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Review article

Polymers and nanoparticles: Intelligent tools for intracellular targeting?

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Abstract

In recent years, a new generation of drugs has entered the pharmaceutical market. Some are more potent, but some are also more toxic and thus, therapeutical efficacy may be hindered, and severe side effects may be observed, unless they are delivered to their assigned place of effect. Those targets are not only certain cell types, moreover, in cancer therapy for example, some drugs even have to be targeted to a specific cell organelle. Those targets in eukaryotic cells include among others endo- and lysosomes, mitochondria, the so-called power plants of the cells, and the biggest compartment with almost all the genetic information, the nucleus. In this review, we describe how the drugs can be directed to specific subcellular organelles and focus especially on synthetic polymers and nanoparticles as their carriers. Furthermore, we portray the progress that has been accomplished in recent years in the field of designing the carriers for efficient delivery into these target structures. Yet, we do not fail to mention the obstacles that still exist and are preventing polymeric and nanoparticular drug carrier systems from their broad application in humans.

Keywords: Drug delivery; Intracellular targeting; Polymers; Nanoparticles; Endosomes; Lysosomes; Mitochondria; Nucleus

1. Introduction

In the context of drug delivery, the requirements on delivery systems can generally be classified into two categories: first to improve the efficacy, and second to reduce toxicity through enhancement of specificity. It has successfully been demonstrated that polymers can form effective delivery systems for drugs [1], proteins [2], and that they can be functionalized with targeting moieties [3]. The linkage between the polymer and the active substance can be designed to control, where and when the drug is released. In order to obtain such bonds, most of the strategies involve incorporation of amino acids, peptides or small chains as spacer molecules. Acid-labile linkages for example are cleaved after the polymer-drug conjugate is taken up into cells by the process of endocytosis [4–6]. Unfortunately, polymer-based carrier systems also have their disadvantages such as their low drug-loading capacity. The payload, that each polymer molecule can carry, depends on the number of reactive groups, where the drug can be attached. Hence, the application of nanoparticles as drug delivery systems may be advantageous for specific applications, as nanoparticles have a higher drug loading capacity. The available nanoparticle toolbox for drug delivery includes inorganic and organic materials, among which liposomes [7,8], micelles [9–11], quantum dots (QDs) [12–14], polymeric nanoparticles [15–17], gold nanoparticles [18,19] and magnetic nanoparticles [20–22] have gained the greatest popularity.

Unfortunately, the human body recognizes drug delivery systems as foreign intruders, and hence, they become easily opsonized and removed from the circulation long prior to completion of their function. Therefore, much effort has been spent on the design of long circulating drug carriers which can slowly accumulate in pathological sites with leaky vasculature via the enhanced permeability and retention (EPR) effect, also termed "passive" targeting [23,24], or can even achieve a better effect of targeted drug

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delivery systems due to a longer interaction time with their target [25]. Poly(ethylene glycol) or PEG is the mostly used polymer to enhance the circulation time of such drug carriers [26,27] by a sterical hindrance of their interaction with blood components as very nicely shown for liposomes [28]. But also other polymers such as *N*-(2-hydroxypropyl)methacrylamide (HPMA) [29,30] can make liposomes long-circulating [30]. Furthermore, the intracellular delivery is one key step in drug delivery. However, the lipophilic nature of the cellular membranes prevents the delivery of macromolecular components. Even more, especially in cancer treatment one has to overcome the problem of multidrug resistance (MDR) [31,32], although new drug delivery technologies have been developed in order to overcome resistance by bypassing the MDR mechanism with for example doxorubicin poly(isohexylcyano acrylate) nanoparticles in hepatocellular carcinoma [33,34]. Therefore, the next step comprised to add the property of a specific target recognition in order to allow for selective drug delivery to certain cell types with the aid of ligands that are specific to cell surface characteristic structures. In some cases, such drug delivery systems can exploit the mechanism of receptor-mediated endocytosis and accomplished their cellular entry via plasma membrane receptors or other cell surface antigens, that are unique to certain cells or related to certain diseases. Upon their binding to cell surface receptors, nanoparticle- and polymer-based drug delivery systems are internalized by a process called endocytosis/phagocytosis and end up in small vesicles called endosomes [35,36]. At this stage, after arrival within the target cell, the process becomes more complicated as endosomes undergo a rapid maturation to late endosomes that are able to fuse with other late endosomes [62] and lysosomes [60]. The luminal pH decreases with the maturation of endocytotic vesicles from early endosomes (neutral pH to ~pH 6), late endosomes (pH ~ 5–6) and lysosomes (pH ~ 4.5). DNase II, for example, is an acidic endonuclease active in lysosomes and may play a role in the destruction of DNA [63], therefore, the accumulation of DNA in lysosomes is not expected to be productive for transfection [3]. So far multiple, but only partially successful, approaches have been made to prevent the degradation of drugs in the lysosomes, as for example to use polymers with buffering capacity or pH-sensitive carriers, which will be described later. After the active component has been released from the carrier system and/or endocytotic vesicles, it must be transported through the cytoplasm to its final destination. One can imagine that the cytosol of mammalian cells, a crowded environment containing soluble proteins and a network of cytoskeletal filaments [37,38], gravidly hampers the transport of macromolecules, and especially nanoparticular systems. Moreover, many drugs or bioactive molecules exert their therapeutic action inside the nucleus or other specific organelles such as lysosomes, mitochondria, or endoplasmatic reticulum. This group of drugs comprises for example DNA which is active in the cell nucleus, or antiapoptotic drugs which have to reach the mitochondria, and enzymes which have to be delivered to lysosomes associated with lysosomal storage disease.

From this description it becomes obvious that pharmaceutical drug delivery systems should be multifunctional in order to facilitate in the first stage a successful delivery to certain cell types or tissues and in the second step to direct the drug carrier to its appropriate intracellular destination. As the first part has been extensively reviewed elsewhere, we will focus on recent approaches in intracellular delivery of drugs and organelle targeting. After a short presentation of the main pathways for the cellular entry of drug delivery systems, this review will focus on targeting strategies to the endo-/lysosomes, cytosol, mitochondria, and the nucleus, and present the first breakthroughs of targeting polymer- and nanoparticle-drug assemblies to these intracellular destinations.

2. Cellular uptake and targeting endosomes/lysosomes

The transport of macromolecular and colloidal carriers from the cell surface to lysosomes starts with a process called endocytosis. Generally, endocytosis occurs by multiple mechanisms that fall into two broad categories, 'phagocytosis' (the uptake of large particles) and 'pinocytosis' (the uptake of fluid and solutes) [35,36]. Phagocytosis is typically restricted to specialized mammalian cells like macrophages, whereas pinocytosis occurs in all cells by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. Besides other pathways into a cell, the receptor-mediated endocytosis is probably the most efficient one for the specific uptake of macromolecules. This process usually begins at specialized regions of the plasma membrane, the so-called clathrin-coated pits, with binding of the macromolecule to complementary cell-surface receptors. This complex then enters the cell in clathrin-coated vesicles known as endosomes. Receptor-mediated endocytosis enables an increase of the intracellular concentration of macromolecules up to 1000-fold. These early endosomes then undergo a rapid maturation to late endosomes and are able to fuse with other late endosomes or lysosomes.

One of the receptors that thus frequently has been used as a tool to increase the concentration of drugs within the cell is the folate receptor. The reduced folate carrier (RFC) is the dominant route for reduced folate cofactors and methotrexate (MTX). 5-Methyltetrahydrofolate (5MTHF), which accounts for 99% of folates in circulation, is believed to be the primary substrate for the RFC. In contrast, folate receptor α (FRα) has a high affinity for folic acid ($K_d \sim 1$ nmol/L) and, to a less extent, for 5MTHF (K_d, 1–10 nmol/L). FRα internalizes folate by receptor-mediated endocytosis [39–41]. Although RFC is ubiquitously expressed in almost all tissues, the biodistribution of FRα is quite limited. FRα is highly overexpressed in various kinds of cancer cells, especially those of ovarian and uterine, because of their augmented

