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Endocytosis and Intracellular Trafficking as Gateways for Nanomedicine Delivery: Opportunities and Challenges

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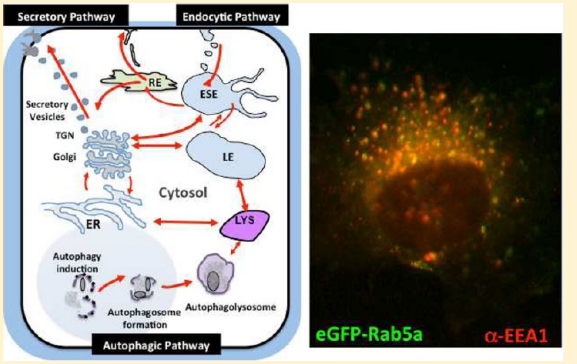
ABSTRACT: More than 40 nanomedicines are already in routine clinical use with a growing number following in preclinical and clinical development. The therapeutic objectives are often enhanced disease-specific targeting (with simultaneously reduced access to sites of toxicity) and, especially in the case of macromolecular biotech drugs, improving access to intracellular pharmacological target receptors. Successful navigation of the endocytic pathways is usually a prerequisite to achieve these goals. Thus a comprehensive understanding of endocytosis and intracellular trafficking pathways in both the target and bystander normal cell type(s) is essential to enable optimal nanomedicine design. It is becoming evident that endocytic pathways can become disregulated in disease and this, together with the potential changes induced during exposure to the nanocarrier itself, has the potential to significantly impact nanomedicine performance in terms of safety and efficacy. Here we overview the endomembrane trafficking pathways, discuss the methods used to determine and quantitate the intracellular fate of nanomedicines, and review the current status of lysosomotropic and endosomotropic delivery. Based on the lessons learned during more than 3 decades of clinical development, the need to use endocytosis-relevant clinical biomarkers to better select those patients most likely to benefit from nanomedicine therapy is also discussed.



KEYWORDS: *endocytosis, nanomedicine, endosome, lysosome, trafficking, lysosomotropic delivery, endosomotropic delivery*

INTRODUCTION

To distinguish them from protein and other nanosized biologics, nanomedicines are defined as specifically engineered, nanosized drugs and drug delivery systems composed of at least 2 components (often they have many more). More than 40 nanomedicines are already in routine clinical use with a growing number entering preclinical or clinical development (reviewed in ref 1). The main goals of nanomedicine design are creation of improved pharmaceutical formulations (e.g., solubilizing a poorly soluble drug and/or enhancing its oral bioavailability), improvement of drug targeting to the desired site of action (at the cellular or, especially for macromolecular drugs, at the subcellular level) with simultaneously reduced access to sites of toxicity, control of the location and rate of drug release rate, and in certain cases the aid of transportation of drug across a biological barrier such as the GI tract, lung, and the blood−brain barrier (BBB). In many cases nanomedicines must navigate the endocytic pathways to achieve these goals. Design therefore relies on a comprehensive understanding of endocytosis and intracellular trafficking pathways in both target and normal cell type(s), and any endocytic disregulation occurring either in disease or due to exposure of cells to the nanocarrier itself will impact on both safety and efficacy. Given the rapidly evolving vision of the role of intracellular membrane dynamics in health and disease,[2] the emergence of ever more complex multicomponent nanocarriers,[1,3,4] and not least, the lessons being learned during clinical evaluation of those first generation nanomedicines designed for lysosomotropic or endosomotropic delivery, it is timely to review the opportunities and challenges for the safe and effective design of those nanomedicines intending to exploit endocytic pathways to deliver improved treatments for life threatening and debilitating diseases. The pros and cons of methods used to quantitate intracellular fate, and the future need to consider endocytosis-relevant clinical biomarkers as tools to better select patients most likely to benefit from nanomedicine therapy, are discussed. At the outset we would like to acknowledge that the bibliography is incomplete: it would be impossible to cite all the excellent reviews and important primary papers relating to this ever growing field. However, the reader is directed to refs 5−8 and the references therein for complementary discussion of specific aspects relating to all the molecular mechanisms involved in the endocytic machinery, the many emerging nanomedicines/nanomaterials[1] and aspects of drug delivery at the intracellular level.

ENDOCYTOSIS AND INTRACELLULAR TRAFFICKING

Endocytosis is not only responsible for the internalization of macromolecules and particles from the extracellular environ-

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ment, it plays a crucial role in many physiological/biochemical processes including removal of cell debris during apoptosis-mediated cell turnover, immune surveillance, neurotransmission, regulation of cell surface receptors and transporters, remodeling of the extracellular environment and intra- and intercellular communication. Moreover, endocytosis is rapidly becoming viewed as the "master organizer of cell signaling".[9,10] The mammalian endomembrane system is characterized by its complexity and the pivotal role it plays in cell homeostasis. Whereas the pathways of endocytosis (from refs 11 and 12), exocytosis/secretion (from ref 13), and autophagy (from refs 14−16) are often studied separately, they always act in concert (Figure 1).

Routing is very carefully regulated with a highly dynamic exchange of both membranes and the cargos translocated within vesicles. The receptor clustering seen within clathrin-coated pits (CCPs) when investigating the uptake of low-density lipoprotein gave the basis for clathrin-mediated endocytosis (CME).[17] This intracellular compartmentalization is critical as it separates

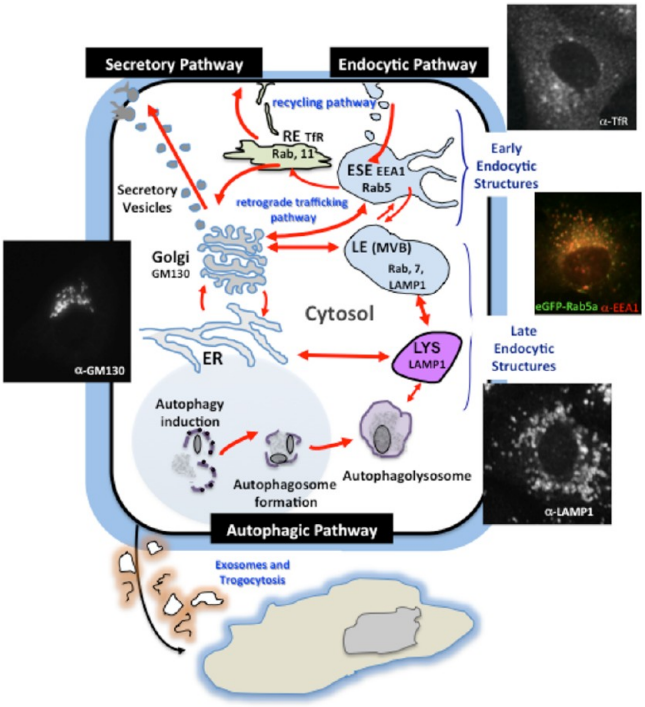

Figure 1. A simplified overview of endomembrane trafficking pathways. Typical markers of organelle identify are shown: early sorting endosomes (ESE; EEA1 and Rab5 positive); late endosomes (LE; Rab7 and LAMP 1 positive), this compartment is also referred to as a multivesicular body (MVB); recycling endosome (RE; Rab11 and transferrin receptor (TfR) positive); lysosomes (LYS; LAMP1 positive) and the Golgi (GM130 positive). This is a highly regulated and dynamic, interconnected network of compartments linked by intermediate hybrid vesicles where vesicle−vesicle fusion and content mixing can occur. Membrane and vesicle shedding is also important in cell−cell communication, e.g., via exosomes and trogocytosis. The fluorescent images depict Vero cells stained using specific organelle markers (for methods see refs 41, 50, and 55). Table 1 gives more details of the constituent organelles and the markers used to identify them. It is important to note that internalized materials usually arrive within the ESE after ∼5−10 min, and in the LE/LYS within ∼30−60 min. Material recycling from the ESE can take several routes back to the surface, but when directed via the RE arrival in a Rab11 positive structure usually occurs after ∼30 min.

biochemically incompatible functions such as anabolism, catabolism and the "cell's memory", the genome. Additionally there is growing awareness that released vesicles (exosomes)[18] and membrane fragments containing proteins (trogocytosis)[19] play a critical role in communication between neighboring cells. The latter will not be discussed at length here, but these processes are clearly important therapeutic targets and potential players in terms of toxicity of novel nanomedicines. Although exocytosis and autophagy play no direct role in cellular entry, the rate of nanomedicine exocytosis governs residence time within intracellular compartments and consequently the window of opportunity available for drug delivery. Moreover, the ability of a nanomedicine to perturb any of the trafficking pathways can potentially elicit toxicity (discussed later in this review).

Throughout, endocytosis and exocytosis are intimately connected both functionally, e.g., through processes such as organelle biogenesis, and mechanistically, e.g., cargo sorting, membrane scission, vesicle transport, membrane fusion and content mixing. Although membrane trafficking may be thought of as both starting and ending at the plasma membrane (PM), in truth it is more of a continuum. An ever-increasing library of coat proteins, adaptors, retrieval proteins, scission proteins (e.g., dynamin), Rab GTPases, and soluble *N*-ethylmaleimide sensitive factor accessory protein receptor (SNARE) proteins have been identified in eukaryotic cells, and they are repsonsible for the regulation of endocytosis and intracellular trafficking. (It is not the intention to discuss the many regulatory proteins individually here (more than 60 Rab GTPases and SNARE proteins have been described), and further detail can be found in refs 8 and 20−22.) The actin−tubulin cytoskeleton also plays a very important functional role in almost all types of endocytosis. While certain endocytic pathways are ubiquitous to all cells, others are cell-specific or play an enhanced role in certain cell types. This is an important consideration when developing a nanomedicine to act within a particular cell type/diseased tissue. Additionally, polarized cells (e.g., the gastrointestinal (GI) tract brush border epithelium, hepatocytes neurones and other cells engaged in chemotaxis and embryogenesis) can exhibit distinct membrane domains and/or controlled basolateral-apical trafficking pathways (see refs 23 and 24). Translocation across cells using the transcytosis pathway has long been seen as a route by which nanomedicines might traverse biological barriers,[25] but it is not evident yet how efficiently this route can be exploited clinically.

Gateways. During endocytosis PM invagination results in the internalization of externally disposed solutes, macromolecules, and pathogens (including bacteria, intracellular parasites, and toxins) via a number of distinct "gateways". Larger particulate matter, including nanomaterial aggregates and opsonized nanoparticles (sizes can be from nm to many μm) are captured by phagocytosis[11,26] (Figure 2a). This mechanism is associated with, but not limited to, professional phagocytic cells such as macrophages, monocytes and dendritic cells. It is triggered by particle binding to the PM (often following particle opsonization), which induces membrane engulfment and then internalization into a membrane-bounded vesicular compartment termed a phagosome. Large particle engulfment has been reported in other cell types, e.g., fibroblasts and endothelial cells, and we even noted that B16F10 murine melanoma cells avidly take up chitosan microspheres[27] (see also Figure 8 later in text). It is interesting to distinguish the physiological mechanism of phagocytosis, induced by particle attachment to specific receptors, from "forced entry", a term used by cell biologists to

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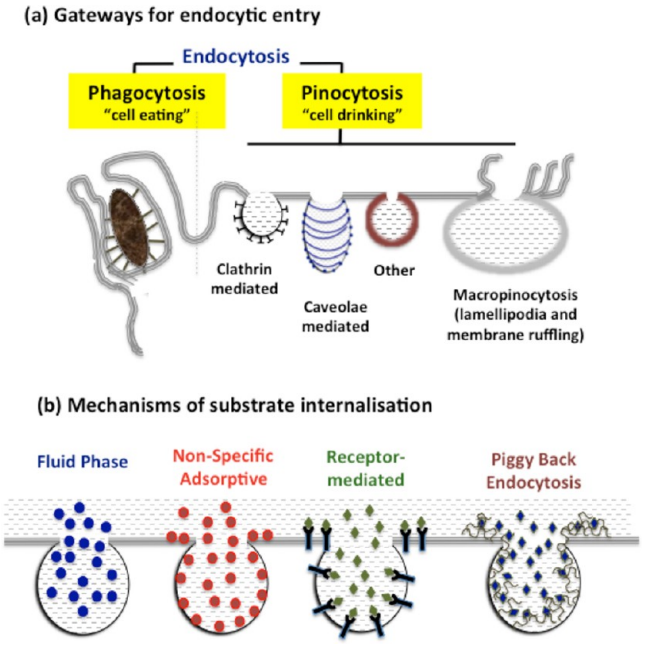

Figure 2. Primary internalization gateways and the fundamental mechanisms of nanomedicine internalization, shown in panel (a) and panel (b) respectively.

describe material internalization driven by the physicochemical attributes of the material surface itself. Initial PM binding encourages further membrane interaction resulting in full or partial engulfment depending on the object's dimensions. Inability to fully internalize (e.g., an asbestos fiber, carbon nanotube, etc.) has also been called "frustrated" endocytosis. Rapid reticuloendothelial system (RES) clearance of intravenously (iv) injected nanomedicines can result in highly efficient drug targeting to these cells, and the liposomal and lipidic amphotericin B products (Ambisome, Abelcet and Amphocil) use this targeting strategy to good effect as treatments for opportunistic infections and leishmaniasis (reviewed in ref 28). However, RES clearance more often constitutes a significant biological barrier reducing access of parenterally administered nanoparticles to more remote target cells/tissues in the body. Many modern strategies seek to circumvent the problem by use of smaller, hydrophilic nanocarriers, or by addition of hydrophilic coatings, the so-called "Stealth" approaches including PEGylation.

