particles in lysosomes. **e** Mitochondria staining (blue) and particles (red) in a macrophage. Figure adapted from [106] with permission

used to identify that ingested particles co-localize in late endosomes. To further confirm the findings, uptake inhibitors were used as controls. In another study, Seydoux and colleagues [108] have examined the size effect of polystyrene NPs in terms of accumulation and co-localization in lysosomes. Results indicate that smaller particles are uptaken at larger numbers compared to their larger counterparts. Again, confocal microscopy was utilized.

A particular uptake mechanism of NPs in cells will most probably affect intracellular kinetics of NPs. Thus live cell imaging and time lapse experiments provide investigators with live information about cellular dynamics [109]. Other fluorescence-based microscopy techniques to monitor kinetics of NPs, which are used are:

Fluorescence Recovery After Photo-bleaching (FRAP)
Fluorescence Resonance Energy Transfer (FRET)
Fluorescence lifetime imaging microscopy (FLIM).

Those techniques are used to measure cellular dynamics and molecular interactions of living cells and will be briefly described hereunder [110]: For example, FRAP experiments can show rapidly interactions of the investigated fluorescently labelled NPs with cellular organelles like mitochondria and other cytoplasmic vesicles (i.e. intracellular fate of the NPs). For example, Hemmerich and von Mikecz examined the cellular uptake mechanisms as well as the deposition of the fluorescently labelled polystyrene NPs at cellular organelles like mitochondria and other cytoplasmic vesicles [109]. Basuki and colleagues have utilized FLIM to visualize polymer coated iron oxide NPs that contain doxorubicin. Nanoparticle uptake and

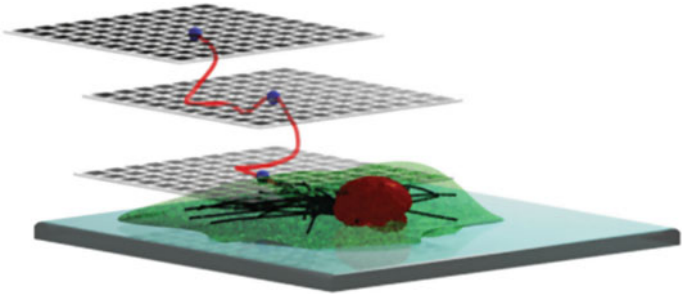

Fig. 8.3 Schematic representation of the 3D multi-resolution microscopy principle. The movement (trajectory) of the nano-probe (blue dot) is depicted in red lines. Then the nano-probe crosses different focal planes until it reaches cell surface (green). Picture adapted from [112]

controlled doxorubicin release was confirmed in cancer cell lines by FLIM [111]. Still, fluorescence microscopy techniques fail to sufficiently measure the early stages of internalization by cells, which would be a key analysis for drug delivery research. Welsher and Yang, proposed a method that peptide conjugated NPs can be visualized with 3D multi-resolution microscopy. Then, imaging software is used to draw the trajectories of the particles into the cells. Multi-resolution 3D visualization utilizes high-speed single-point detection of a nano-probe (i.e. fluorescently labelled NPs). The method allows real-time "target-locking" of the nano-probe, thus "following" the nano-probe at different focal-planes. The method also combines the principles of two-photon laser scanning microscopy. As the nano-probe moves to different focal planes the two-photon laser scanning microscope provides information about the movement of the nano-probe and thus images are built [112] (Fig. 8.3).

Complementary to that, van der Zwaag et al. (2016) have used Two-color Stochastic Optical Reconstruction Microscopy (STORM) and compared their findings with confocal microscopy and electron microscopy. The competitive advantage of STORM is that size and exact location of NPs can be identified inside cells. STORM is based on the principle that activated molecules emit photons. This allows precise localization of the specimen prior to photo-bleaching and beyond the optical resolution limit of 200 nm. Quantitative information can also be extracted via imaging software. The study concludes that STORM can provide additional information regarding cellular uptake of NPs that was missing with confocal and electron microscopy [113].

8.3.11 Super Resolution Microscopy

With the development of super resolution microscopy, imaging at the molecular level has become possible [114]. However, it remains a technically challenging technique, since non-proper stability of the instrument can introduce biasness to the analysis.

