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Invasin-functionalized liposome nanocarriers improve the intracellular delivery of anti-infective drugs

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Experimental

InvA497 overexpression and purification

A volume of *E. coli* BL21 expressing the His-tagged C-terminus 497 of invasin (His_6-InvA497) from *Y. pseudotuberculosis* was extracted and purified as previously described.[1] The bacteria were cultured at 37°C in Luria broth medium (Carl Roth GmbH, Karlsruhe, Germany) to an A600 = 0.4, then shifted to a culture temperature of 17°C and continued with incubation to an A_{600} = 0.6. The expression of His6-InvA497 was induced by adding 100 µM of isopropyl-β-D-thiogalactopyranoside, followed by an overnight incubation of cells at 17°C and subsequent washing. Pelleted cells were then resuspended in 50 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole; pH 8) and protease inhibitor solution (5 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin, 10 mM E46 protease inhibitor, 20 mM leupeptin and 10 mM chymostatin). Cells were subsequently destroyed using a french press. Affinity chromatography with Ni-NTA Agarose (Qiagen, Limburg, Netherlands) was performed to purify the formed His_6-InvA497 protein, followed by two cycles of dialysis against 10 mM Tris buffer (pH 8, containing 300 mM NaCl). Protein quantification was performed using the Bradford protein assay (Pierse, Rockford, USA).

Determination of liposomal phospholipid content

A colorimetric assay based on the ability of phospholipids to form a complex with ammonium ferrothiocyanate[2] was performed to quantify the phospholipid content of liposomes. Liposomes were first lysed in chloroform and mixed 1:1 with the ferrothiocyanate reagent (26 mM of ferric 3-chloride-hexahydrate and 0.39 M of ammonium thiocyanate in 1 l of distilled water, Sigma Aldrich, Steinheim, Germany). The samples were then vigorously vortexed for 20 s and centrifuged for 10 min at 130 g, resulting in phase separation. The optical density of the lower phase, consisting of phospholipids and chloroform, was then measured at 485 nm using a spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer). The obtained absorbance was then applied in conjunction with a calibration curve equation to calculate liposomal phospholipid concentration.

Determination of liposomal cholesterol content

A previously described HPLC method for cholesterol quantification was used, with some modifications.[3] Briefly, a Dionex HPLC system (Thermo Scientific, Germany) composed of a P680 pump, an Elite degassing System, an Asta-medica AG 80 column oven and a UV detector was employed. For separation, a LiChrospher® 100 RP-18 (5 µm) 125 x 4 mm column was used (Merck KGaA, Darmstadt, Germany). The column oven temperature was set at 30°C. A mobile phase of acetonitrile:methanol (70:30 v/v) with a flow rate of 2 ml/min was used, with an analysis time of 15 min and an injection volume of 100 µl. All samples were analyzed in triplicate, with reference to calibration curves created from cholesterol standards. Cholesterol was detected at a wavelength of 210 nm.

Liposome imaging

In order to determine the morphology of non-functionalized liposome formulations, scanning electron microscopy (SEM) was conducted using a Zeiss EVO HD15 SEM (Carl Zeiss Microscopy GmbH, Goettingen, Germany). Liposomes were washed with distilled water to remove any traces of PBS, and then further diluted 1:20 to avoid the formation of aggregates. A 10 µl volume of liposomes was mounted on aluminum stubs using double-sided adhesive carbon tape and silicon wafers in 5 x 5 mm chips (TED PELLA, Inc., California, USA). Samples were dried, then sputter-coated with thick gold film using a Quorum Q150R ES sputter-coater (Gala Instrumente GmbH, Bad Schwalbach, Germany) under argon atmosphere for secondary electron emissive SEM. Samples were analyzed at an acceleration voltage of 5000 kV, and images were processed with SmartSEM® software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Fluorescence imaging of InvA497-functionalized liposomes was also conducted, based on detection of the rhodamine component of liposomal 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss Rhod PE), by linking liposomes to poly-L-glutamic acid-coated glass. Briefly, glass bottomed dish chambers (3.5 cm x 3.5 cm) were coated with poly-L-glutamic acid solution (0.01% in distilled water) for 5 min at room temperature. Chambers were washed with distilled water and incubated with 2 mM N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDC) and 5 mM N-hydroxysuccinimide (NHS) in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6) for 15 min at room temperature, to activate poly-L-glutamic acid carboxyl groups. Unbound EDC/NHS was then removed and chambers were washed with MES buffer. InvA497-functionalized gentamicin-loaded liposomes, diluted 1:1, were then placed in the chambers and incubated for 2 h at room temperature in the dark. The crosslinking reaction was stopped using 50 mM TRIS-HCl buffer for 5 min, and then chambers were washed twice with MES buffer. Images were taken using a Leica DMI6000B microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a metal halogenide lamp and using an oil immersion lens 63x objective. Images were processed using Leica Application Suite Advanced Fluorescence (LAS AF) software.

