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Cellular uptake and toxicity of microparticles in a perspective of polymyxin B oral administration

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ABSTRACT

Alginate/chitosan microparticles with a mean size less than 1 μm, designed in a previous work for the targeting of polymyxin B to M-cells and, then, to the lymphatic system, were assayed for transport ability by enterocytes. Caco-2 cell monolayer model, combined with confocal microscopy, showed that microparticles were endocytosed by the cells through an energy-dependent process, being the process saturable at 6 h incubation. Furthermore, microparticles maintained the biological activity of the antibiotic and decreased the antibiotic cytotoxicity against Vero cell cultures. Therefore, simultaneous pathways *via* both M-cells and enterocytes could be proposed for such a microparticulate carrier.

1. Introduction

Oral administration of micro- or nanoparticles resistant to the gastrointestinal environment and transported through intestinal epithelium cells could represent an effective tool to improve oral bioavailability of drugs characterised by instability in the gastrointestinal tract and poor intestinal permeability. The uptake of inert particles has been shown to occur transcellularly, through enterocytes or M-cells, phagocytic cells on the Peyer's patch (PP) epithelium, and, to a lesser extent, paracellularly (Florence, 1997; McClean et al., 1998; Hussain et al., 2001; des Rieux et al., 2006; Silva et al., 2006; Moyes et al., 2007). Non-fat nutrients and drugs enter the blood capillaries through enterocytes and/or lymphatic vessels through M-cells. Multiple mechanisms of particulate transport for the same material can exist, in function of several factors (particle size, surface charge and hydrophobicity, presence of specific ligands) even if discordant results about effect of size and charge are reported (Desai et al., 1996, 1997; Limpanussorn et al., 1998; McClean et al., 1998; Florence and Hussain, 2001; Hussain et al., 2001). Micro- or nanoparticles taken up selectively by M-cells could represent an useful approach to achieve drug targeting into the lymph providing advantages in terms of avoidance of first pass metabolism, delivery of drugs which treat intestinal lymph pathologies or treat and prevent cancer spreading into the lymph system as well as of peptides and immunostimulating molecules for oral vaccination. Conversely, micro- and nanoparticles taken up selectively or partly by enterocytes would appear more appropriate to improve oral bioavailability of poorly absorbed drugs.

Alginate/chitosan microparticles to target the lymphatic system provided an improved safety when administering polymyxin B (PMB) orally to rats, as stated in a previous work (Coppi et al., 2008). PMB is a cationic peptidic antibiotic, labile in gastrointestinal environment and negligibly absorbed by the intestinal epithelium, used extensively for parenteral and topical treatment of gram-negative infections although the therapy is associated with severe toxicity, mainly nephrotoxicity and neurotoxicity (Hermsen et al., 2003; Falagas and Kasiakou, 2006). Microparticles showed evidence of uptake by both PP and non-PP tissue in a rat animal model supporting the thesis of a simultaneous pathway involving M-cells and villous epithelium (Coppi et al., 2006). By considering that villi with M-cells (lymphocyte filled villi) were found in the small intestine of rats and mice (Moghaddami et al., 1998; Hussain et al., 2001) and, more, a paracellular transport across the narrow gap junctions between enterocytes was described for microparticles owing to the presence of absorption enhancers such as chitosan (Silva et al., 2006). Although this thesis has been ruled out recently (des Rieux et al., 2006), the involvement of enterocytes in this process is not clear. Therefore, the objective of the present work was to evaluate the role of enterocytes in the transport of alginate/chitosan microparticles loaded with PMB through villous epithelium. For this reason,

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Caco-2 cell monolayer model, mimicking enterocytes in terms of cell morphology and biochemistry, was selected and evaluated by cytofluorimetry and confocal microscopy. Furthermore, PMB alone, loaded microparticles and unloaded microparticles were comparatively evaluated for the cytotoxicity on Vero cells cultures.