Pinocytosis provides a large number of alternative gateways for cellular entry. In this case internalizing membrane-bounded vesicles typically have a smaller diameter, i.e., in the range of 50 nm to several 100 nm in the case of macropinocytosis. A growing number of distinct internalization gateways have been identified that are usually named after either the proteins that mediate membrane invagination and/or the physiological cargos being transported. The principal mechanisms are shown in Figure 2a. In each case vesicles pinching-off from the PM are directed to particular intracellular sorting stations after entry (see Table 1). Although the question is often asked, What is the optimum size for entry? it would be unwise to ascribe a specific diameter to each of these entry ports as they vary from vesicle to vesicle, from cell type to cell type and also species to species. Moreover, many profiles are more tubelike than spherical, e.g., the flask-shaped indentations forming caveolae. As a general rule for pinocytosis, "the smaller the better". The most important consideration is to verify the functional ability of target cells (for efficacy or toxicity) to engulf the particular nanomedicine under development.

Commonly discussed gateways include uptake via clathrin-coated pits (CME), caveolae (cavoelae-mediated endocytosis; CavME), and macropinocytosis. The latter is associated with membrane ruffling and formation of lamellipodia and consequently relatively large volumes of extracellular fluid can be internalized (Figure 2b). Membrane microdomains of specific lipid composition, "lipid rafts", can play a pivotal role in CavME and other, non-CavME internalization mechanisms, functioning to assemble trans-membrane and membrane anchored proteins important in the signaling pathways.[29] It is important to note that the many other clathrin- and caveolae-independent pathways (e.g., the flotilin-mediated pathway) and cargo-specific routes have been discussed in detail elsewhere (e.g., refs 6, 7 and 30).

Although endocytosis is common to all cell types (except erythrocytes), certain pathways are cell-specific or display greater expression in certain cells, e.g., CavME in vascular endothelial cells.[31] Even when decorated with ligands designed to enhance receptor-mediated uptake via a particular pinocytic route, a nanomedicine will still enter cells via a number of different gateways at the same time. Gateway defective cell lines and/or cocktails of putative gateway-specific chemical inhibitors have become popular tools when trying to dissect specific nanomedicine or nanomaterial uptake mechanisms *in vitro*. In reality, however, it is difficult to rule out concomitant entry by multiple routes, and the relative importance of different gateways can vary from one clinical target cell type to another. It should be emphasized that many gateways localize cargos to common trafficking pathways thereafter. Moreover, very few *in vivo* or clinical studies are conducted to ascertain whether *in vitro* observations reported are predictive of the clinical setting. Interestingly, it has been suggested that the albumin-paclitaxel nanoparticle Abraxane elicits improved tumor targeting due to interaction with the albumin binding protein SPARC (secreted protein, acidic and rich in cysteine) which promotes gp60 and caveolae-mediated endothelial transcytosis.[32] Although there is preliminary evidence that SPARC expression in head and neck cancer patients may correlate with response to therapy, this theory is still hotly debated.

Whichever entry route is used, it is important to remember that nanomedicines can be internalized either in solution or following interaction with the PM. This attachment in turn can be due to nonspecific (typically due to charge or hydrophobic interaction) or specific receptor-mediated binding (Figure 2). Internalization efficiency over time will depend on the total number of binding sites/receptors per cell, the rate of receptor recycling and the effect of the cargo on receptor up/downregulation. When internalization occurs simply due to the presence of a nanomedicine as a solute in the extracellular fluid the process is called fluid-phase pinocytosis (Figure 2) (discussed in refs 5 and 8). This is sometimes dismissed as poorly efficient mechanism of internalization as uptake is proportional to the extracellular concentration, but fluid-phase pinocytosis can actually be quantitatively very important. Some cells internalize a very large proportion of their surface per hour,[33] and this led to the postulation of membrane recycling long before the phenomenon was experimentally proven.[34] As an example, and contrary to popular opinion that LDL uptake occurs solely via a receptor-mediated pathway in macrophages, high rates of nonsaturable fluid-phase uptake of LDL have been demonstrated, and importantly this can result in the conversion of macrophages into foam cells.[35] Finally, it is important to mention

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Table 1. Intracellular Compartments and the Commonly Used Organelle Markers

Compartment	Characteristics/Function	Markers[a]
The early endosome (ESE)	• cargos typically arrive after ~5 min and those returning to the PM do so via the rapid recycling pathway within 5-10 min • Acidic pH, typically 6.5 maintained by a membrane-associated proton pump • Disassociation of receptor-bound cargo • Material sorting into a (putative) catabolic pathway or recycling pathway • Spatial segregation of membrane, containing proteins and receptors for recycling (into reticular tubes), from solutes due to remodelling of the ESE surface area : volume ratio	• Rab5 • Early Endosomal Antigen 1 (EEA1) • Transferrin receptor (TfR)
The recycling endosome (RE)	• reticular structure that contains recycled material e.g. apotransferrin • cargos typically arrive after ~30 min and those returning to the PM do so within 30-60min	• Rab11 • Transferrin receptor (TfR)
Late Endosomes (LE)	• cargos typically arrive after ~ 60 min • mannose-6-phosphate receptor (M6PR) responsible for translocation of newly synthesised LYS enzymes into the LE. • Rab7 regulates both homotypic and heterotypic fusion • may be documented (by electron microscopy) to contain internal membrane and are also referred to as multi-vesicular bodies (MVB) • they contain LYS-associated membrane proteins (LAMP)s that are highly glycosylated, integral membrane proteins containing a C-terminal sorting signal that directs to LE	• Mannose-6-phosphate receptor (M6PR) • Rab7 • LAMPs 1-3
Lysosomes (LYS)	• these organelles are critically involved in the degradation of materials arriving via endocytosis and autophagy and contain > 60 LYS enzymes	• Rab7 • LAMPs • cathepsin D
Trans-Golgi Network (TGN)	• Sorting of endogenous proteins destined for secretion or other organelles • Rarely accessed by exogenous proteins other than highly evolved toxins	• TGN38 or TGN46 (depending on species) • Ceramide
Cis- and Medial- Golgi	• Sorting of endogenous proteins destined for secretion or other organelles • Rarely accessed by exogenous proteins other than highly evolved toxins	• GM130
Endoplasmic reticulum (ER)	• Quality control of newly synthesised proteins destined for a) secretion, b) other intracellular organelles or c) transmembrane proteins	• Derlin 1 or BiP

[a] More fully reviewed in[41,55]

the phenomenon termed "piggyback" endocytosis. In this case internalization of extracellular solutes, including nanomaterials and nanomedicines, can be driven by interaction with another substrate present in the external milieu (a macromolecule or protein) that itself enters via an adsorptive mechanism, hence the term "piggy-back". As an example, it was found that polycations such as poly(L-lysine) (PLL) do not stimulate pinocytic vesicle formation as originally thought,[36] but enhance cell association of substrates such as colloidal gold and albumin by piggy-back endocytosis.[37] There is a growing debate as to the role of plasma protein−nanomaterial interaction (the "protein corona") and its importance both in determining acute infusion reactions in patients and also in regulating PK and biodistribution *in vivo*.[38] Protein-induced changes in PK may be deleterious, e.g., opsonization resulting in rapid RES clearance, or may be beneficial due to promotion of improved targeting, as suggested for apolipoprotein E (ApoE)-mediated nanoparticle targeting to/translocation across the BBB and hepatocyte-specific localization.

Intracellular Compartments: Features and Functions. The mechanisms responsible for scission of a membrane invagination to form a vesicle, together with the subsequent dissociation of coat proteins, are well documented for CME and CavME (e.g., ref 39). Thereafter inwardly migrating vesicles rapidly relocate to discrete intracellular compartments depending on their origin/cargo. The critical steps responsible for vesicle translocation and fusion are shown in Figure 3. The classes of protein that mediate these events are very carefully regulated through coordinated action, but again it should be emphasized that entry via a particular gateway does not automatically link into a particular trafficking pathway. It is also important to note that it is increasingly being realized that genetic disorders (particularly of the CNS) can occur due to defects in the endomembrane trafficking machinery[40] (e.g., mis-sorting of cargo, defects of vesicle formation, docking or fusion, or in their movement by the cytoskeleton).

Although intracellular vesicles are often labeled as endosomes or LYS based simply on morphological appearance in a TEM or fluorescence microscopy images, intracellular organelle positioning and morphology are often very different in each cell type, making this kind of assumption unhelpful. It is important to identify subcellular compartments using "landmarks" or markers (reviewed in ref 41). As internalized receptors, cargos, and indeed many of the regulatory proteins only transiently associate

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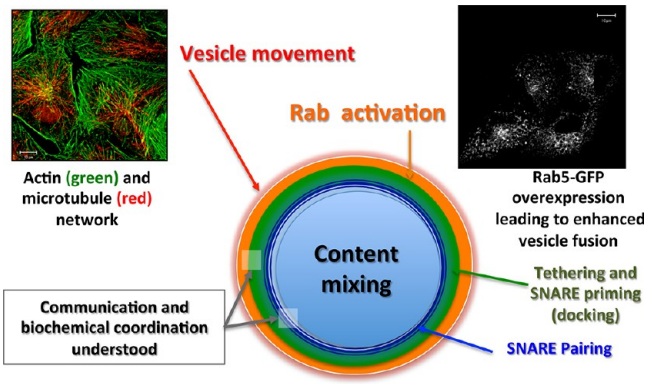

Figure 3. A simplified schematic showing the critical events responsible for intracellular vesicle translocation, and vesicle−vesicle membrane fusion. The fluorescent images are Vero cells. Although much is now known about the biochemical coordination of multiprotein complexes responsible for some of these events, a great deal of research in still ongoing to elucidate all the underlying mechanisms, and their regulation in different cell types, e.g., the role of EHD proteins[180] and deubiquitination.[181]

with a specific compartment (there may be latent protein pools elsewhere in the cell, e.g., the EEA1 cytosolic pool), there is a continuing search for reliable organelle markers applicable to all cell types. Proteins responsible for regulation of fusion (Figure 3 and Table 1) are often chosen as they have the potential to mediate "lock and key" type specificity thus defining organelles and their content through the very nature of their function. However, even such markers can be imperfect as they may catalyze more than one fusion event, e.g., the SNARE protein syntaxin 6, which is primarily associated with the *trans*-Golgi network (TGN) but not uniquely.[42] The principal intracellular compartments accessed by nanomedicines that are frequently critical to their performance, and the markers commonly used to characterize them, are briefly described in Table 1.

Cargo Sorting during Endocytosis. It is important to consider the mechanisms by which physiological cargos are sorted both to gain insights as to the potential regulators of intracellular trafficking of internalized nanomedicines and to consider the opportunities for manipulation of fate to suit the precise therapeutic goal. Multiple mechanisms are responsible for the intracellular sorting "into" or "out of" the default pathway leading to LYS degradation, but the primary sorting decisions are often regulated by protein complexes found on the cytosolic surface of an organelle, for example, the "endosomal sorting complex required for transport" (ESCRT) complex.[43] What happens when a nanocarrier that normally progresses through the EE-LE into LYS compartment is decorated with a targeting ligand that would normally follow a recycling or transcytosis pathway? Which pathway will the vector take? This presents an interesting dilemma, and the outcome is often theoretically impossible to predict in a quantitatively meaningful way. It is important to verify/quantitate experimentally the pathway actually taken in a particular cell type. Regulation of trafficking pathways also requires the modulation of cargo effectors within the lumen of a vesicle, and a transduction system in the form of integral membrane proteins is often deployed, e.g., the reversible ubiquitination of activated epidermal growth factor receptor (EGFR) complexes governs sorting between the recycling and degradation pathways.[44] Although these sorting events work in concert with the route of internalization, they also rarely act as the sole determinant of cargo destination. For example, ricin holotoxin is internalized via multiple entry gateways, and even when both CME and CavME are inhibited, ricin uptake still occurs. Once internalized, ricin is destined for Golgi translocation via the retrograde recycling pathway from the early sorting endosome (ESE) irrespective of entry gateway. Exquisite ability for selective cargo differentiation and sorting is seen during redirection of cargos from the recycling endosome (RE) where destinations include the Golgi for cholera toxin B chain[45] and the PM for transferrin receptor (TfR) which are internalized via CavME and CME respectively.

Alterations of Endocytosis and Trafficking Pathways in Disease. It has long been known that certain intracellular pathogens (e.g., *Leishmania*, *Mycobacteria*, *Salmonella* etc.) can hijack endocytosis and subvert the intracellular trafficking pathways to on one hand avoid LYS degradation and on the other minimize their immunorecognition. Effectively they become "stowaways in the endosomal network".[46,47] There has been significant effort to better understand the biochemical nature of the *Leishmania* parasitophorous vacuole and other intracellular pathogen "protective" compartments with the goal of designing nanomedicines that will be able to localize specifically these compartments.

