Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 298 (2005) 310–314
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

A methodology to study intracellular distribution of nanoparticles in brain endothelial cells☆

Elizabeth Garcia-Garcia[a], Karine Andrieux[a,*], Sophie Gil[b], Hyun Ryoung Kim[a], Trung Le Doan[a], Didier Desmaële[c], Jean d'Angelo[c], Frédéric Taran[d], Dominique Georgin[d], Patrick Couvreur[a]

[a] *Laboratory of Biopharmacy and Pharmaceutical Technology, UMR CNRS 8612, Faculty of Pharmacy, University of Paris-XI, 92296 Châtenay-Malabry, France*
[b] *Clinical Pharmacy UPRES 2706, Faculty of Pharmacy, University of Paris-XI, 92296 Châtenay-Malabry, France*
[c] *Department of Organic Chemistry, Faculty of Pharmacy, University of Paris-XI, 92296 Châtenay-Malabry, France*
[d] *Department of Radiolabelled Molecules, CEA/Saclay, 91191 Gif sur Yvette, France*

Received 7 October 2004; received in revised form 16 March 2005; accepted 21 March 2005
Available online 31 May 2005

Abstract

Cell internalisation and intracellular distribution of PEG-coated polyhexadecylcyanoacrylate (PEG-PHDCA) nanoparticles in rat brain endothelial cells (RBEC) have been investigated. A cell fractionation method has been developed based on the selective permeabilisation of RBEC plasma membrane by digitonin. By interacting with membrane cholesterol, digitonin creates pores allowing the release of soluble and diffusible species outside the cell. The selectivity of plasma membrane permeabilisation was controlled by using compartment markers such as lactate dehydrogenase (LDH) for cytoplasm and cathepsin B for lysosomes. An optimal digitonin concentration of 0.003% (w/v) has been identified to induce a pattern of membrane permeabilisation corresponding to the extraction of 72% LDH and less than 15% of Cathepsin B. Membrane permeabilisation at this digitonin concentration allows one to distinguish between the cell cytoplasm and its endo/lysosomal fraction.

This methodology was applied to investigate the intracellular distribution of the nanoparticles after their incubation with the RBEC. The results showed that PEG-PHDCA nanoparticles were able to be internalised to a higher extent than PHDCA nanoparticles (after 20 min incubation). Additionally, these nanoparticles displayed different patterns of intracellular capture, depending on their specific surface composition: PEG-PHDCA nanoparticles were 48% in the plasma membrane, 24% in the cytoplasm, 20% in vesicular compartments and 8% associated with the fraction of the nucleus, the cytoskeleton and caveolae suggesting that PEG-PHDCA nanoparticle uptake by RBEC is specific and presumably due to endocytosis. Confocal microscopy studies confirmed the cellular uptake of PEG-PHDCA nanoparticles.

Keywords: Pegylated-nanoparticles; Intracellular distribution; Brain endothelial cells; Digitonin

☆ Presented at the 5th European Workshop on particulate systems London 1–3 July 2004.

* Corresponding author. Tel.: +33 1 46 83 59 09; fax: +33 1 46 61 93 34.
E-mail address: karine.andrieux@cep.u-psud.fr (K. Andrieux).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.03.030

Targeting drugs to the brain is a challenge, because this tissue is very efficiently protected by the blood-brain barrier (BBB). This barrier, mainly formed by endothelial cells sealed together by continuous tight junctions, limits the molecular exchange to transcellular transport. However, in vivo studies have recently evidenced that PEG-coated polyhexadecylcyanoacrylate (PEG-PHDCA) nanoparticles were able to penetrate into the brain in a significantly higher proportion than other colloids (Calvo et al., 2001; Brigger et al., 2002). These results have lead to the suggestion that nanoparticles could be internalised by rat brain endothelial cells (RBEC). To investigate this point we have developed a method to study the intracellular distribution of nanoparticles after their internalisation into RBEC.

Collagen S was purchased from Boehringer (Mannheim, Germany); culture medium (EBM-2, fetal bovine serum (FBS)), was from Cambrex (Verviers, Belgium); Digitonin with high purity was obtained from VWR (Fontenay sous bois, France); Nile red was from Interchim (Montluçon, France); PBS, Protease (Type XIV), Nα-benzoyl-DL-arginine-β-naphtylamine (BANA) and all other chemicals were purchased from Sigma (St. Louis, MO, USA). Radiolabelled polymers, poly(hexadecylcyanoacrylate) (^{14}C-PHDCA, 5.8 μCi/mg) and PEG-coated polyhexadecylcyanoacrylate (^{14}C-PEG-PHDCA, 1.5 μCi/mg), were prepared at the CEA (Commissariat à l'Energie Atomique, Saclay, France) according to previously described protocols (Brigger et al., 2002).

