Pharm Res (2015) 32:3018–3028
DOI 10.1007/s11095-015-1683-5

RESEARCH PAPER

Reducing the Visibility of the Vector/DNA Nanocomplexes to the Immune System by Elastin-Like Peptides

Faranak S. Nouri [1] • Xing Wang [1] • Xuguang Chen [1] • Arash Hatefi [1,2]

Received: 12 February 2015 / Accepted: 18 March 2015 / Published online: 31 March 2015

ABSTRACT

Purpose One of the major hurdles facing nanomedicines is the antibody production against nanoparticles that subsequently results in their opsonization and clearance by macrophages. The objective of this research was to examine and identify the sequence of a low-immunogenic peptide based on recombinant elastin-like polypeptides (ELPs) that does not evoke IgG response and can potentially be used for masking the surfaces of the nanoparticles.

Methods Biopolymers composed of a DNA condensing domain in fusion with anionic, neutral and cationic elastin-like peptides were genetically engineered. The biopolymers were used to complex with plasmid DNA and form ELP-coated nanoparticles. Then, the potential immunogenicity of nanoparticles in terms of IgM/IgG response after repeated injections was evaluated in Balb/c immunocompetent mice.

Results The results revealed the sequence of a non-immunogenic ELP construct that in comparison to control group did not elicit any significant IgG response, whereas the vector/DNA complexes that were coated with polyethylene glycol (PEG) did elicit significant IgG response under the same conditions.

Conclusions The identification of the sequence of an ELP-based peptide that does not induce IgG response opens the door to more focused in-depth immunotoxicological studies which could ultimately lead to the production of safer and more effective drug/gene delivery systems such as liposomes, micelles, polymeric nanoparticles, viruses and antibodies.

KEY WORDS elastin · elastin-like polypeptides · ELP · PEG immunogenicity · recombinant polymer

ABBREVIATIONS

ELP	Elastin-like polypeptides
Ig	Immunoglobulin
pCpGfree	Plasmid DNA devoid of CpG islands
pDNA	Plasmid DNA
PEG	Polyethylene glycol
pEGFP	Plasmid DNA encoding green fluorescent protein

INTRODUCTION

One of the major hurdles facing nanomedicines (liposomes, micelles, viruses, etc.) is the recognition of nanoparticles by the immune system and production of IgG antibodies after repeated injections. This results in significant uptake of nanoparticles due to antibody opsonization and their rapid clearance from the blood circulation by the reticuloendothelial system and consequently sub-optimal delivery of the drugs/genes to the target tissues (1). To minimize recognition by immune system and opsonization, the surface of nanoparticles has traditionally been sterically stabilized with the help of hydrophilic polymers such as polyethylene glycol (PEG) (2,3). Although different theories exist (4), some believe that hydrophilic polymers on the surface of particles attract water shells due to their high degree of hydrophilicity resulting in reduced adsorption of opsonins and recognition by reticuloendothelial system. However, recent reports have demonstrated that repeated injections of PEGylated nanoparticles in rats and mice elicit PEG-specific antibodies, which is then responsible for the rapid elimination of subsequent doses

✉ Arash Hatefi
ahatefi@pharmacy.rutgers.edu

[1] Department of Pharmaceutics, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA

[2] Rutgers Cancer Institute of New Jersey, New Brunswick, New Jersey 08903, USA

of PEGylated nanoparticles (5–7). It has been suggested that the immune response against PEG is related to the presence of PEG molecules in high density on the nanoparticles' surfaces that could enhance aggregation followed by opsonization. As a result, there is a growing interest among scientists to develop alternative biocompatible polymers (7).

In recent years, one macromolecule that has received considerable attention is the genetically engineered elastin-like polypeptide (ELP) with a wide variety of applications from tissue engineering to drug delivery (8). ELP sequence is derived from tropoelastin which is an endogenous protein in the body and the possibility of eliciting an immune response to its amino acid sequence is minimal (9–12). Because elastin-like polypeptides based on tropoelastin are shown to be non-immunogenic (12) and biodegradable (13) and their use for shielding the surface of nanoparticles is unexplored, the *objective* of this research was to assemble nanoparticles that are coated with different short hydrophilic ELP sequences and screen for one that does not elicit IgG response.

Elastin is an extracellular matrix protein consisting of several repetitive amino acid sequences, including VPGVG, APGVGV, VPGFGVGAG and VPGG (14). Most recombinant ELPs are based on the repetitive pentapeptide motif (V-P-G-X-G), where the "guest amino acid residue", X, can be any combination of all natural amino acids except proline (15). Depending on the type of X guest residue, the structure of this well characterized biopolymer can be simply manipulated to assume various degrees of hydrophilicity or hydrophobicity (16). To achieve the objective, we genetically engineered biopolymers composed of a plasmid DNA (pDNA) condensing domain, namely RH_3, in fusion with ELP sequences of different hydrophilic properties (RH_3ELP). Our group has previously demonstrated that the RH_3 biopolymer which consists of repeating units of RRVRRSHRRRHT, is able to condense pDNA effectively into nanosize particles with applications in pDNA/siRNA delivery (17,18).