Due to their optical properties (i.e. surface plasmon resonance), metal NPs are mainly used in super resolution microscopy applications. Recently, Peuschel and colleagues have shown interactions of fluorescently labelled silica NPs with human alveolar epithelial type-II like cell line (A549). The authors used stimulated emission depletion (STED) super resolution microscopy [115]. The authors exposed in vitro A549 cells to various concentrations of fluorescently labelled polystyrene NPs of different sizes and used STED in order to quantify uptake. The principle of STED is based on a very narrow and focused excitation spot. STED uses both an excitation and a depletion laser, in order to first excite the fluorescent molecules and immediately after deplete them. Fluorescent molecules are depleted before they can emit light. This takes place only in the focused spot and thus the microscope can emit the fluorescence signals from the surrounding spots. This results in an enhanced signal, which allows super-resolution imaging. Such techniques can be used for quantitative approaches as well as to assure whether the NPs have been internalized or remain at the surface of the cells. Additively, co-localization studies can reveal new insights in terms of internalized particles and endocytic vesicles interactions.

8.3.12 Flow and Imaging Cytometry

Over the last years, we and others have published several articles highlighting the use of multi-color flow cytometry to measure cellular uptake in both in vitro and in vivo settings of various types of NP-related factors like material type, size, surface modification and concentration and associate their uptake with a wide range of functional cell type specific properties (i.e. apoptosis and surface marker expression) and immunological properties (immunophenotyping, antigen uptake, antigen processing and cytokine secretion patterns) [107, 108, 116–118]. Flow cytometry is a technique, which allows analysis of thousands of events within one second, thus high-throughput analysis can be designed. Many parameters could be measured simultaneously and in the data analysis phase, could be tested for correlation with cellular uptake. Complementary to that, imaging cytometry allows real-time bright-field images of single cells in suspension, apart from all the aforementioned capabilities of a conventional flow cytometer. For example, cell monocultures that are treated with fluorescently labelled NPs and labelled for some surface markers could be analyzed and thus the effect of particle uptake on cellular properties could be identified. Additively, extracellular vesicles have gained considerable attention in the field of diagnostics and imaging cytometry is also considered an appropriate method for imaging and quantification purposes [119]. Routine flow cytometry based measurements like cell cycle analysis, quantification of cancer cells, internalization and phagocytosis experiments, surface and intracellular localization can be further enhanced, since apart from the flow cytometry data, real time visual confirmation could be acquired. However, researchers must be cautious since if an experiment requires high resolution microscopy studies imaging cytometry might not be the most suitable option due to lack of high-resolution. Furthermore, since it is a rel-

atively new technique, further investigation and proper training might be needed [120].

Fluorescently labelled nano- and micro-particles have become a standard tool for cellular uptake measurements and pre-clinical studies. Established animal models and advanced in vitro co-culture systems, as well as primary and cell-line monocultures are widely used as model systems to study cellular uptake (i.e. qualitative/quantitative approaches and type of uptake). Laser scanning microscopy, flow cytometry and imaging flow cytometry combined with a suitable antibody panel one may provide a large amount of accurate and reliable data to study dynamics at the level of cell entry and intra-cellular trafficking. This information can be further fortified with the use of sophisticated imaging software and statistics. With the advancement of technology in the field of high-resolution imaging, more capabilities will become possible and accuracy will further increase. Taking together the large body of knowledge of cellular uptake related information with recent trends in computer science (i.e. big data management and computational biology) and 3D biomedical imaging, cellular uptake data can be collected from literature sources in order to develop electronic databases and precise prediction algorithms for cellular uptake. Such approaches may be beneficial for decision making in the drug delivery/nanotoxicology/nanomedicine fields as well as for regulatory and legislative purposes.

8.4 Conclusions and Outlook

With a huge number of new-emerging nanomaterials every year, toxicological risk assessment and the research for novel biomedical treatment approaches are of equal importance. Interaction with the biological interface in general and the involved mechanisms of particle uptake in particular, are of great importance to both fields and require advanced imaging techniques for improved investigation of potential risks and opportunities. In order to investigate how NPs are entering the cell and affect its interior, all of the specific characteristics an NP can show (i.e. size, surface, shape, charge, material, physico-chemical properties etc.), have to be considered. Advanced imaging techniques using fluorescently labelled NPs in combination with laser scanning microscopy have pushed into the super-resolution range already more than a decade ago and are constantly improved and refined. In combination with the long-time established techniques in the field of electron microscopy, super-resolution fluorescence imaging is complementing advanced imaging techniques for NPs by allowing visualization in living samples. Constantly improved imaging techniques at the nano-level will allow a better characterization of potential risks and opportunities of NPs by evaluating a larger number of different pathways of interaction with and uptake by cells and with the possibility to monitor more complex processes. For example, with the new goals of subcellular organelle-level targeting, the field of nanomedicine is now moving to a higher level of complexity [11]. Though the task is challenging, there are promising results highlighting the potential advances that

can be expected from organelle-level targeting. However, the concept of subcellular-targeted NPs is in its infancy, and few strategies have so far been reported organelles like for ER, mitochondria, and nucleus targeting. More detailed investigations are needed to assess the impact and relevance of NP uptake and subcellular targeting for future clinical applications.

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