International Journal of Pharmaceutics 394 (2010) 35–42

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# *In vitro* identification of targeting ligands of human M cells by phage display

V. Fievez[a,b], L. Plapied[a,b], C. Plaideau[a], D. Legendre[a], A. des Rieux[a,b], V. Pourcelle[c], H. Freichels[d], C. Jérôme[d], J. Marchand[c], V. Préat[b], Y.J. Schneider[a,*]

[a] Université Catholique de Louvain, Institut des Sciences de la Vie, Biochimie Cellulaire, Nutritionnelle & Toxicologique, Croix du Sud, 5, 1348 Louvain-La-Neuve, Belgium
[b] Université Catholique de Louvain, Louvain Drug Research Institute, Unité de Pharmacie Galénique, Avenue E. Mounier, 73-20, 1200 Brussels, Belgium
[c] Université Catholique de Louvain, Institut de la Matière Condensée et des Nanosciences, Laboratoire de Chimie Médicinale, Place L. Pasteur, 1, 1348 Louvain-La-Neuve, Belgium
[d] Université de Liège, Centre d'Etude et de Recherche sur les Macromolécules, Sart-Tilman, 4000 Liège, Belgium

ARTICLE INFO

*Article history:*
Received 9 January 2010
Received in revised form 13 April 2010
Accepted 17 April 2010
Available online 24 April 2010

*Keywords:*
Oral delivery
Targeting
M cells
Caco-2 cells
Phage display
Nanoparticles

ABSTRACT

To improve transport of vaccine-loaded nanoparticles, the phage display technology was used to identify novel lead peptides targeting human M cells. Using an *in vitro* model of the human follicle-associated epithelium (FAE) which contains both Caco-2 and M cells, a T7 phage display library was screened for its ability either to bind the apical cell surface of or to undergo transcytosis across Caco-2 cells or FAE. The selection for transcytosis across both enterocytes and FAE identified three different peptide sequences (CTGKSC, PAVLG and LRVG) with high frequency. CTGKSC and LRVG sequences enhanced phage transport across M-like cells. When polymeric nanoparticles were grafted with the sequences CTGKSC and LRVG, their transport by FAE was significantly enhanced. These peptides could therefore be used to enhance the transport of vaccine-loaded nanoparticles across the intestinal mucosal barrier.


## 1. Introduction

Unlike the parenteral vaccines, the mucosal vaccines have the ability to confer a protective local immunity to mucosal barriers, in addition to a systemic immune response. In the recent past, for practical and safety reasons (Lavelle and O'Hagan, 2006; Silin et al., 2007), there has been a shift from traditional mucosal vaccines that are mainly composed of live-attenuated or killed pathogens, towards a new generation of vaccines composed of so-called sub-unit immunogens. However, most of these compounds remain poorly bioavailable upon oral administration (Lavelle and O'Hagan, 2006; Silin et al., 2007). One new approach developed to overcome these problems has involved their association with delivery systems, such as polymeric nanoparticles (des Rieux et al., 2006). These carriers could facilitate an effective immunization by: (i) protecting antigens against degradation and inactivation in the harsh gastro-intestinal environment; (ii) promoting their transmucosal transport; and (iii) acting as mucosal immunomodulatoradjuvants (Slutter et al., 2009). However, despite numerous reports in the literature to show the success of biodegradable antigen carriers to induce an immune response after their oral administration, only few phase 1 human trials for oral vaccines have been realized in the last 10 years (Katz et al., 2003; Lavelle and O'Hagan, 2006; Tacket et al., 1994) and up to now, none of these systems have achieved a commercial status. It is therefore being increasingly questioned whether encapsulated antigens can be taken up in sufficient quantities in human to induce adequate immune responses. An improvement in the efficacy of particles uptake would thus be required before oral vaccination with sub-unit antigens becomes a feasible option.

Diverse strategies have been developed to improve the availability of these polymeric carriers after their oral administration. Some aimed at targeting all intestinal cells (mainly enterocytes), whereas others focused on specialized antigen sampling cells, *i.e.* M cells (des Rieux et al., 2006). M cells are specialized epithelial cells that provide an immuno-sampling function by transporting undegraded particulate matter and presenting it to underlying lymphocytes in the follicle-associated epithelium (FAE) of Peyer's patches (Brayden et al., 2005; Ermak and Giannasca, 1998; Gebert et al., 1996; Kuolee and Chen, 2008). Although these cells are present at a very low density in the gut, they are now considered as a major protagonist to improve the availability of orally administered vaccines. Many

*Abbreviations:* BSA, bovine serum albumin; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; FAE, follicle-associated epithelium; HBSS, Hank's balanced salt solution buffer; NP, nanoparticles; Papp, apparent permeability coefficient; PBS, phosphate-buffered saline; TEER, transepithelial electrical resistance; PCL-PEG, polycaprolactone–polyethylene glycol; PLGA, poly(DL-lactic-co-glycolic) acid; PLGA-PEG, poly(DL-lactic-co-glycolic) acid-polyethylene glycol.

* Corresponding author. Tel.: +32 10 47 27 91; fax: +32 10 47 48 95.
*E-mail address:* yjs@uclouvain.be (Y.J. Schneider).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.04.023

efforts are focused to improve the transcytosis of antigen-loaded nanoparticles by these cells, trying especially to compensate their low number by an efficient targeting (des Rieux et al., 2006). However, the research associated with M cells has long been hampered by the lack of appropriate *in vivo* and *in vitro* experimental models. Recently, the development of a purely human *in vitro* FAE model has provided an important research tool to elucidate the molecular architecture of the apical surface of human M cells (Gullberg et al., 2000, 2005, 2006). It consists in coculturing Caco-2 monolayers with a B lymphocyte cell line, Raji cells. 15–30% of the Caco-2 cells are converted to M-like cells (des Rieux et al., 2007). Nevertheless, currently little is known about surface proteins that could act as specific targets and, up to now, only β1 integrins and recently glycoprotein 2, have been shown to be overexpressed at the apical pole of human FAE (Brayden et al., 2005; Garinot et al., 2007; Gullberg et al., 2006; Hase et al., 2009).

Phage libraries have been used to select peptides that bind immobilized proteins, carbohydrates and peptides displayed by cultured cells (Ivanenkov et al., 1999b; Ivanenkov and Menon, 2000). Phage display peptide libraries are commonly used to obtain defined peptide sequences interacting with a particular molecule. In this system, peptides in as many as $10^9$ permutations are expressed on the phage surface by fusion to one of the phage surface proteins and the desired peptides are selected on the basis of their affinity for the target molecule. The phage display system based on the phage T7 (T7Select; Novagen), is currently the most widely used lytic phage display system and has been employed in numerous applications (Danner and Belasco, 2001; Herrmann et al., 2007; Sharma et al., 2008; Woiwode et al., 2003). This 60 nm phage has a spherical morphology, which is relatively similar to the reference nanoparticles previously used to characterize the *in vitro* model (des Rieux et al., 2007). It displays 415 copies of peptides on the surface of phages. These peptides expressed by the phages could bind to unknown ligands expressed at the surface of enterocytes and/or M cells and could be transported across these cell monolayers. This library expresses seven amino acids with a conformational flexibility that is constrained by cyclisation. This is likely to allow some protection against proteolysis (Smith and Petrenko, 1997).