The ELP sequences in RH_3ELP biopolymers were designed to be short (eight repeats of VPGXG, Mw: ~3 kDa) and have E/G (hydrophilic and negatively charged), S/G (hydrophilic and neutral), A/G (hydrophobic and neutral) and K/G (hydrophilic and positively charged) as guest residues at the fourth position. It has previously been shown by Urry and colleagues that the shorter the length of the ELP sequence, the more hydrophilic and soluble the polymer chain (19). It has also been calculated that the presence of E, K, S and G guest residues in the ELP sequence significantly increases its solubility (hydrophilicity) (15).

MATERIALS AND METHODS

Cloning, Expression and Purification of the Recombinant Biopolymers

The details of cloning and expression methods for similar constructs have previously been reported by our group (18). For simplicity, the recombinant biopolymers are shown as RH_3ELP_{AG}, RH_3ELP_{SG}, RH_3ELP_{EG}, and RH_3ELP_{KG} (Table I). In brief, the genes encoding RH_3ELP_{AG}, RH_3ELP_{EG}, RH_3ELP_{KG} and RH_3ELP_{SG} with N-terminal histags were designed by our group and synthesized by Integrated DNA Technologies (Coralville, IA). A N-terminal NdeI and C-terminal XhoI restriction sites were also designed for cloning purposes. The synthesized genes were double digested with NdeI and XhoI restriction enzymes (New England Biolabs, Ipswich, MA) and cloned into the pET21b expression vector (Novagen, MA). After verifying the sequence, each construct was transformed into BL21(DE3) (Novagen, MA) expression vector. Starter cultures were prepared by inoculation of a single fresh colony of BL21(DE3) harboring expression plasmids in 5 mL of LB medium containing 50 μg/ml carbenicillin incubated at 37°C overnight. The next day, 5 mL of the starter culture was used to inoculate 500 mL of Circlegrow media (MP Biomedicals) containing 50 μg/mL carbenicillin, which resulted in an OD_{600} of 0.1. The culture was grown at 37°C for 3 h to reach the OD_{600} of 1.2. Gene expression was induced by isopropyl β-D-1- thiogalactopyranoside (IPTG) 1 mM at 37°C. The cells were collected after 5 h by centrifugation at 5000 g for 20 min. All biopolymers were then purified using Ni-NTA affinity chromatography. The cell pellets were suspended in lysis buffer (urea 8 M, NaCl 2 M, $NaH_2PO_4$100mM, Tris 10 mM, Triton 1%, imidazole 10 mM) and stirred for 30 min. The lysates were centrifuged at 37,000 g for 1 h to sediment the insoluble fractions. The supernatant was transferred to another tube and incubated with Ni-NTA resins which were equilibrated with lysis buffer for 1 h on ice. The resins were loaded onto a column and washed first with 70 mL of lysis buffer and then with 45 mL of wash buffer (urea 5 M, NaCl 1.5 M, NaH_2PO_4 100 mM, Tris 10 mM, imidazole 40 mM). Finally, the peptides were eluted with elution buffer (urea 3 M, NaCl 0.5 M, NaH_2PO_4 100 mM, Tris 10 mM, imidazole 200 mM).

Solid-Phase Peptide Synthesis of Control Constructs

RH_3 and PEGylated RH_3 (*i.e.*, RH_3PEG3500) were synthesized using solid phase peptide synthesis technique with >95% purity by Biopeptek Inc. (Malvern, PA). PEG3500 was conjugated to RH_3 at C-terminal *via* a covalent bond. R_8ELP_{KG} and R_8ELP_{SG} polymers were also synthesized by using solid

Table I The Amino Acid Sequences and Corresponding Molecular Weights (Mw) of the Genetically Engineered Biopolymers RH_3ELP_{AG}, RH_3ELP_{SG}, RH_3ELP_{EG}, and RH_3ELP_{KG}

Name	DNA condensing motif sequence	ELP sequence	Peptide Mw (Da)
RH_3ELP_{AG}	$(RRVRRSHRRRHT)_3$	VPGAGVPGAGVPGGG VPGAGVPGAGVPGGG VPGAGVPGAG	8006
RH_3ELP_{SG}	$(RRVRRSHRRRHT)_3$	VPGSGVPGSGVPGGG VPGSGVPGSGVPGGG VPGSGVPGSG	8102
RH_3ELP_{EG}	$(RRVRRSHRRRHT)_3$	VPGEGVPGEGVPGGG VPGEGVPGEGVPGGG VPGEGVPGEG	8354
RH_3ELP_{KG}	$(RRVRRSHRRRHT)_3$	VPGKGVPGKGVPGGG VPGKGVPGKGVPGGG VPGKGVPGKG	8349

phase peptide synthesis technique with >95% purity at Rutgers University Chemical Biology core facility (Table II).