Radiolabelled nanoparticles were prepared as described previously (Brigger et al., 2002) using ^{14}C-PHDCA or ^{14}C-PEG-PHDCA polymers. Fluorescent nanoparticles were prepared in the same way as described before except that nile red (25 μg) was added to the organic phase.

Rat brain endothelial cells (RBEC) were obtained as described previously (Garcia-Garcia et al., 2004). At first passage, RBEC were seeded onto collagen-coated Petri dishes (35 mm diameter) in complete growth medium and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

PEG-PHDCA or PHDCA nanoparticles were suspended in the transport medium (TM = EBM-2 and 5% FBS) at a concentration of 20 μg/ml. Nanoparticle suspensions were prewarmed to 37 °C and added to the RBEC monolayers for a 20 min period at 37 °C.

The cell fractionation method used was adapted from a previously described method for vascular smooth muscle cells (Eboue et al., 2003). After 20 min of incubation with nanoparticles, RBEC monolayers were washed three times with 1 ml of cold PBS. After aspirating the PBS, 1 ml of protease solution in protective buffer (PB: 0.25 M sucrose, 20 mM HEPES, 2 mM potassium phosphate, 0.24 mM EGTA and 10 mM $MgCl_2$) was added to the Petri dishes and incubated at 4 °C for 15 min. 100 μl of FBS was added to stop protease activity and cells were flushed with 1 ml pipette five times to ensure that all the cells were in suspension. This suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged (300 g, 5 min, 4 °C, Jouan MR 22i Centrifuge, France). The supernatant was collected and corresponds to the protease fraction and was stored at 4 °C until analysis.

After protease treatment and washing (1 ml of cold PB), the cell pellet was resuspended in 0.5 ml of PB supplemented with low concentrations of digitonin ranging from 0.001 to 0.006% (w/v) for selective permeabilisation of the plasma membrane. The cells were then incubated for 15 min at 4 °C and centrifuged at 500 × *g* for 5 min. The supernatant corresponding to the digitonin fraction was recovered and stored at 4 °C until analysis. The remaining pellet was washed with 1 ml of PB and resuspended in 0.5 ml of PB supplemented with 1% Triton X100 and incubated for 10 min at 4 °C to permeabilise intracellular vesicles. The suspension was then centrifuged for 10 min at 4 °C and the resulting supernatant was referred as the Triton soluble-fraction and was stored at 4 °C until analysis. The pellet was washed and referred as the Triton insoluble-fraction. For each fraction, aliquots were taken and radioactivity was measured (BECKMAN model LS 6000 TA). The radioactivity in digitonin and Triton soluble-fractions were attributed to nanoparticles in the cytoplasmic and vesicular compartments, respectively.

The contribution of cytoplasm compared with the intracellular compartments was evaluated by the determination of lactate dehydrogenase (LDH) and cathepsin B as markers of the cytoplasm and lysosomes, respectively. LDH (Lactate dehydrogenase) is a stable cytoplasmic enzyme. Its activity was determined by an enzymatic test (ROCHE kit, following the manufacturer recommendations (Roche Molecular Biochemicals, Boehringer, Mannheim Germany)). The activity of Cathepsin B, a lysosomal

enzyme, was determined by adding BANA (90 mM) as substrate. BANA was hydrolysed by Cathepsin, liberating 2-naphtylamine which is fluorescent (λ_{exc} = 335 nm and λ_{em} = 410 nm). The 100% of LDH and Cathepsin were determined by the addition of the quantities measured in supernatants of digitonin fraction and Triton soluble-fraction. The quantities of LDH and Capthesine in protease fraction and Triton insoluble-fraction have been considered as negligible.

For confocal microscopy, RBEC were seeded in 10 mm diameter plastic cover-slip at a density of 10,000 cells/ml. The cover-slips were incubated for 1 week to allow cell confluence. The cells were then incubated with a suspension of nile-red nanoparticles (20 μg/ml) in TM for 20 min at 37 °C. At the end of the incubation period, the cell monolayers were rinsed three times with PBS to remove the excess of nanoparticles and fixed with paraformaldehyde (3%) in PBS for 15 min. The samples were analysed with a LSM 510 Zeiss confocal inverted microscope equipped with a Zeiss 63X/1.4 NA oil immersion objective lens. Fluorescence images were acquired using argon (wavelength 488 nm) and helium neon (wavelength 543 nm) lasers.