Although genomics and proteomics research has given much insight into the molecular basis of diseases, the past decade has seen a growing realization that the disregulation of membrane dynamics and intracellular compartmentalization also plays a pivotal role in many of the functional alterations seen. Indeed Sigismund et al.[10] have wisely suggested that we need to "upgrade our vision of endocytosis" from that of a simple degradation and translocation pathway. There is a growing realization that a systems biology approach is needed to understand properly the dynamics of intracellular location of protein effectors and the role of location on their function. Further elucidation of endocytosis and trafficking pathways is seen as an opportunity to generate both new therapeutic targets and a better understanding of current mechanisms of drug resistance (discussed in refs 2 and 48 Mosesson et al.[2] comprehensively reviewed the consequences of "derailed endocytosis" in cancer, discussing the potential for misrouting of junctional proteins (resulting in loss of tight junctions), delayed internalization of growth factor receptors (growth promotion switched on), and modulation of signal transduction thus highlighting the potential for novel therapeutic targets in oncology. So how will this endocytosis disregulation in disease impact nanomedicine design and use? Perhaps this will be a double-edged sword. On one hand there are certainly opportunities for tailor-made nanomedicines that can capitalize on the emerging endocytosis-related therapeutic targets. As their cellular uptake is generally restricted to the endocytic routes, this might seem to be advantageous for targeted delivery in comparison to the use of low molecular weight chemical entities. However, on the other hand, if downregulation of internalization mechanisms and/or nonphysiological trafficking occurs, inhibition of efficient nanomedicine trafficking to the desired intracellular trigger for activation and/or the ultimate pharmacological target might occur (see later discussion on lysosomotropic and endosomotropic delivery. The opportunities and challenges are worthy of careful consideration in each case.

NANOMEDICINE ENDOCYTOSIS: QUANTITATION

Accurate quantitation of nanomedicine endocytic properties is essential in order to define structure−activity relationships accurately, and to optimize a lead candidate design. Before any biological testing, the quality of the material under study must be

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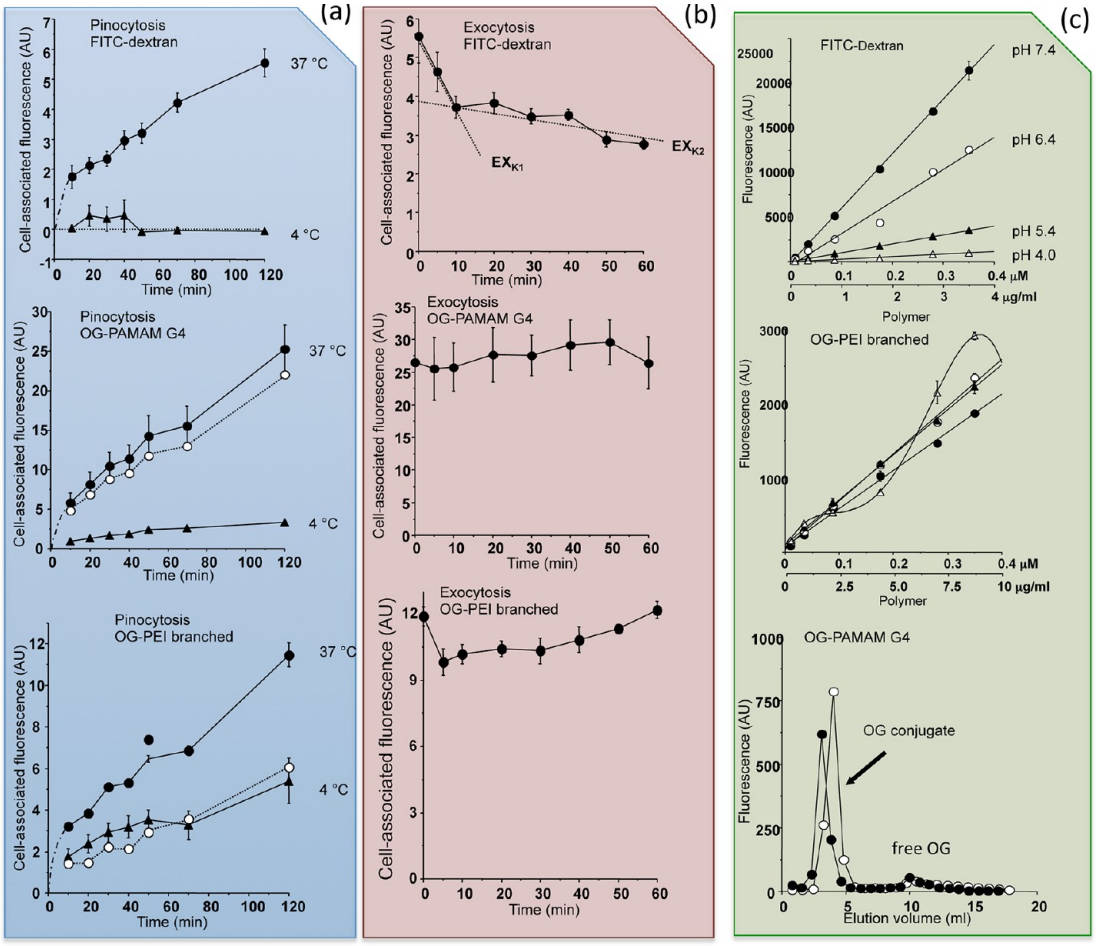

Figure 4. Examples illustrating some key issues relating to the quantitation of endocytosis. (a) Association of polymer/dendrimer−Oregon Green (OG) conjugates with B16F10 cells with time (see ref 60 for full methodological details). Branched PEI−OG exhibits the highest degree of cell association at 4 °C, and this is progressive over time. (b) Exocytosis of the same probes.[60] Whereas FITC-dextran displays exocytosis from a "fast" and a "slow" compartment, neither PEI nor the PAMAM dendrimer was released from cells over this time frame. (c) Illustration of some of the inherent challenges relating to data interpretation in such experiments. FITC-dextran exhibits pH-dependent fluorescence quenching, whereas PEI−OG does not. The PAMAM−OG conjugate releases free fluorophore into the incubation medium over time.[60]

rigorously established, and issues such as inadequate characterization, poor dispersibility and/or poor stability in tissue culture medium as well as the use of appropriate standards have been widely discussed elsewhere.[49] The biological and technical issues listed below are particularly important to consider when studying nanomedicine endocytosis and trafficking.

Cells. The primary cells or cell lines chosen should best represent cell pathophysiology that actually exists in the clinical setting. Although this rarely happens, it is wise to establish the endocytic properties and baseline internalization rates with the aid of a reference marker(s) in advance of studies on particular nanomedicines (e.g., Table 1 and ref 50). Use of an integral endocytic reference marker (an easily quantified substrate) in each experiment also enables monitoring of any day-to-day variability, and allows direct comparison of the rate of uptake of different nanomaterials and nanomedicines with one another in that particular cell type. Further controls can also be performed to show whether the nanomedicine itself impacts on normal cellular endocytosis or intracellular trafficking behavior of the reference substrate.

Culture Conditions. Classical cell biology experiments often make use of serum-free conditions. Nanomedicines administered parenterally will never encounter a serum-free environment, so its value as a tool for evaluation and prediction of behavior *in vivo* is limited. Moreover, it is important to emphasize that many studies investigating the endocytic properties of nanomedicines *in vitro* involve continuous incubation of cells with the probe over many hours. Depending on the substrate concentration in the medium, and the rate of nanomedicine internalization, cells maybe exposed to a high nanomedicine concentration for a long time, i.e., conditions that are irrelevant to the *in vivo* situation. Factors such as substrate concentration, kinetics of uptake (including the potential for receptor saturation/downregulation), and the time dependence of internalization and intracellular trafficking should be carefully considered when setting a protocol for *in vitro* studies in order to maximize relevance of the data obtained to the *in vivo*/clinical setting.

Seeding density, phase of growth, cell-cycle dependence, and degree of confluence can all impact on endocytic behavior, and *in vitro* models should be carefully calibrated with this in mind. Also many nanomaterials/nanomedicines may potentially impact not only cell viability per se but also the machinery responsible for endocytosis, e.g., microfilament and/or microtubule function. Will nanocarriers delivering taxanes disrupt their own internalization pathway before killing the cell? Potentially a great way to induce resistance! Experiments evaluating mechanisms of uptake

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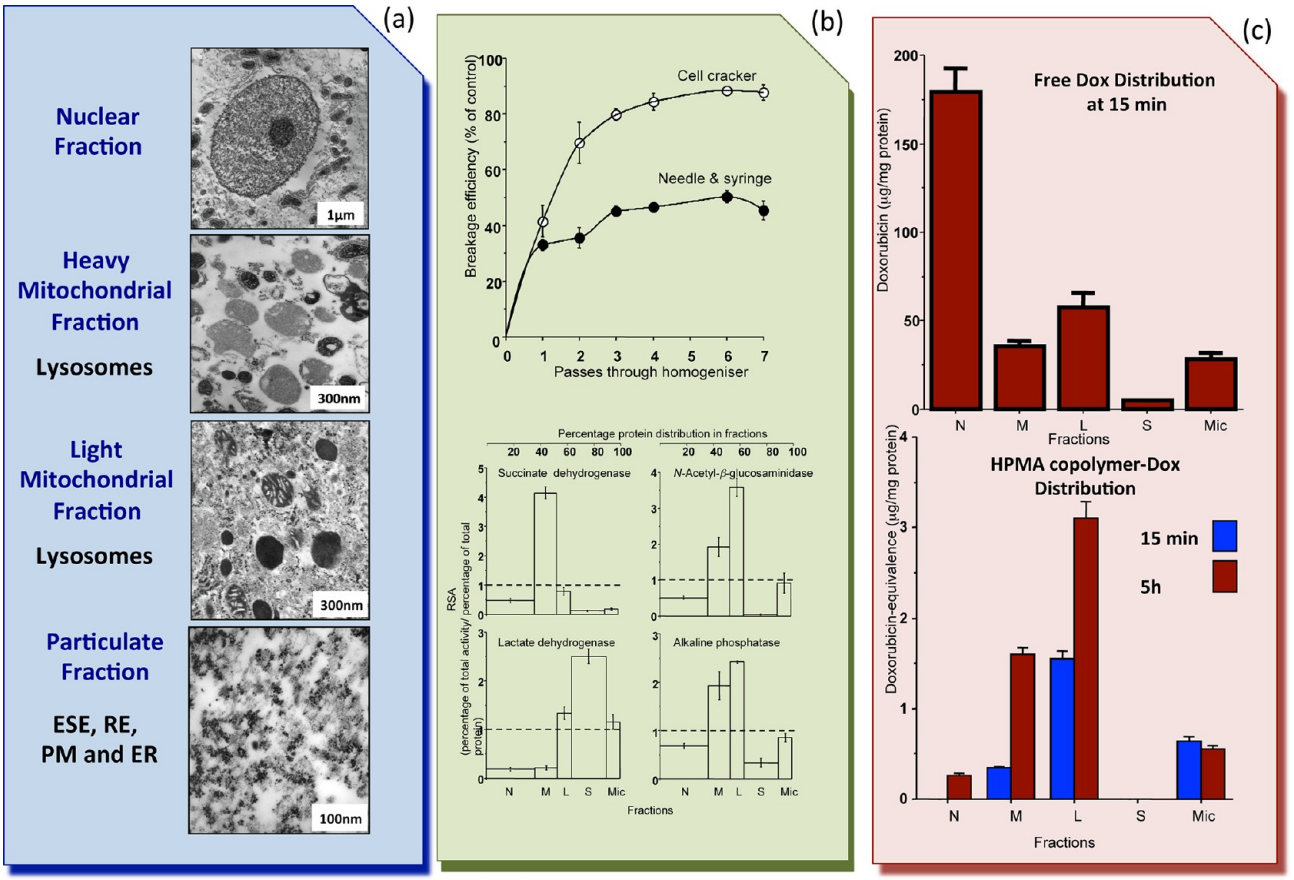

Figure 5. Subcellular fractionation and its use to monitor the fate of a polymer−drug conjugate. Examples are taken from refs 59 and 62 where full methodology can be found. Panel (a) shows the morphology of typical fractions obtained from rat liver,[62] and panel (b) optimization of cell breakage [B16F10 melanoma cells were used and % breakage was determined by measurement of lactate dehydrogenase release following passage of cells through a 25 gauge needle attached to a syringe or using a cell cracker (with a 6 mm gap), and the relative specific activity of marker enzymes recovered in the fractions obtained (typically the enrichment is 2−5-fold[59])]. This value can be significantly increased if methods are used to change the buoyant density of LYS. Panel (c) shows the distribution of HPMA copolymer−doxorubicin and free doxorubicin in fractions isolated from B16F10 melanoma cells at 15 min and 5 h.[59] While free doxorubicin is already present in the nucleus at 15 min, doxorubicin derived from the polymer-conjugated drug is not. The conjugate is already located within vesicular compartments at 15 min, but only after 5 h does doxorubicin liberation in LE/LYS lead to appearance in the nucleus.[59] Key to fractions reported in panels (b) and (c): nuclear (N), mitochondrial (M), lysosomal (L), soluble (S) and microsomal (Mic).

and fate must use substrate concentrations that are established as both nontoxic and without other pharmacological effects that impact endocytosis per se. Similarly, if putative gateway-specific inhibitors are used, it is essential to verify their mechanism of action in the particular cell under study as these substances can exhibit concentration-dependent nonspecific pharmacological effects that can impact cell functioning/endocytosis in general.

Methods of Analysis and Analytical Probes. *Endocytic Uptake.* While a number of new techniques are emerging for live cell imaging, nanoparticle tracking[51] and even methods capable of monitoring LYS enzyme degradation of an endocytic cargo in LAMP1 positive LE and LYS compartments in live cells,[52] fluorescent probes are still the most commonly used to study cell uptake (often coupled with flow cytometry and confocal microscopy techniques). Absolute quantitation is easier using radiolabeled probes or quantitation of inherent material properties, e.g., measurement of Au, Pt, or drug payload by HPLC. Although such techniques have been much less frequently used over the past decade, the search for more quantitative methodology is seeing a renaissance in their use.