The aim of this study was to use the *in vitro* model of the human FAE (des Rieux et al., 2007), previously developed in our laboratory to identify novel peptide ligands specific of human M cells using the phage display technology. In order to find new markers of human M cells, we firstly used a biopanning protocol in which phages were selected at 4 °C for their binding affinity for the apical surface of mono- and cocultures (enterocytes and M-like cells respectively). Phages from the library have also been tested for binding to internalizing receptors, such as integrins, and to undergo an efficient uptake by targeted cells (Ivanenkov and Menon, 2000). Therefore, novel peptide ligands specific to human M and/or enterocytes cells were also searched for their ability to improve the transport of phages by cells. The identified sequences that increased phage transport were then grafted on polymeric nanoparticles to check if their transport across Caco-2 monolayers and/or FAE was enhanced.

## 2. Materials and methods

### 2.1. Phage libraries

#### 2.1.1. Phage library

The library of T7 phages, obtained from Dr. Ruoslahti (Burnham Institute for Medical Research, University of California, Santa Barbara, CA), was prepared as previously described (Essler and Ruoslahti, 2002). It consisted in using NNK-oligonucleotides encoding a random library of cyclic peptides of the general structure CX7C (C, cystein; X, any amino acid). This vector displays peptides in all 415 copies of the phage capsid protein as a C-terminal fusion. Libraries with this structure have yielded numerous high-affinity cell-binding peptides (Danner and Belasco, 2001; Herrmann et al., 2007; Sharma et al., 2008; Woiwode et al., 2003). The library had a diversity of $\sim 3 \times 10^8$ plaque-forming units (pfu).

#### 2.1.2. Phage production and purification

Phages from libraries or from individual clones were amplified in *Escherichia coli BL21* (Amersham, Biosciences, Piscataway, NJ) in liquid lysogeny broth medium (LB medium) (Gibco™, Invitrogen Corporation Carlsbad, CA) at 37 °C for 4 h. Phages were recovered from the culture supernatant by two successive polyethylene glycol precipitations (PEG 6000) and resuspended in TBS (150 mM NaCl, 50 mM Tris at pH 7.5). Between the first and the second precipitations, the phage solutions were filtered through a 0.45-μm filter (Millex-H, Millipore, Billerica, MA). Stock phage concentrations were determined by titration according to the manufacturer's instructions (T7Select® System Manual).

#### 2.1.3. Phage titration

Phages were titrated by plating the infected bacteria on agar plates (Gibco™) according to the manufacturer's instructions (T7Select® System Manual).

#### 2.1.4. PCR amplification and DNA sequencing

Using a sterile loop or pipette tip, a portion of the top agarose of an individual plaque (Nunc™, Leuven, BE) of interest was scraped up, and dispersed in a tube containing 100 μl of LB liquid. After mixing the tube briefly and centrifugation at 14,000 × *g* for 1 min to clarify, the following components were added in a sterile 0.5 ml PCR tube: 5 μl phage lysate, 10 μl Thermofold 10× (Biolabs, UK), 8 μl dNTP mix (5 mM each) (Eurogentec, Angers, FR), 4 μl $MgSO_4$ (Biolabs), 2 μl T7 UP Primer (20 mM) (Eurogentec), 2 μl T7 DOWN Primer (20 mM) (Eurogentec), 1 μl Vent DNA polymerase (2 U/μl) (Biolabs) and to 73 μl deionised water. Then, the DNA was amplified by 35 cycles (10 min at 94 °C, 1 min at 54 °C and 30 min at 72 °C) and a final extension at 72 °C for 5 min in a thermal cycler (PerkinElmer, Courtaboeuf, FR). Amplified DNA was sequenced (B. Purnelle, Institut des Sciences de la Vie, Université Catholique de Louvain, Louvain-la-Neuve, BE) using an ABI3100system (Applied Biosystems Inc., CA).

Up primer: 5′-CGCAATGGGCCAGGGTGCTC-3′
Down primer: 3′- CGATAGACGCCAGAATGTCG-5′

### 2.2. Cell culture

#### 2.2.1. Cell lines and culture media

Human colon carcinoma Caco-2 cell line (clone 1), obtained from Dr. Maria Rescigno (Rescigno et al., 2001) (University of Milano-Bicocca, Milano, IT), from passage $x+12$ to $x+30$, and human Burkitt's lymphoma Raji B line (ATCC, Rockville, MD) from passage 102–110 were grown as previously described (des Rieux et al., 2007; Gullberg et al., 2000).

#### 2.2.2. In vitro model of the human FAE

Caco-2 cells and Raji cells were grown as previously described (des Rieux et al., 2006). The inverted *in vitro* model of the human FAE was obtained as described by des Rieux et al. (2006). Briefly, 3–5 days after Caco-2 cell seeding, the inserts (Transwell® polycarbonate inserts 12 wells, pore diameter of 3 μm, polycarbonate) purchased from Corning Costar (New York, NY) were inverted. After 9–11 days, Raji cells were added in the basolateral compartments. The cocultures were maintained for 5 days. Monocultures

**Table 1**
Polymers characteristics.

| Polymer | Mn (SEC)[a] g/mol | Mn (NMR)[b] g/mol | Mn[c] PEG | Mn[d] PCL | Mol% glycolide | Polydispersity(PDI) index[e] |
|---|---|---|---|---|---|---|
| PLGA | 22,000 | | | | 25 | 1.8 |
| Fluorescent PLGA | 23,600 | | | | 29 | 1.6 |
| PEG-b-PLGA | 29,300 | 4600–16,500(L)/4700(G) | | | 26 | 1.7 |
| PCL-b-PEG | 18,200 | | 5000 | 13,200 | | 1.4 |

[a] Polystyrene calibration.
[b] Determined by NMR by the following formula: $(I_{4.7}/2)/(I_{52}+(I_{4.7}/2)) \times 100$, where $I_{4.7}$ is the signal intensity of the glycolide unit at 4.7 ppm ($CH_2OC{=}O$) and $I_{52}$ is the signal intensity of the lactide unit at 5.2 ppm ($CH(CH_3)OC{=}O$).
[c] Calculated from $^1$H NMR spectrum in $CDCl_3$ at 25 °C by comparing the intensity of the terminal methyl group ($CH_2OCH_3$, 3.2 ppm) with the methylene protons ($OCH_2CH_2O$, 3.6 ppm).
[d] Calculated from $^1$H NMR spectrum in $CDCl_3$ at 25 °C by comparing the intensity of the methylene protons of PEG peak at 3.6 ppm, with the peak of the α-methylene protons of PCL at 4.05 ppm.
[e] PDI = Mw/Mn, determined by SEC by polystyrene standard.

of Caco-2 cells were used as controls. Inserts were used in their original orientation for all the following *in vitro* experiments. Cell monolayer integrity, both in co- and monocultures, was controlled by transepithelial electrical resistance (TEER) measurement using an Endohm™ tissue resistance chamber (Endohm-12, World Precision Instruments, Sarasota, FL) connected to a Millicell®-RES (Millipore, Billerica, MA.) ohmmeter.