need of folic acid [39–41]. Dixit et al. exploited this fact by constructing gold nanoparticles with a thioctic acid (alpha-lipoic acid)-PEG-folate coating, the coating being covalently attached to the particles via the disulfide bonds of the thioctic acid [19]. The particles accumulated significantly in the lysosomes and endosomes of cells overexpressing the folate recepter as shown by transmission electron microscopy (TEM), an effect which could be reversed by addition of free folic acid to the medium. No significant uptake could be determined in cells with normal expression levels of the receptor. The application of liposomes with phospholipid-anchored folate conjugates for the targeting of tumor cells was nicely reviewed by Gabizon et al. in 2004 [42]. Moreover, folate-derivatized adenovirus, cationic polymers, cationic liposomes, and pH-sensitive liposomes have impressive folate receptor selectivity in many cell culture systems and are, therefore, promising delivery systems for gene transfer [43]. However, one of the major limitations of folate-targeted gene therapy lies in the low rate of vector escape after receptor-mediated endocytosis. The transferrin receptor is another example of how receptors can be exploited for the entry to the endosomal pathway and it is as well as the folate receptor overexpressed in many types of cancer cells [44]. Several anti-cancer drugs, such as Doxorubicin, Cisplatin, Chlorambucil, Mitomycin C, Gemcitabine and Daunorubicin, have been coupled to transferrin already. Compared to the uncoupled drugs, they showed higher cytotoxicity [45]. Moreover, they might most likely cause less overall side effects since the drug itself was mainly directed to cells with an abnormal expression of the receptor. Short catalytic single-stranded DNA-molecules (DNAzymes) have also been coupled to transferrin-modified nanoparticles and were efficiently delivered into tumor cells where they could exert their antigene or antisense activity as shown by Pun et al. [46]. Hence, those results suggest that the transferrin receptor might as well serve as a target for intracellular drug delivery. With regard to brain targeting, PEGylated poly(hexadecyl cyanoacrylate) nanoparticles [47] or anionic liposomes loaded with oligonucleotides [48] have been shown to utilize constitutive endocytosis of the low density lipoprotein (LDL) receptor-related protein to enter neurons.

However, the endosomes not only serve as an entry for various kinds of drugs, they can also be exploited as kind of cell tracing marker in animal tissue. Hoshino et al. showed that albumin coupled QDs could be endocytosed by mouse lymphocytes. Thereafter, the QD-labeled cells were intravenously injected into mouse and their bright fluorescence remained even after a period of seven days post-injection. Therefore, endocytosed QDs can serve as endosome marker and might be useful as bioimaging tool for tracing (foreign) target cells over the period of a week *in vivo* [49,50]. This method evades expensive and slow labeling of cells with green fluorescent protein (GFP) or other protein markers and excels labeling with organic fluorophores or antibody staining.

The accumulation of the drug and its carriers in lysosomes can be exploited in association with certain diseases and disorders of lysosomes (e.g., lysosomal infections [51] or even metabolic disorders connected with the deficiency of certain lysosomal enzymes, the so-called storage diseases [52,53]). This topic has received considerable attention in the past.

Yet, one problem with endosomal uptake remains: some drugs and macromolecular therapeutics – such as peptides, proteins, DNAzymes and short interfering RNA and others – can only exert their activity after being released into the cytosol [54] and being transported to their assigned organelles. Since endocytosis is the primary route for their uptake, those molecules accumulate in the endo-/lysosomal compartment, eventually losing therapeutic activity. In the following section we, therefore, describe several methods to achieve the release from the digestive environment innate to endosomes and lysosomes, respectively, by designing the properties of the carriers.

3. Escape from endo-/lysosomes and cytoplasmic delivery

The emergence of macromolecules, such as proteins and nucleic acids, as therapeutics has sparked a renewed interest in pH-responsive drug carriers as a means to enable these macromolecules to reach their subcellular targets. In general, macromolecules and colloids enter cells via an endocytic process. They first reach mildly acidic endosomes (pH 5.0–6.5) and then end up in lysosomes where they face much lower pH conditions and possible enzymatic degradation. Accordingly, therapeutic compounds that are transported to these acidic organelles may be inactivated before reaching their site of action. Hence, pH-responsive carriers are aimed at increasing the intracellular drug bioavailability by rapidly releasing their payload in the endosomes after cell uptake, and/or facilitating the drug transit to the cytoplasm. To achieve this task, several systems have been proposed. They all offer advantages and limitations that vary in importance depending on the desired application. Various approaches have been performed such as the supplementation of fusogenic peptides, the use of polymers, which have an intrinsic endosomolytic escape capacity, or mediate their escape by degradable spacers. Another approach is the use of cell-penetrating peptides (CPP) which have been shown to facilitate the uptake of various cargoes and are described below.

3.1. Fusogenic peptides

The capacity of fusogenic peptides of natural (e.g., N-terminus of hemagglutinin subunit HA-2 of influenza virus) or synthetic (e.g., WEAALAEALAEALAEH LAEALAEALEALAA (GALA), or WEAKLAKALAKA LAKHLAKALAKALKACEA (KALA)) origin has been exploited for the endo-/lysosomal escape of several drug delivery systems [55,56]. These peptides assume a random coil structure at pH 7. Acidification triggers a conforma-

tional transition, which enables their subsequent interaction with the phospholipid membranes, resulting in pore formation or the induction of membrane fusion and/or lysis. Incorporation of synthetic membrane-active peptides into delivery systems has been found to enhance intracellular delivery of drugs including oligonucleotides, peptides, or plasmid DNA. For example, mono-PEGylated KALA has been attached to the surface of plasmid DNA/polyethylenimine (PEI) complexes, in order to improve transfection efficiency due to its fusogenic property [57]. Although encouraging results have been obtained with the use of synthetic peptides to enhance cellular delivery of various compounds, other mechanisms and approaches are probably far more efficient and less expensive.

3.2. Endosomolytic and/or pH-responsive polymers

Endolysosomal escape may also be exemplified by endosomolytic polycationic polymers such as PEI. Behr postultated the so-called 'proton sponge hypothesis' [58], which relates the intrinsic endosomolytic activity of PEI to its capacity to buffer the endosomal environment, prompting the osmotic swelling of the vesicle and finally its rupture, which leads to the liberation of the DNA/PEI complexes (polyplexes) into the cytoplasm. Another mechanism for endolysosomal escape of polyplexes was suggested by Bieber et al. [59]: in electron microscopy studies, endosomal membrane holes have been observed and were related to the direct interaction of high molecular weight (MW) branched PEI (BPEI) (800 kDa) with the endosomal membrane in a non-acidic environment. The authors suggested that low MW PEIs (25 kDa) also induce minor membrane damages, but that those holes may be quickly resealed. In addition to direct membrane interaction, the release of polyplexes may also be attributed to the extension of the polymer network as a result of the increasing electrostatic repulsion of charged groups during acidification [60].

Similarly, polyanionic polymers can also be tailored to interact actively with phospholipid membranes upon external stimulation, such as acidification of the surrounding medium. The pH-dependent conformation of weak polyacids has been studied extensively using poly(methacrylic acid) (PMAA) as a model polymer [61]. In aqueous solution, the conformation of polyelectrolytes bearing pendant carboxylic acid groups is a function of pH. Upon ionization, the polymeric chain becomes more extended as a result of increased electrostatic repulsion between the charged carboxylate groups. Other interacting forces such as hydrophobic interactions and hydrogen bonding, due to the presence of alkyl groups or backbone stiffness, may also influence the conformation adopted by a polyelectrolyte in solution.

Towards this approach, Kim and co-workers attached the amino acid histidine (His) as an endosomolytic agent to poly(2-hydroxyethyl aspartamide) (PHEA-His) and C_{18}-grafted PHEA (PHEA-g-C_{18}-His) via an ester linkage [62]. PHEA-g-C_{18}-His series formed stable self-assembled particles due to the hydrophobic interaction between grafted alkyl chains. After incubation at a pH of 5, the electrostatic repulsion of ionized histidine moieties led to an aggregation of self-assembled particles. Using confocal microscopy, the authors revealed that PHEA-g-C_{18}-His was more uniformly distributed than PHEA-g-C_{18} in HeLa cells after 8 h of incubation. This was attributed to the endosomolytic ability of conjugated histidine moieties. The release of loaded doxorubicin (DOX) in PHEA-g-C_{18}-His was accelerated at pH 5 due to the aggregate swelling, and therefore, most likely enabled DOX to access the nucleus in HeLa cells.

Frechet and co-workers developed acid-sensitive micelles based on PEG-dendrimer hybrids as backbones (Fig. 1) [5]. Their approach involved the attachment of hydrophobic groups to the periphery of the core forming dendrimer block by an acid-sensitive acetal linkage. The system was designed as such, that upon hydrolysis of the linkage and loss of the hydrophobic groups, the core-forming block becomes hydrophilic, thus destabilizing the micelle and enabling escape of the drug from its encapsulating micellar compartment. In comparison with a non-pH-sensitive micelle control, the release of DOX from the pH-sensitive system was much more dependent on pH, although the release of DOX at acidic pH was clearly to some degree due to its increased aqueous solubility [6]. Dynamic light scattering studies revealed that while the pH-sensitive micelles were stable at pH 7.4, acetal hydrolysis at acidic pH resulted in disruption of the micelles and rearrangement to form larger aggregates.