As stressed above, endocytic uptake is only one facet of the highly dynamic endomembrane trafficking system. To define a rate of endocytic uptake it is important to verify the time dependence (not just work at one or two time points) of cell association (most often this is the only parameter measured), the extent of cell surface binding, the rate of exocytosis, and rate of intracellular cargo degradation. Low molecular weight impurities present at the outset or released into the culture medium during incubation will also have a significant impact on data interpretation, as their PK is usually totally different from that of the nanomaterial/nanomedicine whose entry is limited to the endocytic route. Additionally, it is important to consider intracellular degradation which can yield low molecular weight labeled moieties that may either remain within cellular compartments or be released back into the incubation medium (Figure 4). f4

$$\text{total uptake} = \text{cell-associated material} + \text{material exocytosed} + \text{degradation products liberated}$$

It should also be noted that even if uptake is quantified in this way, an internalized nanomedicine (and its drug payload) may become entrapped within a redundant compartment if the pharmacological target is outside the endo/lysosomal system. In

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all cases it is important always to consider the issue of intracellular compartmentalization in relation to "cellular bioavailability" of the active agent being delivered.

Early clinical trials often involve a dose range finding study, so knowledge of the time dependence and substrate concentration dependence of all these processes is also very important. *In vitro* experiments conducted at 4 °C are often used to give an indication of PM binding, and although such experimental conditions may be perfectly valid within the context of cell biological studies undertaken to define cell function and homeostatic regulation, in the context of drug delivery the use of such nonphysiological conditions can lead to spurious conclusions, in this case due to PM fluidity/composition alterations at low temperature (Figure 4). Excluding the fact that the substrate concentration used elicits toxicity (this can be verified in advance), fluid-phase uptake is proportional to extracellular concentration. In contrast, both specific and nonspecific receptor-mediated uptake (Figure 2) will show saturable kinetics. Additional complications associated with the use of fluorescent dyes include their size and hydrophobicity. Both can significantly influence both PM interaction and intracellular fate of the material to which they are attached, even when used with a low loading.

Intracellular Fate. Fluorescent probes and confocal microscopy are most widely used to follow intracellular fate. Microscopy is appealing as it provides an immediate visual result, and video capture of live cell images shows time-dependent changes. But "seeing" should not always be "believing", and independent measurements using other techniques do not always verify the conclusions made using microscopy (discussed in ref 53). Colocalization techniques offer the possibility of organelle identification, and such studies usually use one or more of the following approaches: (i) a pulse-chase experiment using exogenously applied marker substrates, e.g., Transferrin-Texas Red (to mark EE), fluorescein isothiocyanate (FITC)-dextran (to mark LYS) etc., (ii) cells transfected with green fluorescence protein (GFP)-labeled organelle marker proteins, or (iii) following cell fixation use of labeled antibodies against the organelle marker proteins[50] (Table 1). Protocols for, and the pros and cons of, fluorescence microscopy techniques are discussed at length elsewhere.[41,50,54,55] Briefly, the main issues are as follows: these are single cell assays (are the results typical of the whole population?); lack of quantitation; difficulty in obtaining a temporal dissection of the trafficking pathway; and, where fixation is used, the potential for fixation artifacts. Photobleaching may also be a concern, and concentration- and pH-dependent fluorescence quenching of the probe used can significantly bias the conclusions drawn (see examples given in Figure 4). It is wise to establish the pH- and concentration-dependent fluorescence of each probe used as neighboring group effects (depending on the physicochemical properties of the material under investigation) can influence output of even the most widely used fluorophores. Organelle location of a nanomaterial/nanomedicine is often assumed without substantive proof, and moreover, as stressed above, the amount of cell-associated material at any time is only one factor to consider when quantitating endocytic uptake rate. Finally, with increasing use of *in vitro* models as screening tools, it is essential to carefully consider whether the conditions used, namely, seeding density, growth media, transfection with GFP-labeled marker proteins etc., have an inherent effect on the cells' endocytic behavior or modulate inherent membrane trafficking pathways.

Subcellular Fractionation. Historically subcellular fractionation has been used, albeit by a smaller number of groups, to follow the intracellular fate of nanomedicines. The advantage of this approach is the ability to quantitate the intracellular fate using cells cultured *in vitro* or target cells/tissues harvested after nanomaterial/nanomedicine administration *in vivo*. For example, studies have reported the intracellular fate of anthracycline−DNA complexes and anthracycline−human serum albumin conjugates *in vitro* (reviewed in ref 56), and the time-dependent trafficking of HPMA copolymer−anthracycline conjugates ± galactosamine (and released drug) in liver cells following iv administration,[57,58] or in B16F10 cells *in vitro*[59,60] (Figure 5). f5
The technique has also been used to determine the intracellular fate of nonviral vectors designed for gene and siRNA delivery, e.g., polyethyleinimine (PEI),[61] linear poly(amimidoamines) (PAAs)[62] (Figure 6), and cationic lipid vectors[63] (discussed in f6
the section on lysosomotropic and endosomotropic delivery). It is possible to follow the migration of nanomedicine from PM to LYS, the impact of the vector on LE-LYS membrane permeability by monitoring cytosolic appearance of LYS enzymes, e.g., *N*-acetyl-*β*-glucosaminidase (NAG), the long-term fate of the carrier (does it persist or is it degraded?) and the intracellular PK and localization of any low molecular weight or macromolecular drug being released (see the section on endosomotropic delivery and Figure 5 for examples).

Subcellular fractionation is often viewed as a challenging technique. Indeed it can be labor intensive while establishing and validating a new protocol for a particular cell type/tissue. For *in vitro* experiments a large number of cells are required. However, this may be considered also an advantage as the results obtained represent an average of a large cell population. There is a need to carefully standardize the assays used to verify the organelle integrity throughout the process, and the final composition of each fraction obtained. An important issue to consider is the fact that the internalized material may itself change the buoyant density of the organelles in which it resides. This in turn can influence the fraction to which it is localized, a fact that becomes evident via changes in the overall pattern of organelle marker distribution. However, the ability to purposely alter vesicle density does provide a purification tool that has been used very successfully to increase enrichment of given vesicle type (e.g., prior administration of the detergent TWR1339 is used to aid purification of rat liver LYS, so-called "Tritosomes"). The recent use of a magnetic separation system to enrich vesicles containing iron dextran coupled with an in-line laser-induced fluorescence detection system to monitor individual vesicle pH values provides a new analytical tool that maybe be useful in determining the effect of nanomedicines on the pH of vesicles in which they reside.[64]

There are 3 main steps involved in subcellular fractionation and each needs careful validation: (i) cell breakage, as many of the cells in the population as possible must be broken open without damage to their intracellular organelles (Figure 5); (ii) optimization of the fractionation procedure, differential or density gradient centrifugation, and (iii) characterization of the purity and composition of the subcellular fractions obtained using specific organelle markers (Figure 5). Typical protocols can be found in refs 59, 62 and 65. The growing use of subcellular fraction is fueled not only by the need to quantify intracellular fate of nanomedicines but also by the increasing interest in organelle/LYS proteomics.[66−68] Subcellular fractionation has been used to monitor the fate of LYS-targeted liposomes in HeLa cells with a goal of treatment of lysosomal storage diseases,[69]

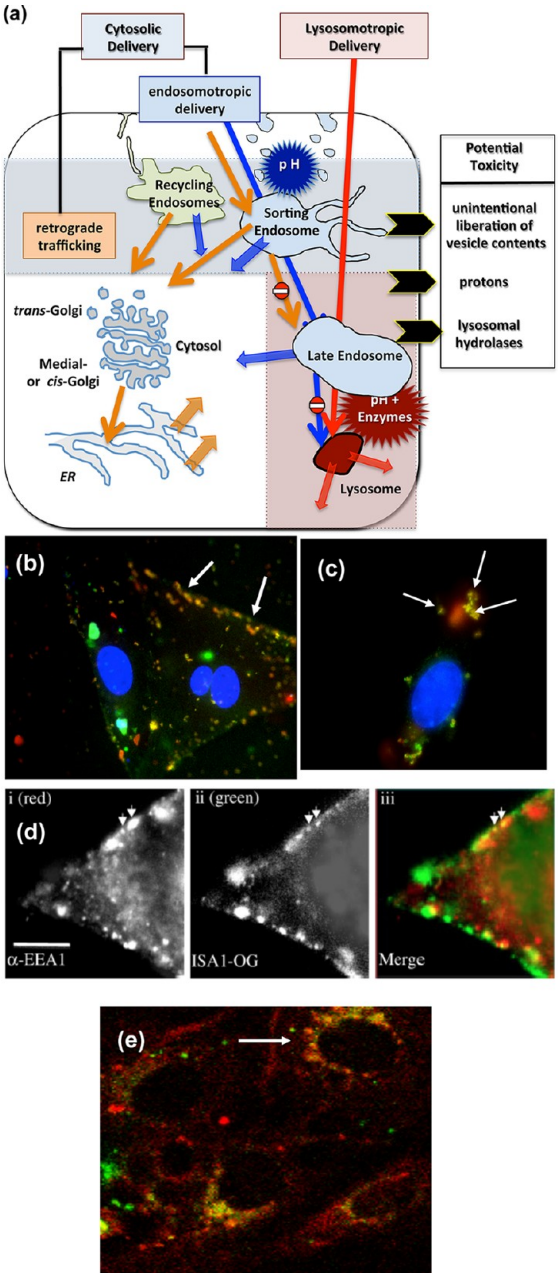

Figure 6. Lysosomotropic and cytosolic delivery. Panel (a) is a simplified scheme illustrating (i) lysosomotropic delivery (red line) (pH or lysosomal enzymes are often used as a trigger for drug release), (ii) endosomotropic delivery (blue line) (pH is used to provoke endosomal exit, ideally before transfer to LYS) and (iii) the retrograde trafficking pathways (orange line) in which transfer to the cytosol can occur via the RE or ER. Transient permeabilization of organelle membranes can also potentially lead to release of vesicle contents, and this may subsequently induce toxicity. As examples, panels (b)–(d) show the intracellular distribution of the putative endosomolytic polymer ISA 1-OG (green) and gelonin-TR (gelonin is a toxin that does not inherently access the cytosol) (red) in B16F10 cells. Large yellow vesicles at the margin of the cell (white arrows) represent ISA 1/gelonin colocalization, and the red halo to these vesicles is evidence of gelonin liberation into the cytosol (c). Panel (d) shows ISA1-OG within EEA1 positive EE in B16F10 cells. In each case nuclei are stained blue. Panel (e) shows the endocytic exit of Shiga Toxin B Chain, colocalizing with a fluorescent Golgi marker (ceramide) in live Vero cells. (The methodology used is given in full in refs 50 and 62.)

determination of the binding, uptake and intracellular fate (cytosolic vs vesicular distribution) and nuclear localization of cell penetrating peptides in CHO cells,[70,71] and in studies on the intracellular trafficking of an antitumor anti-CD30 monoclonal antibody linked to monomethyl auristatin E (MMAE)[72]

General Features and Emerging Models. Before discussing specific nanomedicines and their therapeutic goals, it is important to highlight recent studies that have real potential to bring a paradigm shift to the improved design of next generation nanomedicines. Although the molecular mechanisms responsible for internalization and trafficking of natural substrates including macromolecules, viruses, and toxins are increasingly well understood,[73,74] over recent decades relatively few investigators have carefully documented the endocytic properties of their first generation nanomedicines during their optimization. Some exceptions include studies on the effect of features such as molecular weight/size, charge, hydrophobicity and chemical composition on PM binding and endocytic uptake of synthetic polymers,[75] liposomes,[76] nanoparticles,[77] micelles and quantum dots,[78,79] and gold particles and gold nanorods.[80] Most historical studies have simply screened pharmacological activity of nanomedicine *in vitro* and *in vivo* without documenting endocytic fate.

With the advent of so many new classes of nanomaterials, and also development of innovative techniques able to engineer controlled size, shape and surface properties, e.g., the PRINT technology of DeSimone and colleagues,[81] there has been a dramatic increase in the number of studies on endocytosis, "screening" large libraries of nanomaterials in order to determine the effect of size, shape, elasticity of surface (modulus), and protein corona on PK at the cellular and whole organism level. It has been suggested that all these features impact internalization rate,[80,82] and even the aspect ratio of both gold and polymeric rods seems important. Do long thin objects have higher rates of internalization in all cell types? Some studies on the cellular uptake of carbon nanotubes suggest that they "pierce" the cell membrane and translocate directly into the cytosol—a matter of "frustrated endocytosis"? A potential role for carbon nanotubes in cytosolic delivery has been suggested.[83] In contrast, other investigators suggest that single walled carbon nanotubes (SWCNT) are lysosomotropic with trafficking to mitochondria at higher doses. These observations led to the conclusion that cellular uptake of SWCNT occurs by endocytosis[84] and not PM penetration. As many different cell lines have been used in all these experiments, often coupled with some of the analytical methodological limitations discussed above, it is still too early to be sure if the structure–activity relationships noted will be widely applicable to all cells of clinical relevance. Nevertheless, the realization that endocytosis is one of "the" key factors controlling safety and efficacy of emerging nanomedicines is a major breakthrough and will no doubt assist improved design of clinically more effective products in the future.

