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Bacteriomimetic invasin-functionalized nanocarriers for intracellular delivery

Hagar Ibrahim Labouta [a,b,1], Sara Menina [a], Annika Kochut [c], Sarah Gordon [a], Rebecca Geyer [c], Petra Dersch [c], Claus-Michael Lehr [a,d,*]

[a] Dept. of Drug Delivery (DDEL), Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Campus A4 1, D-66123 Saarbrücken, Germany
[b] Department of Pharmaceutics, Alexandria University, Alexandria 21521, Egypt
[c] Department of Molecular Infection Biology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany
[d] Department of Pharmacy, Saarland University, D-66123 Saarbrücken, Germany

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ABSTRACT

Intracellular bacteria invade mammalian cells to establish an infectious niche. The current work models adhesion and subsequent internalization strategy of pathogenic bacteria into mammalian cells to design a bacteriomimetic bioinvasive delivery system. We report on the surface functionalization of liposomes with a C-terminal fragment of invasin (InvA497), an invasion factor in the outer membrane of *Yersinia pseudotuberculosis*. InvA497-functionalized liposomes adhere to mammalian epithelial HEp-2 cell line at different infection stages with a significantly higher efficiency than liposomes functionalized with bovine serum albumin. Covalent attachment of InvA497 results in higher cellular adhesion than liposomes with physically adsorbed InvA497 with non-specific surface protein alignment. Uptake studies in HEp-2 cells indicate active internalization of InvA497-functionalized liposomes via β_1-integrin receptor-mediated uptake mechanism mimicking the natural invasion strategy of *Y. pseudotuberculosis*. Uptake studies in Caco-2 cells at different polarization states demonstrate specific targeting of the InvA497-functionalized liposomes to less polarized cells reflecting the status of inflamed cells. Moreover, when loaded with the anti-infective agent gentamicin and applied to HEp-2 cells infected with *Y. pseudotuberculosis*, InvA497-functionalized liposomes are able to significantly reduce the infection load relative to non-functionalized drug-loaded liposomes. This indicates a promising application of such a bacteriomimetic system for drug delivery to intracellular compartments.

1. Introduction

Functionalizing nanoparticles with peptide tags to initiate or enhance nanoparticles uptake by mammalian cells have significantly increased over the past years. Yet, impact on clinical praxis remains disappointing. One of the promising approaches, yet not much explored, is nanoparticle functionalization with bacterial outer membrane adhesins or invasins, i.e. proteins essential for cell adhesion and invasion of bacterial pathogens. Early trials indicate efficient cellular delivery using lectin [1,2] and invasin (InvA497) [2–5] surface treated particles. This is the first in-depth mechanistic study investigating the cellular interaction of bacteriomimetic nanocarriers decorated with a truncated bacterial protein ligand in the presence of the modeled bacterial counterpart.

Yersinia species (*Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) are facultative intracellular pathogens that elaborate several surface bacterial ligands involved in virulence, one of which is invasin (InvA) [6,7]. InvA is an outer membrane protein required for the efficient cellular uptake of enteric *Yersinia* species by binding to members of the β_1-integrin receptor family (e.g. $\alpha_5\beta_1$). Integrins are heterodimeric integral membrane proteins mediating communication between the extracellular environment and the cytoskeleton by binding to cytoskeletal components and either extracellular matrix proteins or other cell surface proteins. InvA binding to β_1-integrins leads to a reorganization of the host cytoskeleton and formation of pseudopods that migrate around the bacteria and enclose them into a membrane-bound vacuole [8]. This study makes use of the detailed knowledge of the InvA-mediated cell adhesion and subsequent internalization of the Gram-negative bacterium *Y. pseudotuberculosis* to design a "bacteriomimetic" delivery system; InvA497-functionalized liposomes.

