Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 26–32
BBRC
www.elsevier.com/locate/ybbrc

Targeting of nanoparticles to the clathrin-mediated endocytic pathway

Oshrat Harush-Frenkel [a,b], Nir Debotton [a], Simon Benita [a], Yoram Altschuler [b,*]

[a] Departments of Pharmaceutics, The School of Pharmacy, The Hebrew University of Jerusalem, POB 12065, Jerusalem 91120, Israel
[b] Department of Pharmacology, The School of Pharmacy, The Hebrew University of Jerusalem, POB 12065, Jerusalem 91120, Israel

Received 6 November 2006
Available online 6 December 2006

Abstract

Nanoparticles (NPs) are considered attractive carriers for gene therapy and drug delivery owing to their minor toxic effect and their ability to associate and internalize into mammalian cells. In this study, we compared the endocytosis into HeLa cells of NPs exposing either a negative or positive charge on their surface. The exposed charge significantly affected their ability to internalize as well as the cellular endocytosis mechanism utilized. Negatively charged NPs show an inferior rate of endocytosis and do not utilize the clathrin-mediated endocytosis pathway. On the other hand, positively charged NPs internalize rapidly via the clathrin-mediated pathway. When this pathway is blocked, NPs activate a compensatory endocytosis pathway that results in even higher accumulation of NPs. Overall, the addition of a positive charge to NPs may improve their potential as nanoparticulate carriers for drug delivery.

Keywords: Endocytosis; Clathrin; Macropinocytosis; Nanoparticles; HeLa cells

Nanoparticles (NPs) can be prepared from a variety of polymers using different techniques [1] for the purpose of drug delivery. The size and surface charge of the NPs are the most important factors in determining their *in vivo* metabolism, distribution and internalization [2]. When injected into the bloodstream, NPs are rapidly cleared by the reticulo-endothelial system (RES), resulting in a marked decrease in pharmacological action [1]. The rapid RES uptake of NPs can be significantly reduced by anchoring polyethylene glycol (PEG) to the NP surfaces. The PEG moieties, oriented towards the aqueous solution, confer steric stabilization and prevent NP opsonization and uptake by the RES. These stealth NPs exhibit a prolonged half-life in the blood circulation [3,4] which is a prerequisite for NP targeting to tumors that are often characterized by badly formed and leaky vasculature due to neovascularization [5]. The encapsulation of anti-tumor agents in NPs has been widely investigated since NPs are a suitable means of improving the therapeutic index of potent drugs while greatly reducing their side effects. Utilization of NPs for the delivery of anti-tumor agents such as doxorubicin and paclitaxel has exhibited some promising results [6,7].

While different aspects of NPs and new potential therapeutic applications have been reviewed in detail elsewhere [2,8,9], information about the mechanism of the endocytic machinery involved, as well as about NP intracellular trafficking, is sparse [2]. Endocytosis of NPs may occur through various endocytic processes which have not been fully identified [5–7]. Qaddoumi and colleagues have shown that in primary cultures of rabbit conjunctival epithelial cells (RCEC), endocytosis of poly (DL-lactide-co-glycolide) (PLGA) NPs occurs mainly via clathrin- and caveolin-1-independent pathways [10]; these authors suggested that NP uptake occurs by adsorptive endocytosis [11]. Internalization of cationic chitosan NPs appears to occur predominantly by adsorptive endocytosis and in part by a clathrin-mediated process [5,12–16]. Although current PEG-NPs are capable of improved accumulation in target tissues compared to unmodified NPs, there is clearly a therapeutic need to improve intracellular uptake of PEG-coated NPs. Understanding the molecular mechanisms that underlie

* Corresponding author. Fax: +972 2 6758927.
E-mail address: yoram11@md.huji.ac.il (Y. Altschuler).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.135

the interactions of NPs with the cell, their endocytic pathway and the mechanism of retention on the plasma membrane (PM) is crucial for the successful development of these vehicles. In the present investigation, we performed a comparative study on the mechanism of internalization of negatively versus positively charged PEGylated NPs. In contrast to all the cited studies that have made use of metabolic inhibitors to evaluate NP uptake, in this study, using an adenoviral delivery system, we expressed specific dominant negative polypeptides of the endocytic machinery in HeLa cells and gained mechanistic insight into the endocytic pathway of differently charged PEGylated NPs. We show that a positive surface charge targets the NPs to the clathrin-mediated endocytic pathway and results in greater internalization and accumulation of the NPs in cells.

Materials and methods

Materials. D,L-Lactide (Purasorb) was purchased from Purac (Gorinchem, Netherlands). Benzyl alcohol, DMSO, MTT, methoxy-PEG MW 5000, stannous 1-ethylhexanoate, and polysorbate 80 (Tween 80) were acquired from Sigma (St. Louis, MO). Acetone, ethyl acetate, methylene chloride and water were from J.T. Baker (Deventer, Netherlands). PEG-15-hydroxystearate (Solutol® HS 15) from BASF (Ludwigshafen, Germany). Coumarin-6 from Polysciences (Warrington, PA). Cell culture reagents from Biological Industries (Beit Haemek, Israel). T7-tag antibody obtained from Novagen (San Diego, CA). 12CA5 antibody from Covance (Indianapolis, IN). Secondary antibodies from Jackson Immunolabs (Bar Harbor, ME).

