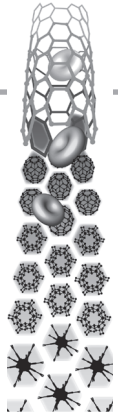
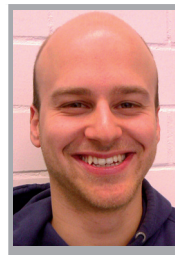


Pharmacology of nanocarriers on the microscale: importance of uptake mechanisms and intracellular trafficking for efficient drug delivery

"*The route of endocytosis and the differential sorting into endosomal compartments determines the effects the cargo will have.*"

KEYWORDS: drug delivery ■ endocytosis ■ intracellular trafficking ■ nanoparticle uptake



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A thorough understanding of nano–bio interactions on a microscale is essential for successful drug delivery

Nanocarriers continue to be one of the most promising tools as drug delivery devices for improving the pharmacokinetics of substances. Huge efforts have been made to optimize nanospheres for the efficient delivery of unstable drugs or biomolecules, such as siRNA and peptides. The loading of such nanospheres enables, in principal, the delivery of high doses of effector molecules to the target site with simultaneous control of their release. On a macroscopic level, surface modifications (e.g., by polyethylene glycol) prolong the circulation time within the body and pledge to enhance the effective dose in the desired cells (e.g., tumor cells). To exploit the full potential of nanocarriers, several macroscopic obstacles need to be overcome such as aggregation of nanocarriers in blood or undesired particle accumulations in the liver and spleen. Besides these objectives, it is equally essential to understand the microscopic and submicroscopic interactions with the cell for the optimal design of nanocarriers, such as drug delivery devices. The route of endocytosis and the differential sorting into endosomal compartments determines the effects the cargo will have. This has been demonstrated in other fields, such as for the presentation of bacterial antigens [1] and for different early endosomal compartments [2].

The docking of nanospheres to a cell membrane initializes a variety of endocytotic processes that mediate their intracellular uptake. The endocytotic effector proteins have a preferential mode of action towards uptake of distinct molecules and nanoparticles [3]. Therefore, nanocarriers should target the most efficient uptake pathway. Endocytosis mechanisms initiate the inverse budding of a vesicle entrapping the nanocarrier and the encapsulated cargo in an endosomal compartment. While the target location for most payloads is found in the cytoplasm, the majority of nanoparticles are first trapped in membrane-surrounded vesicles prone to degradation of the nanocarriers. Endosomal release by opening of the nanocarrier and triggering the endosomal release are the major challenges for drug release. The opening of loaded nanospheres is intended to occur by intracellular and extracellular triggers (e.g., pH changes, enzymes, light and electromagnetic fields) that may require large knowledge of the endosomal milieu. Depending on the opening strategy, release either leads to diffusion of the payload through the endosomal membrane into the cytosol or to the destruction of the endosome [4,5].

Focusing on the less prominent pathways

Currently, it is well accepted that nanoparticle uptake is mediated by several endocytotic mechanisms that are used simultaneously to different extents [6]. Various endocytotic pathways have been successfully studied in approaches utilizing specific inhibitors or siRNA knockdown identifying a major endolysosomal route for several nanospheres [3]. Well-characterized mechanisms of nanoparticle endocytosis involve clathrin and caveolin proteins, as well as several GTPases. GTP-binding proteins such as cdc42, RhoA and Arf6 define and control the uptake pathways of nanomaterials in a distinct manner [7]. Less prominent endocytotic pathways such as flotillin/reggie-dependent endocytosis are upcoming pathways of high interest in the field. Flotillins appear in a wide range of cellular processes and build microdomains associated

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with cholesterol and sphingomyelin that are part of the intracellular lipid transport. Flotillins also play an important role in nanoparticle endocytosis. Braeckmans and colleagues depict an early uptake of polyplexes mainly by Rab5- and flotillin-2-positive vesicles [8]. Further reports associate flotillins with the uptake of silica nanoparticles in a caveolae- and clathrin-independent fashion [9]. In addition, whether the fusion of flotillin vesicles to the lysosome is always occuring or, if so, the presence of flotillin on these early endosomes trafficks them to other pathways has to be investigated. This could raise interest in targeting further unknown endocytotic pathways.

