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Supporting Information for Proteomics

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Supporting information

NSOM- and AFM-based nanotechnology elucidates nano-structural and atomic-force features of a *Y. pestis* V immunogen-containing particle vaccine capable of eliciting robust response

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Full materials and methods

Bacterial strains and growth conditions Strains and plasmids used in this study were summarized in Table 1. *L. lactis* NZ 9700 was used for production of nisin. *L. lactis* PA1001, lacking *acmA* and *htrA*, was used for the production recombinant antigen. *L. lactis* strains were grown at 30℃ in M17 broth containing 0.5% (wt/vol) glucose (GM17) and when necessary, supplemented with 5 μl/ml of chloramphenicol for plasmid selection. Induction for P_{nisA}-driven gene expression was done by adding two times (OD600 0.5 and 1.0) of the sterilized (by 0.45μm filter) culture supernatant of the nisin-producing organism *L. lactis* NZ9700, containing secreted nisin A, at a 1:1000 dilution, and cultured overnight.

Production of the GEM microparticles *L. lactis* MG1363 was used for production of the GEM microparticles. The Chemical pretreatment of *L. lactis* MG1363 to generate GEM microparticles was performed follow the method from Leenhouts K[1]. Briefly, 5ml of fresh overnight cultured *L. lactis* MG1363 was added to 500ml of pre-warmed fresh GM17, and continuously incubated at 30℃ overnight in the standing culture. Cells were collected by centrifugation and washed once with 250ml of phosphate-buffered saline (PBS pH 7.2). Subsequently, 100ml acid solution (0.1M HCl pH 1) was added to the pellet and re-suspended the cells. The cell suspension was placed in boiling water for 30 minutes under atmospheric pressure. The cells were then washed three times in 250ml sterile PBS with vigorous vortexing. After the last washing step, cells were re-suspended in 50ml sterile PBS. Numbers of GEM microparticles per milliliter were determined by microscopy with a Burker-Turk counting chamber. One unit (U) is defined as 2.5×10^9 GEM microparticles, and standard stock solutions containing 10U/ml were stored at -80℃. The diameter of a GEM microparticle is about 800 nm, as determined by AFM.

Cloning of DNA encoding V antigen into *L. lactis* vector plasmids The gene encoding V-antigen was isolated by PCR from genomic DNA of *Y. pestis* and then cloned into pPA3 and pXmh, *lactococcal* vactors. pPA3 reconstructed plasmics named pPA3-V were used for the expression of V-antigen fusion with protein anchor (PA) under the control of the nisin A inducible promoter P_{nisA}. The secreted recombinant fusion proteins named V-PA, respectively. In addition, pXmh vector was used for expression of V-antigen which tagged by six histidine at C-terminal. V-His fusions are purified by His-tag isolation for coating of ELISA plates. The primers used for PCR are listed in Table 2. Plasmids were constructed by ligating a *NcoI*- and *SphI*-cleaved PCR-amplified fragment into the *NcoI* and *SphI* sites of plasmids. Electrotransformation of *L. lactis* PA1001 was performed by using a gene pulser (Bio-Rad) at 2.5 kV, 25μF and 200 Ohm.

Preparation of subunit GEM microparticle vaccine After construction, the expression vector plasmid was electrotransformed into *L. lactis* PA1001. The host cells were incubated in GM17 medium with chloramphenicol (5μg/ml) and incubated at 30℃ standing culture overnight, induced twice by adding nisin A containing supernatant (1:1000 dilution) at OD600 is 0.5 and 1.0. The supernatant was collected by

centrifugation and sterilized by 0.45μm filter, then used as a source of secreted fusion proteins. GEM microparticles were incubated with the cell-free supernatant containing immunogen-PA fusion proteins at a concentration of 2×10^8 GEM microparticles per ml at room temperature for 1 hour with slowly rotary shaking. Then, the immunogen-loaded GEM microparticles were collected by centrifugation, washed twice with PBS and stored in 20μl PBS per unit (2.5×10^9 microparticles), saved in −20℃ freezer until further use.

Protein purification for AFM-based single-molecule force-binding and ELISA analyses For protein purification by FPLC-F10, unpurified 0.2ml V antigen protein or 0.2ml V-PA protein was applied onto a RESOURCE 15Q column (10 × 64 mm) (BioRad BioLogic DuoFlow system FPLC-F10) equilibrated with start buffer 20mM-Na_2HPO_4 (pH 8.5). The column was washed with start buffer to remove proteins not adsorbed. The proteins bound to the ion-exchanger were eluted by 60% starting buffer and 40% elution buffer supplemented with 1M NaCl at a flow rate of 5 $mL\cdot min^{-1}$ for V antigen protein and 2 $mL\cdot min^{-1}$ for V-PA protein. The fractions containing the desired protein were collected, desalted, and concentrated by ultra-filtrating in a Millipore Amicon ultra equipped with a 30 kD membrane. Purified proteins were subjected to SDS-PAGE analysis. The molecular weight of the resulting V and V-PA proteins determined by SDS-PAGE coincided with the calculated value of 38.95 kD and 64.36 kD respectively. For the purification of His-Tagged V antigen, the C-terminal His-tagged antigens were purified by using Ni-NTA agarose (Qiagen) at room temperature after concentrating the protein containing supernatant. 4 volume concentrated protein containing supernatant mixed with 1 volume 5× native binding buffer (250 mM NaH_2PO_4/ 2.5 M NaCl/ Imidazole 15 mM, pH 8.0), allowing the His-tagged proteins to bind to Nickel-Chelating Resin at room temperature for 2 hours in a rotator. Columns were washed with washing buffer (50 mM NaH_2PO_4/ 500 mM NaCl/ Imidazole 20 mM, pH 8.0). The His-tagged proteins were eluted with elution buffer (50 mM NaH_2PO_4/ 500 mM NaCl/ Imidazole 250 mM, pH 8.0). Desalting and concentration of proteins were performed by 10 kD centrifugal filter (Millipore). Then the FPLC purified proteins were used for AFM-based single-molecule force measurement whereas His-tagged proteins were used for ELISA analyses.