### 2.3. Cell binding or transcytosis of phages in the in vitro model of human FAE

#### 2.3.1. Screening a phage library using cell binding assay

The cell monolayers were washed twice and incubated in serum free HBSS for 1 h at 37 °C. 400 μL of phage solution ($6 \times 10^{10}$ pfu/ml) in HBSS/BSA (0.1%, w/v) were then added to the apical compartment of cell monolayers for 1 h on ice and under horizontal rotation at 200 rpm. Then, the phage solution was removed and the cells washed six times with 500 μl HBSS/BSA/Tween 20 (0.2%, v/v) (Sigma–Aldrich, Schnelldorf, DE) for 5 min each, followed by 2 washes of 5 min with 500 μl HBSS/BSA. Phages were eluted in 150 μl of 0.1 M HCl/BSA (0.1%, w/v) for 10 min and neutralized with 22 μl of 1 M Tris (pH 9.0). Finally inserts were washed with 500 μl of HBSS/BSA to collect remaining phages. This solution was pooled with the 150 μl previously recovered. The number of HBSS/BSA/Tween 20 washes increased along the rounds of selection, from 6 for the first round to up to 12 for the last round. Subsequent rounds of selection were performed in the same manner using phage isolated and amplified from the previous round. After five rounds of selection and propagation, phage clones were sequenced.

#### 2.3.2. Transcytosis mechanisms of phage transport by M-like cells (des Rieux et al., 2007)

To evaluate whether the phages are transported by an energy-dependent mechanism, their transport by mono- and cocultures was evaluated at 4 and 37 °C. Inserts were pre-incubated for 30 min in HBSS at these temperatures. To control cell monolayer integrity, 0.9 μCi/ml of $^{14}$C-mannitol (a paracellular marker) was added to the phage suspension ($6.5 \times 10^{10}$ pfu/ml). This solution was first pre-incubated at the desired temperature and added to the apical side of the cell monolayers (400 μl). Inserts were incubated at 4 or 37 °C for 60 min. Finally, the basolateral solutions were sampled and the number of transported phages was determined by titration while the amount of transported $^{14}$C-mannitol was measured by liquid scintillation.

The phage transport by mono- and cocultures was evaluated between the apical to the basolateral compartment and inversely. Inserts were pre-incubated for 30 min in HBSS at 37 °C. The phage suspension ($6.5 \times 10^{10}$ pfu/ml) was added to the apical side (400 μl) or the basolateral side (1200 μl) of the cell monolayers and inserts were incubated at 37 °C for 90 min. Finally, the apical or basolateral solutions were sampled and the number of transported phages was measured by titration.

Transport experiments were also conducted with an inhibitor of macropinocytosis such as EIPA. Cell monolayers were first pre-incubated apically with 150 μl of EIPA (50 μM) in HBSS for 20 min at 37 °C, before adding the phage suspension ($6.5 \times 10^{10}$ pfu/ml) containing 50 μM EIPA at the apical side (400 μl) of the cell monolayers. Inserts were incubated at 37 °C for 90 min. Finally, the basolateral solutions were sampled and the number of transported phages was measured by titration.

Results are expressed as apparent permeability coefficient (Papp) (Artursson, 1990; des Rieux et al., 2007) as a mean ± standard error of the mean (SEM). The apparent permeability coefficient (Papp, cm s$^{-1}$) was calculated using the following equation:

$$\text{Papp} = \frac{dQ}{dtAC_0}$$

where d$Q$/d$t$ is the number of phages (pfu) or the amount of $^{14}$C-mannitol (dpm) present in the basal compartment (output) in function of time (s), $A$ the area of Transwell ($A = 1.13\ \text{cm}^2$) and $C_0$ the initial concentration of phages (input) or mannitol in apical compartment (pfu/ml or dpm/ml).

#### 2.3.3. Screening of a phage library for peptides that mediate phage transcytosis

After equilibration in HBSS at 37 °C, the apical medium of the cell monolayers was replaced by the phage suspension (400 μl, $7.5 \times 10^{10}$ pfu/ml) and inserts were incubated at 37 °C during 90 min. The basolateral solutions were then sampled and the number of transported phages was measured by titration. Recovered phages were then propagated by infection of bacteria and purified. After five rounds of selection and propagation, phage clones were sequenced.

### 2.4. Formulation of peptide-conjugated nanoparticles

#### 2.4.1. Polymers synthesis and characterization

Poly(DL-lactic-co-glycolic) acid (PLGA), fluorescent PLGA, poly(DL-lactic-co-glycolic) acid–polyethylene glycol (PLGA–PEG) and polycaprolactone–polyethylene glycol (PCL–PEG) were all prepared by ring-opening polymerization as previously described (Fievez et al., 2009; Garinot et al., 2007). They were characterized by $^1$H NMR (400 MHz, Bruker DRX400) spectra and by size-exclusion chromatography (SEC) (Fievez et al., 2009). The polymers used for the preparation of nanoparticles and PCL–PEG used for grafting are described in Table 1.

#### 2.4.2. Photografting of peptides on PCL–PEG

CTGKSC, LRVG and PAVLG peptides (>95% purity) were purchased from Eurogentec (Seraing, BE). The grafting of these

peptides on PCL–PEG (13,200–5000) was carried out as previously described (Garinot et al., 2007; Pourcelle et al., 2009; Fievez et al., 2009). Briefly, polymer was solubilized in methylene dichloride or acetonitrile with O-succinimidyl 4-(p-azidophenyl) butanoate (0.6 mmol per gram of PCL–PEG). After solvent evaporation, the sample was irradiated at 254 nm in a quartz flask under an argon atmosphere for 20 min. After washing, the "activated" sample was immersed in a 1 mM solution of ligand in phosphate buffer (0.1 M): acetonitrile (1:1, v/v) at pH 8 and shook for 24 h at 20 °C. Then the sample was washed and dried under a vacuum at 40 °C to constant weight. As described elsewhere (Fievez et al., 2009; Pourcelle et al., 2009) the grafting rates were controlled with a tritrated probe (L-[4,5-$^3$H] lysine monohydrochloride). Rates of 3300 nmol of covalently fixed probes per gram of copolymer were attained.

*2.4.3. Preparation of PEGylated PLGA-based nanoparticles*

Fluorescent nanoparticles were prepared by the "water-in-oil-in-water" solvent evaporation method as reported by Garinot et al. (2007). Briefly, 50 μL of PBS buffer were emulsified with 1 ml of methylene dichloride (Acros, Beerse, BE) containing 50 mg of polymers (70% PLGA/15% PLGA–PEG/15% PCL–PEG) with or without the ligand with an ultrasonic processor (70W, 15 s). The second emulsion was performed with 2 ml of 1% (w/v) sodium cholate (Sigma) aqueous solution. The double emulsion was then poured into 100 ml of a 0.3% sodium cholate aqueous solution, and stirred at 37 °C for 45 min. The nanoparticle suspension was then washed twice in PBS by centrifugation at 22,000 g for 1 h. Particle size and zeta potential were determined as previously described by Garinot et al. (2007) using the Zetasiezer Nano Series Malvern (Worcestershire, UK). For targeted nanoparticles a concentration of 24.75 nmol of peptidic sequence per formulation was attained.

