Electronic Supplementary Information

EXPERIMENTAL SECTION

The polypeptide-block-peptide block copolymer $PBLG_{50}$-K was prepared and characterized as described previously.[17-19] Influenza hemagglutinin (HA) antigen (H3N2 Wisconsin strain) was obtained from Solvay (Weesp, The Netherlands). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). ELISA plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were ordered from Southern Biotech (Birmingham, USA). Chromogen 3,3′,5,5′-tetramethylbenzidine (TMB), substrate buffer for ELISA, GM-CSF and interleukin-4 (IL4), were provided by Biosource-Invitrogen (Breda, the Netherlands). Hank's Balanced Salt Solution (HBSS), fetal bovine serum (FCS) and all culture media, including penicillin/ streptomycin (PEST) and trypsin were supplied from Gibco (Invitrogen, Carlsbad, CA). Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands) and Rompun® (20 mg/ml Xylazine, Bayer B.V.,Mijdrecht, The Netherlands) were obtained from the pharmacy of Leiden University Medical center. 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were acquired from Sigma-Aldrich (Zwijndrecht, NL), unless stated otherwise.

$PBLG_{50}$-K polymersomes were prepared by a solvent evaporation method as was previously described.[19] Briefly, $PBLG_{50}$-K (0.02 µmol) was dissolved in 2 ml tetrahydrofuran (THF) in a 50 ml round flask, then 3 ml of HEPES sucrose buffer (Hepes 20 mM, sucrose 10% (w/w), pH 7.4) were added all at once to the polymer solution, and the mixture was homogenized by vortexing for 1 minute (200 rpm). Finally, the THF was removed by rotary evaporation at 30 kPa, 25°C for 10 minutes. HA loaded polymersomes were prepared by adding the HA stock solution (453 µg/ml) to the preformed $PBLG_{50}$-K polymersome suspension (100 µg/ml), resulting in a final HA concentration varying between 2.5 to 50 µg/ml.

Particle size distributions were determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments). The zeta potential of the particles was measured by laser Doppler velocimetry on the same instrument. The $PBLG_{50}$-K concentration was determined with a BCA protein assay (Pierce) according to the manufacturer's instructions, using albumin standard. Transmission electron microscopy (TEM) was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing 5 µL of solution on carbon-coated copper grids. After ~5 min, the droplet was removed from the edge of the grid. A drop of 2% phosphotungstic acid (PTA) or 2% osmium tetraoxide (OsO_4) stain was applied and removed after 2 min.

The degree of HA association to the polymersomes was determined by filtration as described previously [28]. For this study we used labeled HA (IRDye® 800CW, Licor). Briefly, the polymersomes/HA complexes and free HA were filtered through polycarbonate membranes (Whatman, Nucleopore) of 0.1 µm pore size, using an extruder (T001 10 ml, Thermobarrel Extruder Lipex Biomembrane). Under these conditions the polymersomes are retained on the filter and free HA passes through. The amount of HA in the filtrate was quantified with an Infinite M100 microplate reader (Tecan).

Toxicity of the formulations on Caco-2 cells was assessed using the MTT method[29]. Caco-2 cells (10,000/well) were seeded in a 96-well plate (Nunc) and maintained for 2 days at 37°C and 5% CO_2. After 48 h exposure to a range of concentrations of the $PBLG_{50}$-K polymersomes, the cells were washed twice with Hank's Balanced Salt Solution (HBSS) and incubated for 3 h with 0.5 mg/ml MTT in DMEM. Medium was removed and the purple formazan crystal was dissolved in 100 µL DMSO. Absorbance at 570 nm was measured using a µQuant ELISA plate reader (Biotek).

The immunogenicity study was achieved with female C57-BL/6 mice, 8-weeks old at the start of the vaccination study, were purchased from Charles River, and maintained under standardized conditions in the animal facility of the Leiden/Amsterdam Center for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands. The mice received two subcutaneous injections of 200 µl vacine: a prime (day 1) and a boost (day 21). We used two different HA dosages: 0.5 µg and 2 µg HA/injection. The antigen was either injected alone or mixed with polymersomes (100 µg/ml). Blood samples were taken one day before prime and boost, and 3 weeks after the boost. IgG titers were determined by ELISA.

The IgG subtype profile of influenza-specific antibodies was checked on day 20 and 42 by sandwich ELISA as previously described.[30] Briefly, ELISA plates (Greiner) were coated overnight at 4°C with 100 ng/well of influenza subunit antigen (H3N2) in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.6 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37°C. Thereafter the plates were washed three times with PBST. Two-fold serial dilutions of sera from individual mice were applied to the plates and incubated for 2 h at 37°C. Plates were incubated with HRP-conjµgated goat antibodies against either mouse IgG, IgG1 or IgG2a (Invitrogen) for 1.5 h at 37°C. After washing, plates were incubated with TMB and the reaction was stopped with sulfuric acid (2M). The detection was done by measuring optical density at 450 nm. Antibody titers were expressed as the reciprocal of the sample dilution that corresponds to half

of the maximum absorbance at 450 nm of a complete s-shaped absorbance–log dilution curve.

Hemagglutination inhibition (HI) titers in serum were determined as described by Amorij et al.[31] Briefly, serum was inactivated at 56°C for 30 min. In order to reduce nonspecific hemagglutination, 25% kaolin suspension was added to inactivate sera. After centrifµgation at 1,200×*g*, 50 µL of the supernatant was transferred in duplicate to 96-well round-bottom plates (Greiner) and serially diluted twofold in PBS. Then, four hemagglutination units of A/Wisconsin influenza inactivated virus were added to each well, and the plates were incubated for 40 min at room temperature. Finally, 50 µL of 1% guinea pig red blood cells were added to each well and incubated for 2 h at room temperature. The highest dilution capable of preventing hemagglutination was scored as the HI titer.

Antibody and HI titers were logarithmically transformed before statistical analysis. Unpaired Student's t-test analysis was performed for each antigen dosage, in order to demonstrate significant differences between the two experimental groups (HA alone and the polymersome/HA mix). The statistical analysis was carried out using Prism (Graphpad) and a p value less than 0.01 was considered to be significant.

References

28. D. Lemoine and V. Preat, *J Control Release*, 1998, **54**, 15-27.
29. J. Weyermann, D. Lochmann and A. Zimmer, *Int J Pharm*, 2005, **288**, 369-376.
30. M. Amidi, S. G. Romeijn, J. C. Verhoef, H. E. Junginger, L. Bungener, A. Huckriede, D. J. Crommelin and W. Jiskoot, *Vaccine*, 2007, **25**, 144-153.
31. J. P. Amorij, V. Saluja, A. H. Petersen, W. L. Hinrichs, A. Huckriede and H. W. Frijlink, *Vaccine*, 2007, **25**, 8707-8717.