Cell culture and adenovirus-mediated expression. HeLa tet off cells were kindly provided by Hermann Bujard (Heidelberg, Germany) [17] and grown in DMEM supplemented with 10% (v/v) fetal calf serum and antibiotics.

Recombinant adenoviruses encoding the dominant negative mutant dynamin-I K44A (HA tag at the N terminus) and dominant negative clathrin hub (T7 tag at the N terminus) were as previously described [18,19]. Protein levels were regulated by the amount of virus and length of incubation time (16–24 h) to prevent high levels of expression and adenovirus-related toxic side effects.

Preparation of nanoparticles. The polymers: D,L-polylactide (PLA) and poly(ethylene glycol-co-lactide) (mPEG–PLA) were synthesized using the ring-opening polymerization method in the presence of stannous 1-ethylhexanoate as catalyst [20]. The crude polymers were dissolved in methylene chloride and precipitated twice in 4 l of a cold propyl ether/petroleum ether mixture (3:2, v/v). Prior to characterization and MW determination, the polymers were vacuum-dried. Then, the NPs were prepared by the solvent-displacement method [21]. The polymers PLA MW 100,000 and mPEG–PLA MW 100,000 (2:1) were dissolved in 50 ml acetone containing 0.2% (w/v) Tween 80, at a concentration of 0.6% (w/v), and acetone coumarin-6 solution at a concentration of 3×10^{-4}% (w/v) was added to the organic phase. The organic phase was added to 100 ml of an aqueous solution of 0.25% (w/v) Solutol® HS 15. The suspension was stirred at 900 rpm for 1 h and subsequently concentrated by evaporation to 10 ml. All formulations were diafiltrated with a 100-ml solution of 0.1% Tween 80 (Vivaspin 300,000 MWCO, Vivascience, Stonehouse, UK) and filtered through a 1.2-μm filter (FP 30/1.2 CA, Schleicher & Schuell, Dassel, Germany). A typical blank cationic NP formulation consisted (in % w/w) of $PLA_{100,000}$ 2, mPEG-$PLA_{100,000}$ 2, Solutol® HS 15 2.5, stearylamine 0.2, Tween 80 1 and doubled-distilled water to 100. The composition of the negatively charged NP formulation was identical to that of the positively charged one with the exception of the cationic lipid stearylamine.

Characterization of nanoparticles. Particle-size distribution and mean diameter measurements were carried out utilizing an ALV Noninvasive Back-Scattering High Performance Particle Sizer (ALV-NIBS HPPS, Langen, Germany).

Zeta potential measurements were carried out using the Malvern Zetasizer (Malvern Instruments, Ltd., Malvern, UK). Three batches of each NP formulation were prepared and all experiments were performed in triplicate.

Cytotoxic evaluation of nanoparticles. NP cytotoxicity was assayed using the MTT method [22]. During the MTT assay, four consecutive dilutions (100–2000) into HBSS were prepared from each formulation and five replicates were performed for each dilution point.

Evaluation of nanoparticle cellular uptake by confocal laser scanning microscopy. HeLa cells were seeded on cover slips for 1 day. Subsequently, cells were extensively washed. NPs were diluted in serum free media (1:1000, v/v) and incubated for 0–60 min with HeLa cells. Cells were then extensively washed with cold PBS, fixed, mounted and examined with an Olympus 1×70 confocal laser scanning microscope (Olympus Co. Ltd., Tokyo, Japan). Ten images of each time point containing 25–30 cells, were analyzed. All images were compiled using Adobe Photoshop and Image J software. Images are representative of the original data.

Fluorescence-activated cell sorter (FACS) analysis of nanoparticle cellular uptake. Cells were incubated with NPs for the indicated time points and then washed with cold PBS. Cells were harvested using trypsin and analyzed for cell-associated NPs by FACS (Cellquest software; Becton–Dickinson & Co., Franklin Lakes, NJ).

Elucidation of the mechanism of nanoparticle internalization by expression of dominant negative endocytic polypeptides. Cells were infected with the indicated adenoviruses and incubated for 16–24 h to allow expression of clathrin hub and dynamin I K44A. Subsequently, the cells were incubated with NPs for 60 min. NP uptake was examined by confocal microscopy. Levels of protein expression were assayed by Western blotting.