*2.4.4. Nanoparticle transport*

The concentration of stock nanoparticle solutions were checked by FACS analysis (FACScan, Becton Dickinson, Erembodegem, BE) and adjusted by dilution in HBSS to a final concentration of $2.7 \times 10^{10}$ nanoparticles/ml. After equilibration in HBSS at 37 °C, the apical medium of the cell monolayers was replaced by the nanoparticle suspension (400 μl) and inserts were incubated at 37 °C during 90 min. The basolateral solutions (1200 μl) were then sampled and the number of transported particles was measured by FACS analysis. The measurements were based on both fluorescence and particle size.

*2.5. Statistics*

The phages and nanoparticle transport across the co- and monocultures cell monolayers was compared using Kruskal–Wallis non-parametric tests ($p < 0.05$).

## 3. Results

*3.1. Screening the T7 phage library for cell binding assay*

To check if phages could specifically bind to enterocytes and/or to M cells, monocultures (Caco-2 monolayers) or cocultures (monolayers of Caco-2 and 15–30% of M cells) (des Rieux et al., 2007) were incubated at 4 °C with the 77 phage library. The phages bound to cells were recovered by washing. After five selection rounds in the mono- as well as in the cocultures, a large proportion of the recovered phages did not display any specific peptide sequence (wild type) (data not shown). These wild type phages are however known to amplify more efficiently than phages bearing peptides in fusion with their own surface proteins. So, their presence in the phages selected after four rounds of selection may indicate that no sequence had a high affinity for the apical surface of the cells. In addition, most of the sequenced phages possessed a smaller sequence due to the presence of a stop codon in the first part of the insert, which could provoke a higher amplification. The evolution of the number of phages recovered on the apical side of mono- and cocultures after each round of selection could also confirm that no sequence had been selected since no enrichment of the library was observed. It was possible, however, that experimental conditions may have been unfavourable in terms of selection quality since this experiment was performed at 4 °C – a temperature at which cells are the most sensitive.

*3.2. Mechanisms of phage transcytosis in the in vitro FAE model*

In order to overcome these potential problems encountered at 4 °C, a second selection was performed at 37 °C. At this temperature, cells should be able to bind and transport the phages from the apical towards the basolateral compartment. So, in this second selection, peptides could be selected for their ability to induce a specific transmucosal transport of the phages. Before performing transepithelial selections, however, the transport mechanism of the phage library by the two cell monolayers first needed to be characterized. We have previously shown that the transport of reference carboxylated nanoparticle of 200 nm in the *in vitro* FAE model was mediated by a transcellular energy-dependent mechanism (des Rieux et al., 2007). To check whether it was also the case for the phage transport, cell monolayers were also incubated at 4 and 37 °C in the presence of $^{14}$C-mannitol, a paracellular marker. Phage transport by monocultures was not influenced by temperature whereas it was dramatically increased in the cocultures at 37 °C (Fig. 1A). Irrespective of the temperature, the Papps of mannitol were higher for co- than for monocultures (Fig. 1B) and were in the same range that the values previously obtained (des Rieux et al., 2007), demonstrating that the tight junctions were functional and that cell monolayers were unaffected by phages.

Phage transport between the apical and basolateral compartments was also investigated. Phages were 10 times more transported across cell monolayers in the apical-to-basal direction than in the opposite direction (Fig. 2). In addition, as previously shown for polystyrene and polymeric nanoparticles (des Rieux et al., 2007; Fievez et al., 2009; Garinot et al., 2007), the number of transported phages after 90 min of incubation at 37 °C was very significantly higher in the cocultures than in monocultures. These results suggest that the uptake of phages like that of reference nanoparticles by M cells occurred by an energy-dependent mechanism.

As internalization of reference nanoparticles by M cells is inhibited by EIPA a non-specific macropinocytosis inhibitor (des Rieux et al., 2007), phage transport was studied in presence of EIPA. Phage transport was not significantly inhibited in the mono- as in the cocultures by this inhibitor (Fig. 3) which is consistent with the presence at the phage surface of random peptide sequences that might mediate the phage transport via receptor-mediated transcytosis.

*3.3. Selection of phages that undergo transcytosis in the human FAE in vitro model*

The phages selection that undergoes the transcytosis was thus achieved in HBSS at 37 °C during 90 min in the absence of any inhibitor. In order to enrich sufficiently the library for phages that preferentially improve its transport *in vitro*, five rounds of selection were performed, after which it consisted of *ca.* $3 \times 10^8$ different peptides. The number of recovered phages on mono- and cocultures increased as compared to the first transport experiment (Fig. 4).

Phages obtained after 5 rounds of selection were then sequenced. Results are presented in Table 2. Three identical clones

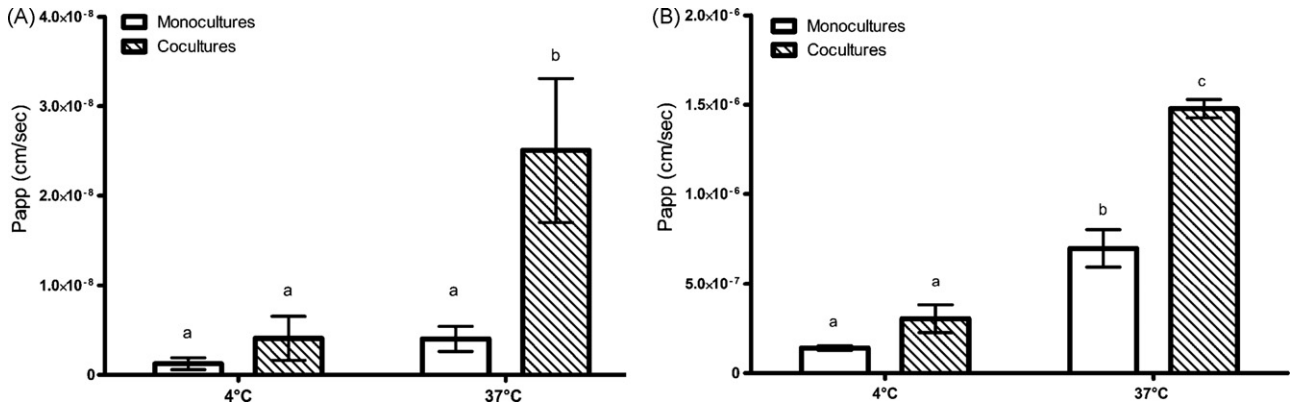


**Fig. 1.** Temperature dependence of phage transcytosis across mono- and cocultures of Caco-2 cells. Mono- and cocultures of Caco-2 cells were incubated in HBSS at 4 or 37 °C with $6 \times 10^{10}$ pfu/ml of phages suspended in HBSS further containing 0.9 μCi/ml of $^{14}$C-mannitol, pre-incubated at 4 or 37 °C, and added to apical pole of cell monolayers. Mono- and cocultures were incubated for 30 min at 4 °C and then for 60 min at 37 °C ($n = 6$). (A) Titration of phages by plating bacteria on LB medium according to the manufacturer's instructions (T7Select® System Manual). (B) $^{14}$C-Mannitol was quantified by liquid scintillation. Results are expressed as apparent permeability or Papp (cm/s) as a mean ± standard error of the mean (SEM). Groups a, b and c were significantly different ($p < 0.05$).

