Chapter 3

Polymersomes enhance the immunogenicity of influenza subunit vaccine

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Abstract

In this study, peptide polymersome based on the peptide polymer –K are tested as an immune adjuvant for an antigen, influenza hemagglutinin (HA). The polymersomes are loaded with HA antigens and the in vivo immunogenicity of the resulting hybrid assemblies was tested in vivo, resulting in an improvement of the immune response for the influenza antigen co-administered with the polymersomes.

Introduction

Vaccination against influenza remains the most effective method to prevent infection by the virus and to reduce the associated morbidity and mortality.1 Seasonal influenza vaccines currently in use are mostly subunit formulations, consisting of hemagglutinin antigens (HAs) from a mixture of strains. The downside of these vaccines is their relatively low immunogenicity, which can necessitate their administration with an adjuvant, i.e. a component, added to the antigen to enhance its immunogenicity, although the currently marketed seasonal influenza vaccines do not contain any adjuvant. Well known examples of adjuvants that are licensed for use in humans are colloidal aluminum salts and emulsions, such as MF59. Adjuvants can act in several different ways, e.g. by creating an antigen depot at the injection site, by protecting the antigen from enzymatic degradation, by improving the delivery of the antigen to dendritic cells (DCs) or by activating DCs.[2] Various types of nanoparticles have been shown to be able to act as antigen delivery systems which can combine several of these mechanisms.[3,4] Polymer as well as lipid based nanoparticles with HA have been successfully tested, enhancing antigen uptake by the DCs and resulting in enhanced antigen-specific acquired immune responses.[5,6] Nanoparticles can range in size from 10 to 1000 nm, and some studies have shown that the uptake of particles by DCs and their immune-stimulating effect is dependent on their size.[7] Nanoparticles can vary in several other properties, such as composition, surface charge and hydrophobicity. The nanoparticles can be loaded with antigens by adsorption, covalent attachment, or encapsulation. However, the elicited immune responses of the current formulations do not offer adequate protection and there is still a need for new alternatives. Polymersomes are self-assembled polymer shells composed of block copolymers.[8–10] These block copolymers have amphiphilic properties similar to lipids, but they have much larger molecular weights, and for this reason they have been compared with viral capsids, composed of large polypeptide chains. Depending on the choice of the block copolymer, its molecular weight and biocompatibility, polymersomes can be used as delivery systems with a broad range of tunable properties.[11–13] Polymersomes have shown to be stable, in terms of size and structure,[14] they enable the encapsulation of both hydrophilic and hydrophobic species and can carry functional moieties, such as structures with cell penetrating capabilities.[15] Moreover, polymersomes based on the degradable di-block polymer polyethylene glycol–polybutadiene functionalized with an HIV-derived Tat peptide successfully enhanced, in vitro, the cellular delivery of nanoparticles to DCs.[16] These results highlight the potential use of polymersomes as robust, virus like antigen delivery systems, but they have not been tested for vaccination yet. Recently, we developed a new class of polypeptideblock- peptides which self-assemble into polymersomes.[17–19] These particles were shown to be stable for several months. The hydrophilic peptide block is composed of a specific amino acid sequence that is

able to form a coiled–coil complex [20,21] allowing for the non-covalent functionalization of the polymersome surface with functional moieties. The ability of this recognition motif was indicated by the development of non-covalent triblock copolymers and model systems for membrane fusion.[22,23] The hydrophobic block is composed of poly(g-benzyl-L-glutamate) and both blocks adopt an a-helical conformation when the amphiphiles are assembled into polymersomes.

The aim of this study was to investigate whether polymersomes can enhance the immunogenicity of a HA subunit vaccine. The polypeptide- block-peptide used in this study was the rod–rod block copolymer $PBLG_{50}$-K,[17] where $PBLG_{50}$ is the hydrophobic poly-(γ-benzyl-L-glutamate) block with an average degree of polymerization of 50, and K is a hydrophilic designed peptide with amino acid sequence $G(KIAALKE)_3–NH_2$. This amphiphilic block copolymer has been shown to self-assemble into polymersomes with a size of 250 nm. The HA antigen was from a H3N2 A/Wisconsin strain, which is currently used for seasonal vaccination in combination with HA from two other strains. The association of the antigen with the polymersomes was investigated, and the DC-stimulating capacity in vitro and the immunogenicity in mice of the HA–polymersomes were compared with that of plain HA.