Results and discussion

Physicochemical and cytotoxicity properties of nanoparticles

The NPs elicited a positive surface charge following the incorporation of the cationic fatty amine stearylamine. Positively and negatively charged NPs exhibited zeta potential values of $+32.8 \pm 8.19$ and -26 ± 1 mV and average diameters of 89.8 ± 4 and 96.4 ± 6 nm, respectively (Table 1). There was no marked change in either surface charge or NP size over 60 days of storage at 4 °C, indicating that both NPs were stable (Table 1). The manufactured NPs were in the size range of caveolae or clathrin-coated endocytic vesicles, suggesting the possible involvement of these endocytic machineries in NP uptake [10,11,23,24].

Irrespective of their concentration, the NPs did not exhibit a significant cytotoxic effect on HeLa cells, as revealed by the MTT assay shown in Supplementary Fig. 1. A minor increase in cellular toxicity was triggered by the positively charged NP formulation, which may be explained by their increased cellular internalization (Figs. 2 and 3). Therefore, the difference in surface charge did not significantly affect the cytotoxicity of either NP formulations.

Positively charged nanoparticles reveal a higher rate of internalization

To evaluate the putative differences between positively and negatively charged NPs, we incubated fluorescently labeled NPs with HeLa cells. Both charged NPs

Table 1
Physical stability of positively and negatively charged NPs stored over 60 days at 4 °C under nitrogen atmosphere

Storage (day)	Cationic nanoparticles		Anionic nanoparticles	
	Droplet diameter (nm ± SD)	Zeta potential (mV ± SD)	Droplet diameter (nm ± SD)	Zeta potential (mV ± SD)
0	89.8 ± 4	32.8 ± 8.19	96.36 ± 6	−26 ± 1
15	ND	39.47 ± 10.31	ND	−21.2 ± 8.73
30	91.86 ± 8	45.46 ± 2	93.52 ± 5	−22.17 ± 0.4
45	ND	34.9 ± 7.08	ND	−29 ± 7.34
60	73.24 ± 5	37 ± 1.3	91.56 ± 3	−24.57 ± 0.67

(mean ± SD, $N = 3$); ND, not determined.

internalized into the cells in a time-dependent manner and accumulated in perinuclear punctate staining (Fig. 1). Furthermore, NPs were mostly endocytosed and did not localize on the PM. However, positively charged NPs were observed in cells within 5 min of incubation and accumulated to a much higher degree than their negatively charged counterparts (Fig. 1A vs. B). Internalization of positively charged compared to negatively charged NPs was approximately doubled, as observed from densitometric analyses of the cells (Fig. 1C). FACS analysis quantitation of the NPs accumulated in the cells supported our previous data of enhanced internalization of positively charged NPs. Positively charged NPs exhibited much higher uptake values than the negatively charged ones at all time points tested (Fig. 2). It should be noted that cell harvesting for FACS analyses involved cell trypsinization resulting in reduced fluorescence of the PM-associated NPs that had not been internalized into the cells. In addition, the

