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

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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

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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