The group of Kataoka performed a similar assay by attaching an anticancer drug adriamycin (ADR) to a micelle-forming copolymer by an acid-sensitive hydrazone linkage [4]. The micelle was a nanosized supramolecular assembly from the self-assembling amphiphilic block copolymers, poly(ethylene glycol)–poly(aspartate hydrazone adriamycin) [PEG-p(Asp-Hyd-ADR)] (Fig. 2a). The anticancer drug was afterwards conjugated to the core-forming segments through the hydrazone linkers that were stable under physiological conditions, but cleavable at pH values below 6 and, therefore, enabled the release of ADR with decreasing pH (Fig. 2b). The drug loaded micelles were taken up by cells *in vitro* and the released drug most likely accumulated in the cell nuclei (Fig. 2c).

A very promising approach was the attachment of pyridyl disulfide acrylate (PDSA) to the amphiphilic copolymer consisting of methacrylic acid (MAA) and butyl acrylate (BA), which resulted in a glutathione- and pH-sensitive, membrane-disruptive terpolymer with functional groups, that allowed thiol-containing molecules to be readily conjugated [63]. The pH sensitivity could be tuned by controlling the length of the hydrophobic alkyl group substituted on the pH-sensitive monomer and by the incorporation of a hydrophobic BA monomer [64]. The authors

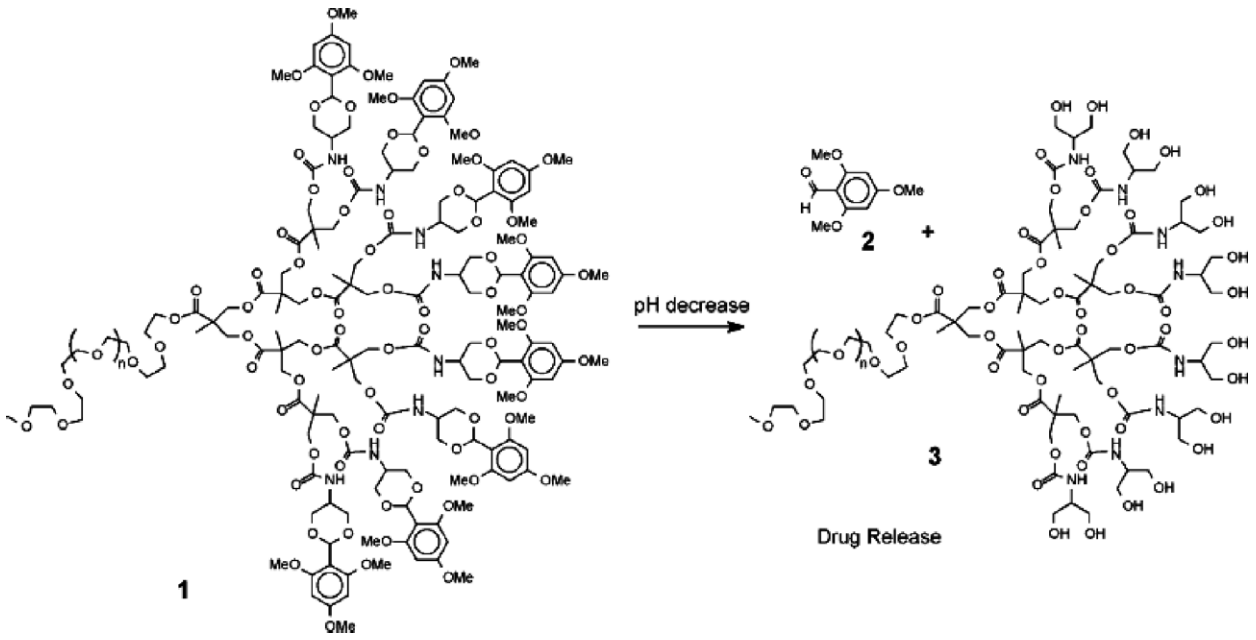

Fig. 1. Hydrolysis of acetals on the dendrimer periphery of the micelle-forming coplymer (1) leads to a solubility change designed to disrupt micelle formation and trigger the release of drug. Reprinted with permission from [5]. Copyright (2007) American Chemical Society.

showed that the membrane destabilizing activity was related to the pK_a of the carboxylic groups incorporated in the polymer composition. Several pH-sensitive, membrane-destabilizing, and glutathione-reactive polymer compositions were identified as a result of these structure–activity studies including poly(ethylacrylic acid (EAA)-co-BAco-PDSA) and poly(propylacrylic acid (PAA)-co-BA-co-PDSA) terpolymers, and poly(PAA-co-PDSA) copolymer. In future experiments, the drug loading capacity and suitability in cell culture have to be studied with this novel kind of polymer.

3.3. pH-responsive liposomes

As described above, endosomes are a kind of sluice from the cell outside to the cell cytoplasm. It has been evaluated that also liposomes can play on the pH drop in endosomes. Phosphatidylethanolamine in liposomes undergoes for example a transition from lamellar to inverted micelle structures at low pH which allows for fusion of liposomal and endosomal membrane and consequently a destabilization of endosomes. Among various other drugs, pH-sensitive liposomes have successfully been applied for the delivery of antisense oligonucleotides into the cytoplasm [65]. However, the design and application of pH-sensitive liposomes has been extensively reviewed elsewhere [65–68].

3.4. Cell-penetrating peptides

Despite the successful application of the described strategies, a direct delivery to the cytoplasm is more desirable as it reduces the chance to lose the bioactivity of the transported drug. A novel approach to deliver macromolecules to intracellular compartments of the cell is tethering them to peptides that transport them through cellular membranes. Several "cell-penetrating peptides" (CPPs) or "protein transduction domains" (PTDs) for the transport of various cargoes with molecular weights several times greater than their own have been described in the last decade and nicely reviewed by Futaki [69] and others [70–72]. Among the most frequently used peptides are the Tat [72], Antennapedia (Antp) [72], poly-arginine peptides [72], Tp10, a variant of transportan [73], YTA2 [74], and penetratin [75]. The exact mechanism of internalization of these peptides, which gain direct access to the cell cytoplasm independent of the cell type, and the nucleus, is still unclear, but seems to differ from peptide to peptide [76,77]. More than one particular mechanism is assumed to be responsible for intracellular delivery of various cargo molecules [77–79]. Tat-mediated delivery has been proposed to proceed via energy-dependent macropinocytosis with subsequent escape from the endosome into the cytoplasm and eventually with the transport into the nucleus [80], while other CPPs penetrate cells via electrostatic interactions and hydrogen bonding in non-energy dependent mechanisms [81].

The use of CPPs as ferries for macromolecules such as proteins [82], DNA [83], antibodies [84], and liposomes [85] into mammalian cells has been reviewed by Gupta et al. [71]. Here, we want to elucidate the use of CPP for the delivery of polymers and nanoparticles. Kopecek and co-workers, for example, synthesized HPMA copolymer-cell penetrating peptide Tat conjugates and evaluated their subcellular distribution in A2780 human ovarian carcinoma cells by confocal fluorescence microscopy and subcellular fractionation. They described the transport of