Parallel studies defining the inherent endocytic properties of specific target cells/diseases are beginning to ensure that *in vitro* models will in the future be more realistic in terms of the *in vivo* and clinical setting. For example, whereas polymeric micelle endocytosis occurred efficiently via CavME in epithelial cancer cells, the same micelles were not taken up by normal epithelial cells able to form tight junctions.[85] Another example relates to the development of nanomedicines for vascular endothelial cell targeting. The effect of vascular blood flow on endocytosis is rarely considered, but recent experiments showed an effect of "flow" on the uptake of ICAM-1-targeted nanocarriers by

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Table 2. Some Examples of Nanomedicines Designed To Exploit Endocytosis[a]

product	composition	mechanism	disease	status
AmbiSome	liposomal amphotericin B	RES clearance and local drug release to reduce nephrotoxicity	opportunist infections; leishmaniasis	market
Adcetrist	anti-CD30-MMAE conjugate	receptor-mediated targeting and intracellular drug release	cancer	market
Xyotax/Opaxio	PGA-paclitaxel	cathepsin B degradation of PGA to mediate drug release	cancer	phase III
En 489/145	folate-anticancer conjugates	folate receptor targeting with a self-immolative linker for intracellular drug delivery	cancer	phase III
CALAA-01	nanoparticle of PEG-cyclodextrin	cytosolic delivery of siNRA	cancer	phase I
BR96-DOX, SGN-15	antibody doxorubicin conjugates	targeting LewisY antigen, hydrazone or cathespsin B cleavable linkers	cancer	phase I/II
FCE28068/9	HPMA–doxorubicin conjugates	cathepsin B degradation of GFLG polymer–drug linker	cancer	phase I/II

[a]A full list of products in routine used and clinical development can be found in ref 1.

endothelial-like EAhy926 and human umbilical vein endothelial cells (HUVECs) previously grown *in vitro* for 24 h under laminar shear stress.[86] Endocytosis in flow-adapted cells was ~35% slower under flow than in the same cells incubated under static conditions. These observations are corroborated *in vivo* where uptake is higher in capillaries where the shear stress is lower. Such observations not only are important for the design of nanomedicines for use where the physiological target (capillaries vs arterioles/venules) or the pathological target (ischemia, inflammation) may present very different patterns of blood flow, they also underline the need to consider carefully flow and physical/mechanical forces in other clinical settings, e.g., GI transit, the lung mucociliary escalator, lymphatic flow, etc. A recent study has also shown that nanoparticle attachment and endocytic uptake by HUVECs is affected by the substrate on which they are grown,[87] again emphasizing the need to carefully consider the experimental conditions used for cell culture depending on route of administration and clinical application. There is growing understanding of the importance physical/mechanical forces between cells and their microenvironment as controllers of pathophysiological behavior, and this in turn may impact signal transduction and endomembrane trafficking. Although discussion is beyond the scope of the article, the topic is nicely overviewed in relation to the physics of cancer metastases.[88] The basic concepts are very important to bear in mind when considering both appropriate models and nanomedicine design.

NANOMEDICINES: CURRENT STATUS AND KEY ISSUES FOR DESIGN

Over several decades the main goals for development of advanced drug delivery systems have remained essentially the same. They are as follows:

1. The creation of improved pharmaceutical formulations that are able, by solubilizing a poorly soluble drug and/or enhancing its oral bioavailability, to create a "practical to use" medicine where otherwise it would be impossible.
2. Enhanced targeting of the bioactive agent to the desired site of action. This can be realized at four distinct levels: (i) the organ, (ii) the target diseased cell with in an organ, (iii) a target intracellular organelle/compartment, e.g., cytosol, and (iv) most recently efforts are being made to localize to intraorganelle compartments (e.g., mitochondria)
3. Controlling release of the bioactive at a rate and location that will optimize the concentration at the site of pharmacological target for a duration that will realize optimal therapeutic effect and minimize toxicity.
4. Improved transportation of drug across a biological barrier it would otherwise be unable to penetrate, e.g., the GI tract, lung, the BBB, etc.

Endocytosis and intracellular processing play a central role in the realization of points 2 and 3, and they are discussed below in the context of lysosomotropic and endosomotropic delivery. As mentioned at the outset, some 40 nanomedicines have already been transferred safely into routine clinical use with many following in clinical development (products are listed in ref 1). Selected examples are given in Table 2 to illustrate the relationship with endocytosis, but there is a much larger, and rapidly growing, database of clinical experience in this area. To date the products primarily fall into these categories:

- *iron nanoparticles*, used to treat anemia and as MRI imaging agents, e.g., Feridex, Venofer;
- *liposomes and lipidic drug carriers*, e.g., Ambisome, Doxil/Caelyx;
- *nanocrystals* largely, but not only, used to improve the oral bioavailability of poorly soluble drugs, e.g., Rapamune, Emend;
- *nanoparticles* (polymeric or albumin), e.g., the albumin nanoparticle Abraxane;
- *antibody– and protein–drug (and radioisotope carrying) conjugates*, e.g., Zevalin, Mylotarg;
- *polymer therapeutics*, polymeric drugs (e.g., Renagel, Vivagel), polymer–protein conjugates (e.g., Peg-intron, Neulasta), PEG-aptamer conjugates (e.g., Macugen), polymer–drug conjugates and block copolymer micelles

Although each class has a significant pipeline of products following in clinical development (with some 70 nanomedicines in clinical trials as anticancer agents alone[89]), of particular interest are the novel, and often multicomponent, nanomedicines that have entered preclinical or clinical development over the past decade; e.g., the PEGylated-gold-TNF antitumor agent Aurimmune (CYT-6091),[90] the liposomal/lipidic systems (Atu027;[91] Sirna-027;[92] ALN-VSP[93]) and the polymer conjugate-based nanoparticle CALAA-01[94] designed for siRNA delivery, and the liposomal carrier CPX-351[95] containing both cytarabine and daunorubicin. This product based on the "CombiPlex Technology" is already showing promise in phase II clinical trials in acute myeloid leukemia (AML) patients.[96] This particular study is an important landmark as nanomedicines delivering combination therapy will undoubtedly play a major role in the future. Biotechnology/recombinant approaches are

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Table 3. Summary of the Opportunities and Challenges Relating to Design and Use of Nanomedicines for Lysosomotropic Drug Delivery, Endosomotropic Delivery and Retrograde Trafficking

Goal/Opportunity	Potential Challenges
Receptor-mediated endocytosis improved disease-specific delivery with reduced drug concentration in sites of toxicity	• low selectivity (% dose) arriving to target cells • heterogeneity in receptor expression on target cells • receptors on off-target cells that are particularly sensitive to drug toxicity • binding-site barrier preventing penetration to all target cells in tissue • receptor down-regulation • dose-dependant receptor saturation resulting in loss of targeting *in vivo* • side-effects associated with inappropriate receptor activation • inappropriate trafficking of receptor-bound nanomedicine resulting in inadequate exposure to conditions for linker degradation • the pharmacological mechanism of action of the drug delivered should not impair endocytosis e.g. anti-microtubule inhibitors
Intracellular trafficking delivery of drugs and/ or macromolecules to a specific intracellular compartment; bypass PM membrane efflux pumps; improve organelle-specific localisation; cytosolic delivery of macromol-ecular drugs	• rapid recycling leading to premature nanomedicine efflux • inappropriate trafficking causing failure to localise to target compartment • impairment/alteration of the physiological trafficking pathways • permeabilisation of endomembranes causing leakage • LYS accumulation/creation of LSD† syndrome
Controlled drug release triggered drug release (or cytosolic delivery of macromolecular drugs) mediated by pH, LYS enzymes, or the intra-vesicle reducing environment	• failure to access the required compartment • heterogeneity of enzyme expression/ concentration in target organelle • alterations of activating conditions in disease - changes within the target organelle, changes extracellularly resulting in premature drug release • the bioactive being delivered must be stable in the endosomal/LYS compartment, and in the case the pharmacological target is outside the LYS compartment have the ability to cross the LYS membrane

†LSD; lysosomal storage disease

making an increasingly important contribution, not only for the generation of recombinant proteins, but also for the manufacture of polymers, e.g., polyglutamic acid, hyaluronic acid and alginates.

By far the most striking recent development has been the diverse library of novel nanomaterials now being proposed for drug delivery and/or imaging; e.g., fullerenes, carbon nanotubes, quantum dots, novel polymers, polymeric architectures (including dendrimers), self-assembling and core−shell nanoparticles, nanoparticles based on iron oxide, gold, silver and silicon (reviewed at length in ref 1 and contained in the bibliography). These will create the nanomedicines of the future. The following sections discuss issues relating to nanomedicines as they try to exploit the endocytic pathways for lysosomal and cytosolic delivery. The opportunities and challenges are summarized schematically in Figure 6 and Table 3). Of course all technologies must first circumvent the many other biological barriers in the body before arrival at the target cell, and this issue has been very thoughtfully considered elsewhere.[1,97]

NANOMEDICINES FOR LYSOSOMOTROPIC DELIVERY

Lysosomotropic Delivery Drug Delivery. The term "lysosomotropic delivery" was coined by De Duve and colleagues in the 1970s,[98] and it is used to describe both low molecular weight drugs, typically a weak base, able to penetrate cell membranes and accumulate within acidic vesicles and those nanosized carriers that localize to LYS via endocytic pathways. At that time efforts began in earnest to realize drug targeting via LYS using macromolecular drug conjugates based on proteins (albumin[56] and antibodies[99]), and water-soluble synthetic polymers (reviewed in refs 100 and 101), liposomal carriers (reviewed in ref 102), and polymeric nanoparticles/nanocapsules.[32,103] As discussed in ref 104, the early objectives were simple, to limit access of drugs of poor pharmacological selectivity that normally distribute widely in the body (e.g., cytotoxic anticancer drugs) to cellular uptake via endocytosis, and thus improve therapeutic index by increasing drug targeting to the diseased cells and decreasing access to sites of toxicity. An increasing number of antibody− and polymer−drug conjugates specifically designed for lysosomotropic delivery have entered clinical development, but progress to routine clinical use has been slow. When recently discussing "nanomaterial strategies from the point-of-view of the cell"[97] it was suggested that "the ability to target nanoparticles to cancer cells (secondary targeting) and to influence their uptake into specific cellular compartments (tertiary targeting) is now feasible".[97] This overarching statement is however rather an oversimplification if we look at the clinical evidence before us today. Perhaps the amalgamation of leading edge discoveries in cell biology and engineered design of improved nanomedicines will help to realize these goals more efficiently in the future. It is important to address the issues raised in Table 3. Although a large proportion of the experimental literature relating to lysosomotropic drug delivery has focused on anticancer therapies, the underlying principles relate equally to other therapeutic indications.

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Receptor-Mediated Targeting. Whichever gateway is used for cellular entry, nanomedicines arriving at the target cell that are small enough to be internalized frequently inherently traffic to LYS via the degradation pathway. Greater challenges arise when trying to use receptor-mediated endocytosis to elicit cell specificity and/or increase uptake rates. Phase I/II trials[105] have evaluated an HPMA copolymer−doxorubicin−galactose conjugate as a treatment for hepatocellular carcinoma. This conjugate was designed to target the hepatocyte asialoglycoprotein receptor (FCE28069) using a multivalent galactose-receptor binding. Administration iv led to ∼17% of the dose localized in the liver at 24 h. However in all other cases it has proved difficult to localize a high percentage of the dose of a nanomedicine to a clinical target using receptor-mediated endocytosis. Even for immunoconjugates <0.01% of the dose administered is actually delivered to the tumor tissue in the clinical setting.[106] Although PEGylated liposomal-doxorubicin targeted using the Fab fragment of mAb C225 (cetuximab)[107] or Herceptin (trastuzumab),[108] folate receptor-targeted conjugates (e.g., EC0489 and EC0145)[109,110] and FCE28069 have shown some evidence of anticancer activity in early clinical trials, most anticancer nanomedicines in clinical development still rely on passive solid tumor targeting by the enhanced permeability and retention (EPR) effect[111] or direct intratumoral administration (e.g., thermal ablation of glioblastoma using iron oxide (Fe_3O_4) nanoparticles[112]) for selectivity. Well-known challenges associated with receptor-mediated targeting include the limited number of specific antigens/receptors on the target cell, heterogeneity of their expression across the cell population, inefficient receptor internalization leading to suboptimal drug release rates intracellularly, and also clinically relevant factors such as receptor saturation at the clinical dose used and/or receptor downregulation on repeated dosing. Many of these points are now being more carefully scrutinized during preclinical experimentation for all receptor-targeted lysosomotropic nanomedicines.

Even the realization of safe and effective antibody−drug conjugates and recombinant immunotoxins has been challenging.[113] Two products are currently approved for human use: Ontak,[114] a recombinant protein composed of sequences from diphtheria toxin and human interleukin-2 used to treat cutaneous T-cell lymphoma, and Adcetrist, an anti-CD30−MMAE conjugate used to treat patients with Hodgkin's lymphoma. The anti-CD33−calicheamicin conjugate Mylotarg, developed to treat AML, was actually first to market, but this conjugate was withdrawn in 2010 due to the high incidence of side effects. Mylotarg clearly shows activity in AML and non-Hodgkin's lymphoma patients, and clinical studies are ongoing to try to identify the subset of patients that can best benefit from this therapy.[115] Perhaps nanomedicine/endocytosis biomarkers have a role to play here?