2. Materials and methods

2.1. Materials

Polymyxin B sulfate (PMB) and chitosan (CS) (low $M_w \cong 70{,}000$) were purchased from Fluka Chemie (Buchs, Switzerland). Sodium alginate (Na-A) ($M_w \cong 147{,}000$, containing 62% mannuronic acid and 38% guluronic acid) was donated by ISP Alginates (Girvan, UK). Fluorescein isothiocyanate (FITC) was purchased from Sigma–Aldrich (Milan, Italy). Culture reagents for intestinal Caco-2 cell lines were purchased from Euroclone-Celbio (Milan, Italy). All the other chemicals were of analytical grade.

2.2. Microparticles preparation and in vitro characterisation

The microparticles were developed and characterised by using the techniques described previously in more detail (Coppi et al., 2004, 2006). Shortly, fluorescent calcium alginate/chitosan microparticles were prepared by spray-drying a 0.5% water solution of NaA/PMB/FITC (3:1:0.01) and by crosslinking the microparticles with calcium chloride and chitosan under mechanical stirring. The same technique was performed by using NaA/FITC (3:0.01) to obtain unloaded microparticles. Microparticle fluorescence was observed by epifluorescence videomicroscopy using a FITC filter (N-400NF, Optika Microscopes, M.A.D. Apparecchiature Scientifiche, Bergamo, Italy). The obtained microparticles were characterised for morphology, by a scanning electron microscope (SEM, XL-40, Philips, Eindhoven, The Netherlands), and for size, by computerized image analysis (IMG-WIEW, CIGS, University of Modena and Reggio Emilia). Microparticle PMB content was determined by placing the samples in 6% sodium citrate water solution and assaying the PMB concentrations in supernatant, after centrifugation, spectrophotometrically ($\lambda = 750$ nm) (Lambda 3B, Perkin-Elmer, Norwalk, CT, USA) by the Lowry assay (Lowry et al., 1951). The PMB *in vitro* release was performed in saline solution at pH 3.0 for 2 h and, subsequently, in phosphate buffer solution at pH 7.4 for 4 h, determining spectrophotometrically ($\lambda = 750$ nm) the concentration of released drug at fixed time intervals. Microparticle microbiological activity was determined by agar-well diffusion method, using *Escherichia coli* ATCC 10536 as standard strain, on samples, dispersed in water or disaggregated in 6% sodium citrate water solution, and compared with standard PMB solutions. The reported data were averaged on three determinations.

2.3. Microparticle uptake by Caco-2 cells

Caco-2 cells, used at passage 43, were cultured at 37 °C in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% non-essential amino acids. Cells were seeded 48 h before the incubation with the microparticle suspension. The microparticles suspended in water (10 mg/5 ml) and diluted (1:1) in DMEM were applied to the cell monolayer and then incubated at either 4 °C or 37 °C for 3 h, 6 h or overnight (24 h). Cells were washed twice with Phosphate Buffer Saline (PBS) and then collected by trypsinization for the cytometry analysis. Flow-cytometry evaluation of microparticle intracellular uptake versus untreated cells, as control, was performed by a Coulter Epics XL flow cytometer equipped with 488 nm argon laser (Beckman Coulter, Fullerton, CA, USA). FITC fluorescent cells were expressed as a percentage of the total cell population. Alternatively Caco-2 cells were fixed using the p-formaldehyde 3% and, after washing by PBS, examined by epifluorescence confocal laser scanning microscopy (mod. DM IRE2, Leica Microsystems Heidelberg GmbH, Germany) at 1.11 μm steps.

2.4. Cytotoxicity test

Vero cells (kidney epithelial cells from African green monkey) were cultured in Eagle's minimum essential medium (EMEM) (Lonza, Milan, Italy) enriched with 10% foetal bovine serum (FBS) (Lonza) and 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml) and 1% L-glutamine in sterile conditions and maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. Once cells were grown to confluence (around 70%), they were transferred together with the culture medium into disposable sterile plates with 24 wells. Living cells were counted by the Trypan blue exclusion test to assure an initial inoculum of 35×10^6 cells. The plates were then incubated for 24 h under the same conditions and subsequently the programmed cytotoxicity test was performed.