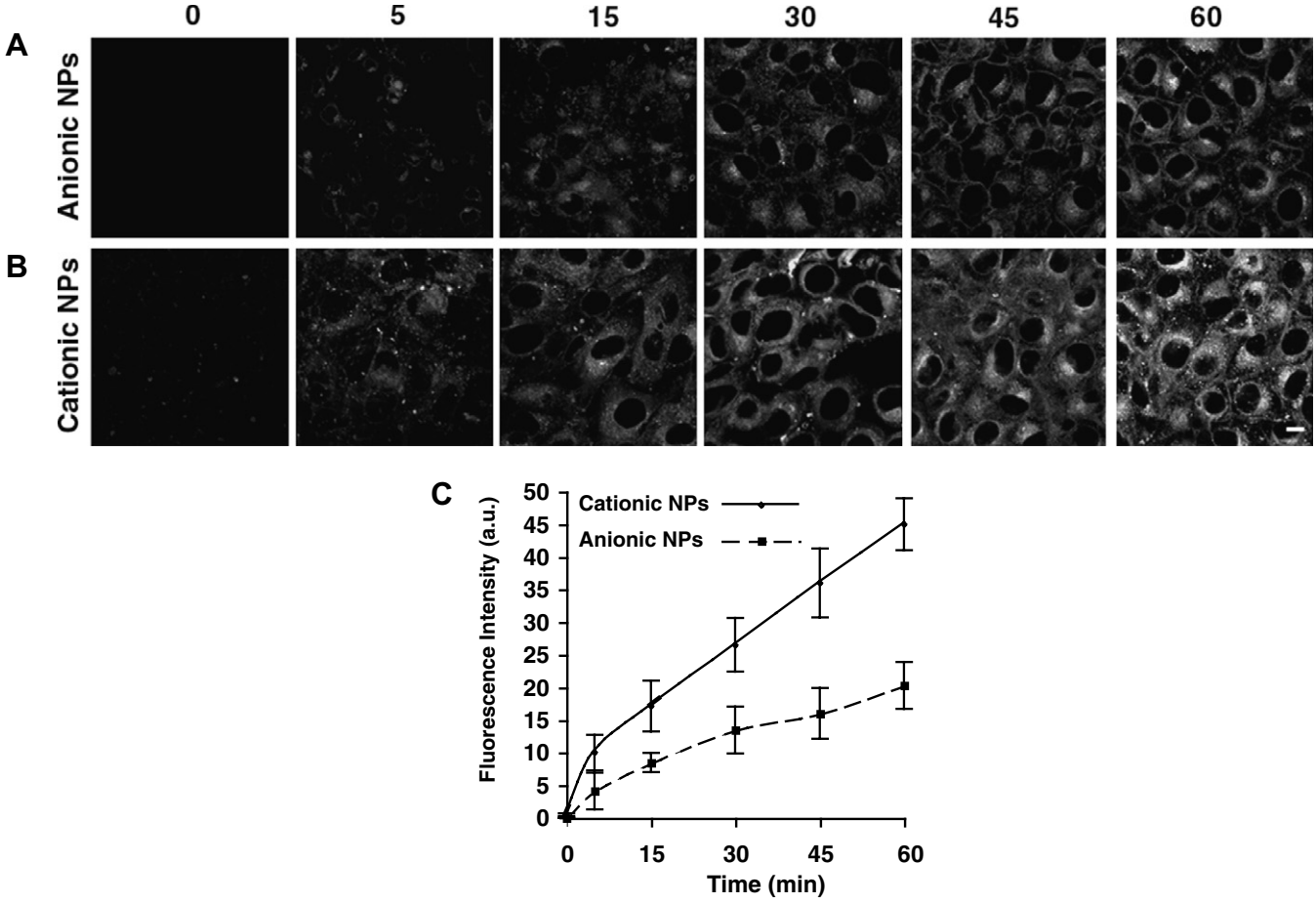

Fig. 1. Endocytosis kinetics of charged NPs into HeLa cells. HeLa cells were incubated for the indicated time periods with either negatively (A) or positively (B) charged NPs. Subsequently, cells were fixed and processed for fluorescence microscopy. (C) Fluorescent intensities of internalized NPs in (A,B) were quantified and used to calculate the amount of NPs endocytosed. Ten images of each time point, each containing approximately 25–30 cells, were analyzed by Image J software. NPs appear to accumulate in a perinuclear compartment and positively charged NPs exhibit twice more uptake than negatively charged NPs. Bar 5 μm.

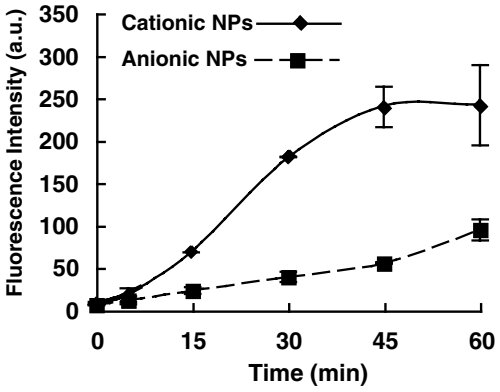

Fig. 2. Uptake of charged NPs by HeLa cells as revealed by FACs analysis. HeLa cells were incubated for the indicated time periods with either negatively (broken line ■) or positively (unbroken line ♦) charged NPs diluted 1:500 (v/v) in serum-free media. Subsequently, cells were trypsinized, extensively washed of unbound NPs and processed for FACs analysis. Control HeLa cells incubated with serum-free media were used to calculate time zero. Results show that positively charged NP uptake is two- to three-fold higher than that of negatively charged NPs. Uptake of the positively charged NPs reaches a plateau within 45–60 min, indicating saturated binding sites on HeLa cells. The experiment was repeated three times and a representative experiment is shown.

rate of positively charged NP uptake at 45 min reached a plateau, indicating possible NP recycling and or binding saturation which might result in an inability to internalize additional NPs.

Mechanism of nanoparticle internalization

The observed differences in NP formulation internalization rates may have been due to differential targeting of positively vs. negatively charged NPs to different endocytic internalization mechanisms. To clearly identify the mechanism utilized by charged NPs for their internalization into HeLa cells, we expressed well-established specific dominant negative polypeptides known to inhibit the clathrin and caveolin endocytic pathways.

We used the dominant negative mutant of dynamin I (K44A) which lacks the GTPase activity of dynamin. This mutation enables dynamin-dependent constriction of clathrin-coated pits and caveolar invagination but inhibits the scission event, leaving the invaginated pits on the PM. This mutant has been shown by us and others to specifically inhibit two main endocytosis pathways: clathrin- and caveolin-dependent pathways [18,25–27]. HeLa cells expressing the dynamin I dominant negative mutant exhibited a moderate increase in cellular staining of negatively charged NPs (Fig. 3). A closer look revealed some punctate staining of negatively charged NPs on the PM that could be localized to dynamin-constricted pits on the PM (Fig. 3A, arrows). On the other hand, expression of an N-terminal-deleted mutant of clathrin heavy chain, known to moderately inhibit the clathrin-mediated budding event, had no effect on the amount of negatively charged NPs taken up. These results imply that internalization of negatively charged NPs occurs mainly through pathways other than clathrin and caveolin.