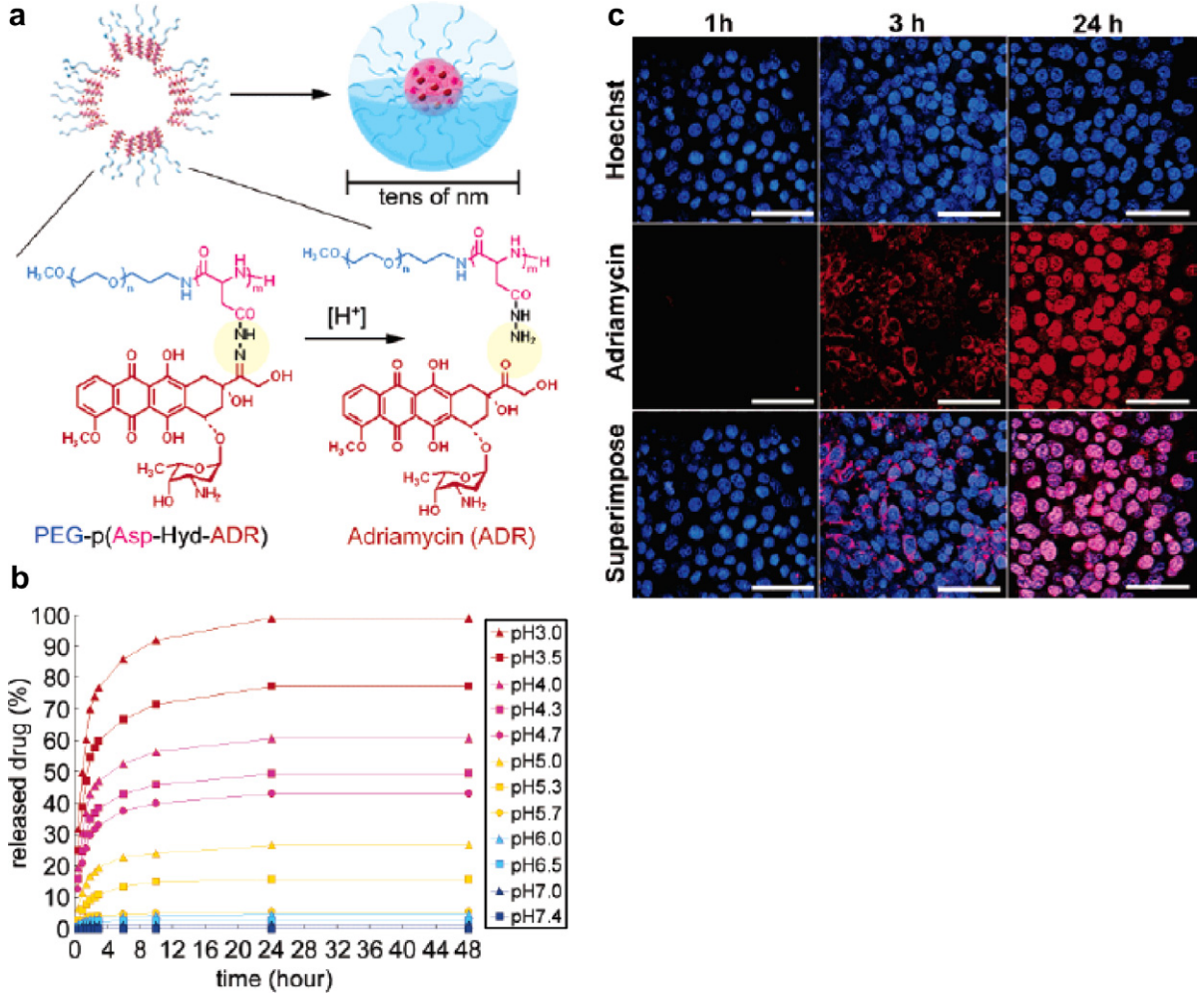

Fig. 2. Preparation of polymeric micelles with intracellular pH-sensitivity. (a) Micelles were prepared from self-assembling amphiphilic block copolymers, PEG-p(Asp-Hyd-ADR), in which the anticancer drug, adriamycin (ADR), was conjugated through acid sensitive hydrazone linkers. (b) The micelles released the loaded drugs under acidic conditions below pH 6.0 corresponding to intracellular space, but remained stable under the conditions of vascular and extracellular space (pH 7.4–7.0). (c) The intracellular drug release and localization of the micelles in each cell of a multicellular tumor spheroid (MCTS) were observed in detail using a high-magnification 63× objective. The images clearly demonstrated that the micelles were internalized into the cells and released drugs, and that the released drug eventually accumulated in the cell nuclei (staining with Hoechst dye). Bar 50 μm. Reprinted with permission from [4]. Copyright (2007) American Chemical Society.

these conjugates by a single Tat molecule to both the cytoplasm and nucleus via a non-endocytotic and concentration independent process. In contrast, HPMA copolymers lacking the Tat peptide were internalized solely by endocytosis [86]. In another example, concerning the area of gene delivery to the lung, an oligopeptide related to the protein transduction domain of HIV-1 Tat was covalently coupled to 25 kDa PEI via a heterobifunctional PEG spacer resulting in a Tat-PEG-PEI conjugate [87]. Polyplexes were made with either PEI only or Tat-PEG-PEI using luciferase as a reporter gene. While *in vitro* gene expression in A549 cells was much lower for Tat-PEG-PEI (0.2 ng/mg protein) than for PEI (2 ng/mg), significantly higher transfection efficiencies for Tat-PEG-PEI were detected after *in vivo* (intratracheal instillation) transfection studies in mice. Thus, the authors speculated that Tat-PEG-PEI represents a new approach to non-viral gene carriers for lung therapy, comprising protection for plasmid DNA, low toxicity and significantly enhanced transfection efficiency under *in vivo* conditions.

Furthermore, various groups also accomplished the cellular uptake of nanoparticles using CPPs. Feldheim and co-workers described the cellular uptake of gold nanoparticles carrying the protein transduction domain from HIV Tat protein into 3T3/NIH or HepG2 cells, which was temperature dependent, and, therefore, they suggested an endosomal pathway of uptake [88]. However, their microscopic observations revealed that the conjugates did not enter the nuclei (Fig. 3).

Weisseleder and co-workers demonstrated a cell labeling approach using short HIV-Tat peptides attached to superparamagnetic nanoparticles. The particles were efficiently internalized into hematopoietic and neural progenitor cells in quantities up to 10–30 pg of superparamagnetic iron per cell, and cell populations were successfully localized *in vivo* by magnetic resonance imaging or they were recovered on

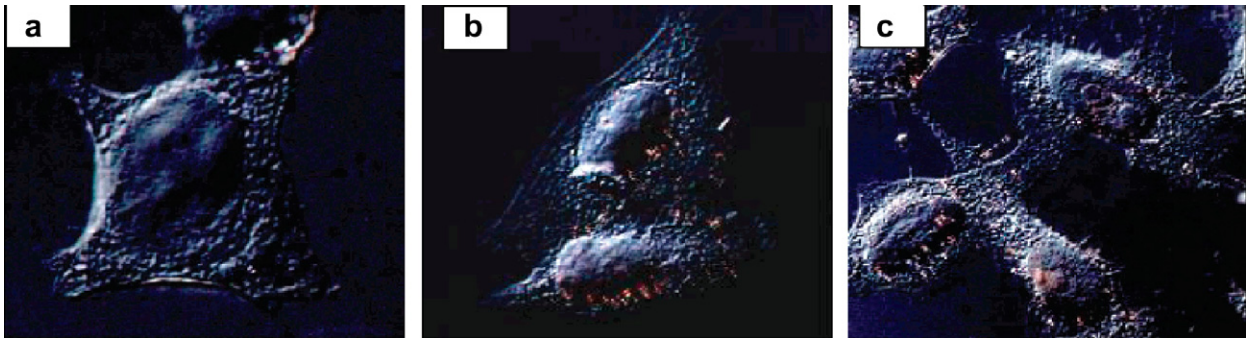

Fig. 3. Incubation of HeLa cells with 20 nm diameter gold nanoparticles observed by VECDIC (video-enhanced color, differential interference contrast microscopy). Cells were incubated for 0.5, 1.5, and 3 h (a, b, and c, respectively) with gold nanoparticles carrying peptide M2 (peptide sequence from HIV Tat protein). Nanoparticles were found in the cytoplasm of HeLa cells after 1 h, after 3 h only minimal nuclear localization was observed. Reprinted with permission from [88]. Copyright (2007) American Chemical Society.

magnetic separation columns after sacrifice of the animal and subsequent collection of hematopioetic and neural progenitor cells [89].

4. Mitochondrial targeting

4.1. Properties of mitochondria

Mitochondria occupy a substantial volume of the animal cell cytoplasm and, as they provide the bulk of the cellular ATP, they are entitled the power plants of the cell. Mitochondria are distinct organelles consisting of two subcompartments, the intermembrane space and the internal matrix space, which are formed by two membranes. The porous outer membrane is characterized by the presence of a channel-forming protein, termed voltage-dependent anion channel (VDAC), which limits the passage of molecules to the intermembrane space to a MW of 5000 Da or less [90,91]. The inner membrane is highly convoluted, forming folds called cristae, and containing proteins with different, vitally important functions: the ATP synthase, proteins of the oxidation reactions of the respiratory chain, and specific transport proteins regulating the passage in and out of the matrix. The matrix contains a highly concentrated mixture of hundreds of enzymes, in addition to the special mitochondrial ribosomes, tRNA, and several copies of the mitochondrial genome. The major functions of the enzymes include oxidation of pyruvate and fatty acids, and the accomplishment of the citric acid cycle. The ATP synthesis by oxidative phosphorylation establishes a proton electrochemical gradient across the inner membrane [92] comprising a membrane potential (−130 to −150 mV) and a pH gradient (pH 8 inside). Besides their central role in the energy metabolism, mitochondria are also involved in the regulation of apoptotic cell death, in the complex process of cardioprotection, the formation of free radicals, and the calcium metabolism. Consequently, mitochondrial dysfunction has been associated with a variety of pathophysiological disorders, especially neurodegenerative diseases and cancer, as well as cardiovascular diseases, and diabetes [93–97]. Therefore, mitochondria are a promising intracellular target for drug delivery [94,98,99].