The key steps of the methodology developed here for studying the intracellular distribution of nanoparticles in RBEC are summarized in Fig. 1. Protease treatment permitted the detachment of RBEC from the culture

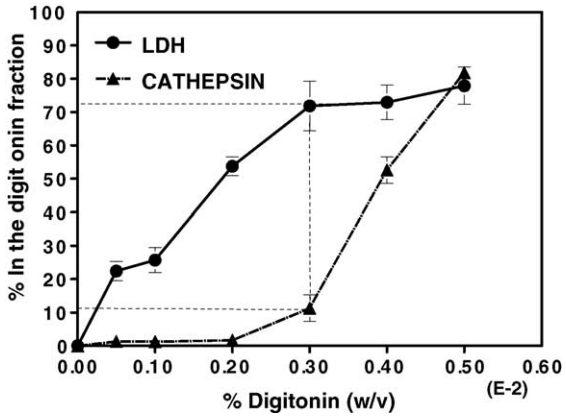

Fig. 2. Effect of digitonin concentration on the release of the markers into the digitonin fraction. Data are means (n = 3) ± S.D.

dish and removal of nanoparticles which just adhered at the cell surface (step1, Fig. 1). Then the fractionation method was mainly based on the selective permeabilisation of the plasma membrane by digitonin (step 2, Fig. 1). The effect of the digitonin concentration on the release of the markers into the digitonin fraction is shown in Fig. 2. Increasing the concentration of digitonin increased the LDH release, but above 0.004% w/v, high concentrations of Cathepsin (>50%) were also found in this fraction. It is worth noting that 72 ± 7.5% of the total cellular LDH leaked at a digi-

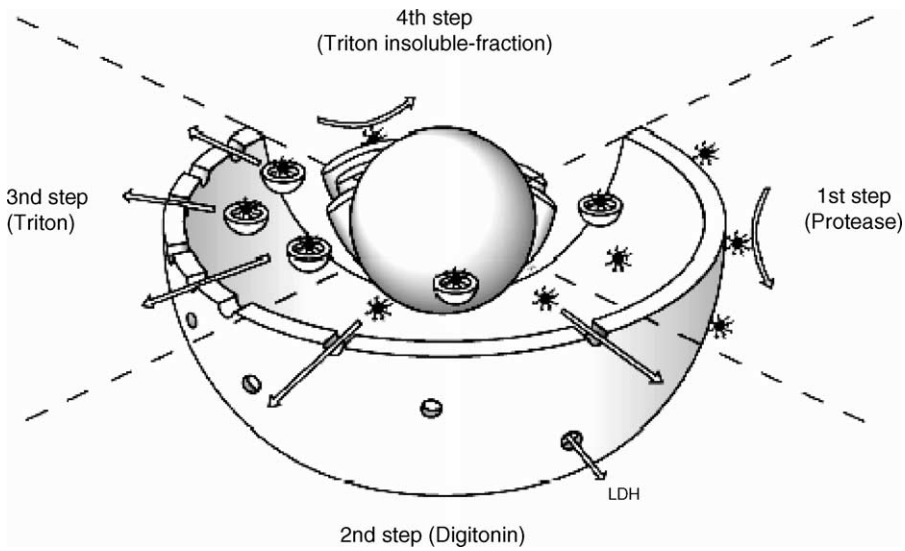

Fig. 1. Schematic representation of the key steps for the cell fractionation method.

tonin concentration of 0.003% w/v. On the contrary at this concentration, the cathepsin B release was not significantly affected ($11 \pm 4\%$). Therefore, this was the optimal concentration of digitonin allowing the selective extraction of the soluble cellular cytosolic content, without a significant disruption of the intracellular vesicles (Fig. 2). The lysis of the intracellular vesicles was obtained by adding 1% of Triton X100 which allowed the recovery of 80% of the capthepsin (and $13 \pm 3.4\%$ LDH) from RBEC (step 3, Fig. 1). The remaining Triton insoluble-fraction presumably contained structures such as the nuclear material, cytoskeleton elements and caveolae (step 4, Fig. 1). Thus, a digitonin concentration of 0.003% w/v and a Triton concentration of 1% were applied to futher explore intracellular distribution of nanoparticles.

First, PEG-PHDCA nanoparticles were more efficiently internalised than PHDCA nanoparticles after 20 min of incubation (Fig. 3). It has to be noted that PHDCA and PEG-PHDCA nanoparticles were similar in size (166 and 171 nm, respectively) and zeta potential (-20 ± -1 mV). In these conditions, both nanoparticle types display very different intracellular distributions: 80% of PHDCA nanoparticles have remained in the plasma membrane and only 10% were found both in cytoplasm and vesicular compartments. On the contrary, only 48% of PEG-PHDCA nanoparticles were associated with the plasma membrane, 24% being in cytoplasm, 20% in vesicular compartments and 8% in Triton insoluble-fraction (Fig. 3). This result demonstrates that PEG-PHDCA nanoparticle uptake by RBEC is specific and presumably due to endocytosis. Moreover, the presence of nanoparticles in the Triton insoluble-fraction suggests that PEG-PHDCA nanoparticles could be associated with structures such as the nucleus, the cytoskeleton or caveolae. These data clearly demonstrate that the nature of the nanoparticle surface (presence of the hydrophilic and flexible PEG) is a key parameter for intracellular uptake and delivery.