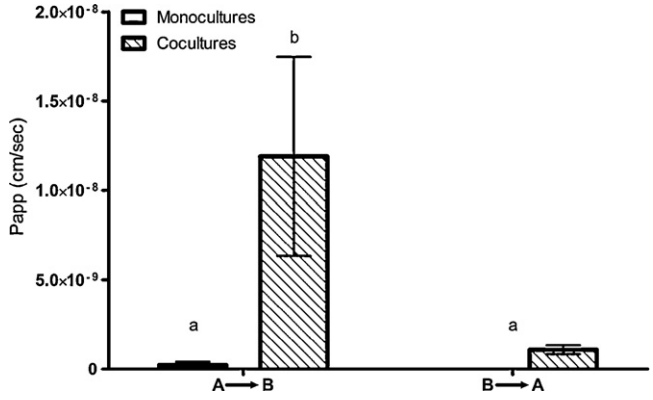


**Fig. 2.** Unidirectional apical-to-basal phage transport in mono- and cocultures of Caco-2 cells. Cells were cultured on 12 wells inserts ($n = 16$). $2 \times 10^0$ phages were applied either into the apical or basal compartments of culture inserts. Following the incubation at 37 °C for 3 h, aliquots were taken from the opposite chambers and the phage titer was determined as above. Results are expressed as apparent permeability coefficient (Papp) as a mean ± standard error of the mean (SEM). Groups a and b were significantly different ($p < 0.05$). A → B: transport from the apical towards the basolateral compartment; B → A: transport from the basolateral towards the apical compartment.

(CTGKSC, PAVLG and LRVG) appeared at high frequency after selection on both mono- and cocultures (Table 2), whereas one consensus sequence of amino acids was found only upon selection on cocultures (SGTS and SGGTS), although to a lesser extent than the others clones. Consensus sequences of amino acids appearing in different contexts and particular peptide sequences present with high frequencies are commonly considered as an important evidence of the phage display selection. The progressive enrichment in phage titers along the succeeding rounds of selection is also commonly observed.

To evaluate the ability of the different clones selected with a very high frequency to improve their transmucosal passage, the transport by mono- and cocultures of phages displaying these peptides was compared (Fig. 5A). For the phages bearing the CTGKSC or LRVG peptides as well as for the library, the transport across cocultures was significantly higher than that across monocultures ($p < 0.05$). LRVG peptide increased the phage transport by cocultures 14 times when compared to the library. CTGKSC peptide was also able to increase phage transport by the cocultures, but to a lesser extent than LRVG peptide, since this transport was improved by only 3 times. Surprisingly, the transport of phages bearing the PAVLG sequence was the same in the mono- and cocultures and

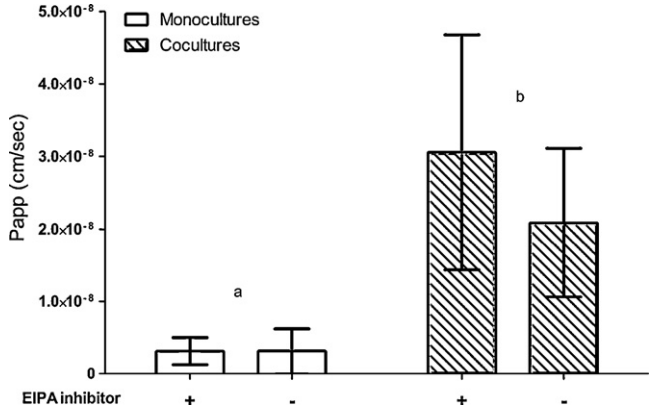


**Fig. 3.** Effect of an endocytosis inhibitor on phage transport by mono- and cocultures of Caco-2 cells. Cell monolayers ($n = 20$–25) were first apically pre-incubated with EIPA in HBSS for 20 min at 37 °C, before adding phage suspension ($7.5 \times 10^{10}$ pfu/ml). The inhibitor was present throughout the whole experiment (90 min at 37 °C). Cocultures incubated in HBSS were used as control. The number of transported phages was evaluated by titration as above. Results are expressed as apparent permeability coefficient (Papp), as a mean ± standard error of the mean (SEM). Groups a and group b were significantly different ($p < 0.05$).

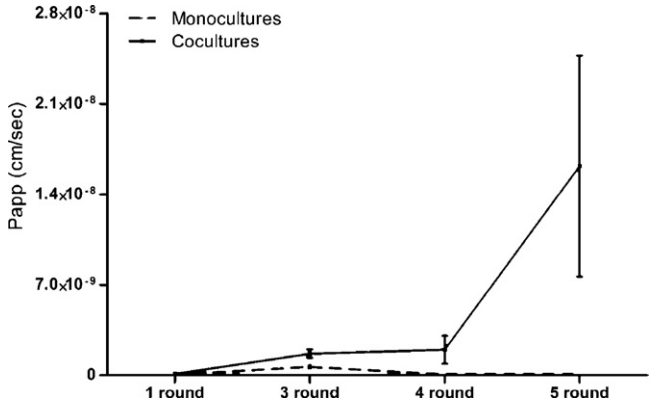


**Fig. 4.** Evolution of transported phages in function of the selection round. Mono- and cocultures were incubated with $7.5 \times 10^{10}$ pfu/ml of phage suspension and transcytosis was assessed at 37 °C during 90 min. Basolateral solutions were sampled and the number of transported phages was measured by plating as above. Recovered phages were then propagated by infection of bacteria and purified. After five rounds of selection and propagation, individual phage clones were sequenced. Results are expressed as apparent permeability coefficient (Papp) as a mean ± standard error of the mean (SEM). *$p < 0.05$.

**Table 2**
Phage selection. Clones were sequences after five rounds of selection in the human FAE *in vitro* model. T5C were clones selected for their abilities to induce phage transcytosis on cocultures and T5M on monocultures. Numbers 1 and 2 represent both sequencing in which 12 and 50 clones were analyzed respectively.