4.2. Targeting mitochondria

Some anticancer agents, already in clinical use such as paclitaxel, vinblastine, lonidamine, etoposide, and arsenic trioxide, directly target, permeabilize mitochondria and trigger apoptosis [100]. Moreover, the large membrane potential across the inner membrane and the organelle's protein import machinery may facilitate the selective targeting of drugs and biologically active substances to mitochondria. In future, the mitochondrial DNA (mtDNA) could be an appealing target as deletions or point mutations have been found to be associated with neuromuscular diseases or mitochondrial myopathies [101,102]. Approaches such as those involving cytoplast fusion and mitochondrial microinjection are essential for gene therapy of diseases caused by these mutations [103]. The delivery of nucleic acids to mitochondria will be a challenge as cationic polymer- or lipid-associated plasmid DNA accumulates around the nucleus in the absence of targeting moieties. In the following section we will describe some current approaches (for an overview, please see Table 1) towards targeting mitochondria, of which low molecular weight substances are probably more applicable as compared to high molecular weight substances and nanoparticles. To date, the concept of targeting mitochondria is still in its infancy, and its feasibility has still to be proven.

4.3. Import mediated by (lipophilic) cations

Due to the negative membrane potential of 130–150 mV, which is lower compared with others in living cells, lipophilic cations such as rhodamine 123 [92] or tetraguanidinium oligomers [104], accumulate selectively within mitochondria. Especially, the cation triphenylphosphonium (TPP) has been applied in various studies for mitochondrial targeting of antioxidants with the aim of protecting them from oxidative damage [105,106]. This principle has also been successfully exploited to transport peptide nucleic acid

Table 1
Overview of various approaches for mitochondrial targeting

Targeting moiety	Transport of	Reference
Nonpeptidic targeting sequences		
Triphenylphosphonium (TPP)	TPP-based antioxidants	[105,106]
	Peptide nucleic acids (PNA)	[107]
	HPMA	[108]
Dequalinium	Plasmid DNA	[111]
Peptide targeting sequences		
Cell-permeable, mitochondrial targeted peptide	Peptides themselves have antioxidant capacity	[97,117,118]
Presequence of cytochrome *c* oxidase (COX) subunit VIII	PNA	[119]
Presequence of COX subunit VIII	QDs	[121]
Signal sequence of COX subunit IV	Dihydrofolate reductase (DHF)	[120]
GH3 domain from Grim protein	QDs	[122]

(PNA) of 3.4–4 kDa to mitochondria [107]. Callahan and Kopecek tested whether HPMA copolymer, possessing a single terminal TPP moiety and fluorescent labels, would be transported to the mitochondria of target cells [108]. The fluorescent dyes fluorescein (FITC) or BODIPY FL (negatively charged or uncharged, respectively, at physiological pH) were used as model drugs and as a means to visualize the trafficking of the polymer at the same time (Fig. 4). They presumed that the conjugate should additionally possess a molecular weight less than 4 kDa and be otherwise electrically neutral and relatively nonpolar. Uptake studies using free isolated mitochondria indicated that only TPP-HPMA-BODIPY FL with an average molecular weight below 5 kDa showed significant localization to mitochondria. Higher molecular weight BODIPY FL copolymers above 10 kDa showed little if any localization and none of the FITC-labeled polymers yielded significant localization. Therefore, they suggested that the cationic TPP is only able to direct the movement of electrically neutral and very low molecular weight conjugates. However, the applied assay did not distinguish between fluorophores only adsorbed to the surface or taken up into the membranes of mitochondria. Even more surprising was, that after polymer microinjection into the cytosol, none of the TPP polymers exhibited colocalization with mitochondria. As the polymer is freely diffusable within the cytosol, Callahan and Kopecek speculated that the attraction of the lipophilic cation TPP may be overcome by other forces that hinder the polymer from entering the mitochondria.

Dequalinium (DQA), a dicationic amphiphilic compound, has also been shown to exclusively localize in mitochondria [109]. Moreover, DQA forms liposome-like aggregates in aqueous media, the so-called DQAsomes, which have also been shown to bind plasmid DNA [110]. In a very nice study of the group of Weissig, it has been demonstrated that the DQAsomes complexed with plasmid DNA can enter cells, approach mitochondria and even release the transported nucleic acid after interacting with the mitochondrial membrane [111]. These very promising properties of DQA have also been reviewed in [109,112].

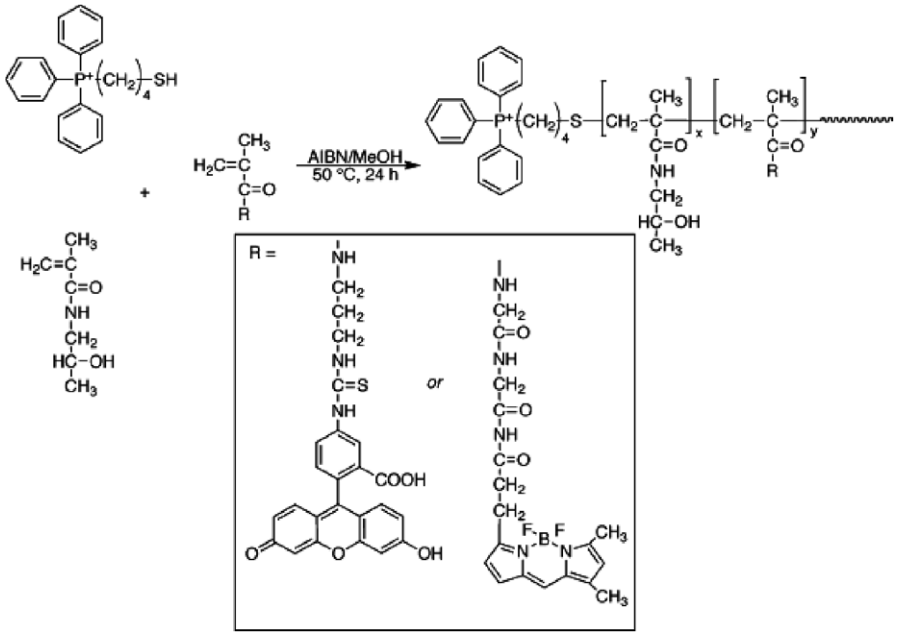

Fig. 4. General synthesis of TPP-functionalized HPMA copolymers labeled with FITC and BODIPY FL (R) comonomers. Reprinted with permission from [108]. Copyright (2007) American Chemical Society.

Interestingly, it has been shown that also TAT fusion proteins with green fluorescent protein (TAT-GFP) can traverse the mitochondrial membrane through a non-regular import pathway [113], probably due to electrostatic interaction. In order to avoid non-specific targeting to a different organelle, the mitochondrial targeting sequence (MTS) from mitochondrial malate dehydrogenase (mMDH) was added, resulting in TAT-mMDH-GFP fusion protein. Only the fusion protein with the MTS allowed the intramitochondrial processing of the fusion protein and was necessary for its persistence in mitochondria over time.

4.4. Import mediated by peptide- or protein-based targeting sequences

Most mitochondrial proteins are encoded in the nucleus and carry a targeting signal that enables their proper delivery into the organelle. The common feature of such matrix signal sequences is to fold in an amphipathic α helix, in which positively charged clusters are on one side of the helix, while hydrophobic residues are clustered on the opposite side [114]. This conformation is more important than a precise amino acid sequence. Signal sequences [115] are usually located at the N terminus of the transported protein and cleaved off after successful import. Protein translocation across the outer membrane is mediated by the translocases of the outer membrane of mitochondria (TOM) complex. Two translocase of the inner membrane of mitochondria (TIM) complexes enable the transport across the inner membrane [114,116]. A series of small, cell-permeable, mitochondria-targeted peptides which have antioxidant capacity and can protect mitochondria from oxidative damage was recently reported [99,117,118]. The structural motif of these Szeto–Schiller (SS) peptides centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides). These small peptides freely penetrate cells despite carrying a 3+ net charge at physiologic pH, concentrate by >1000-fold in the inner mitochondrial membrane, and prevent oxidative cell death with EC_{50} in the nanomolar range. Furthermore, in another study, a presequence of cytochrome *c* oxidase (COX) subunit VIII, which is located in the inner membrane of mitochondria, successfully enabled the PNA import into isolated mitochondria [119]. A fusion protein of dihydrofolate reductase (DHF) and signal sequence of COX subunit IV was imported into isolated mitochondria [120]. In future studies, these peptides could be a promising approach for targeting macromolecules to mitochondria.

Yamamoto and co-workers made an auspicious approach by conjugating a peptide-based mitochondrial targeting sequence to QDs [121]. The sequence was attached to *n*-trioctylphosphine oxide (TOPO)-capped QDs in a multistep process (Fig. 5A): by a thiol-exchange method, 3-mercaptopropanoic acid (MPA) was used to generate carboxyl-QDs, which were then coupled with the amino acid cysteine to get free sulfhydryl groups on the surface of QDs. The amino group of the mitochondrial targeting sequence Mito-8 (NH_2-MSVLTPLLLRGLTG SARRLPVPRAKIHWLC-COOH) was then attached using the heterobifunctional linker sulfo-SMCC. The number of peptides per particle was calculated to be 62. In contrast to QDs covered with a control peptide (Fig. 5B, d–f), QD520-Mito8 (Fig. 5B, a–c) exhibited a strong mitochondrial localization in living cells, which was expressed by the merge of the green signal from QD520-Mito8 and the red signal from mitochondrial staining using confocal images.