$PBLG_{50}$-K has been shown previously to assemble into well-defined polymersomes in aqueous buffered solutions.[17–19] The polymersome size can be tuned from 200 to 2000 nm with low polydispersity by varying the conditions during the self-assembly process, such as ionic strength and temperature, or the preparation method used.[18,19] As previously stated the interaction of nanoparticles with DCs and the resulting immune-stimulating effect is dependent on their size,[24] with optimum DC uptake for particles with a diameter of 0.5 micron and below. Therefore for this study, polymersomes at the lower end of this size range were selected. In HEPES sucrose at 20 °C $PBLG_{50}$-K self-assembled into polymersomes with a hydrodynamic diameter of about 250 nm, a polydispersity index of 0.1 (Fig. 1) and a zeta potential (ZP) of -40 mV. The polymersomes were stable, with no sign of turbidity/sedimentation and no detectable aggregation, as observed with dynamic light scattering (DLS) and electron microscopy, for at least 4 weeks. The HA proteins are elongated molecules that extend 13 nm from the exterior of influenza viruses, being anchored in the viral membrane by means of a hydrophobic domain. This hydrophobic domain causes free HA to aggregate in aqueous solutions. The HA used in this study formed clusters with an average hydrodynamic diameter of about 50 nm in PBS, as measured by DLS. The binding of HA in these clusters is proposed to be relatively weak, as no clear aggregates were observed with transmission electron microscopy (TEM) when sampleswere stained with either OsO_4 (pH7), or PTA (pH 2 or 7.4) (Fig. 2B inset). Upon the addition of the HA solution to the preformed $PBLG_{50}$-K polymersomes an immediate particle size

increase was observed by DLS. Higher final concentrations (from 0.5 to 50 mg.mL^{-1}) of HA resulted in larger HA/polymersome aggregates (Fig. 1).

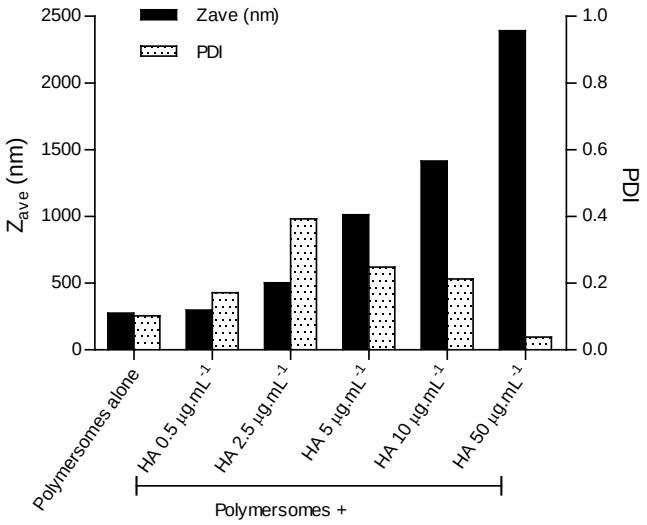

Fig. 1: Average hydrodynamic diameter (Z_{ave}) and polydispersity index (PDI) of a fixed amount of $PBLG_{50}$-K polymersomes (100 µg.mL^{-1}), mixed with a raising amount of HA (final concentration ranging from 0.5 to 50 µg.mL^{-1}).

A comparative study with another kind of polymersomes, based on a PBLG-E polymer,[18] with a different hydrophilic peptide sequence $G(EIAALEK)_3–NH_2$, was conducted and showed no sign of aggregation (data not shown), indicating that the nature of the peptide has a direct impact on the HA/polymersome interaction. TEM revealed that the plain $PBLG_{50}$-K polymersomes did not aggregate which is in accordance with the DLS data (Fig. 2A). For the polymersome/HA mixtures, clustering was observed, with the HA presumably acting as a non-covalent crosslinker (Fig. 2B and D). The TEM images also revealed that the HA proteins interact with the polymersomes in a relatively weak manner as the shape and size of individual polymersomes did not change. The association of HA with the polymersomes was further investigated by filtering the suspension through 0.1 mm filters. Under these conditions the $PBLG_{50}$-K polymersomes were retained on the filter. The filtration of free HA showed a recovery of 88%, whereas for the polymersome/HA complexes it dropped to 28%, showing that most of the HA was associated with the polymersomes.

