

## ELECTRONIC SUPPLEMENTARY MATERIAL

### Synthesis and immunological evaluation of virus-like particle-milbemycin A<sub>3</sub>/A<sub>4</sub> conjugates

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#### Part II. Synthesis of milbemycin-M-L4-BSA conjugate

For synthesis of covering antigen M-L4-BSA, milbemycin hemisuccinate **M-L4** (6.5 mg) was dissolved in dimethylformamide (DMF; 100  $\mu$ l) and added to a solid mixture of EDC (1-ethyl-3- [3-dimethyl-aminopropyl] carbodiimide; 2.3 mg) and sulfo-NHS (N-hydroxysulfo-succinimide; 2.6 mg). The resulting reaction mixture was shaken for 4 h at room temperature. Then the excess of EDC was neutralized with mercaptoethanol (1.4  $\mu$ l).

The reaction mixture from previous step (100  $\mu$ l) containing freshly prepared sulfo-NHS activated ester was slowly added to bovine serum albumin solution (1 ml; BSA, Serva, Heidelberg, Germany; 10 mg/ml in 200 mM sodium borate, pH 8.0) and incubated for 16 h at 4 °C on a rotator mixer. The reaction was stopped by adding Tris·HCl (20  $\mu$ l; 1M, pH 7.0, 1M). Visual inspection of the sample after reaction revealed a slight turbidity. Then, the M-L4-BSA conjugate was dialyzed against 200 volumes of 1xPBS buffer at 4 °C for 16 h, clarified by centrifugation (16,000 g, 10 min), and analysed by UV-spectroscopy and mass spectrometry. (Fig. SII-1).

As can be seen in Fig. SII-1, the UV spectrum of BSA was considerably changed after coupling of **M-L4**. Characteristic for milbemycins absorbance peak at 245 nm was observed after coupling of milbemycin hemisuccinate **M-L4**. Moreover, mass spectrometry analysis demonstrated that all BSA molecules were modified: no molecules with molecular weight of free BSA (66.5 kDa) were detected. Predominant molecular weight of the reaction product was 78.5 kDa (Fig. SII-1, B) corresponding to a molecular weight of BSA with approximately 17 coupled **M-L4** residues.

The M-L4-BSA conjugate was used as covering antigen in ELISA tests and as a control antigen in immunization experiments.

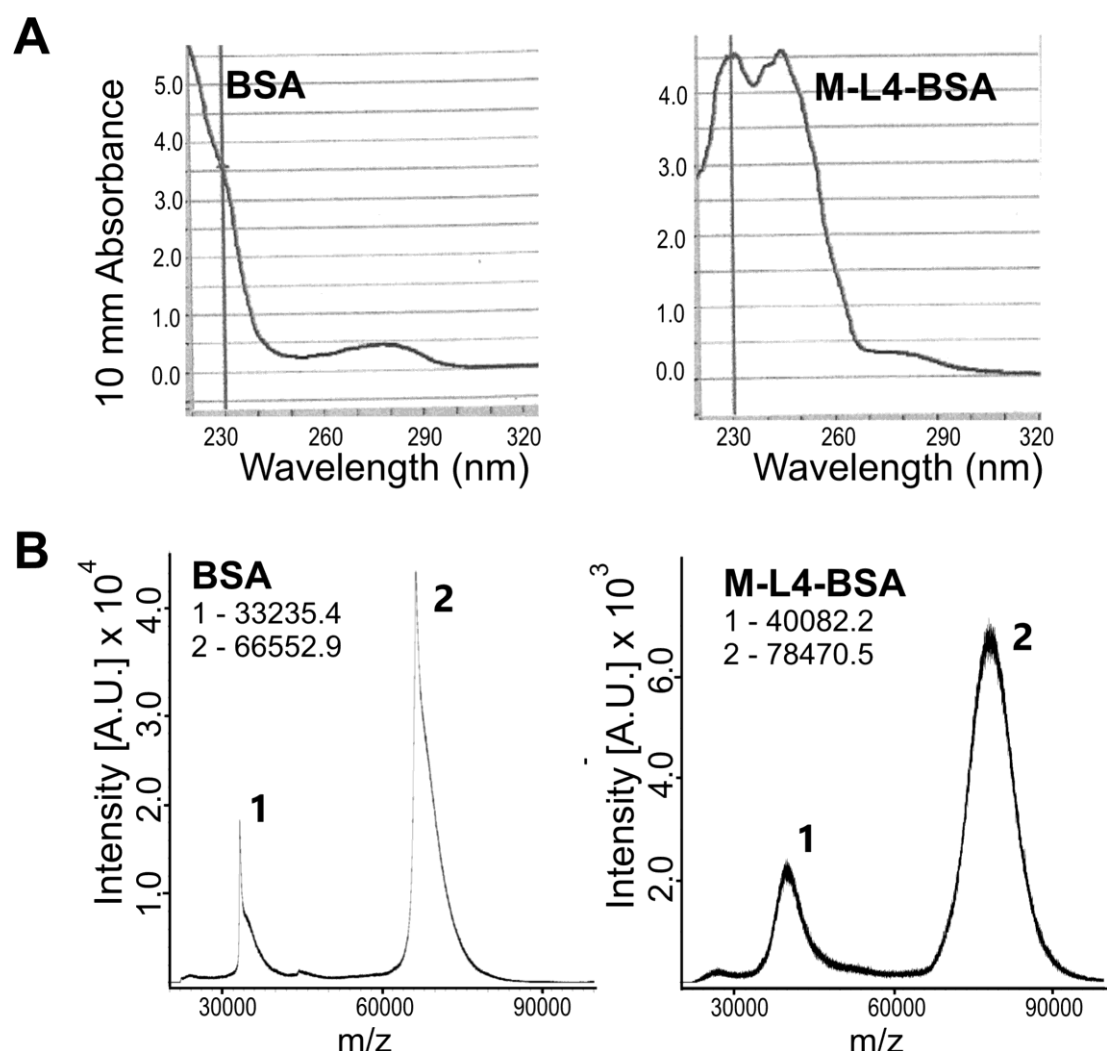


Fig. SII-1. Comparison of UV and mass spectra of unmodified bovine serum albumin (BSA) and milbemycin-M-L4-BSA conjugate (M4-BSA). A - UV spectra of BSA and M-L4-BSA derivative. The UV spectrum of protein solution (1 mg/ml) was recorded using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). B – Mass spectrometric analysis. BSA samples (1 mg/ml) were diluted with a 3-hydroxypicolinic acid matrix solution and were spotted onto an MTP AnchorChip 400/384TF. Matrix-assisted laser desorption/ionization (MALDI)-TOF MS analysis was carried out on an Autoflex MS (Bruker Daltonik, Bremen, Germany). The protein molecular mass calibration standard II (22.3–66.5 kDa; Bruker Daltonik) was used for mass determination.