In contrast, HeLa cells expressing the dynamin I K44A dominant negative mutant and incubated with positively charged NPs showed a significant increase in cellular staining (Fig. 3B). Though most of the NPs were observed in the intracellular perinuclear compartment, a significant amount decorated the PM in a punctate fashion (Fig. 3B and C, arrows). The dots observed at the PM were most likely NPs localized within clathrin or caveolar pits that had been banned from entering the cell. Furthermore, circular organelles whose surfaces were decorated with NPs were observed at the cell periphery, looking like macropinosomes (Fig. 3D and E, arrowheads). Expression of clathrin hub significantly inhibited endocytosis of positively charged NPs (Fig. 3B). The effects driven by the expression of both dynamin and clathrin dominant negative mutants imply that the main portal for positively charged NPs into the cells is clathrin- and possibly caveolae-mediated endocytosis.

The increased uptake of both NPs into cells expressing the dynamin I mutant may be a consequence of the extensive impact of this mutant on both clathrin- and caveolin-mediated endocytic pathways. To maintain cell, and particularly PM, homeostasis, a compensatory endocytic pathway is stimulated through which positively and negatively (although to a lesser extent) charged NPs internalize into HeLa cells [28–31]. Specifically, inhibition of both clathrin- and caveolae-mediated endocytosis results in stimulation of macropinocytosis. This phenomenon has been shown in HeLa cells expressing these dominant negative constructs in a study of adenovirus endocytosis [32]. Similar to positively charged NPs, the TAT protein transduction domain exposes a positive charge owing to arginine residues that are critical for its endocytosis. A study on the mechanism of TAT endocytosis revealed its entry by macropinocytosis [33]. We hypothesize that in HeLa cells, macropinocytosis operates at a low basal rate. The inhibition of clathrin- and caveolae-mediated endocytic pathways results in the stimulation of macropinocytosis, which is a preferred entry mechanism for positively charged NPs.

The endocytic machinery of PLA NPs is of great interest due to their application in gene therapy and drug delivery. It has previously been shown that NP uptake occurs through the endocytic system in a time- and dose-dependent manner. Moreover, in the presence of serum in the medium, a fraction of the internalized NPs recycle back to the PM and then exocytose [13,14]. Surface modification of NPs has been shown to affect their uptake into arterial tissue, suggesting that NPs may initially associate with membrane proteins and lipids, subsequently be delivered to the endocytic machinery, and finally, escape from the endosomal compartments into the cytoplasm [2].

In our experiments, we attempted to follow the initial events in NP endocytosis and therefore, we performed short incubation times of up to 60 min, during which