Drug Release. Critical for a successful outcome is stability of the nanomedicine in transit, avoiding drug inactivation en route and/or premature release from the carrier. To achieve these goals a variety of biodegradable linkers have been used to create drug conjugates (reviewed in refs 116 and 117) and inherently bioresponsive/biodegradable carriers (e.g., liposomes, nanoparticles and block copolymer micelles) have been explored.[118] Following internalization the prerequisites for activity usually include drug release from the carrier at a rate that is optimal for the pharmacological mechanism of action, e.g., taking into account cell cycle dependency, drug stability in harsh EE-LE-LYS environment (low pH and hydrolytic enzymes) and, not least where the pharmacological target is extralysosomal, ability of the drug to escape from the vesicular compartment (Table 3).

Systematic optimization of peptidyl linkers for cleavage by LYS proteases began >3 decades ago for both albumin conjugates[119] and HPMA copolymer−drug conjugates.[120] Early experiments involved incubation of libraries of conjugate bearing different peptidyl spacers incubated with mixtures of isolated lysosomal enzymes (Tritosomes) and those sequences degraded by the thiol-dependent proteases emerged as most promising. The HPMA copolymer conjugates containing doxorubicin bound via a cathepsin B-sensitive GFLG linker (FCEC28068, FCE28069) showed significantly reduced anthracycline toxicity in phase I/II clinical trials and antitumor activity in some chemotherapy resistant patients.[105,121] Clinical PK of HPMA copolymer-bound and free drug in plasma and urine was consistent with the preclinical PK seen in rodents. Dose proportionality was observed in patients, but given the very low doxorubicin levels detected in plasma (% dose) it is difficult to know if this also represents a linear relationship between dose and intracellular degradation of the conjugate. Patient response to FCE28068 was certainly more evident at relatively lower doses, but whether or not these observations reflect a higher rate of doxorubicin bioavailability in the tumor tissue is a matter of conjecture as the patient cohort was so small.[121] It is interesting to consider at what dose an intracellular activating enzyme might become saturated? The PGA polymer backbone of the paclitaxel conjugate Xyotax/Opaxio is also degraded by cathepsin B,[122] in this case to yield mono- and diglutamyl-paclitaxel from which drug is liberated by further ester bond hydrolysis. Elegant experiments using cathepsin B knockout mice confirmed a pivotal role for cathepsin B in determining antitumor activity *in vivo*, but as discussed later, clinical evaluation has revealed the complexity of cathepsin B activation.[123]

To capitalize on the drop in pH driven by the vesicular H(+)-ATPase (V-ATPase) proton pump, pH-triggered degradable linkers (e.g., *cis*-aconityl and hydrazone) have also been popular (reviewed in ref 116). However, the slow drug liberation rate from the hydrazone linker coupled with the potential for the conjugate to recycle through an acidic intracellular compartment was in part blamed for poor clinical activity of BR96-Dox in phase I clinical studies. There is still considerable effort ongoing to optimize antibody and polymer conjugate conjugation chemistry. Disulfide/bioreducible linkers have become popular[124,125] in constructs designed for both lysosomotropic and endosomotropic delivery. However, a recent study using antibodies engineered to include a single cysteine (for drug conjugation via maleimide linkers) at different sites in the molecule has clearly illustrated the subtlety of neighboring group effects on both the linker hydrolysis rate and conjugate performance.[126] The behavior of any linking chemistry (stability/drug release rate) is highly dependent on the physicochemical properties of both the carrier platform and the particular drug to which it is attached.

Recent advances include the growing library of novel self-immolative linkers,[127] and the use of the redox-active conditions in LYS (they contain iron and copper arising from autophagic degradation of metalloproteins, as well as a high concentration of thiols and low pH) to exploit ROS-activatable systems.[128] In addition, certain drug release mechanisms have been chosen to take advantage of additional target disease selectivity, e.g., aggressive tumors can overexpress cathepsins B and D, and the osteoclast enzyme cathepsin K has be used to activate nanomedicines designed to treat bone disease/osteoporosis.

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But premature extracellular drug release from macromolecular prodrugs and nanoparticle carriers can also be a concern. For example, if drug release is mediated by the extracellular proteases and/or low pH in the tumor interstitium, this could be a disadvantage given that antitumor nanomedicines internalized via endocytic pathways do have the potential to bypass the PM efflux pumps responsible for drug resistance, a benefit that would be lost if the drug payload was liberated extracellularly.

Of course on arrival within the LE/LYS antibodies, proteins, and degradable polymeric carriers can release bound drug simply due to degradation of the carrier, itself irrespective of any linking chemistry. Thus it is important to consider which cleavage event will be the rate-limiting step. Carriers that noncovalently entrap a drug payload, e.g., liposomes, albumin nanoparticles and biodegradable polymer nanoparticles, can also be degraded in LYS enabling drug liberation. Their design can be further enhanced by inclusion of bioresponsive elements such as pH sensitive lipids, polymers or peptides to provide an additional rate control of drug release.

Clinical studies have highlighted some of the challenges for design of effective lysosomotropic conjugates. Poor stability of ester linkers, e.g., in HPMA copolymer−paclitaxel and camptothecin conjugates, led to premature drug release in the circulation, or in the case of the camptothecin conjugate in the bladder following renal excretion, with the latter leading to dose-limiting cystitis (reviewed in ref 129). Phase III clinical studies involving Opaxio (Xyotax/CT-2103) showed significantly increased survival in female patients with non-small cell lung cancer (NSCLC) but not male patients.[123] Correlation between estrogen levels and cathepsin B activity was postulated as an explanation for the difference, and a plasma estradiol concentration of >25 pg/mL was proposed as optimal for conjugate efficacy. However, a recent study involving patients with metastatic prostate cancer (their disease had progressed with hormone therapy) treated with low dose transdermal estradiol in combination with Opaxio showed no therapeutic benefit, so the situation is obviously more complex.[130]

Theoretically, a number of factors may impact linker performance and consequently pharmacological activity in the clinical setting. These include the following: (i) failure to traffic appropriately, e.g., to the LYS (Figure 6), (ii) variability of the concentration of target enzyme in disease/in individual patients (gender differences?), (iii) the impact of enzyme kinetics on the rate drug release when working at specific clinical doses (enzyme saturation) (few studies define enzyme kinetics *in vitro* and or *in vivo*), (iv) impact of the nanomedicine (all components of the carrier and drug) on enzyme activity, (v) effect of the nanomedicine (all components) on the EE-LE-LYS pH, vesicle fusion and content mixing, and finally (vi) the ability of released drug to traverse the LE-LYS membrane. It is evident that identification of clinically relevant biomarkers relating to each of the above may have an important role to play in disease characterization.

Treatment of Lysosomal Storage Diseases. A heterogeneous mixture of naturally occurring macromolecular substrates are continually arriving in LYS via both the autophagic and endocytic pathways en route to degradation (Figure 1). Some 40−70 lysosomal storage diseases (LSDs) have been identified in which materials accumulate within LYS often as a result of missing single or multiple LYS enzymes and/or deficiencies in the normal trafficking pathways.[5,131] Major deleterious pathophysiological changes result, often visible as cell vacuolation. Although disabling multiorgan failure can result, the cell type(s) most susceptible to first signs of damage vary according to the specific LSD. Indeed, many LSDs have such serious consequences that they are lethal before birth. Others are progressive and often become evident within the CNS over time. Moreover, defective lysosomal function has been implicated in diseases of aging, e.g., intraneuronal accumulation of proteins in Alzheimer's disease, and lipofuscin accumulation in age related macular degeneration (AMD).

In recent years enzyme replacement therapy has become the clinical standard for treatment of LSDs, particularly through the successful use of recombinant glucocerebrosidase to treat type 1 (the non-neuropathic form) Gaucher disease. In this case the target cells, such as macrophages of the liver and spleen, can be easily accessed repeatedly by iv administration. However, inability of the enzyme to cross the BBB prevents adequate treatment of type 3 Gaucher disease (this has CNS involvement), and in general enzyme replacement has been less successful for other LSDs. Alternative approaches are thus being explored including gene therapy, cell therapy and not least the use of lysosomotropic nanomedicines to aid delivery of either the missing enzymes or "pharmacological chaperones", molecules that stimulate enzyme activity (reviewed in ref 132). A variety of different vectors are currently under study including fusion proteins designed to promote enzyme targeted delivery with enhanced rates of cellular uptake, liposomes designed to promote internalization and lysosomal delivery, and various nanoparticles including an intercellular adhesion molecule 1 (ICAM-1) targeted nanoparticle containing α-galactosidase A (α-Gal)[133] designed as a treatment for Fabry disease which typically leads to premature mortality due to renal failure, cardiovascular disease and/or cerebrovascular disease.

In theory, once the target cells are reached it might be expected that access to the LE-LYS compartments to deliver the cargoes needed to rectify storage disease problems will easily follow. However, this may not necessarily be the case as accumulation of natural macromolecules or partial degradation products in these compartments is now being shown to severely disrupt normal endomembrane trafficking. For example, in Niemann−Pick (NP) disease the accumulation of luminal lipids within vesicles alters Ca^{2+} homeostasis, resulting in impaired trafficking to LYS,[134] and in neurodegenerative diseases accumulation of cholesterol in LYS impairs SNARE complex function, resulting in defective membrane fusion.[135] Such observations are not only an important finding for improved understanding of the molecular basis of diseases such as Alzheimer's and Huntington's diseases but they have major implications for the ability of any externally arriving nanomedicine to penetrate the vesicular network and effectively deliver their therapeutic payload.

NANOMEDICINES FOR CYTOSOLIC DELIVERY

Generally therapeutics of molecular weight >1,000 Da do not cross the PM and cellular access is limited to the endocytic route. Like all incoming physiological macromolecules, macromolecular therapeutics are candidates for inactivation by acidic pH and/or rapid hydrolysis by LYS enzymes; e.g., antisense oligonucleotides have a $t_{50\%}$ = 30−40 min when incubated with Tritosomes *in vitro*.[136] By the mid 1980s, advances in the Biotech Industry began to highlight the urgent need for vectors able to both protect emerging "molecular medicines" during transit and promote their efficient delivery to the desired intracellular compartment on arrival at target cells. At the outset the main emphasis was on clinical realization of human gene therapy, but over the years new classes of "molecular medicines" have

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continued to emerge including natural and engineered target-specific proteins and peptides, antisense oligonucleotides, ribozymes, peptide nucleic acids (PNAs), and most recently small interfering RNAs (siRNAs). Demand for effective cytosolic delivery systems continues to increase. The potential advantages of synthetic "nonviral" vectors compared to viruses for gene therapy have long been discussed.[137] Although polymer-based vectors (e.g., PLL,[138] PEI[139] and dendrimers[140]), liposomes and lipidic complexes[141] were developed in the 1980s/1990s, and indeed a good number have been commercialized as *in vitro* transfection reagents, few have progressed into clinical trial essentially due to their toxicity, low or irreproducible *in vivo* transfection efficiency, and/or difficulties of reproducible manufacture to a specification suitable for clinical use.

Despite >20 years of continuing R&D there is still no FDA clinically approved gene therapy, and almost all clinical studies have used using viral vectors. However, the current drive to realize the therapeutic potential of siRNAs[138,142] has revived interest in the use of synthetic vectors for cytosolic delivery. Although most studies on siRNA-based nanomedicines are conducted *in vitro* and often simply screen for pharmacological end points, >14 clinical trials have been documented (reviewed in ref 143). Some involve naked siRNA, but liposomal and lipidic vectors[91,93] and the transferrin targeted polymer-cyclodextrin-based nanoparticle CALAA01[92] have given some early clinical proof of concept.[94,144,145] To improve disease targeting, local administration is often used, e.g., to the eye to treat AMD (RTP801/PF-655) or intranasally to prevent respiratory syncytial virus infection (ALN-RSV01), but other strategies involve iv administration, e.g., a PEGylated liposomal vector that seems to associate with circulating apolipoprotein E that promotes receptor-mediated liver hepatocyte targeting is being explored as a treatment for metastatic liver cancer (ALN-VSP02) and severe hypercholesterolemia (ALN-PCS). The TfR targeted system CALAA01 and the lipoplex Atu027 are both being evaluated clinically as treatments for solid tumors. It is still too early to understand the significance of these promising early clinical results as siRNA can cause off-target effects and nonspecific gene silencing, and in certain cases vector-induced toxicity can be a concern. Ultimately the therapeutic index (risk:benefit) will be critical in each disease setting.

Successful design of any cytosolic delivery system will be governed by the clinical target (frequency of dosing duration of administration), the therapeutic cargo and the route of administration. Also important to note is that a vector designed for gene therapy will require different attributes compared to, for example, one delivering siRNA. Individual siRNA molecules are >100-fold smaller than a gene, and the final destination is cytosol rather than nucleus. However, many of the biological barriers are essentially similar for all nanomedicines signposted for cytosolic delivery (widely discussed in the context of siRNA delivery in refs 8, 146 and 147. They also add to those challenges facing constructs designed for lysosomotropic delivery (summarized in Table 3).

Endosomotropic Delivery. Endosomotropic delivery is inherently more challenging than lysosomotropic delivery. The goal is simple, a discrete bioresponsive change in the vector triggered by the lowering of pH following internalization (usually pH 6.5 is discussed) that is able to transiently permeabilize the EE-LE membrane allowing a cargo to enter the cytosol. This must occur within a specific temporal window, i.e., ideally prior to the translocation of cargo to the LE and/or the fusion of the LE with Lys (Figure 6.) in order to prevent degradation of the cargo and/or the release of catabolic LYS enzymes and/or protons into the cytosol where they can initiate apoptosis.[148] Also if the construct is destined for a recycling pathway, efficient endosomal escape must occur before return to the PM. Many viruses and naturally occurring protein toxins are exquisitely equipped to pop out of the vesicular compartments into the cytosol within the given time frame.[7] The question however remains: Can a synthetic viral vector ever hope to mimic these mechanisms of endosomal escape? Will they ever be able to do so efficiently during repeated dosing? To explain the bioactivity of naked siRNA, it has been suggested that there may be "leakage" from the vesicle lumen during vesicle−vesicle fusion.[8] However, if true it is debateable whether this would be physiologically helpful given the normal tight regulation of intravesicle pH and LYS hydrolase trafficking to avoid self-harm.