Early trials to enhance uptake into mammalian cells on coupling InvA to nanoparticles [1,3–5,9] and microparticles [10,11] used polymers. However, modeling the bacterial flexibility and the bacterial membrane structure, liposomal carriers hold more promise. Yet, from the delivery aspect, the impact of InvA functionalization on rather more physiologically compatible, easily loadable nanoparticulate systems for therapeutic applications remains to be shown. To the best of

* Corresponding author at: Department of Pharmacy, Saarland University, D-66123 Saarbrücken, Germany.
E-mail address: Claus-Michael.Lehr@helmholtz-hzi.de (C.-M. Lehr).
[1] Present Address: Department of Chemistry, Faculty of Science & Cellular and Molecular Bioengineering Research Laboratory (CMBRL), Schulich School of Engineering, University of Calgary, Calgary, Canada.

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the authors' knowledge, this is the first study reporting the potential of bacteriomimetic liposomes functionalized with a C-terminal fragment of the InvA protein (Inv497) and their uptake mechanism and kinetics in comparison to their natural bacterial counterparts. The system invasion efficacy was challenged in the presence of *Y. pseudotuberculosis*, the bacteria expressing InvA. As a proof of concept, the therapeutic effect of InvA497-functionalized liposomes was demonstrated when loaded with the hydrophilic and poorly permeable aminoglycoside antibiotic gentamicin as a model anti-infective. Gentamicin-loaded InvA497-functionalized liposomes were employed in a human epithelial cell model preinfected with an invasive *Y. pseudotuberculosis* serotype O:3 strain, and assessed for their ability to affect intracellular bacterial killing.

2. Experimental section

2.1. Overexpression and purification of the cell-surface exposed C-terminal domain of invasin (InvA497)

InvA497 was extracted and purified according to previous studies [6]. Two liters of *E. coli* BL21 expressing the His-tagged C-terminal 497 amino acids of InvA (His6-InvA497) from *Y. pseudotuberculosis* was grown at 37 °C in Luria broth medium (LB) (Carl Roth GmbH, Karlsruhe, Germany) to an A600 = 0.4. The culture temperature was then shifted to 17 °C and grown to an A_{600} = 0.6. Isopropyl-b-D-thiogalactopyranoside was added to a final concentration of 100 μM to induce the expression His6-InvA497. Cells were allowed to grow overnight at 17 °C, followed by washing in order to produce a cell pellet. The cell pellet was then resuspended in 50 ml cold lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8) together with a protease inhibitor cocktail containing 5 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin, 10 mM E64 protease inhibitor, 20 mM leupeptin and 10 mM chymostatin. The cells were disrupted using a French press (2× at 1000 psi). The His6-InvA497 protein was purified by affinity chromatography with Ni-NTA Agarose (Qiagen, Venlo, Limburg, The Netherlands), eluted in elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole (pH 8) and dialyzed twice against 10 mM Tris buffer (Tris aminomethane, pH 8) containing 300 mM NaCl. Protein concentrations were determined by the Bradford protein assay (Pierce, Rockford, IL, USA).

2.2. Preparation of fluorescent liposomes containing carboxylic groups

Liposomes were prepared by lipid film hydration followed by membrane extrusion to obtain monodispersed unilamellar vesicles following a previously published method after modification [12]. In detail, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma-Aldrich, Steinheim, Germany) and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (sodium salt) (Avanti Polar Lipids, Inc., Alabaster, USA) in a molar ratio of 6:3:0.6 were dissolved in 5 ml chloroform-methanol mixture, 2:1. A 100 μl of 0.5 mg/ml chloroformic solution of the fluorescent dye "1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)" (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was added. The final lipid mixture (19.2 mM) was dried in a rotary evaporator (Büchi, Essen, Germany) at 70 °C, 200 mbar and 145 rpm for 1 h to form a thin uniform lipid film. Complete evaporation of the remaining solvents was achieved by further heating at the same temperature under a pressure of 40 mbar with 145 rpm for further 30 min. The lipid film was then hydrated with 5 ml 100 mM MES (4-Morpholineethanesulfonic acid) buffer, pH 6 at a speed of 55 rpm for 1 h at 50 °C. Unilamellar liposomes were prepared by extruding the resulting multilamellar vesicles through 200 nm polycarbonate membrane (AMD Manufacturing Inc., Ontario, Canada) at 60 °C under high pressure using nitrogen flow in a sealed stainless steel jacketed extruder (liposoFast L-50, Avestin, Mannheim, Germany). Liposomal dispersions were diluted 1:10 with MES and stored at 4 °C prior to further use.