In a similar study, QDs were covered with an apoptotic trigger, the 21 amino acids long GH3 domain from the Grim protein, via streptavidin-biotin linkage [122], which is known to induce mitochondrial-mediated cell death. The GH3-conjugated QDs were additionally pre-incubated with Pep-1, a peptide carrier, which is extremely efficient in delivering substances into cells. HeLa cells internalized the Pep-1-/GH3-conjugated QDs and exhibited severe morphological changes such as membrane blebbing and nuclear condensation after 18 h. These changes are known markers of apoptosis and a sign for successful mitochondrial delivery of the QDs. On the one hand, the results indeed suggest that QDs conjugated with the GH3 domain were targeted to the mitochondria and able to cause cell death, but on the other hand, QDs without Pep-1 coating also induced cell death to a significant lower part. This means it has still to be proven whether the cytotoxic effects are due to mitochondrial targeting of the QDs or due to the toxicity of the QDs themselves.

4.5. Import without targeting sequences

Maysinger and co-workers made interesting observations concerning the intracellular distribution of block copolymer micelles that were made of poly (caprolactone)-b-poly(ethylene oxide) (PCL-b-PEO) block copolymer with tetramethylrhodamine-5-carbonyl azide (TMRCA) covalently attached to the PCL end of the polymer [123]. In triple-labeling confocal microscopy studies, they stained the nucleus and either lysosomes, Golgi apparatus and endoplasmatic reticulum (ER), mitochondria and ER, or mitochondria alone. The third fluorescence signal came from the micelles. Unexpectedly, the studies revealed that in PC12 cells, the micelles localized in several cytoplasmic organelles, especially mitochondria, but not in the nucleus. Therefore, they suggested that these micelles are worth exploring for their potential to selectively deliver drugs to specified subcellular targets.

5. Nuclear targeting

The nucleus is the largest and one of the most important subcellular structures in eukaryotic cells since it is the place where DNA replication and transcription occur. It is separated from the cytosol by two membranes which are periodically interrupted by the nuclear pores, large protein complexes of about 120 MDa in vertebrates [124] and

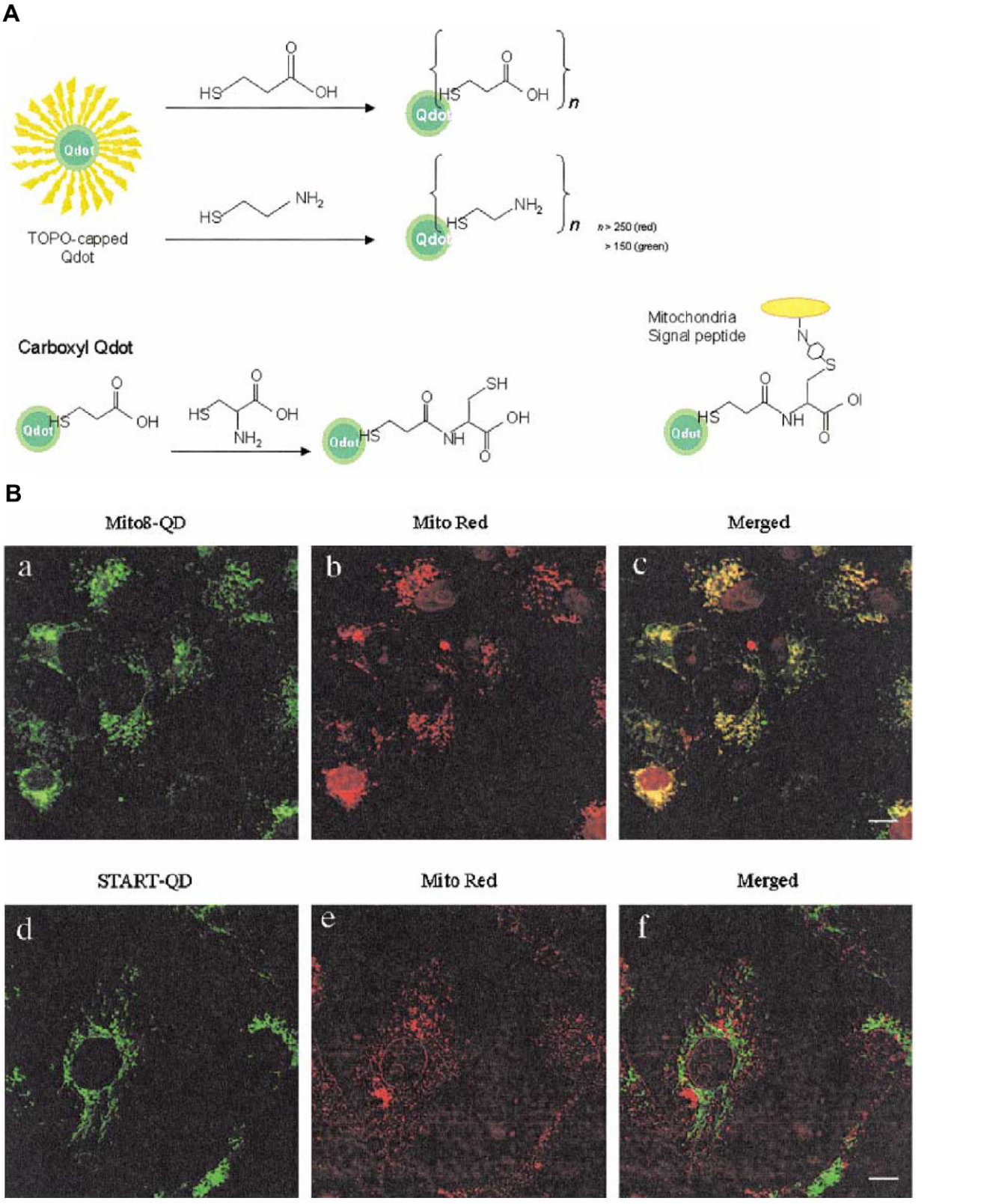

Fig. 5. Schematic illustration of peptide conjugated QDs for organelle targeting and imaging. (A) Chemically synthesized TOPO-capped QDs were replaced by MPA or cysteamine using thiol-exchange reactions. After reaction, QDs were covered with approximately 250 carboxy or amino groups per particle. A two-step strategy of QD-oligopeptide probes. MPA-QD was primarily coupled with the amine groups of cysteine or serine by using EDC coupling reagents. Then the acid coated QDs were secondarily conjugated with target peptide. (B) Living mitochondria images by a confocal microscope. Vero cells were cultured with 1 μM QD520-Mito8 (a–c) or QD520-START (control peptide, d–f), as indicated by the green color, for 12 h and then stained with MitoRed®, as indicated by red. Bars indicate 20 μm. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.) Reprinted with permission from [121]. Copyright (2007) Rights and Permission Department for Academic Publications Japan.

50 MDa in yeast [125] which have a basket like appearance. The pores allow free diffusion of molecules up to a size of 9 nm diameter or 40–45 kDa [124], all other molecules, including the RNA produced in the nucleus and the nuclear proteins sythesized on the ribosomes in the cytosol, have to be actively transported into or out of the nucleus. There exist several nuclear localization signals (NLS) for different kinds of proteins. The most simple classification is dividing them into classic (basic) NLS and non-classic NLS (for a database of all up to now known NLS, see [126]).

5.1. Mechanism of nuclear import

The classic NLS consist of four or more repeats of basic amino acids at the N-terminus of the transported protein. This recognition sequence forms a helical structure that fits into a cleft at the α-subunit of the importin-α/importin-β dimeric nuclear import receptor. After binding to the nuclear import receptor, a trimeric cargo complex is formed out of which the importin-β subunit subsequently interacts with the phenylalanine/glycine (FG) repeats of the FG-proteins on the cytosolic side of the nuclear pore complex (NPC) (Fig. 6: 1), moving the whole cargo complex through the nuclear pore [127]. Once arrived in the nucleoplasm, a Ran-GTP complex binds the cargo complex (Fig. 6: 2) inducing a conformational change in the importin subunit which triggers the release of the cargo protein (Fig. 6: 3). Thereafter, the importin Ran-GTP complex leaves the nucleus, following its concentration gradient, by interactions with the FG-proteins (Fig. 6: 4). Back in the cytoplasm, a GTPase accelerating protein (GAP), which is an essential part of the nuclear pore complex, catalyzes the exchange of GTP by GDP (Fig. 6: 5) triggering a conformational change in Ran and releasing its bound importin (Fig. 6: 6). Afterwards, a nuclear transport factor 2 (NTF2) dimer binds Ran-GDP (Fig. 6: 7 and 8) transporting it back into the nucleoplasm along the FG repeats of the nuclear pores (Fig. 6: 9). The Ran guanine nucleotide exchange factor (Ran-GEF) subsequently catalyzes the exchange of GDP by GTP (Fig. 6: 10) reconstituting the active Ran-GTP and releasing the NTF2 dimer (Fig. 6: 11) which diffuses back into the cytosol (Fig. 6: 12) [128,129].