After a further incubation period (24 h) the cytotoxicity test was performed by direct addition of PMB alone in water solution, loaded and unloaded microparticles in water suspension. Cultures were treated with PMB increasing concentrations (0.1 μM, 0.5 μM, 1.0 μM and 10.0 μM) as well as unloaded microparticles in the same amount as the loaded ones. Following a further incubation period of 48 h, the MTT cell proliferation assay was performed according to the method described by Mosmann (1983). The MTT test was performed in triplicate and the results were expressed as percentage of cell growth with respect to the control wells (only the cells in the medium).

2.5. Statistical analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA). Significance was indicated by $P < 0.05$.

3. Results and discussion

3.1. Microparticle characteristics

As observed previously (Coppi et al., 2004, 2006), the obtained microparticles showed a nearly spherical shape with a wrinkled surface, a size ranging from 0.1 μm to 2.5 μm (mean size 0.75 ± 0.55 μm) (Fig. 1), being about 75% of population less than 1.00 μm and a marked fluorescence (Fig. 2). The antibiotic loaded into the microparticles (loading level of 10.55 ± 0.68% (w/w) and encapsulation efficiency of about 42%) was found associated with the alginate chain by an electrostatic interaction between PMB cationic charge and the polyanionic alginate as demonstrated by rheological analysis of PMB/alginate water solutions after PMB/alginate complex removal (Coppi et al., 2004). PMB release was negligible in simulated gastric fluid at pH 3.0, due to a restricted alginate neutralization that could determine a partial PMB displacement from the complex whereas the polymer backbone structure would remain intact, and it was gradual in simulated intestinal fluid at pH 7.4, due to the disruption of Ca^{2+} linkage by sequestrating ions present in the release medium (Coppi et al., 2006). Furthermore, microparticles were still fluorescent following the release assay in gastrointestinal fluids.

The biological activity of PMB loaded in microparticles was preserved, as demonstrated by the microbiological assay (Fig. 3). The inhibition zone provided by the microparticles disaggregated in sodium citrate solution, extrapolated on the best fit-line, was found in agreement with the theoretical amount of the antibiotic without significant difference ($P > 0.05$). On the contrary, microparticles

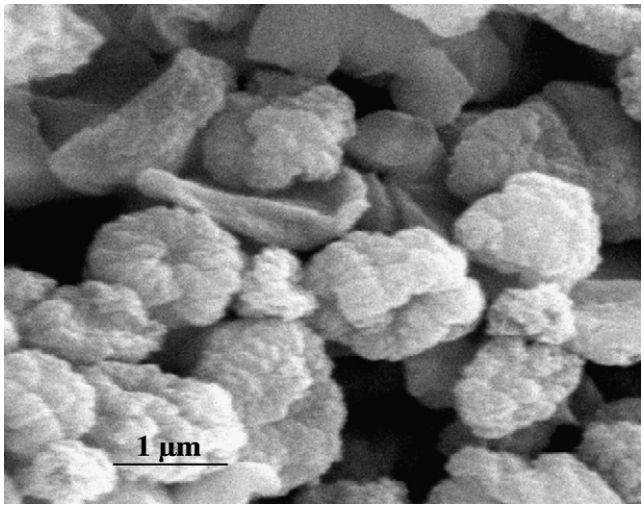

Fig. 1. SEM image of microparticles.

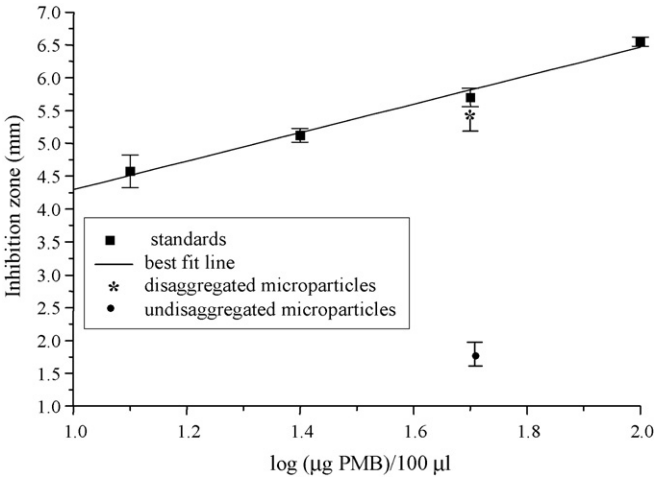

Fig. 3. Microbiological assay of disaggregated and non-disaggregated microparticles in comparison with PMB standards.