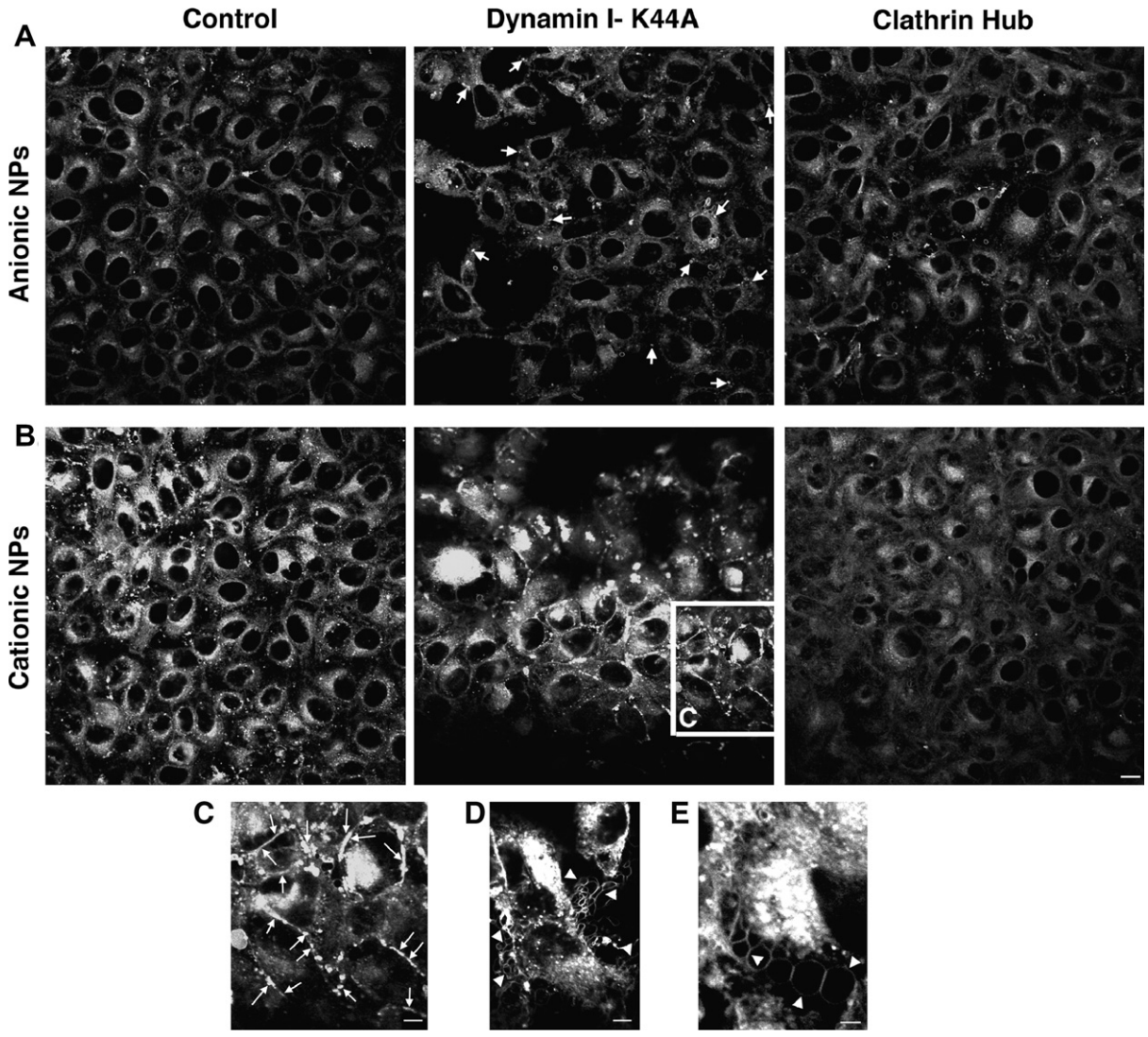

Fig. 3. Positively charged NPs are endocytosed through dynamin- and clathrin-dependent endocytic pathways. HeLa cells were either not infected (control) or infected with adenoviruses to express the dominant negative dynamin I K44A mutant for 18 h or the clathrin hub dominant negative mutant for 24 h. Subsequently, cells were incubated in the presence of negatively charged (A, anionic NPs) or positively charged (B, cationic NPs) NPs for 60 min. Consequently, cells were fixed and processed for fluorescence microscopy. (C) Enlargement of a section in (B) showing punctate PM accumulation of NPs. (D,E) Enlargement of cells expressing either dynamin I K44A (D) or clathrin hub mutant (E), revealing circular organelles, decorated at their surface with NPs, which resemble macropinosomes (arrowheads). Arrows point to accumulation of NPs at the PM. (A,B) Bar 5 μm, (C–E) bar 2 μm. Positively charged NPs rapidly move into the clathrin pit and thus reveal higher rate of internalization. Inhibition of endocytosis by the dynamin dominant negative mutant results in accumulation of punctate staining at the PM and increased internalization of NPs. Positively charged NPs reveal higher uptake under these conditions. This compensatory endocytosis is most likely through macropinocytosis.

NPs endocytosed and may also have recycled back to the extracellular medium. In the 60-min time frame, positively charged NPs exhibited enhanced intracellular accumulation. NPs accumulated mostly within a perinuclear compartment. The lack of elevated PM staining at the short incubation time points of either positive or negatively charged NPs indicates that at any time point, the cell exposes a limited number of NP binding sites and that following association with the membrane, NPs rapidly endocytose. This explanation is supported by the accumulation of NPs at the PM in cells expressing dynamin K44A, where endocytosis is severely inhibited. The time-dependent appearance of new binding sites, followed by uptake, indicates that this process takes place through endocytic machinery that operates in a constitutive manner. Clathrin-mediated endocytosis, which we showed here to be one of the main entry pathways for positively charged NPs, operates in HeLa cells in a constitutive manner and is the main port of entry of receptors in nonpolarized cells, as reported for example, in iron internalization through the transferrin–transferrin receptor complex [34].

Clathrin-mediated endocytosis of different NPs in various cellular models has also been shown by other researchers [2,13,16]. For example, uptake of PLGA NP by vascular smooth muscle cells was significantly reduced after inhibition of clathrin-mediated pathways, but not of

caveolae-dependent pathways, using metabolic inhibitors such as sucrose hypertonic treatment [14]. It is interesting to note that also fluorescein isothiocyanate-conjugated mesoporous silica NPs were internalized into mesenchymal stem cells and 3T3-L1 cells following short-term incubation via a clathrin-mediated endocytosis pathway [35]. Additionally, polyethylenimine (PEI)-coated NPs with superparamagnetic iron oxide resulted in enhanced uptake into HeLa, epithelial BEAS-2B and HEP-G2 cells, apparently through clathrin- and caveolae-mediated endocytic pathways [36]. The high internalization efficiency was most likely attributable to the cationic nature of the PEI NPs. This was also confirmed by two other studies investigating the internalization of cationic NPs. The positive charge was acquired by the incorporation of chitosan into NPs and uptake by epithelial Caco-2 cells was through clathrin-mediated endocytosis pathways [37]. In contrast, other authors have shown that internalization of chitosan NPs occurs predominantly by adsorptive endocytosis initiated by nonspecific interactions between NPs and cell membranes, and in part by a clathrin-mediated process [10,12,14,35–38]. It should be emphasized that knockdown of clathrin heavy chain by transfection with antisense oligonucleotides did not alter PLGA NP endocytosis from the apical PM of RCEC cells, leading the authors to conclude that NP cellular uptake is mostly clathrin-independent [10]. It is important to note that similar NPs have exhibited different mechanisms of entry into various cells and that this can be explained by the different mechanisms of endocytosis utilized by polarized compared to nonpolarized cells.