Fusion proteins (perhaps inherently not nanomedicines, but when conjugated chemically to a therapeutic payload they may be considered so) and the complex, hybrid synthetic nanosized systems being developed as carriers typically use one of three approaches to gain cytosolic access:

(i) cell penetrating peptides to translocate cargos across the endosomal membrane (mechanisms discussed in refs 149 and 150)
(ii) bioresponsive polymers/fusogenic liposomes to promote escape by osmotic effects, membrane binding or membrane fusion
(iii) the use of location signals to promote retrograde trafficking via Golgi and endoplasmic reticulum (ER) to cytosol (discussed later)

Most widely studied have been bioresponsive synthetic vectors designed to take advantage of the acidic lumen of ESE-LE. Because of their inherent membrane activity, polycations (e.g., PLL, PEI, and polyadmidoamine (PAMAM) dendrimers) have long been popular. They display multivalent interaction with anionic lipids both externally, i.e., with the PM, and following internalization to the previously exofacial leaflet that faces the lumen of the EE. The mechanism of PLL-induced membrane disruption has been studied since the 1950s.[151,152] Nonspecific interaction probably accounts for the molecular weight-dependent toxicity of seen for many polycations and cationic lipoplexes. It also probably explains, at least in part, lipoplex-mediated deliveryof siRNA due to fusion with the PM rather than selective transfer via endocytic pathways. Many chemical approaches, including PEGylation, creation of new architectures, use of block copolymers etc., have been used to try to ameliorate polycation-induced toxicity and to improve *in vivo* biodistribution, but this has usually resulted in concomitant reduced transfection/delivery efficiency. This has brought growing interest in the use of polyplexes and lipoplexes that contain pH-sensitive or bioreducible linkers to facilitate detachment of polymer coatings or complex destabilization following internalization to enhance delivery efficiency.

The mechanism of polycation-induced permeabilization of the EE-LE-LYS membrane is still not completely clear. The "proton sponge hypothesis" was first proposed in ref 139 to explain PEI-induced effects, and many still regard this as a primary mechanism of action. It occurs due to the substantial buffering capacity of polycations (dependent on their pK_a) which, following internalization, has potential to raise intravesicle pH, induce chloride influx, and cause vesicle swelling and consequently membrane destabilization. It is known that V-ATPase-mediated vesicular acidification is required for both

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homotypic and heterotypic endocytic fusion[153] so any reduction of acidification would not only reduce cargo degradation but also inhibit vesicle−vesicle fusion. Direct evidence in support of the proton sponge hypothesis came from observations of chloride accumulation and endosome swelling when exposed to buffering PEI- and PAMAM dendrimer-polyplexes, which did not occur on addition of nonbuffering PLL-polyplexes.[154] Universal applicability of the proton sponge mechanism is still hotly debated due to the design of polymer chemistries tailored to exhibit the effect that fail to do so, and the underlying validity of the biological basis on the following counts:

(i) There is a requirement for transporters (and channels) for ions and aquaporins for water during normal cellular homeostasis, as neither water nor ions move readily over membranes of their own volition.[155,156]
(ii) The osmotic pressure required for the rupture of endosomes has been calculated to be greater than that generated by internalized PEI.[157]
(iii) There exists excess membrane, associated with the limiting membrane of ESE (and internal to LE). In the instance of early sorting endosomes, where a high ratio of limiting membrane to volume is necessary for cargo sorting, the capacity for vesicle expansion decreasing the surface area to volume ratio as a response to water influx (rather that rupture) is self-evident. This is also true of many of the reticular structures seen to house fluorescent endocytic cargo often observed within the cell.

Given that different cell types exhibit different trafficking pathways, and may well also exhibit subtly different EE-LE-LYS pH, it is not surprising that the proton-sponge contributes differently according to the vector tested and cellular model. Other mechanisms offered to explain polycation-induced membrane permeabilization include direct membrane interaction in the EE-LE-LYS, induction of pore formation, and colloidal osmotic effects. For cationic liposome/lipoplexes it has also been suggested that destabilization arises due to formation of nonbilayer lipid structures such as the inverted hexagonal (H(II)) phase.[158] Whichever mechanism(s) are operational, it is interesting to consider the potential impact of any such phenomena on the normal pathophysiological functioning within the endosomal−lysosomal target or other cells.

Better quantification of the cellular PK and the intracellular fate of putative delivery systems and their cargos *in vitro* and *in vivo* and direct measurement of the alterations in membrane permeability over time are essential to advance this field. Wattiaux and colleagues have made an important contribution over several decades using subcellular fractionation of rat liver to quantify trafficking of radiolabeled cationic lipids/lipoplexes, polymers and protein toxins (e.g., gelonin) after iv administration (e.g., refs 63, 159 and 160). In certain studies biodistribution was also measured and liver cell separation was used to define which liver cell type was responsible for uptake. Incubation of isolated LYS *in vitro* was also used to study membrane permeability of vesicles entrapping different cargos. For example, it was shown that free [^{35}S]DNA and a cationic lipid *N*-(1-(2,3-dioleoxyloxy)propyl)-*N*,*N*,*N*,-trimethylammonium-methyl-sulfate (DOTAP):[^{35}S]DNA complex were both rapidly captured by the liver after iv injection, but that their intracellular fate was markedly different. Free [^{35}S]DNA transferred rapidly to LYS where degradation occurred, but the lipoplex remained for a long time in low density organelles that were not positive for the LYS marker arylsulfatase. It was concluded that retention there, perhaps due to inhibition of vesicle−vesicle fusion, might explain protection against degradation and favor DNA endosomal escape.[63] Addition of DOTAP to isolated LYS led to increased release of the beta-galactosidase,[160] and likewise incubation of HepG2 cells with methyl-cyclodextrin (MCD), an agent known to remove cholesterol from biological membranes, produced a reversible increase in the buoyant density of the lysosomal fraction isolated from these cells. These isolated LYS were more fragile (as judged by LYS enzyme release) when incubated *in vitro* in isotonic glucose, or hypotonic sucrose, and less resistant to increased hydrostatic pressure.[159] As CALLA01 contains pendant cyclodextrin moieties, it is interesting to consider whether these observations have implications for its mechanism of action?

We have used similar techniques to investigate the endosomolytic properties of linear, amphoteric, poly(amidoamine)s (PAAs), reviewed in ref 161. PAA chemistry can be tailored to give a polymer that is neutral or slightly anionic outside the cell and consequently >100-fold less toxic than PLL). However, the PAA backbone is able to protonate during translocation through the EE-LE system, and the polymer coil expansion that occurs as pH falls has been demonstrated *in vitro* using small angle neutron scattering, SANS.[162] The ability of PAAs to mediate gene and nonpermeant toxin (ricin A chain and gelonin) delivery *in vitro* has also been shown.[161] Although certain PAA structures were optimized to avoid liver accumulation and enable tumor targeting by the EPR effect, a hepatotropic ^{125}I-labeled PAA (ISA 1) was used as a tool to study the dose and time dependence of PAA subcellular localization. Simultaneously the distribution of NAG was followed.[62] ISA1 (radioactivity) and NAG showed time- and dose-dependent changes in intracellular distribution (Figure 7), and NAG appeared in the cytosolic fraction after 30 min indicating LE-LYS membrane permeabilization. At 1 h there was 2-fold more radiolabeled NAG (∼10%) in the cytosolic fraction than PAA (∼5%) (Figure 7a) although most PAA remained associated with the vesicular fractions throughout. The putative mechanism for membrane permeabilization involves direct membrane interaction for this polymer. The effects of ISA1 were dose-dependent. In agreement with the observations made with the (DOTAP):[^{35}S]DNA lipoplexes, an increase in the buoyant density of PAA-containing vesicles was seen after 0.5−1 h, but this was transient with a return to normal over 3 h. Isolated PAA-containing vesicles showed a clearly visible change in vesicle morphology (Figure 7c,d) and a PAA dose-dependent increase in membrane permeabilization evidenced by NAG liberation *in vitro* (Figure 7e). Notably addition of PAA to the outside of isolated vesicles did not enhance NAG release where addition of the polycation PEI did.[62]

Given that the key triggers for cytosolic delivery are vesicle acidification and/or reducing capacity in the context of bioreducible constructs, it is surprising that these key parameters are rarely defined in target cells. Moreover, although ref 163 measured the effect of various DNA delivery systems (including PLL, PEI and Lipofectamine) on intravesicle pH and discussed the correlation of pH with construct trafficking to LYS, studies are rarely undertaken to assess whether or not the vector itself has any impact on pH. Methodology, although often complex to validate, is available, e.g., pH-dependent fluorescent probes such as FITC that have been widely used by cell biologists to monitor vesicle acidification during phagocytosis. Recent studies in nonprofessional phagocytic cells, Madin−Darby canine kidney cells (MDCK) and human intestinal Caco-2 epithelial cells have also used 2 μm polystyrene beads bound to either Alexa488 (a

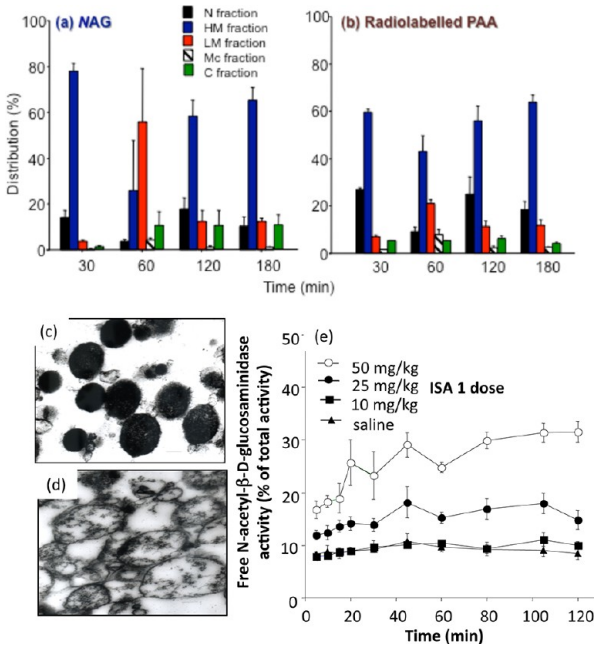

Figure 7. The subcellular distribution of NAG and the putative endosomolytic polymer ^{125}I-labeled ISA1 in rat liver at various times after iv administration of ^{125}I-labeled ISA1 is shown in panels (a) and (b) respectively. TEM of the isolated lysosomal fraction is shown in panel (c) for the saline control and in panel (d) following iv administration of ISA 1 (25 mg/kg). Panel (e) shows the release of NAG from isolated rat liver lysosomes *in vitro* that were obtained following iv administration of ISA 1 at the doses stated. Full details of the methodology can be found in ref 62.

pH-independent dye) or FITC to measure pH changes with time during vesicular trafficking.[164] Knowledge of intravesicle pH in the clinical target cell population and/or V-ATPase expression could be an important clinical biomarker in this context.

Retrograde Trafficking through the Endomembrane System. Although the endosomal compartments have been most widely considered as the entry port for cytosolic access, and those nanomedicines designed for cytosolic delivery in clinical trials have been based on this putative mechanism for delivery, retrograde trafficking pathways provide an interesting alternative, discussed in ref 54 and shown schematically in Figure 6. Materials can move not only in an anterograde (forward) direction but also a retrograde (backward) direction through both the endocytic and the secretory pathways.

The secretory pathway includes the ER, the ER-Golgi intermediate compartment (ERGIC), and the *cis*- (CGN), medial- (MGN) and *trans*-Golgi network (TGN). During normal cell functioning materials from the Golgi are trafficked either to the PM for secretion or to other destinations such as the EE or LE. Retrograde transport can facilitate receptor recycling, e.g., the mannose-6-phosphate receptor (M6PR). Importantly, this pathway can also be exploited by some viruses and toxins as the route for cellular invasion.[45]

The AB family of protein toxins localize to the EE (EEA-1 positive vesicles) after internalization. Although some pass to the cytosol via the LE or RE, others undergo retrograde trafficking via the Golgi to the ER where cytosolic translocation of the catalytic protein domain occurs. This can result in inhibition of protein synthesis for ribosome inactivating proteins such as ricin toxin (RT) and Shiga toxin (ST), interference with cellular communication as in the case for anthrax toxin (AT) lethal factor, or disregulation of the ion balance, e.g., for cholera toxin (CT). Common to all is the inbuilt ability of the toxin to deliver a bioactive macromolecule into the cytosol from the ER (Figure 6). RT also exploits a different retrograde trafficking step. It can translocate directly out of the ESE resulting in accumulation in the Golgi and then eventually through a variety of mechanisms transfer to the ER (Figure 6). In this case wild-type toxin releases the RTA chain into the cytosol leaving the RTB chain in the ER.