Confocal imaging For confocal microscopy imaging, GEM loaded *Y. pestis* V-PA, V or control protein Ysc

were first fixed by 2% formalin PBS solution and labeled by mouse monoclonal biotin-anti-Myc antibody (Sigma, MO) to label Myc motif between PA and V followed by quantum dot (QD) streptavidin conjugates 655 (Invitrogen, CA). For each labeling step, PBS was used to wash twice to remove any unbound antibody according to manufacturer's instruction. Before labeling, QD streptavidin conjugates were filtered by Millipore Ultrafree filter (diameter 100 nm) to remove any QD aggregations, as described by our recent work[2]. Finally, GEM were suspended in dd water and loaded on slides for confocal imaging on a Carl Zeiss LSM510 Meta5 laser scanning confocal microscope equipped with a 63x1.2 water-inmmersion objective or a 100x1.30 oil-immersion objective.

AFM-tip functionlization Briefly, the cleaned AFM cantilevers (Veeco, CA) were immediately floated on 1% APTES (Sigma, MO) in toluene solution for 2 h at room temperature. And then tips were rinsed thoroughly with the solvent to wash out the unbound silane. Then the tips were immersed into 2% glutaraldehyde solution in dd water for 20 min and then rinsing thoroughly with dd water. After that, tips were immersed in protein solutions for 2 h followed by floating in glycine solution to block the free CHO groups. Finally, theses tips were washed by PBS twice to remove any unbound chemical components or proteins.

Animals, immunization, sample collection, and ELISA measurement of the titer of V immunogen-specifice antibody response 6-8 week old Balb/C female mice (180-200g) were purchased from University of Illinois at Chicago (UIC) and housed under biosafety level 2 conditions. All animal experiments were performed with approval of the Animal Experimentation Committee of the UIC. After anesthesia by intraperitoneally injecting 0.4mg/kg of Fentanyl, mice were intranasally immunized with GEM without V antigen (control group) or 20μl of GEM loading V immunogen (test group) at week 0 (prime), week four (boost), and week eight(re-boost), respectively. Blood samples of about 1 ml were collected by retro-orbitally bleeding at weeks 2, 4, 5, 6, 9, and 10 to glass tubes and supplemented with heparin. The clot was allowed to contract overnight at 4°C before centrifugation and placing the serum into 1.7-ml labeled Eppendorf tubes. ELISA was then used to determine the titers of anti-V IgG antibody in circulating blood as described by our recent publication[3].

Table 1. Bacterial strains and plasmids used in this study

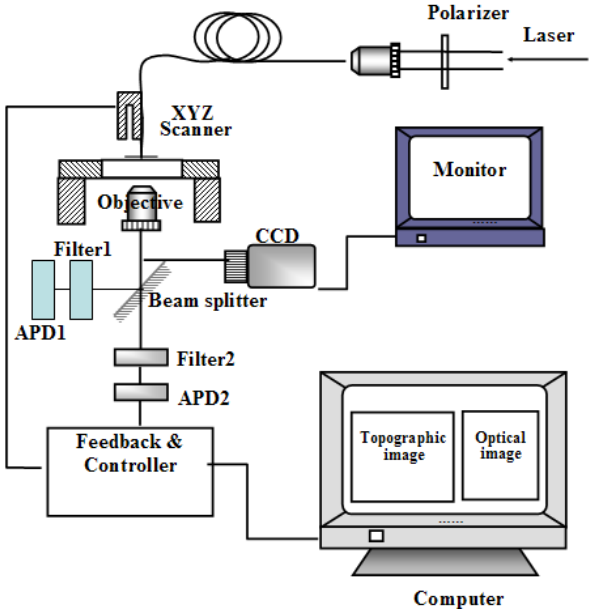
Strains	Relevant phenotype(s) or genotype(s)	reference
L. lactis PA1001	Derivative of *L. lactis* lacking *acmA* and *htrA*	[1]
L. lactis NZ9700	Nisin producer	
L. lactis MG1363	Plasmid-free derivative of *L. lactis* for making GEM	
Plasmids	Relevant phenotype(s) or genotype(s)	reference
pPA3	Cm^r, containing c-*myc*, the *acm*A PA under control of P_{nisA}, and $usp45_{ss}$	[1]
pXmh	pPA3 derivative containing Cm^r, c-*myc*, the *acm*A PA under control of P_{nisA}, and $usp45_{ss}$ adding a his-tag and a stop code before the *acm*A PA	This study

Table 2 Primers used for PCR

primers		Enzyme	Vector plasmid
F1p1	5'- gcccatggcagatttaactgcaagc -3'	NcoI	pPA3 and pXmh
F1p2	5'- gagcatgcttggttagatacggttac -3'	SphI	
Vp1	5'- gcccatggtgattagagcctacgaac -3'	NcoI	
Vp2	5'- gagcatgctttaccagacgtgtcatc -3'	SphI	
yscFp1	5'- gcccatggtgagtaacttctctggat -3'	NcoI	pPA3
yscFp2	5'- gagcatgctgggaacttctgtaggatg -3'	SphI	
yscFpa	5'- gtgcggccgcaatgagtaacttctctggat -3'	NotI	pXmh
yscFpb	5'- gatctagaaatgggaacttctgtaggatg -3'	XbaI	

Supplementary Figure

Fig S1 Schematic depiction of the single-molecule-sensitive NSOM.



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