Cellular invasion assays establishment and optimization

In order to clarify the optimal parameters for testing the anti-infective efficacy of liposome formulations, epithelial cells of the HEp-2 cell line were infected with either *S. enterica* or *Y. pseudotuberculosis*. Cells were cultured one day before the experiment in 24 well-plates, following exchange of cell culture medium for binding buffer (RPMI 1640 medium with 20 mM hydroxyethyl-piperazineethane-sulfonic acid buffer (HEPES) and 0.4% BSA). Bacteria were cultured 24 h prior to experiments in glass tubes containing 5 ml of Lennox broth (LB) medium (Carl Roth GmbH, Karlsruhe, Germany). Tubes were kept overnight in a shaking incubator (Infors AG, Bottmingen, Switzerland) at 37°C in the case of *S. enterica*, and 25°C for *Y. pseudotuberculosis*. *S. enterica* was then freshly diluted 1:100 with LB medium and incubated at 37°C for a further 3 h, to allow for growth to the late exponential phase and corresponding induction of Salmonella invasion proteins (Sips) gene expression. Cultured bacteria were then washed once and resuspended in PBS (Medicago, Uppsala, Sweden). The invasion load was assessed by using first different multiplicities of infection (MOI - bacteria:HEp-2 cell ratio) - 10, 25, 50 and 100 - following by centrifugation to facilitate sedimentation of bacteria onto the cells (6708 g for 5 min, Eppendorf 5810 R Centrifuge, Hamburg, Germany). Culture plates were incubated for 0.5 or 1 h in a humidified incubator at 37°C and 5% CO_2 atmosphere in order to allow for bacterial adhesion and cellular invasion. Cells were then washed twice with PBS and incubated for 1 h or 2 h with binding buffer containing 50 µg/ml of gentamicin solution (Sigma-Aldrich, Steinheim, Germany) for extracellular bacteria killing (EBK). Killed extracellular bacteria together with extracellular gentamicin were then removed by duplicate washing of infected cells with PBS. HEp-2 cells containing solely intracellular *S. enterica* or intracellular *Y. pseudotuberculosis* were then lysed, and the cell lysate was plated in sterile agar plates in serial dilutions. Plated lysates were incubated overnight at 37°C for *S. enterica* and for 48 h at 25°C for *Y. pseudotuberculosis*. Following incubation, bacterial colonies were counted and multiplied by the appropriate dilution factor and expressed as a percentage of the number of colonies from the inoculum, referred to as the percentage of invasion.

Results

Liposome Morphology

SEM visualization of liposomes after preparation via LFH revealed the presence of spherical vesicles of approximately 200 nm in diameter, confirming the results of photon correlation spectroscopy investigations (see main manuscript, Figure 1A). A representative SEM image of gentamicin loaded liposomes prepared by the LFH method can be seen in Figure S1A. InvA497-functionalized liposomes could be visualized using fluorescence microscopy, facilitated by the presence of rhodamine (Liss Rhod PE) in the formulation. Distinct points rather than a diffuse pattern of fluorescence were observed in imaged samples, reflecting an acceptable dispersion and integrity of liposomes (Fig. S1B).

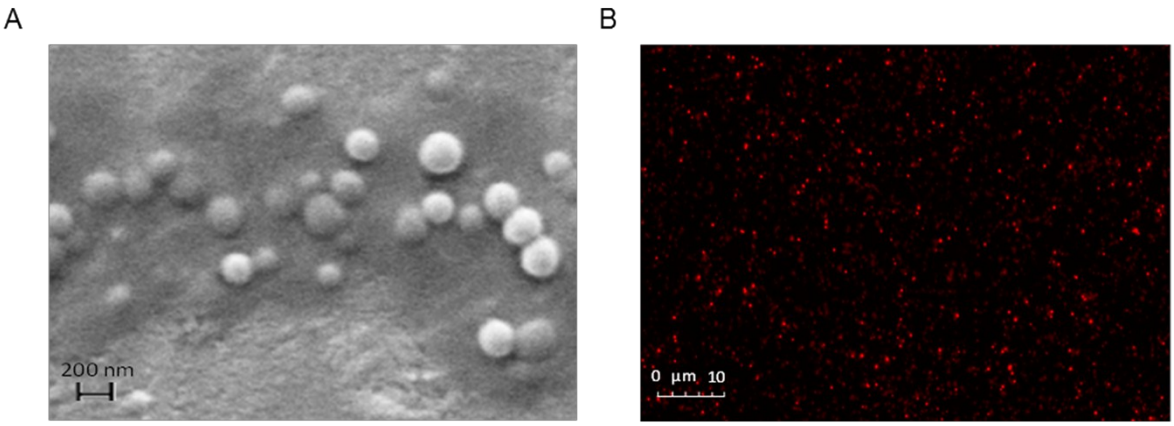

Fig. S1 (A) Representative SEM image of gentamicin-loaded liposomes prepared by LFH method showing spherical vesicles of approximately 200 nm in diameter. (B) Fluorescence image of InvA497-functionalized, gentamicin-loaded liposomes.