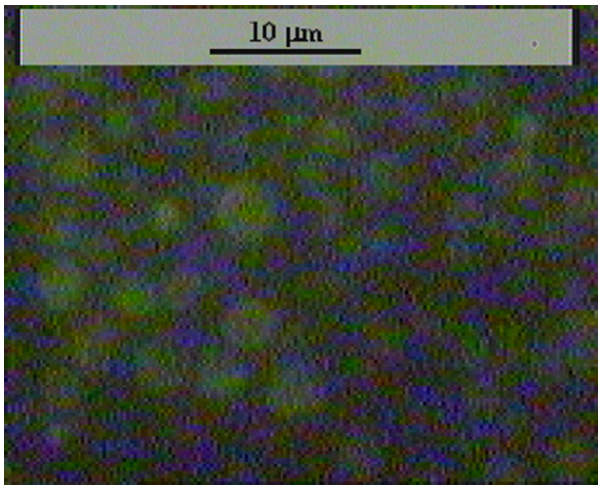

Fig. 2. Epifluorescence microscopy image of microparticles.

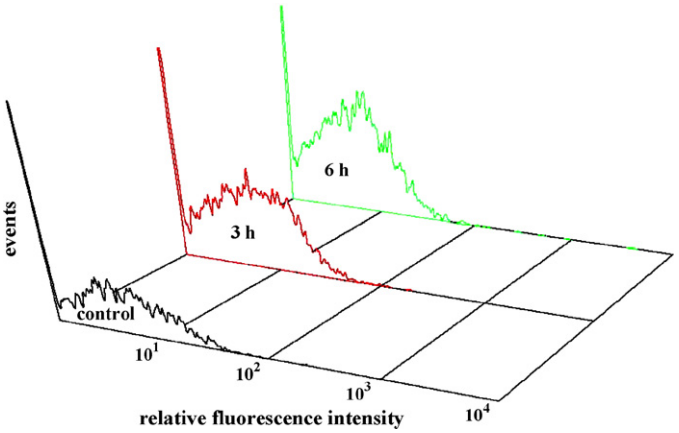

Fig. 4. Caco-2 cell cytofluorimetric analysis at 4 °C of microparticles after 3 h and 6 h incubation in comparison with the control.

dispersed in water provided a sparing strain inhibition in comparison with the standard solutions ($P < 0.05$) because PMB was not extracted by water owing to the resistance of both the polymer network and the antibiotic interaction with the alginate chain (Coppi et al., 2004, 2006).

3.2. Microparticle uptake by Caco-2 cells

Caco-2 cell monolayer model was used to study the microparticle uptake because it is an established *in vitro* tool to evaluate the intestinal permeability by enterocytes, useful to explain the mechanism of absorption without using laboratory animals (Hilgers et al., 1990; McClean et al., 1998; Ma and Lim, 2003; Sambuy et al., 2005; Silva et al., 2006). Particles can be transcytosed by normal enterocytes and the transcytosis begins with an endocytic process that takes place at the cell apical membrane. Then, particles are transported across the cells and released at the basolateral pole (des Rieux et al., 2007). Cellular uptake efficiency was expressed as a percentage of fluorescent cells on the total cell population (Table 1) and as flow-cytometry histograms (Figs. 4 and 5), in function of the temperature (4 °C and 37 °C) and the incubation time (3 h and 6 h at 4 °C; 3 h, 6 h and 24 h at 37 °C). Temperature-dependence experiments at 4 °C and 37 °C were carried out to investigate the processes involved in the interaction of the particles with Caco-2 cells. In fact, reducing the temperature from 37 °C to 4 °C, energy-dependent endocytic processes and, consequently, binding and uptake by the cells, will be reduced. Cellular binding data obtained at 4 °C following 24 h incubation were not reported owing to the relevant cell suffering. At 37 °C, the increased cellular binding efficiency values (Table 1), of about 7 fold at 3 h and of about 30 fold at 6 h, has arisen from the shift in the fluorescence intensity of the treated cells, compared with the control, revealed that microparticles were associated to Caco-2 cells following 3 h incubation, this process being

Table 1
Cytofluorimetric analysis: cellular binding efficiency in function of the incubation temperature and time (percent values ± S.D.).