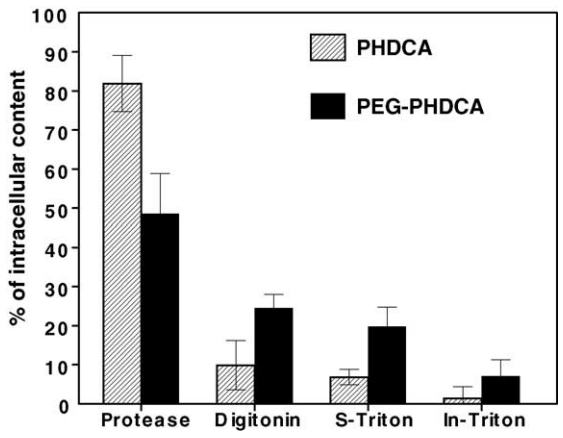

Fig. 3. Intracellular distribution of PHDCA and PEG-PHDCA nanoparticles. Data are expressed as percentage of radioactivity in the protease, digitonin, Triton soluble- (S-Triton) and Triton insoluble- (In-Triton) fractions. Values means ($n = 3$) ± S.D.

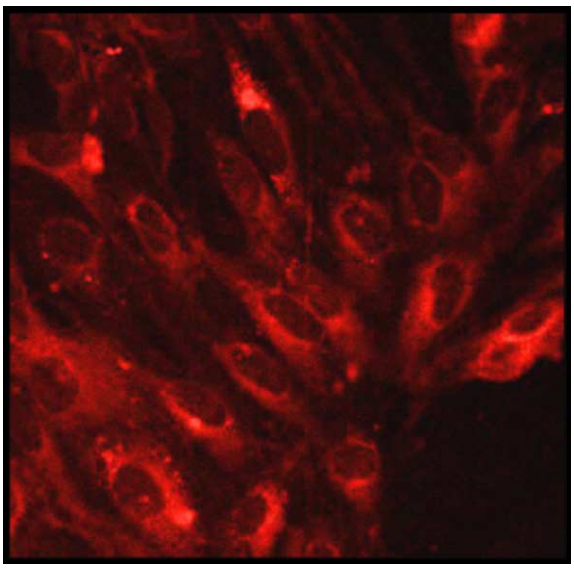

Fig. 4. Confocal microscopy image of PEG-PHDCA nanoparticles labelled with nile red after incubation with RBEC (20 min).

Confocal microscopy studies confirmed the cellular uptake of PEG-PHDCA nanoparticles by RBEC. After 20 min incubation, cell uptake of PEG-PHDCA nanoparticles was shown as highly fluorescent punctuated patterns, preferentially located in the cytoplasm or around nucleus (Fig. 4).

In conclusion, the proposed fractionation method has permitted study of the intracellular distribution of nanoparticles in RBEC, and in particular, their significant localisation in cytoplasm, vesicular and the Triton insoluble-fraction. Further investigations on the mechanisms of nanoparticles translocation in RBEC are in progress in order to investigate the influence of nanoparticle surface properties on colloïd translocation through the BBB.

References

Brigger, I., Morizet, J., Aubert, G., Chacun, H., Terrier-Lacombe, M.J., Couvreur, P., Vassal, G., 2002. Poly(ethylene glycol)-coated hexadecylcyanoacrylate nanospheres display a combined effect for brain tumor targeting. J. Pharmacol. Exp. Ther. 303, 928–936.

Calvo, P., Gouritin, B., Chacun, H., Desmaele, D., D'Angelo, J., Noel, J.P., Georgin, D., Fattal, E., Andreux, J.P., Couvreur, P., 2001. Long-circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery. Pharm. Res. 18, 1157–1166.

Eboue, D., Auger, R., Angiari, C., Le Doan, T., Tenu, J.P., 2003. Use of a simple fractionation method to evaluate binding, internalization and intracellular distribution of oligonucleotides in vascular smooth muscle cells. Arch. Physiol. Biochem. 111, 1–8.

Garcia-Garcia, E., Gil, S., Andrieux, K., Devocelle, A., Espinasse, C., Appel, M., Chacun, H., Desmaele, D., D'Angelo, J., Andreux, J.P., Roux, F., Couvreur, P., 2004. A novel primary rat in vitro blood–brain barrier to study transport of colloidal carriers. Pharmaceutical Sciences World Congress (PSWC2004). Poster 1E-IV-085.