Cellular Invasion assays

Investigation of invasion parameters revealed that the employment of MOIs of 10 and 25 with 1 h infection time and either 1 or 2 h EBK resulted in the highest invasion rates for *S. enterica* (Fig. S2A) and also *Y.*

pseudotuberculosis (Fig. S2B). Using these results, further invasion assays were performed in order to determine the optimum conditions for establishment of intracellular infection (see main manuscript).

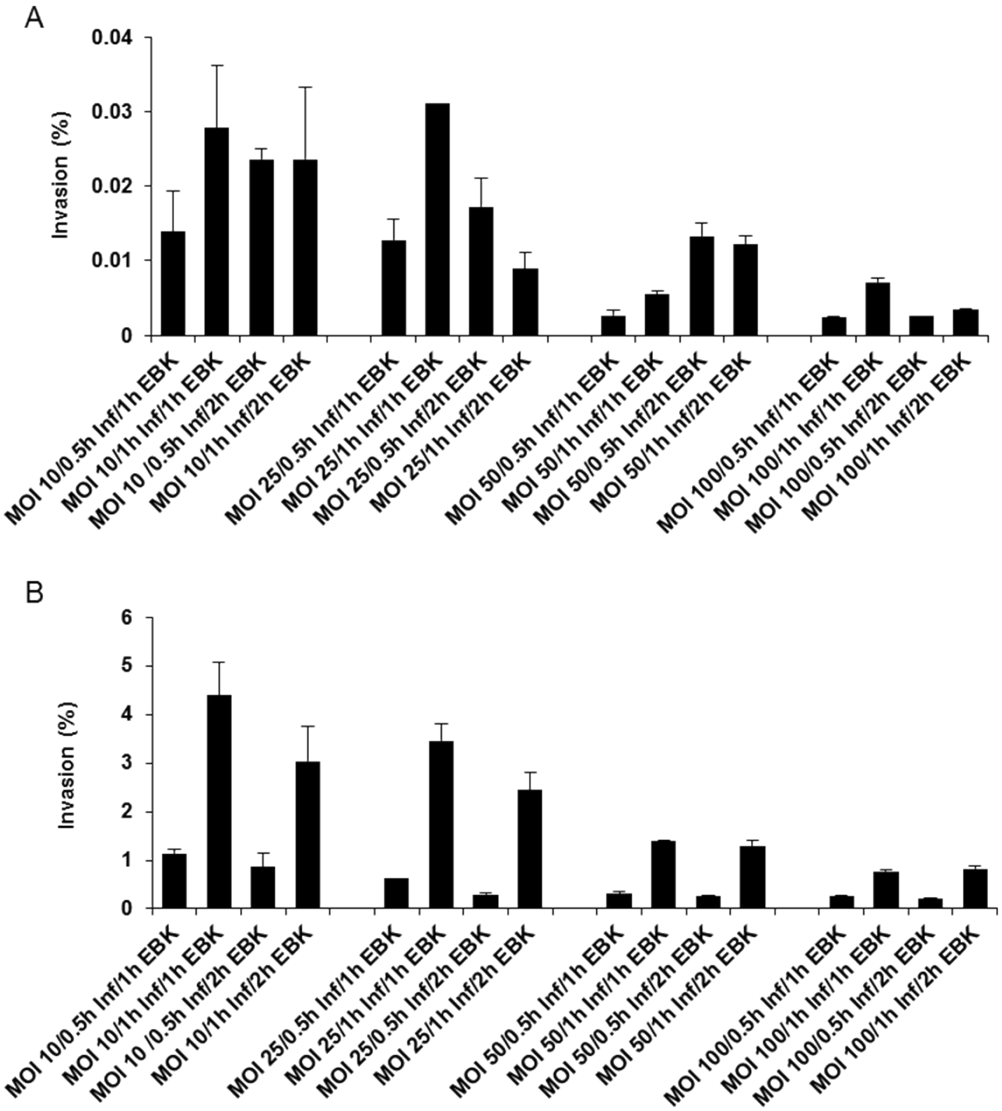

Fig. S2 Invasion percentage of *S. enterica* (A) and *Y. pseudotuberculosis* (B) tested in HEp-2 cells at MOIs of 10, 25, 50 and 100, with either 0.5 or 1 h infection time (Inf) followed by either 1 or 2 h of extracellular bacterial killing (EBK). Data represents the mean ± SEM of three independent preparations.

References

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