Samples	4 °C		37 °C		
	3 h	6 h	3 h	6 h	24 h
Control	1.22 ± 0.46	3.39 ± 1.18	2.58 ± 0.81	2.40 ± 0.80	1.31 ± 0.35
Microparticles	4.25 ± 0.39	6.75 ± 1.65	19.24 ± 7.24	72.96 ± 15.52	88.96 ± 8.57

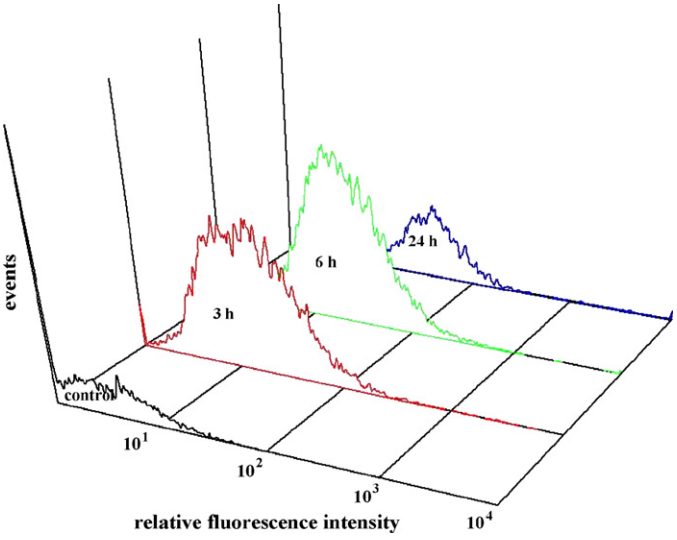

Fig. 5. Caco-2 cell cytofluorimetric analysis at 37 °C of microparticles after 3 h, 6 h and 24 h incubation in comparison with the control.

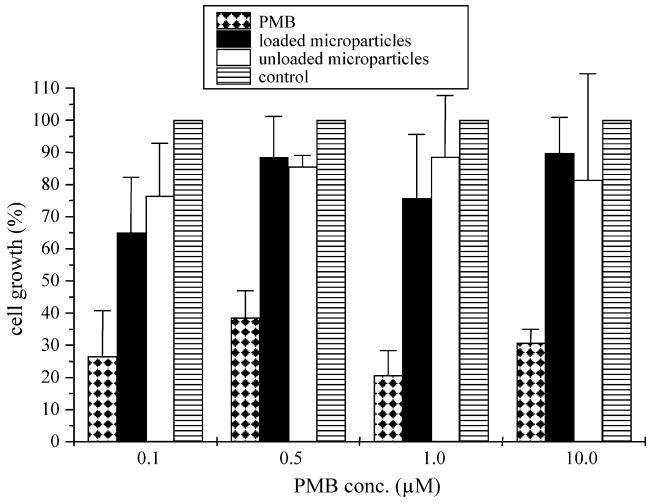

Fig. 7. Percentage of cell growth inhibition evaluated by the MTT assay on Vero cell cultures treated with increasing concentrations of PMB alone, PMB loaded and unloaded microparticles.

saturable at 6 h approximately since no significative increase was observed at 24 h incubation ($P < 0.05$ between the values at 3 h and 6 h incubation; $P > 0.05$ between the values at 6 h and 24 h incubation). Decreasing the temperature from 37 °C to 4 °C resulted in a significant ($P < 0.05$) reduction of about 5 and 11 fold at 3 h and 6 h incubation, respectively, in the average cell fluorescence, i.e. in the number of microparticles associated with Caco-2 cells, suggesting that microparticle binding by these cells would be an energy-dependent process.