5.2. Polymeric gene delivery systems

One of the key interests in nuclear targeting is obviously delivering DNA for gene therapy or transfection. In recent years several attempts have been made to develop polymer-based non-viral delivery methods [130,131]. Some of the utilized polymers are supposed to promote endosome disruption (see above). However, DNA in the cytoplasm is susceptible to degradation by DNases, as well as the mobility of bigger DNA fragments is restricted due to the crowded environment. Additionally, entry of the DNA into the nucleus still heavily relies on the breakdown of the nuclear envelope during mitosis. Polymer-based gene delivery is only successful if the gene is delivered to nucleus in a required quantity and transcribed to the corresponding mRNA, resulting in a high transfection efficiency [131,132]. As the delivery of the DNA to the nucleus is one of the major bottlenecks in non-viral gene delivery, the coupling of peptides containing an NLS to plasmid or linear DNA is one approach towards increased delivery of these cargo molecules to the nucleus. Strategies for coupling peptides to DNA have involved electrostatic interaction [133,134], PNA-mediated hybridization [135,136] or covalent coupling to plasmid DNA [137,138]. Various NLS, among other the SV40 large T-antigen derived peptide [133,135,139], the NLS from HIV Tat [140] or the M9 sequence from the heterogeneous nuclear ribonucleoprotein [134,141], have been tested. Despite the promising concept, there is a lack of consensus concerning the potency of NLS-peptides in nucleic acid delivery. Some studies have shown the successful nuclear delivery of oligonucleotides or linearized plasmids functionalized by an NLS [135,138], but the transfer of intact plasmids was less efficient [142]. Furthermore, the mechanism of plasmid delivery by synthetic polymers has not yet been elucidated. There is disaccord, if the polyplexes of DNA and polymer have to disassemble before the nuclear entry of the DNA or if they both enter the nucleus. Therefore, at this point, one cannot be sure, if attachment of the NLS to the DNA or to the polymer is the more promising approach. For example, if the NLS is attached to the DNA, but the DNA is complexed inside the polymeric carrier, no enhancement of transfection efficiency can be expected. On the other hand, if the NLS is on the polymer, but the polyplexes disassemble before the nuclear entry, it would be more useful to have the NLS on the DNA, in order to enhance its transport into the nucleus. Thus, before more and more studies are carried out to determin the effect of the attachment of an NLS to either DNA or polymer, it would be desirable to know more about the exact mechanism of DNA delivery by polymeric carriers.

Non-dividing cells might benefit more from nuclear targeting strategies compared to cell lines, because of their lack of nuclear breakdown during mitosis and therefore also the lack of passive nuclear entry by means of diffusion. Additionally, the amount of nuclear localization sequences per plasmid DNA molecule which is required for efficient nuclear import remains controversial. The addition of either an excess [135] or only one NLS [138] linked to DNA has proved to be successful.

The work of the Kopecek and co-workers on HPMA copolymers is not limited to the coupling of cell penetrating peptides [86] or targeting polymeric drug carriers to the mitochondria [108], in another article they described a way of directing polymers into the nucleus. They used HPMA-copolymers and monitored their cellular distribution after addition to the culture medium or after microinjection into the cytoplasm via confocal laser scanning microscopy (CLSM). They demonstrated that the polymers alone were able to promote endosomal escape. In follow up

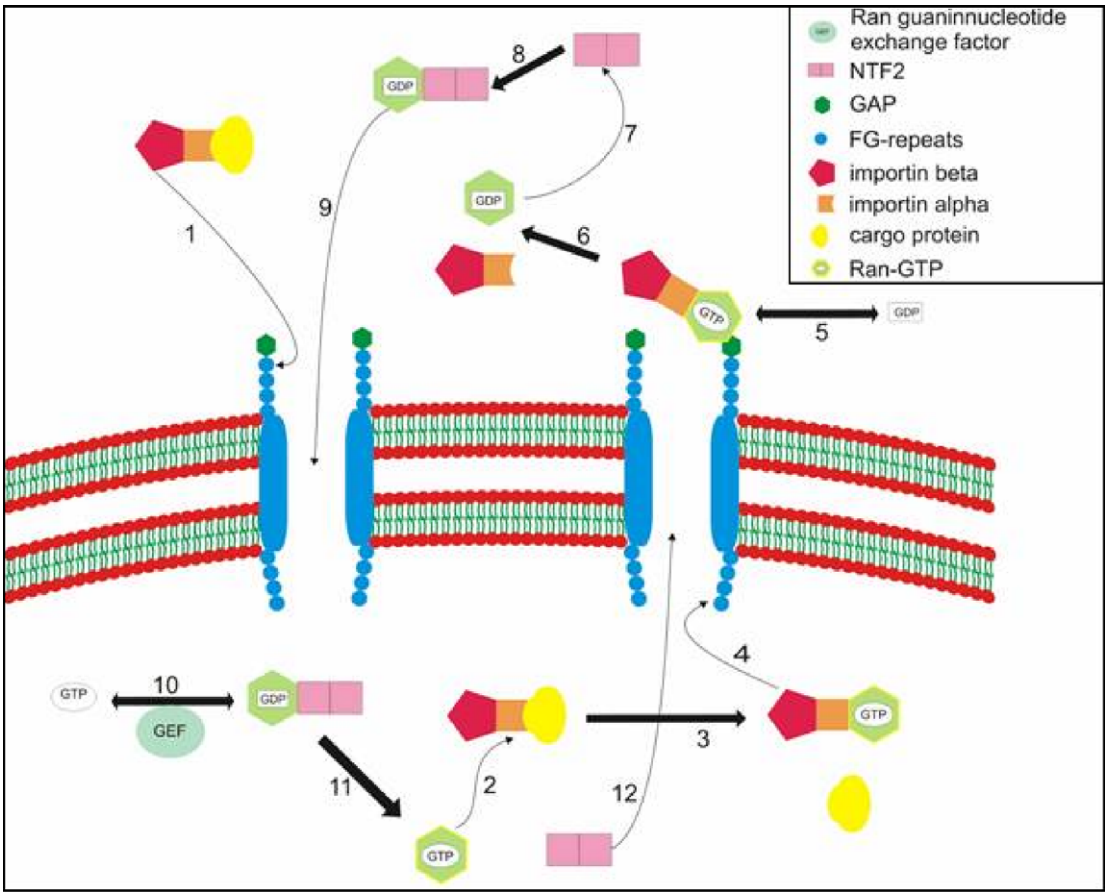

Fig. 6. Mechanism of the nuclear import. (1) Transport of cargo into nucleus through nuclear pore complex. (2 and 3) Exchange of cargo by Ran-GTP. (4) Transport of importin α/β-Ran-GTP out of nucleus. (5 and 6) Exchange of GTP by GDT and subsequent release of Ran-GDP. (7, 8 and 9) Backhaul of Ran-GDP into the nucleus with the help of NTF2. (10 and 11) Exchange of GDP by GTP and release of Ran-GTP. (12) Backhaul of NTF2 into the cytoplasm.

experiments, they linked fluorescently labeled antisense oligonucleotides via a degradable spacer to the polymers. Surprisingly, they could observe the oligonucleotides mainly in small vesicles, in the cytoplasm and in the nuclei, whereas the polymers were restricted almost exclusively to the vesicles. Jensen et al. concluded that to promote a detectable antiviral activity by the oligonucleotides, they had to be delivered in a more efficient way to the nucleus, and thus the minimum NLS, was conjugated to the polymer. The polymers targeted in this fashion were able to increase the amount and the speed of the delivered oligonucleotides to the nucleus. However, the majority of the complexes were still excluded from cytoplasmic or even nuclear entry [143].

5.3. Targeting of nanoparticles to the nucleus

In recent years, some very promising experiments have been carried out and have shown that it is possible to direct inorganic gold particles and QDs specifically to the nucleus. Gold nanoparticles and QDs were used as a kind of model nanoparticle, as they can be easily detected by TEM or fluorescence-based techniques, and moreover, they can be easily synthesized in various size distributions. With the use of such inorganic nanoparticles, it might be possible to derive rules for a maximum size and the amount of targeting sequences per particle necessary for a successful nuclear delivery.