| Clones name | Sequence/peptide | Frequency |
|---|---|---|
| T5C1-2/3/5/11-T5C2-1/4 | **L-R-V-G-stop** | **6** |
| T5C1-1 – T5C2-3/5/8/10/12/14/17/19 /22/24/26/28/38/40/42/45/47 | C-**T-G-K-S**-C-stop | **25** |
| T5C1-4 | C-stop | 1 |
| T5C1-6 | C-E-G-P-L-K-P-stop | 1 |
| T5C1-7 | C-G-G-X-D-N-S-C | |
| T5C1-8 – T5C2-2/13/20/30/41 | S-stop | 6 |
| T5C1-9 | C-A-P-I-L-F-P-R-C | 1 |
| T5C1-10 – T5C2-7/29/34/36/37 | P-A-stop | 6 |
| T5C1-12 – T5C2-9 | C-L-E-S-K-K-K-T-C | 2 |
| T5C2-21/27 | **P-A-V-L-G** | **2** |
| T5C2-6 | C-R-M-K | 1 |
| T5C2-18 | C-E-K-R | 1 |
| T5C2-25 | C-I-G-K-R-D-A-K-H | 1 |
| T5C2-32 | C-R-R-stop | 1 |
| T5C2-33 | C-K-**S-G-G-T-S**-A-C | 1 |
| T5C2-35 | C-R-**S-G-T-S**-R-S-C | 1 |
| T5C2-46 | C-R-D-stop | 1 |
| T5M1-2/4/8 – T5M2-9/11/18/23 | **P-A-V-L-G** | **7** |
| T5M1-1 – T5M2-1/3/7/20/24 /26/28/30/35/38/45/48/49 | C-**T-G-K-S**-C | **25** |
| T5M1-3 – T5M2-4 | S-A-stop | 2 |
| T5M1-5 – T5M2-16/21 | P-A-stop | 2 |
| T5M1-6 | C-I-E-V-P-C | 1 |
| T5M1-7 | C-G-E-K-K-M-R-C | 1 |
| T5M1-9 | C-**G-K-S**-T-K-N-W | 1 |
| T5M1-10 – T5M2-5/8/10/17/22 | S-stop | 6 |
| T5M1-11 | P-A-R-L-A-R-L | 1 |
| T5M2-2/13/14/19/25 | **L-R-V-G** | **5** |
| T5M2-36/46/47 | C-P-F-D-S-stop | 3 |
| T5M2-6/12 | C-K-stop | 2 |
| T5M2-29 | L-V-G-G-H-C-G-E-C | 1 |
| T5M2-15 | C-Q-E-A-T-N-R-K-C | 1 |
| T5M2-37 | C-**T-G-K**-R | 1 |

Bold type represents identical clones recovered at high frequency after selection on mono- and cocultures.

was not significantly enhanced as compared to the transport of the library.

### 3.4. In vitro transport of targeted nanoparticles

To check if the selected sequences could also influence the transmucosal transport of vaccine-loaded nanoparticles, these selected peptides were covalently grafted on the surface of PLGA polymeric nanoparticles as previously described for others ligands (Fievez et al., 2009; Garinot et al., 2007) and their influence on the nanoparticle transport was studied *in vitro*. Formulations were first characterized in term of size and zeta potential (Table 3). As previously shown (Fievez et al., 2009; Garinot et al., 2007), all formulations presented a homogenous size around 200 nm, which is commonly considered as optimal size for nanoparticles to be taken up by M cells (des Rieux et al., 2005). In addition, the presence of PEG chains in the formulation probably shielded negative charges present at the nanoparticle surface. Consequently, the zeta potentials of the four populations were close to zero, and as previously reported, formulations close to zero benefited from a preferential uptake by M cells when compared to ionized formulations (Florence, 2005).

Grafted and non-grafted nanoparticles were added at the apical side of the mono- and cocultures, at 37 °C, for 90 min. For all groups the transport across the cocultures was significantly higher than that across monocultures ($p < 0.05$) (Fig. 5B). As previously observed for the phage transport, the presence of LRVG and CTGKSC peptides at the nanoparticle surface significantly increased their transport across cocultures by 8 and 4 times respectively when compared to non-conjugated nanoparticles. The transport of PAVLG-conjugated nanoparticles by cocultures was the same than that of non-conjugated nanoparticles.

## 4. Discussion

The aim of the current study was to identify novel targeting ligands for human M cells of Peyer's patches using the *in vitro* phage

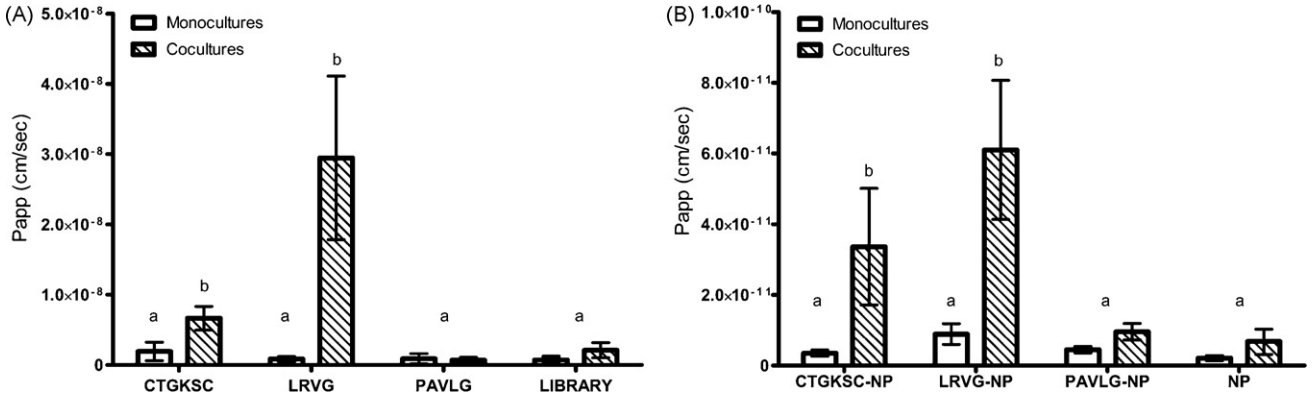


**Fig. 5.** Influence of selected sequences on phage and nanoparticle transport by mono- and cocultures of Caco-2 cells. (A) Cell monolayers were incubated for 90 min at 37 °C with $1 \times 10^{10}$ phages/ml of each formulation suspended in HBSS. The number of transported phages was evaluated by titration as above and is expressed as apparent permeability coefficient. (Papp) as a mean ± standard error of the mean (SEM). Groups a and b were significantly different ($p < 0.05$). (B) Cell monolayers ($n = 9$) were incubated for 90 min at 37 °C with $2.7 \times 10^{10}$ nanoparticles/ml of each formulation suspended in HBSS. The number of transported nanoparticles was evaluated by flow cytometry and is expressed as apparent permeability coefficient. (Papp) as a mean ± standard error of the mean (SEM). Groups a and b were significantly different ($p < 0.05$). A second experiment ($n = 8$) showed the same tendency (data not shown).

**Table 3**
Physicochemical properties of the nanoparticles.

| | Non-targeted | LRVG | PAVLG | CTGKSC |
|---|---|---|---|---|
| Size (nm) | 186 ± 30 | 193 ± 22 | 186 ± 25 | 179 ± 24 |
| Pdi | 0.110 | 0.170 | 0.130 | 0.110 |
| Zeta potential (mV) | −12 ± 5 | −10 ± 5 | −11 ± 4.5 | −12 ± 6 |

display methodology. The screening of phage-displayed peptide libraries on intact mammalian cells has proven to be a fruitful method to identify peptides that recognize certain cell types with high affinity and cell-specificity (Ivanenkov et al., 1999a,b; Ivanenkov and Menon, 2000). This technique does not require prior knowledge of the cellular receptor and, therefore, allows the isolation of targeting peptides for cell types for which little is known about their cell surface architecture. Since it is the case for human M cells, phage display appeared thus ideal to identify a new ligand specific of their apical surface.

We firstly used a protocol of biopanning in which phages were selected at 4 °C for their binding affinity for the apical surface of mono- and cocultures. However, after four rounds of selection, no sequence had really shown a high affinity for the apical surface of M cells, probably due to the harsh experimental conditions, which could have a negative effect on the quality of the selection.