The exact location of the microparticles, i.e. whether they were intra- or extracellular, however, cannot be discerned by this method. Therefore, confocal microscopy was carried out on cells incubated at 37 °C for 6 h in order to discriminate between material bound to the cells extracellularly and that internalized by the cells. As shown in Fig. 6, fluorescent spots inside the cells at different depths from the apical surface, attributable to the microparticles, were noticed. Conversely, only a slight natural fluorescence could be detected from the control cells. This finding could reasonably indicate the microparticle property to be internalized by Caco-2 cells.

3.3. Cytotoxicity test

The viability of Vero cells after incubation with PMB alone, loaded and unloaded microparticles was expressed in percent by comparison with a non-treated control having 100% viability (Fig. 7). No significant differences were found between cell viability provided by the same sample at different PMB concentration or unloaded microparticle amount ($P > 0.05$). The employment of microparticles as drug carrier results in a significant ($P < 0.05$) reduction of PMB cytotoxicity (from 20–40% to 65–90% of cell viability) and cell viability in the cultures treated with microparticles loaded with PBM is very similar to the unloaded microparticles. This finding indicates that microparticles could be considered safe and able to protect tissue cells against drug toxicity.

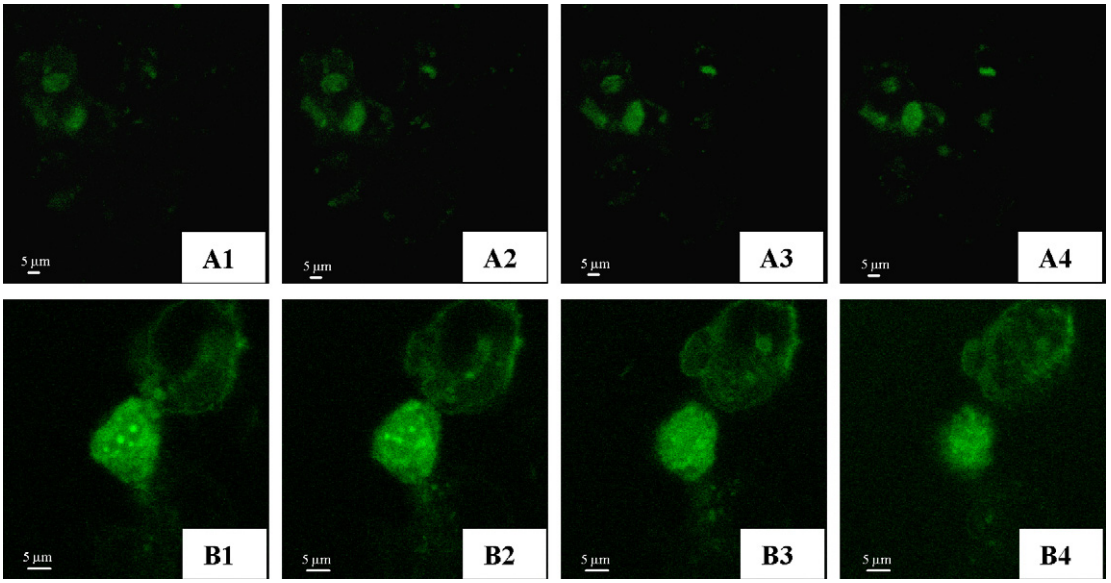

Fig. 6. Confocal scanning laser microscopy images of Caco-2 cells at four different focal planes: (A1–A4) control cells; (B1–B4) cells incubated for 6 h with microparticles which appeared inside the cells as fluorescent spot with a diameter less than 1 μm. The images were collected from the fluorescein isothiocyanate channel.

4. Conclusions

The uptake study by Caco-2 cell monolayers showed that alginate/chitosan microparticles could be taken up by enterocytes, this process combining with that by Peyer's patch tissue, observed previously in an *in vivo* experiment (Coppi et al., 2008). Although the low endocytic activity of enterocytes (des Rieux et al., 2006), by considering their high number, a transcellular pathway across enterocytes could contribute significantly to the overall translocation process of particulate matter by the intestinal epithelium. Therefore, this microparticulate system could represent an effective tool to promote the oral bioavailability of drugs negligibly absorbed by the intestinal epithelium, such as polymyxin B.

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