Our finding that positively charged NPs are targeted primarily to clathrin-mediated pathways as well as to macropinocytosis requires additional study to fully elucidate the decisive NP factor for the preferred endocytic pathway. These factors may differ between nonpolarized cells such as HeLa cells and the polarized epithelial cells lining the lungs and intestinal system, which are the main drug portals into the body. This clarification of the endocytic pathway involved in NP uptake is of crucial importance for advanced studies aimed at further enhancing total NP uptake into cells, manipulating their intracellular trafficking and minimizing possible toxic effects [12]. In the comparative study described here, we show that the exposed charge on the NP surface significantly affects the amount of NPs internalized and the endocytic pathway utilized for their internalization into nonpolarized HeLa cells. Whereas negatively charged NPs exhibit a poor rate of endocytosis, positively charged NPs internalize rapidly and accumulate to a much higher extent. These latter NPs utilize mainly the clathrin-mediated endocytosis pathway, although they may use caveolae-mediated endocytosis as well. Interestingly, when the clathrin-mediated endocytosis pathway is blocked by expression of either dynamin or clathrin dominant negative constructs, a fraction accumulates in puncta at the PM in what looks like clathrin-coated pits that have been banned from entering the cell. Additionally, positively charged NPs activate a compensatory endocytosis pathway that results in enhanced accumulation of NPs. Overall, the addition of a positive charge to NPs may improve their potential as nanoparticulate drug-delivery carriers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.135.

References

[1] M.L. Hans, and A.M. Lowman, Biodegradable nanoparticles for drug delivery and targeting, Curr. Opini. Solid State Mater. Sci. 6 (2002) 319-327.
[2] J. Panyam, V. Labhasetwar, Biodegradable nanoparticles for drug and gene delivery to cells and tissue, Adv. Drug Deliv. Rev. 55 (2003) 329–347.
[3] C. Passirani, G. Barratt, J.P. Devissaguet, D. Labarre, Long-circulating nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate), Pharm. Res. 15 (1998) 1046–1050.
[4] M.T. Peracchia, S. Harnisch, H. Pinto-Alphandary, A. Gulik, J.C. Dedieu, D. Desmaele, J. d'Angelo, R.H. Muller, P. Couvreur, Visualization of in vitro protein-rejecting properties of PEGylated stealth polycyanoacrylate nanoparticles, Biomaterials 20 (1999) 1269–1275.
[5] J. Davda, V. Labhasetwar, Characterization of nanoparticle uptake by endothelial cells, Int. J. Pharm. 233 (2002) 51–59.
[6] D. Missirlis, R. Kawamura, N. Tirelli, J.A. Hubbell, Doxorubicin encapsulation and diffusional release from stable, polymeric, hydrogel nanoparticles, Eur. J. Pharm. Sci. 29 (2) (2006) 120–129.
[7] J.S. Park, T.H. Han, K.Y. Lee, S.S. Han, J.J. Hwang, D.H. Moon, S.Y. Kim, Y.W. Cho, *N*-Acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: Endocytosis, exocytosis and drug release, J. Control Release 115 (1) (2006) 37–45.
[8] A.A. Moshfeghi, G.A. Peyman, Micro- and nanoparticulates, Adv. Drug Deliv. Rev. 57 (2005) 2047–2052.
[9] S.M. Moghimi, A.C. Hunter, J.C. Murray, Long-circulating and target-specific nanoparticles: theory to practice, Pharmacol. Rev. 53 (2001) 283–318.
[10] M.G. Qaddoumi, H.J. Gukasyan, J. Davda, V. Labhasetwar, K.J. Kim, V.H. Lee, Clathrin and caveolin-1 expression in primary pigmented rabbit conjunctival epithelial cells: role in PLGA nanoparticle endocytosis, Mol. Vis. 9 (2003) 559–568.
[11] M.G. Qaddoumi, H. Ueda, J. Yang, J. Davda, V. Labhasetwar, V.H. Lee, The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers, Pharm. Res. 21 (2004) 641–648.
[12] M. Huang, Z. Ma, E. Khor, L.Y. Lim, Uptake of FITC-chitosan nanoparticles by A549 cells, Pharm. Res. 19 (2002) 1488–1494.
[13] J. Panyam, V. Labhasetwar, Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells, Pharm. Res. 20 (2003) 212–220.
[14] J. Panyam, W.Z. Zhou, S. Prabha, S.K. Sahoo, V. Labhasetwar, Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery, FASEB. J. 16 (2002) 1217–1226.
[15] K.A. Foster, M. Yazdanian, K.L. Audus, Microparticulate uptake mechanisms of in-vitro cell culture models of the respiratory epithelium, J. Pharm. Pharmacol. 53 (2001) 57–66.
[16] H. Suh, B. Jeong, R. Rathi, S.W. Kim, Regulation of smooth muscle cell proliferation using paclitaxel-loaded poly(ethylene oxide)-poly(lactide/glycolide) nanospheres, J. Biomed. Mater. Res. 42 (1998) 331–338.