Complexity of intracellular trafficking impedes the clear identification of different intracellular compartments

The maturation of endosomes during the different stages of intracellular trafficking leads to an increase in pH and a recruitment of characteristic proteins onto the vesicle. If the nanoparticles are entering the recycling pathway and are exocytosed, the endosomal enrichment of lysosomal proteases and the increasing intravesicular acidification defines the final destination of the majority of nanoparticles. Once trapped in the lysosomal compartment, the nanosphere stays there until degradation or cell death occurs. Besides this, several studies report crosstalks of nanoparticle-containing endosomes with components of the autophagosomal pathway [8,10]. These rare events mirror the possible diversity of vesicular fusion events complicating subcellular drug delivery. In cases of autophagy, vesicles of the endosomal pathway (early/late endosomes) merge with autophagosomes forming an amphisome [11]. This opens up a pathway with an intermediate vesicle normally ending up in an autolysosome. The markers LC3-II as well as Rab11 and Rab7 play major roles in the biogenesis of amphisomes autolysosomes, impeding the precise distinction of late endosomes, recycling endosomes and amphisomes [11]. These roles have to be examined in more detail, since autolysosomes are built by completely different pathways fulfilling different functions [12].

The route that the cargo takes mainly depends on the surface proteins on the vesicle. Further on, components of the endosomal sorting complex required for transport direct biological freight from early endosomes to multivesicular bodies. These control centers may decide whether the cargo is exocytosed or processed for lysosomal degradation. Some previous works report the exocytosis of nanoparticles in several cell lines [13,14]. The mechanisms of nanoparticle exocytosis remain unclear. Moreover, ejection of cargo is also mediated by $Rab11^+$ recycling endosomes. Data suggest that a small portion of nanoparticles are transported to $Rab11^+$ vesicles, revealing the possible ejection of nanocarriers using a recycling pathway. However, one has to be careful to interpret Rab11 as a unique marker for recycling endosomes. Since Rab11 was also found on amphisomes, a misinterpretation of autophagosomal pathways in not excluded [11].

Technical approaches to examine intracellular trafficking

Experimental detection of intracellular trafficking pathways is limited to a handful of markers applicable at the same time. Incomplete knowledge regarding vesicular fusion events in nanoparticle transport also restricts research in this field. Recent approaches try to bypass these hurdles by using co-localization experiments utilizing overexpressed fluorescent proteins, as well as immunostaining, to gain an understanding of the events occurring during intracellular trafficking [15]. Further approaches use pH-sensitive dyes to measure the acidification of intracellular vesicles [16]. Since nanoparticle uptake happens simultaneously by different mechanisms, the tracking of co-expressed markers remains difficult. Often, marker proteins are natively expressed in a low copy number, making their detection via fluorescence-based methods challenging. These circumstances force us to work with systems overexpressing proteins with the possible pitfall of altering the vesicular transport [17]. Therefore, new methods have to be established to measure nanoparticle tracking under native conditions, thereby also detecting rare trafficking events.

Future perspective: finding the right approaches for controlled intracellular release

Several approaches demonstrate a controlled release of nanoparticle content. Reports of degradation of polymeric nanospheres due to redox reactions and enzymes have been published [4]. The next step will be to overcome the endosomal membrane barrier in order to enable subcellular targeting. Approaches using GALA peptides, and other peptides, can mimic the endosomal escape sequence of viral proteins, inducing a direct release of nanocargo into the cytosol [18]. Bräuchle and colleagues demonstrated a photochemical rupture of the endosomes thereafter releasing a

disulfide-bound dye molecule due to the reducing conditions in the cytoplasm [4]. Our group used biodegradable poly(butyl)cyanoacrylate for a controlled endosomal release of oligonucleotides for specific subcellular targeting to mitochondria [19]. Further approaches utilize lipid-coated calcium phosphate nanoparticles that are disassembled due to increased endosomal acidification, leading to the release of the payload into the cytosol [20]. Each of these systems achieves the delivery of cargo to the cytosol in a different way and on different time scales. This demonstrates the powerful potential of nanoparticle drug delivery and strengthens the vision of a directed subcellular targeting someday becoming fully controllable.

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