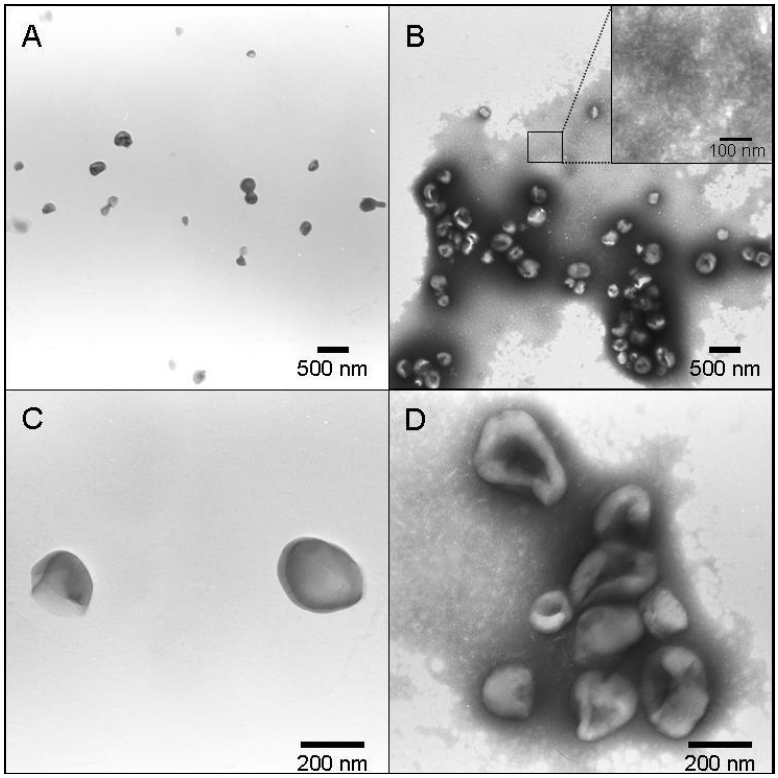

Fig. 2: TEM images (PTA staining) of the polymersomes (A, C) and the mixture of polymersome with 10 μg.mL^{-1} HA (B, D).

The adjuvant effect of PBLG$_{50}$-K polymersomes (mixed with HA antigen) was investigated in an immunization study with mice. In order to study the effect of the polymersomes without any masking from the antigen alone, we used HA doses of 0.5 and 2.0 mg per immunization (corresponding to HA concentrations of 2.5 and 10 mg.mL^{-1} in the formulations). The polymersome concentration was kept constant for all the formulations (100 mg.ml^{-1}), resulting in a final HA/polymersome weight ratio of 1/40 and 1/10, respectively. The two doses of HA were tested in the presence and absence of polymersomes. The HA-specific serum IgG, IgG1 and IgG2a were assessed after the first (prime) and the second (boost) immunization, and hemagglutination inhibition (HI) titers, as ameasure for the level of functional antibodies, were measured after the boost. After both the prime and the boost (Fig. 3A and B), PBLG$_{50}$-K polymersomes significantly enhanced the IgG titers compared to non-adjuvanted HA for the high-dose (2 mg HA) group. In the low-dose group (0.5 mg) there was also a trend toward higher IgG responses for the polymersome formulation as compared to free HA, although the differences were not statistically significant. The IgG1 titers closely followed the total IgG titers, while the IgG2a titers (after prime and boost) were below the detection limit for all groups (results not shown). The HI titer was assessed by measuring the inhibition

by the mouse sera of HA-induced red blood cell agglutination. The sera from mice immunized with non-adjuvanted HA showed a dose dependent HI titer, which was close to the detection limit of the assay for the low-dose group (Fig. 3C). For both HA doses polymersomes acted as adjuvant, as higher HI titers were found, ca. 20 fold for the low HA dose and 8 fold for the high dose, although the latter increase was not statistically significant.