[17] M. Gossen, H. Bujard, Tight control of gene expression in mammalian cells by tetracycline-responsive promoters, Proc. Natl. Acad. Sci. USA 89 (1992) 5547–5551.
[18] Y. Altschuler, S.M. Barbas, L.J. Terlecky, K. Tang, S. Hardy, K.E. Mostov, S.L. Schmid, Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms, J. Cell Biol. 143 (1998) 1871–1881.
[19] Y. Altschuler, S.-H. Liu, L. Katz, K. Tang, S. Hardy, F. Brodsky, K. Mostov, ADP-ribosylation Factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells, J. Cell Biol. 147 (1999) 7–12.
[20] D. Bazile, C. Prud'homme, M.T. Bassoullet, M. Marlard, G. Spenlehauer, M. Veillard, Stealth Me. PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system, J. Pharm. Sci. 84 (1995) 493–498.
[21] H. Fessi, F. Puisieux, J.-P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4.
[22] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[23] Y. Dong, S.S. Feng, Poly(d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs, Biomaterials 26 (2005) 6068–6076.
[24] K.Y. Win, S.S. Feng, Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs, Biomaterials 26 (2005) 2713–2722.
[25] Q. Yao, J. Chen, H. Cao, J.D. Orth, J.M. McCaffery, R.V. Stan, M.A. McNiven, Caveolin-1 interacts directly with dynamin-2, J. Mol. Biol. 348 (2005) 491–501.
[26] P.U. Le, G. Guay, Y. Altschuler, I.R. Nabi, Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum, J. Biol. Chem. 277 (2002) 3371–3379.
[27] T. Baba, H. Damke, J.E. Hinshaw, K. Ikeda, S.L. Schmid, D.E. Warnock, Role of dynamin in clathrin-coated vesicle formation, Cold Spring Harbor Symp. Quant. Biol. 60 (1995) 235–242.
[28] M. Wienisch, J. Klingauf, Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical, Nat. Neurosci. 9 (2006) 1019–1027.
[29] C. Smith, E. Neher, Multiple forms of endocytosis in bovine adrenal chromaffin cells, J. Cell. Biol. 139 (1997) 885–894.
[30] C.R. Artalejo, A. Elhamdani, H.C. Palfrey, Calmodulin is the divalent cation receptor for rapid endocytosis, but not exocytosis, in adrenal chromaffin cells, Neuron 16 (1996) 195–205.
[31] C.R. Artalejo, J.R. Henley, M.A. McNiven, H.C. Palfrey, Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin, Proc. Natl. Acad. Sci. USA 92 (1995) 8328–8332.
[32] O. Meier, K. Boucke, S.V. Hammer, S. Keller, R.P. Stidwill, S. Hemmi, U.F. Greber, Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake, J. Cell Biol. 158 (2002) 1119–1131.
[33] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, Nat. Med. 10 (2004) 310–315.
[34] S.D. Conner, S.L. Schmid, Regulated portals of entry into the cell, Nature 422 (2003) 37–44.
[35] D.M. Huang, Y. Hung, B.S. Ko, S.C. Hsu, W.H. Chen, C.L. Chien, C.P. Tsai, C.T. Kuo, J.C. Kang, C.S. Yang, C.Y. Mou, Y.C. Chen, Highly efficient cellular labeling of mesoporous nanoparticles in human mesenchymal stem cells: implication for stem cell tracking, FASEB. J. 19 (2005) 2014–2016.
[36] S. Huth, J. Lausier, S.W. Gersting, C. Rudolph, C. Plank, U. Welsch, J. Rosenecker, Insights into the mechanism of magnetofection using PEI-based magnetofectins for gene transfer, J. Genet. Med. 6 (2004) 923–936.
[37] Z. Ma, L.Y. Lim, Uptake of chitosan and associated insulin in Caco-2 cell monolayers: a comparison between chitosan molecules and chitosan nanoparticles, Pharm. Res. 20 (2003) 1812–1819.
[38] C.C. Berry, S. Rudershausen, J. Teller, A.S. Curtis, The influence of elastin-coated 520-nm- and 20-nm-diameter nanoparticles on human fibroblasts in vitro, IEEE Trans. Nanobioscience. 1 (2002) 105–109.