Since phage particles displaying ligands can bind to internalizing receptors, such as integrins and undergo an efficient uptake by targeted cells (Ivanenkov & Menon, 2000), a second experiment in which peptides ligands were selected for their ability to induce the transmucosal transport of the phages was realized. Before performing the selection, the mechanism of transport of the phage library by cell monolayers was first characterized. This transport was essentially unidirectional, driven between the apical towards the basolateral compartments and occurred by an energy-dependent mechanism as previously observed using MDCK cells (Ivanenkov and Menon, 2000). In contrast to the transport of carboxylated reference nanoparticles that was inhibited by the presence of EIPA, an inhibitor of macropinocytosis (des Rieux et al., 2007), that of phages was not influenced by its presence, which suggests that this transport occurs by a mechanism other than macropinocytosis. Then, after five rounds of selection to enrich the phage library in phages that preferentially improve their transport *in vitro*, three different sequences (LRVG, PAVLG and CTGKSC) were selected with a high frequency on mono- and cocultures. The selection of similar and common sequences in both cultures is in agreement with the fact that only 15–30% of enterocytes were converted into M-like cells in the *vitro* model of the human FAE (des Rieux et al., 2007). *In vivo*, phage display has already been used to select peptides that improve the transmucosal transport of phages across the murine intestine (Kang et al., 2008; Kim et al., 2006; Takagi et al., 2007) and to identify novel peptide ligands targeting M cells on rat Peyer's patches (Higgins et al., 2004). None of the sequences found in this work seemed to have similarity with those selected in the previous studies, which is however consistent with the complexity and the important variability in the intestinal cell surface proteins that exists across different species.

The influence of the 3 sequences identified on the transport of phage and grafted nanoparticles was investigated. The comparison of the transport of these clones in the human *in vitro* FAE model, showed a significant enhancement of the phage transport across the cocultures for phages bearing the LRVG and the CTGKSC peptide. The transport of PAVLG-phages was the same across mono- and cocultures and identical to the phage library transport across monocultures. In order to check if these three sequences were also able to influence the transport of vaccine-loaded nanoparticles, they were grafted at the surface of polymeric nanoparticles. The transport of conjugated nanoparticles in the human *in vitro* FAE model was significantly improved for the LRVG and CTGKSC peptide while it remained the same for PAVLG-nanoparticles and non-conjugated nanoparticles, confirming therefore the previous data obtained with the phages.

The enhancement of the *in vitro* transport by M-like cells using the LRVG and the CTGKSC peptides could be explained either by the overexpression of a receptor at the FAE apical surface or by their influence on the particle surface properties. Several studies have shown that even if some receptors are not specific to M cells, they could be overexpressed in the FAE and be used to improve the bioavailability of vaccine particles (Jain and Vyas, 2005, 2006). Furthermore, the reduced glycocalyx overlaying the FAE should facilitate the interaction between the targeted particles and M cells, as compared to enterocytes. The surface properties of particles have also been identified as crucial for their uptake by intestinal epithelial cells although the conclusions regarding optimal properties still remain controversial, depending furthermore on the type of intestinal cell targeted (des Rieux et al., 2006) (enterocytes *vs.* M cells). As these peptides are present in 415 copies at the phage surface, their properties could play a role in the interaction of phages with the intestinal epithelium, influencing the selection and the transport process. Regarding the hydrophobicity profile of selected peptides with the Kyte–Doolittle scale (Kyte and Doolittle, 1982) PAVLG sequence has a rather hydrophobic profile whereas the LRVG and CTGKSC profiles were more amphiphilic. The CTGKSC and LRVG sequences display a positive charge whereas the PAVLG is neutral (CLC Main Worbench 4.0.1 free software). Non-ionized nanoparticles have been shown to be more transported than positively charged ones (Shakweh et al., 2005; des Rieux et al., 2005). According to Jung et al. (2000), the best combination favouring non-specific absorption across M cells would be an hydrophobic, neutral particles as displayed by the PAVLG peptide. That suggests that the positive, amphiphilic peptides (LRVG and CTGKSC), which increased the phage and nanoparticle transport across cocultures, might specifically interact with M cells.

The similar transport of PAVLG-conjugated and non-conjugated nanoparticles could result from the mode of linkage of the ligand on the polymer since they are grafted by their N-terminal moiety. The method used allows the covalent coupling via an amide linkage resulting from the N hydroxyl-succinimide displacement (Pourcelle et al., 2009). In general, the ligands display primary amines (as CTGKSC or LRVG) but the PAVLG peptide has a cyclic secondary amine, which induces some steric hindrance that could limit the reaction. Therefore, the covalent grafting of PAVLG by clip chemistry would be more difficult and less effective.

## 5. Conclusion

This study allowed the selection of two peptidic sequences that improved the transport of phages and polymeric nanoparticles in an *in vitro* model of the human FAE. As far as we know, it is the first time that this model was used to perform a selection using the phage display technique. To confirm the hypothesis that these sequences could be used to increase the availability of vaccine-loaded nanoparticles after oral administration, immunizations study should be performed *in vivo* with antigen-loaded grafted nanoparticles or with phages displaying these sequences used as vectors.

## Acknowledgments

The authors thank A. Tonon from Ludwig Institute Cancer Research (Brussels, BE) for his support in the realization of FACScan analysis as well as Prof. P. Soumillion (Institut des Sciences de laVie, UCLouvain, Louvain-la-Neube, BE) for his valuable comments on this work. This work was supported by the FNRS as well as by the DGTRE (Walloon Region Government) with both the projects FIRST EUROPE (no EPH3310300R58F/415847) and VACCINOR (WINNOMAT).

## References

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J. Pharm. Sci. 79, 476–482.