Recombinant technology has been used to inactive the "catalytically active motifs" of such toxins, by either mutation of specific amino acids or removal of entire domains, such that the resulting "attenuated toxins" can be used as functional components of nanomedicines designed for cytosolic delivery (discussed in refs 45, 165 and 166). This strategy has been successfully applied to a STB chain fused to a tumor antigen to localize peptide to dendritic cells to enable MHC class I presentation,[167] and in other studies using a STB chain fusion protein antitumor immunity was demonstrated.[168] However, efforts to use the retrograde trafficking pathways to deliver macromolecular cargos has so far had limited success. A CTB chain covalently linked to poly(D-lysine) was able to enhance transfection of nerve growth factor-differentiated rat PC12 pheochromocytoma cells,[169] but the relative contributions of the polycation and CTB chain are difficult to assess.

EXPLOITING ENDOCYTIC PATHWAYS: SAFETY CONSIDERATIONS

Although comprehensive discussion of all the safety issues relating to nanomedicines is beyond the scope of this article (see refs 170 and 171), technologies specifically designed to navigate the endocytic pathways do have every opportunity to disturb normal functioning of all physiological endomembrane trafficking pathways (Figures 1 and 6). Nondegradable or slowly degradable nanomedicine components obviously have the potential to accumulate in LYS thus creating a LSD-like syndrome. For example, engorgement of B16F10 cells *in vitro* following incubation with chitosan microspheres or cationic dendrimers with a diaminoethane (DAE) core was clearly visible by TEM after only 72 h of incubation (Figure 8). The resultant toxicity could be quantified.

Early clinical evidence of intracellular accumulation was seen following use of the synthetic polymer poly(vinylpyrolidone) (PVP) as a plasma expander during World War II which led to liver cell vacuolation, detected later in tissue taken from patients during autopsy (discussed in ref 170). The potential for intracellular accumulation of nonbiodegradable nanomaterials in general and nondegradable polymers like PVP[172] and PEG (increasingly seen to induce vacuolation in preclinical studies[173]) has become a hot topic for discussion today.

This background highlights the need to take special care when optimizing the design of any nanomedicine in the context of the proposed clinical use. It is essential to avoid high intravesicle concentrations of any nanomedicines during either acute or chronic use. In addition, if a charged or bioresponsive endosomotropic polyplex or lipoplex is administered, there is a real possibility of inducing uncontrolled leakage of protons or LYS enzymes resulting in apoptosis (Figure 6). This may not be a problem if it occurs in a target cancer cell, but could pose a real risk of inducing acute or chronic toxicity in off-target cells. As discussed above, and shown in Figure 7, there is also the capability of some systems to inhibit vesicle–vesicle fusion which could have a major deleterious impact on normal cellular homeostasis.

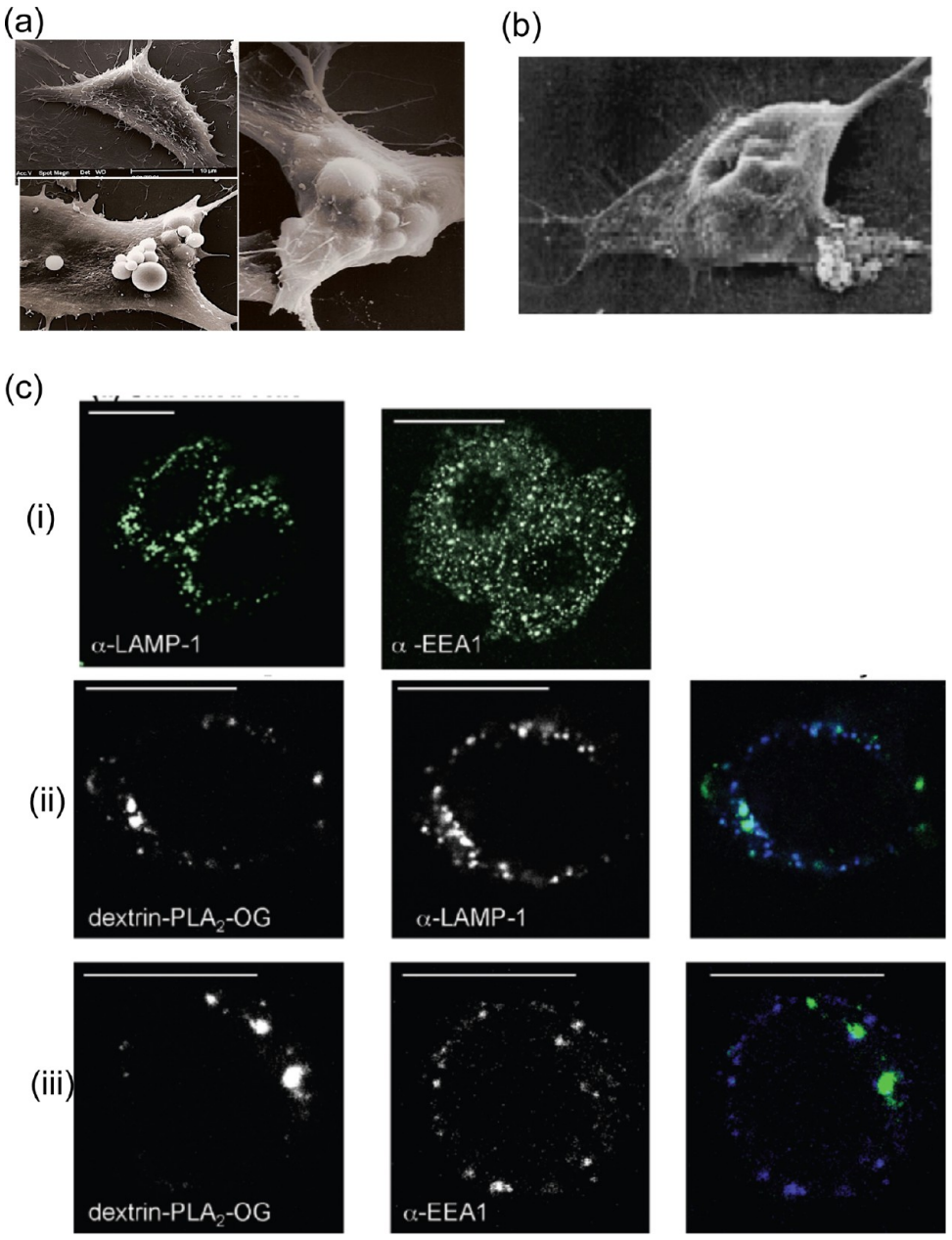

Figure 8. Examples to illustrate the potential cellular effects of polymers/nanomedicines internalized by endocytosis. SEMs of B16F10 cells incubated (72 h) with, in panel (a), chitosan microspheres (1 mg/mL) (control cells incubated without addition are shown in the upper left of pane),[27] or diaminoethane (DAE) dendrimers (10 μg/mL).[182] Panel (c) shows confocal micrographs of MCF-7: line (i) shows the distribution of LAMP-1 and EEA1 in untreated cells; line (ii) and line (iii) show the distribution of LAMP-1 and EEA1 respectively after incubation of MCF-7 cells with a dextrin-PLA_2−OG conjugate (for full technical details see ref 176).

Induction of cell vacuolation by nanomaterials, e.g., quantum dots, rare earth oxides, and fullerenes, and/or changes in autophagy markers have been interpreted as nanomaterial induction of autophagy, e.g., ref 174. From a cell biological viewpoint the mechanism(s) responsible for direct stimulation of autophagy are difficult to envisage. Autophagy, mitochondria and oxidative stress are however biochemically interconnected,[175] and as shown in Figure 1 all the trafficking pathways are inextricably linked. It is not unlikely that modulation of the autophagic pathway is a secondary event resulting from one or more of the following: (i) accumulation in the endolysosomal system altering membrane trafficking per se, (ii) induction of EE-LE-LYS membrane permeabilization leading to leakage of endogenous and exogenously applied materials into the cytosol, and (iii) free radical generation (e.g., iron oxide nanoparticles) leading to oxidative stress. In addition, subversion of the normal trafficking pathways has potential to modulate MHC-I presentation of macromolecular peptidyl drugs and/or targeting ligands, and this could lead to enhanced antigenicity.

The fact that nanomedicines themselves may also cause a change in distribution of intracellular vesicles might be expected to impact normal cell functioning. For example, when examining the mechanism of action of putative anticancer dextrin−phospholipase A_2 (PLA_2) conjugate in MCF-7 cells,[176] it was shown that whereas a reference dextrin−OG conjugate was seen in BSA-Texas Red-containing and LAMP-1 positive vesicles (i.e., LE and LYS) in the perinuclear region, incubation with the dextrin−PLA_2−OG conjugate led to a marked change in the

intracellular pattern of fluorescence distribution with location predominantly in vesicles in the cell periphery that exhibited no colocalization with EEA1 or LAMP-1 (Figure 8). Moreover, recent observations that PAMAM dendrimers transfer across perfused human placenta (albeit at a low rate),[177] and that silica and titanium dioxide nanoparticles can cross the placenta and cause pregnancy complications in mice,[178] highlight the need to carefully monitor placental transcytosis and/or any perturbation of normal maternofetal transport when designing and developing nanomedicines.

Rigorous preclinical quantitation of whole body PK, distribution, including rate and route of elimination, as well as quantitation of cellular PK and intracellular distribution *in vitro* is important not only with respect to nanomedicine optimization for efficacy but also to guide safety evaluation and the clinical protocol with respect to the issues discussed above.[170] While assessment of safety markers per se is important, the experimental time frames used in such experiments must not be so short that it will be impossible (*in vitro* or *in vivo*) to adequately define the effects of long-term chronic exposure to the nanomedicine and/or the components that may be liberated during intracellular metabolism. Quantitative cellular PK studies can at least go some way to defining how much material is retained, for how long and in which intracellular compartment to guide the design of such safety studies.

EXPLOITING ENDOCYTIC PATHWAYS: PATIENT BIOMARKERS

Use of patient-specific molecular biomarkers to improve success in early clinical trials is widely discussed. There is hope that this will bring a greater success rate knowing that ~95% of all drugs entering clinical testing today fail to make it into routine clinical use (discussed in refs 1 and 179). Moreover, their is a belief that the use of personalized medicines in general will minimize side effects and maximize efficacy improving the patient's experience. However, almost all discussions focus on biomarkers relating to the use of low molecular weight chemical entities, and in certain cases those biotech drugs that act via PM localized target effectors, e.g., Herceptin and the human epidermal growth factor receptor 2 (HER2). Given the recent advances in the understanding of the molecular mechanisms of trafficking pathways in health and disease that are discussed here, coupled with the known requirements for safety and efficacy of lysosomotropic and endosomotropic nanomedicines with respect to their endocytosis, trafficking and activation (e.g., pH, LYS enzymes and reducing environment), it is essential to proactively consider the disease biomarkers that are relevant to nanomedicine performance. This would improve the selection of those patients (disease subtypes) most likely to benefit when conducting early clinical trials with nanomedicines.

There are a number of obvious opportunities for use of clinical nanomedicine biomarkers, and these have been pointed out throughout the text. One simple example is the need to verify cathepsin B status of those patients treated with anticancer nanomedicines activated by this enzyme prior to their selection for therapy. Another, more complex opportunity is to type patient tumors for the known endocytic machinery protein alterations (see ref 2) in order to better the correlation with nanomedicine performance. Most often tumor biopsies are explored for markers of drug response and/or resistance but no thought is given to the known aberration of trafficking markers. In addition, as nanomedicines rely on a functional endocytic machinery for cellular access and activation there is a real opportunity to consider more carefully both disease features of and treatment-induced changes in the endolysosomal system that would also be expected to impart resistance to nanomedicines. Obviously there is also still much to do preclinically. There is a need to verify that all *in vitro* cell models and *in vivo* disease models are properly validated with respect to their functional endocytosis and trafficking behavior, not just the pharmacological drug target, established mechanisms of drug resistance, and/or the metabolism markers relating to the use of low molecular weight chemical entities as medicines.

CONCLUSIONS

The opportunities are there for all to see. The beginning of the 21st Century is bringing remarkable advances in molecular cell biology/systems biology, on one hand, and across the nanosciences on the other. This in turn has produced an extraordinary array of possibilities for design of novel nanomedicines with real potential to provide improved diagnostics and treatments for debilitating and life-threatening diseases. These will build on the successes so far seen with the first generation technologies already in routine clinical use. Of course challenges abound. Nanomedicines must be cost-effective, amenable to reproducible manufacture and validated characterization; no easy task given the complexity of many technologies. Not least there is a need to demonstrate that targeted nanomedicines can actually reach the diseased cells of a patient at sufficiently high therapeutic concentrations to mediate therapeutic benefit.

Successful exploitation of lysosomotropic and endosomotropic nanomedicines will in the future rely on interdisciplinary collaboration from the outset, thus ensuring that advances in the understanding of the molecular and functional basis of the endocytic machinery in health and disease are fully engaged to guide improved design of practical-to-use nanomedicines. There is a real need for *quantitative* (even GLP) evaluation of cellular PK *in vitro* and *in vivo* (including studies on concentration dependence), definition of intracellular compartmental distribution and metabolic fate. For experimental design, there must be an appreciation that endocytosis is only one endomembrane trafficking route within the complex array of interconnected intra- and even extracellular membrane trafficking pathways (Figure 1). Although many others factors are certainly critical points for success, ability to harness *the endocytic machinery* in *the target disease cell* will be the ultimate determinant of efficacy, will define whether or not resistance to therapy will emerge, and also will govern the safety of a nanomedicine. Finally, it is important to stress that it is becoming apparent that the appropriate selection of patients most amenable to nanomedicine therapy (using nanomedicine-relevant biomarkers) is needed during both early clinical evaluation and subsequent routine clinical use if the undoubted potential of novel nanomedicines is to be fully realized.

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