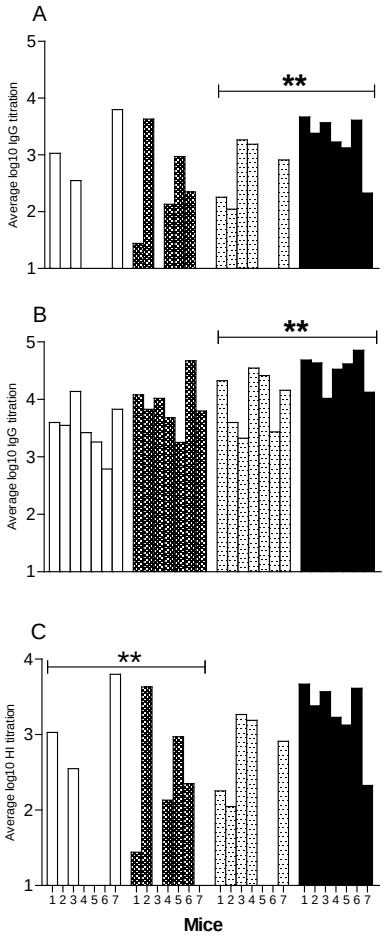

Fig. 3: Immune response in mice after subcutaneous injection of HA formulations: HA-specific serum IgG titers after prime (A) and boost (B), and HI titers after boost (C). Each bar represents the titer of an individual mouse. The formulations tested are: ▭ 2.5 μg.mL^{-1} HA, ▩ 2.5 μg.mL^{-1} HA + PBLG$_{50}$-K, ⁘ 10 μg.mL^{-1} HA, ■ 10 μg.mL^{-1} HA + PBLG$_{50}$-K. (**: significant difference between the average titers of each group, for $p < 0.01$).

The cytotoxicity of the polymersomes has been tested in vitro. The cell viability was evaluated in Caco-2 cells using the MTT assay. The cells were exposed for 48 hours to a polymersome concentration range of 0.5 to 10 mg.mL^{-1} (the necessary

dilution in the cell culture media did not allow the testing of higher polymersome concentrations). The resulting percentages of cell viability showed no sign of toxicity for any of the polymersome concentrations tested (results not shown).

In summary, the immunization study shows an increase of the serum IgG and HI titers, when the antigen is co-administered with the polymersomes. The improvement of the immune response against HA, when associated with the polymersomes demonstrates that the polymersomes can act as an adjuvant. The HA/polymersome hybrid has been characterized with DLS and electron microscopy showing that HA forms complexes with the polypeptide-block-peptide based polymersomes. The HA/polymersome association is presumably a combination of both electrostatic and hydrophobic interactions, arising from the hydrophobic membrane-anchoring domain of the HA, the localized charge on the HA, and the charged corona of the polymersomes. This is confirmed by the difference observed in the DLS study between PBLG-K and PBLG-E. The presence of three protonated lysine residues (at pH 7.4) in K, instead of three glutamic acids in E, had changed the electrostatic interactions between HA and the polymersome surface, resulting in different aggregation behaviors. The mechanism by which polymersomes act as an adjuvant is unknown, but could include a depot effect,[5] the ability to target the antigen presenting cells (APCs) with the antigen/adjuvant complexes and enhancement of the antigen uptake by APCs.

As detailed in the introduction, the antigen used in this study is one of the main components of the current subunit seasonal influenza vaccines (which consist of a mixture of HA from different strains). Since neutralizing antibody levels expressed as HI titers are considered to be the main protective immune component for parentally administrated subunit vaccines,[25,26] and as this assay is also the test of reference according to the industry standards, the increase of the systemic immune response against HA formulated with $PBLG_{50}$-K polymersomes, as observed in our study, is a clear improvement compared to the current formulation (HA alone). However, in order to provide a superior level of protection against the influenza infection than HA alone, it has been shown previously that an IgG2a response (indicative of a Th1 immune response) is the strongest isotype in response to viral infection.[27] Our formulations induced low IgG2a titers, therefore our future research will be directed at enhancing the IgG2a response of the polypeptide-*block*-peptide polymersomes by coencapsulation of immuno-modulators.

In conclusion, the work detailed in this paper demonstrates, for the first time, that polymersomes are able to enhance the immunogenicity of an antigen. Moreover, it shows the proof of concept that they can be used as a delivery tool for influenza subunit vaccine with enhanced immune response and no sign of cellular toxicity.

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 $\mu g.mL^{-1}$) to the preformed $PBLG_{50}$-K polymersome suspension (100 $\mu g.mL^{-1}$), resulting in a final HA concentration varying between 2.5 to 50 $\mu g.mL^{-1}$.

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% CO2. 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 $\mu g.mL^{-1}$ 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 C57BL/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 $\mu g.mL^{-1}$). 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

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