In 2002, Panté and Kann have already carried out experiments with gold nanoparticles that had been coated with nucleoplasmin (NP) from *X. laevis* [144]. They applied the model nanoparticles in order to elucidate how large molecules were able to translocate through the nuclear pore complex (NPC) because up to that point it was thought that molecules no larger than 26 nm were able to cross the NPC. Therefore, they made three batches of differently sized gold nanoparticles, 22 ± 2 nm, 26 ± 3 nm and 36 ± 4 nm. They coated the particles with NP and used BSA as a control. As observed by TEM, BSA coated particles were not transported into the nucleus, whereas of the NP coated particles only the largest batch was excluded from the nucleus, indicating the too large size. However, they were not able to determine the exact size of the imported particles and thus they added importin α and importin β to the gold-NP particles before microinjection into *X. laevis* oocytes. The addition of NP, importin α

and importin β added an extra 13 nm to the particle diameter as verified by negative staining and examination under TEM. The surprising conclusion was that the particles which accumulated most in the nucleoplasm were from the middle sized batch, with a diameter of approximately 39 nm including all the protein coat.

Tkachenko and co-workers developed a method of delivering gold nanoparticles into the nucleus. They conjugated bovine serum albumin (BSA) to several viral and adenoviral peptides, e.g., to SV40 large T NLS, to adenoviral NLS, to adenoviral receptor-mediated endocytosis signal (RME) and to adenoviral fiber protein (contains both RME and NLS). The gold particles were then coated with the BSA-peptide conjugate via adsorptive forces. Only the adenoviral fiber protein showed the ability to translocate the gold particles across the cell membrane as well as translocating them into the nucleus, verified by video-enhanced color and differential interference contrast microscopy (VECDIC). However, the amount of particles found in the nucleus was greater when both the adenoviral NLS and the adenoviral RME peptides were conjugated to the gold particles, showing the need for an efficient uptake mediator in addition to the NLS [145].

A more promising approach was carried out by de la Fuente et al. They synthesized gold nanoparticles with a covalently attached tiopronin (2-(2-sulfanylpropanoylamino)acetic acid) protective coat (Au@tiopronin) which was further functionalized by covalent attachment of HIV Tat peptide (Au@Tat) (Fig. 7a). As visualized by TEM, few of the particles with only the tiopronin protective coat could be found inside the cell body of human fibroblasts and none was found in the nucleus (Fig. 7a), whereas particles with the Tat functionalization were not only able to translocate across the cell membrane, but also located to a great extent in the nucleus (Fig. 7a) [146].

Not only gold nanoparticles have been directed to a specific organelle, Hoshino et al. also managed to direct fluorescent quantum dots to the nucleus [121]. They prepared

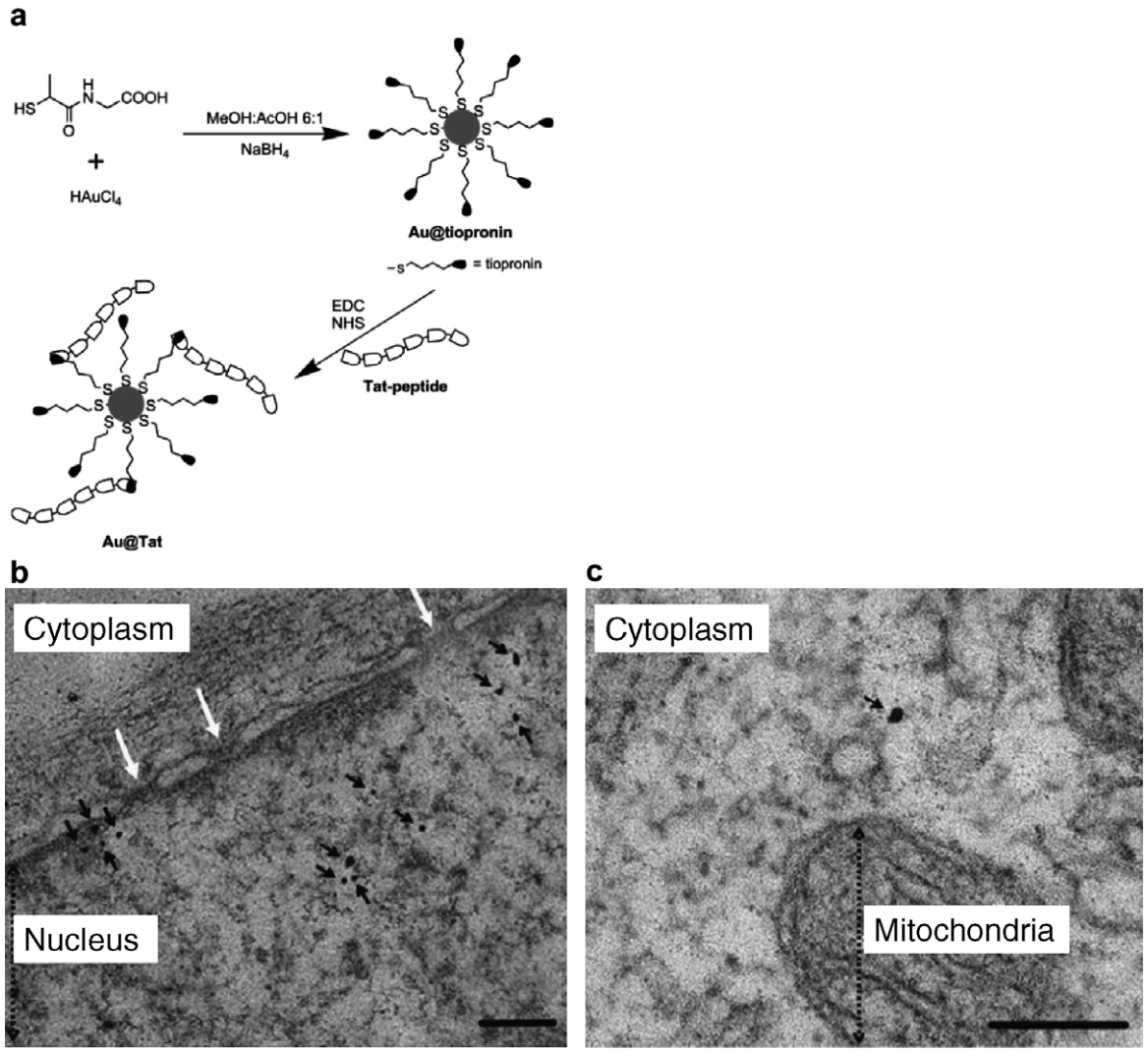

Fig. 7. (a) Preparation of Au-Tat-nanoparticles; transmission electron micrographs of human fibroblasts incubated with Au@Tat (b) and Au@tiopronin (c) nanoparticles. The black dots indicated with black arrows are nanoparticles, and white arrows show nuclear membrane pores (scale bars 50 nm). Reprinted with permission from [146]. Copyright (2007) American Chemical Society.

the QDs as described in the section about mitochondria, but instead of a mitochondrial targeting sequence they added a fluorescein labeled nuclear localization peptide, namely NH_2-RRRRRRRRRRKC-COOH, to the TOPO-capped QD. Fluorescence microscopy time lapse experiments showed that after as short as 1 min the QDs had accumulated in the nucleus and after 15 min even nucleolar accumulation could be verified by CLSM.

6. Conclusions

Significant effort in research has been dedicated to the development of polymeric and/or nanoparticular drug carriers in the past 10 years, but there are still obstacles that have to be overcome. Because of the high toxicity of drugs nowadays it is counterproductive if the drug is taken up by the wrong cell population. Likewise, since the uptake of nanoscale particles occurs mainly via endocytosis, in order to exert its activity, the drug must be released from the endo- and lysosomes. Additionally, it must be transported to the assigned organelle. Polymers and nanoparticles do not naturally possess these requirements so they have to be functionalized. They can be targeted to a specific cell population by the attachment of molecules that are mainly recognized by these cells. Furthermore, they can be delivered to only one type of cell organelle by attaching signaling peptides that can also be found in the cell's own proteins.

Recent studies have shown that QDs and proteins of different origin were successfully transported into mitochondria. This is a particularly interesting discovery because mitochondrial dysfunction is central to a range of human disorders. For the treatment of these diseases we need to develop strategies to selectively target bioactive compounds to mitochondria. Exploiting the membrane potential or the protein import machinery may be of particular use, but their feasibility has still to be proven.

Polymers and nanoparticles are interesting tools to deliver DNA and/or drugs successfully and efficiently to the nucleus. However, to broaden the applicability of these carriers, it will be necessary to construct particles which are not only endocytosed by the cells, but are also released from the endosomes and translocated to the nucleus. It can be hypothesised that these particles have an outer shell which enables efficient endocytosis and is degraded upon the change of pH in the endosomes revealing one of the above-described molecules to promote endosomal escape and, additionally, either an NLS or PTD to faciliate nuclear entry.

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