2.3. Liposome surface functionalization

Liposomes were surface functionalized with either InvA497 or bovine serum albumin (BSA; Sigma Aldrich, Steinheim, Germany). A volume of 2 ml liposomal dispersion was incubated overnight with 300 μl of crosslinking agent, consisting of 48 mM EDC/19 mM NHS (EDC: N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; Sigma Aldrich, Steinheim, Germany; NHS: N-hydroxysuccinimide 99%; Carbolution Chemical GmbH, Saarbrücken, Germany) in MES buffer (pH 6) with gental shaking at room temperature. Liposomes were centrifuged (Rotina 420R; Hettich Zentrifugen, Tuttlingen, Germany) in Centrisart® tubes 300,000 MWCO (Sartorius, Goettingen, Germany) at 3270 g, 4 °C for 30 min to remove excess free reagent followed by three successive washing steps during which the MES buffer was gradually exchanged with phosphate buffer saline (PBS) (Sigma Aldrich, Steinheim, Germany), pH 7.4. The total volume was then completed to 2.5 ml with PBS. A 300 μl volume of 1 mg/ml InvA497 or BSA in PBS was added and coating process was continued overnight in ice bath with gentle shaking. This was followed by centrifugation and washing steps in Centrisart® tubes 300,000 MWCO to remove unbound protein.

2.4. Measurement of colloidal characteristics

Liposomes, 0.2 μM, were characterized for mean diameter, polydispersity index (PDI) and zeta potential using the ZetaSizer Nano (Malvern Instruments Ltd., Malvern, UK) before and after covalent protein attachment. Colloidal stability of liposomes in biological media was also investigated. Measurements were performed in triplicates.

2.5. Determination of protein functionalization efficiency

The amount of InvA497 or BSA coupled to the surface of liposomes was quantified using a bicinchoninic acid (BCA) kit, in accordance with the manufacturer's instructions (QuantiPro™; Sigma-Aldrich, Steinheim, Germany). This assay is suitable to measure even proteins covalently bound to surfaces [13]. Absorbance was measured at 562 nm and concentration was determined reference to preconstructed calibration curves of InvA497/BSA in the presence of control liposomes to correct for lipid interference. Protein quantification was also performed using SDS-PAGE and Western blot following a protocol by Schulze et al. [14].

2.6. Cell cultures and treatments

The human epithelial type 2, HEp-2 cell line (CCL-23 ™; ATCC, Manassas, VA, USA) originating from a human laryngeal carcinoma and expressing the β_1 integrin receptor was cultivated in Roswell Park Memorial Institute medium (RPMI 1640) (Gibco by Life Technologies™, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Lonza, Cologne, Germany) and kept in culture for a maximum of 2 months after thawing.

Caco-2 cells, clone C2Bbe1 (CRL-2102™; ATCC, Manassas, VA, USA) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 1% non-essential amino acids (PAA cell culture company, Pasching, Austria) and used at passages 58–72.

2.7. Cell viability assay

An ATP bioluminescent assay (ViaLight™ Plus, Lonza, Basel, Switzerland) was used to determine the viability of both HEp-2 and Caco-2 cells in response to treatment with liposomal formulations. This assay is based on enzymatic determination of ATP present in all metabolically active cells. Luciferase enzyme is employed to catalyze