Brayden, D.J., Jepson, M.A., Baird, A.W., 2005. Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov. Today 10, 1145–1157.
Danner, S., Belasco, J.G., 2001. T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. Proc. Natl. Acad. Sci. U.S.A. 98, 12954–12959.
des Rieux, A., Fievez, V., Garinot, M., Schneider, Y.J., Préat, V., 2006. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J. Control. Release 116, 1–27.
des Rieux, A., Fievez, V., Theate, I., Mast, J., Preat, V., Schneider, Y.J., 2007. An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells. Eur. J. Pharm. Sci. 30, 380–391.
des Rieux, A., Ragnarsson, E., Gullberg, E., Préat, V., Schneider, Y.J., Artursson, P., 2005. Transport of nanoparticles across an *in vitro* model of the human intestinal follicle associated epithelium. Eur. J. Pharm. Sci. 25, 455–465.
Ermak, T.H., Giannasca, P.J., 1998. Microparticle targeting to M cells. Adv. Drug Deliv. Rev. 34, 261–283.
Essler, M., Ruoslahti, E., 2002. Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. Proc. Natl. Acad. Sci. U.S.A. 99, 2252–2257.
Fievez, V., Plapied, L., des Rieux, A., Pourcelle, V., Freichels, H., Wascotte, V., Vanderhaeghen, M.L., Jérôme, C., Vanderplasschen, A., Marchand-Brynaert, J., Schneider, Y.J., Préat, V., 2009. Targeting nanoparticles to M cells with non-peptidic ligands for oral vaccination. Eur. J. Pharm. Biopharm. 73, 16–24.
Florence, A.T., 2005. Nanoparticle uptake by the oral route: fulfilling its potential? Drug Discov. Today: Technol. 2, 75–81.
Garinot, M., Fievez, V., Pourcelle, V., Stoffelbach, F., des Rieux, A., Plapied, L., Theate, I., Freichels, H., Jérôme, C., Marchand-Brynaert, J., Schneider, Y.J., Préat, V., 2007. PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. J. Control. Release 120, 195–204.
Gebert, A., Rothkotter, H.J., Pabst, R., 1996. M cells in Peyer's patches of the intestine. Int. Rev. Cytol. 167, 91–159.
Gullberg, E., Keita, A.V., Salim, S.Y., Andersson, M., Caldwell, K.D., Soderholm, J.D., Artursson, P., 2006. Identification of cell adhesion molecules in the human follicle-associated epithelium that improve nanoparticle uptake into the Peyer's patches. J. Pharmacol. Exp. Ther. 319, 632–639.
Gullberg, E., Leonard, M., Karlsson, J., Hopkins, A.M., Brayden, D., Baird, A.W., Artursson, P., 2000. Expression of specific markers and particle transport in a new human intestinal M-cell model. Biochem. Biophys. Res. Commun. 279, 808–813.
Gullberg, E, Velin Keita, A., Salim, S.Y., Andersson, M., Caldwell, K., Söderholm, J.D., Artursson P., 2005. Increased CD9 and B1-integrin expression in human follicule associated epithelium is linked with selective transport into human Peyer's patches. Particle Transcytosis Across the Human Intestinal Epithelium (Thesis).
Hase, K., Kawano, K., Nochi, T., Pontes, G.S., Fukuda, S., Ebisawa, M., Kadokura, K., Tobe, T., Fujimura, Y., Kawano, S., Yabashi, A., Waguri, S., Nakato, G., Kimura, S., Murakami, T., Iimura, M., Hamura, K., Fukuoka, S., Lowe, A.W., Itoh, K., Kiyono, H., Ohno, H., 2009. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. Nature 462, 226–230.
Herrmann, S., Leshem, B., Lobel, L., Bin, H., Mendelson, E., Ben-Nathan, D., Dussart, P., Porgador, A., Rager-Zisman, B., Marks, R.S., 2007. T7 phage display of Ep15 peptide for the detection of WNV IgG. J. Virol. Methods 141, 133–140.
Higgins, L.M., Lambkin, I., Donnelly, G., Byrne, D., Wilson, C., Dee, J., Smith, M., O'Mahony, D.J., 2004. In vivo phage display to identify M cell-targeting ligands. Pharm. Res. 21, 695–705.
Ivanenkov, V.V., Felici, F., Menon, A.G., 1999a. Corrigendum to: 'Uptake and intracellular fate of phage display vectors in mammalian cells'. Biochim. Biophys. Acta 1451, 364.
Ivanenkov, V.V., Felici, F., Menon, A.G., 1999b. Targeted delivery of multivalent phage display vectors into mammalian cells. Biochim. Biophys. Acta 448, 463–472.
Ivanenkov, V.V., Menon, A.G., 2000. Peptide-mediated transcytosis of phage display vectors in MDCK cells. Biochem. Biophys. Res. Commun. 276, 251–257.
Jain, S., Vyas, S.P., 2005. Mannosylated niosomes as carrier adjuvant system for topical immunization. J. Pharm. Pharmacol. 57, 1177–1184.
Jain, S., Vyas, S.P., 2006. Mannosylated niosomes as adjuvant-carrier system for oral mucosal immunization. J. Liposome Res. 16, 331–345.
Jung, T., Kamm, W., Breitenbach, A., Kaiserling, E., Xiao, J.X., Kissel, T., 2000. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? Eur. J. Pharm. Biopharm. 50, 147–160.
Kang, S.K., Woo, J.H., Kim, M.K., Woo, S.S., Choi, J.H., Lee, H.G., Lee, N.K., Choi, Y.J., 2008. Identification of a peptide sequence that improves transport of macromolecules across the intestinal mucosal barrier targeting goblet cells. J. Biotechnol. 135, 210–216.
Katz, D.E., DeLorimier, A.J., Wolf, M.K., Hall, E.R., Cassels, F.J., van Hamont, J.E., Newcomer, R.L., Davachi, M.A., Taylor, D.N., McQueen, C.E., 2003. Oral immunization of adult volunteers with microencapsulated enterotoxigenic *Escherichia coli* (ETEC) CS6 antigen. Vaccine 21, 341–346.
Kim, S.H., Lee, K.Y., Kim, J., Park, S.M., Park, B.K., Jang, Y.S., 2006. Identification of a peptide enhancing mucosal and systemic immune responses against EGFP after oral administration in mice. Mol. Cells 21, 244–250.
Kuolee, R., Chen, W., 2008. M cell-targeted delivery of vaccines and therapeutics. Expert Opin. Drug Deliv. 5, 693–702.
Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105–132.
Lavelle, E.C., O'Hagan, D.T., 2006. Delivery systems and adjuvants for oral vaccines. Expert Opin. Drug Deliv. 3, 747–762.
Pourcelle, V., Freichels, H., Stoffelbach, F., Auzely-Velty, R., Jérôme, C., Marchand-Brynaert, J., 2009. Light induced functionalization of PCL–PEG block copolymers for the covalent immobilization of biomolecules. Biomacromolecules 10, 966–974.
Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. 2, 361–367.
Shakweh, M., Besnard, M., Nicolas, V., Fattal, E., 2005. Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by Peyer's patches in mice. Eur. J. Pharm. Biopharm. 61, 1–13.
Sharma, N., Oikonomopoulou, K., Ito, K., Renaux, B., Diamandis, E.P., Hollenberg, M.D., Rancourt, D.E., 2008. Substrate specificity determination of mouse implantation serine proteinase and human kallikrein-related peptidase 6 by phage display. Biol. Chem. 389, 1097–1105.
Silin, D.S., Lyubomska, O.V., Jirathitikal, V., Bourinbaiar, A.S., 2007. Oral vaccination: where we are? Expert Opin. Drug Deliv. 4, 323–340.
Slutter, B., Plapied, L., Fievez, V., Alonso-Sande, M., des Rieux, A., Schneider, Y.J., Van Riet, E., Jiskoot, W., Préat, V., 2009. Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. J. Control. Release 138, 113–121.
Smith, G.P., Petrenko, V.A., 1997. Phage display. Chem. Rev. 97, 391–410.
Tacket, C.O., Reid, R.H., Boedeker, E.C., Losonsky, G., Nataro, J.P., Bhagat, H., Edelman, R., 1994. Enteral immunization and challenge of volunteers given enterotoxigenic E. coli CFA/II encapsulated in biodegradable microspheres. Vaccine 12, 1270–1274.
Takagi, T., Arisawa, T., Yamamoto, K., Hirata, I., Nakano, H., Sawada, M., 2007. Identification of ligands binding specifically to inflammatory intestinal mucosa using phage display. Clin. Exp. Pharmacol. Physiol. 34, 286–289.
Woiwode, T.F., Haggerty, J.E., Katz, R., Gallop, M.A., Barrett, R.W., Dower, W.J., Cwirla, S.E., 2003. Synthetic compound libraries displayed on the surface of encoded bacteriophage. Chem. Biol. 10, 847–858.