Biopolymer Desalting and Preparation of Stock Solution

To desalt, the G-25 sepharose column (GE Heathcare) was first conditioned with HEPES buffer (100 mM, pH 7.4) and then loaded with the biopolymer solution. The elute fractions were collected and the concentrations of the desalted biopolymers were measured by Bradford protein assay (Hercules, CA) according to manufacturer's protocol. Briefly, four dilutions of bovine serum albumin including 0.125, 0.25, 0.5 and 1 mg/ml were prepared as the standard samples. 250 µl of Bradford dye was transferred to each well of 96-well plate and 5 µl of each standard and test samples were added to the dye and incubated at room temperature for 5 min. The absorbance was measured at 595 nm by spectrophotometer. The standard samples were used to plot a standard curve and from that the test sample concentrations were calculated.

DNA Neutralization and Condensation

The neutralization of negatively charged plasmid DNA (pDNA) by positively charged biopolymers was examined by a gel retardation assay. Different amounts of desalted biopolymers and 1 µg pDNA (pEGFP, Clontech, CA, USA) were each diluted to 50 µL with HEPES buffer (100 mM pH 7.4) in separate tubes. The biopolymer solution was then added to pEGFP solution and incubated at room temperature for 15 min before loading onto the agarose gel. The mobility of pDNA was visualized on an agarose gel by ethidium bromide staining.

The pDNA neutralization data from the gel retardation assay was used to study the ability of the biopolymers to condense pDNA effectively into nanosize particles. Predetermined amounts of each biopolymer were mixed with 1 µg of pEGFP to make nanoparticles at different weight/weight ratios. Desired amounts of biopolymer and pDNA were each diluted to 50 µl with HEPES buffer (100 mM pH 7.4) in separate tubes. Nanoparticles were formed by addition of biopolymer to pEGFP solution using the flash mixing method. In this method (nanoprecipitation), biopolymer solution is rapidly added to pEGFP solution all at once. Nanoparticles were incubated at room temperature for 15 min before measuring hydrodynamic radius by dynamic light scattering (173° angle detector) using Malvern NanoZS Zetasizer (Malvern Instruments, U.K). Three independent batches of each biopolymer/pDNA complexes were prepared and the results are presented as mean ± s.d ($n=3$).

Particle Surface Charge and Conductivity Analysis

Nanoparticles were prepared as described above and the zeta potential of the nanoparticles and conductivity of the suspending media were measured by Laser Doppler Velocimetry using Malvern NanoZS Zetasizer (Malvern Instruments, U.K). Three independent batches of each biopolymer/pDNA complexes were prepared as mentioned

Table II The Amino Acid Sequences and Corresponding Molecular Weights (Mw) of the Synthetically Made Peptides RH_3, RH_3-PEG3500, R_8ELP_{SG} and R_8ELP_{KG}

Name	DNA condensing motif sequence	ELP sequence	Peptide Mw (Da)
RH_3	$(RRVRRSHRRRHT)_3$	–	4983
RH_3PEG_{3500}	$(RRVRRSHRRRHT)_3$–PEG3500	–	4983
R_8ELP_{SG}	RRRRRRRR	VPGSGVPGSGVPGGG VPGSGVPGSGVPGGG VPGSGVPGSG	4387
R_8ELP_{KG}	RRRRRRRR	VPGKGVPGKGVPGGG VPGKGVPGKGVPGGG VPGKGVPGKG	4633

above in HEPES buffer (100 mM) and the results are presented as mean±s.d ($n=3$). To plot the standard curve for conductivity, NaCl was dissolved in HEPES buffer (pH 7.4) to make 1.5 to 100 mM solutions.

Immunogenicity Study

Balb/c immunocompetent mice (6–8 weeks old) with an average weight of 20 g were purchased from Jackson Laboratories Inc. (Maine, USA). All animals were cared for in accordance with the Institutional Animal Care and Use Committee approved protocols. Mice were divided randomly into different groups (5mice/group) and injected with pDNA alone (3 μg) or in complex with RH_3, RH_3-PEG_{3500}, RH_3ELP_{AG}, RH_3ELP_{EG}, RH_3ELP_{KG}, RH_3ELP_{SG}, R_8ELP_{KG} and R_8ELP_{SG} (equivalent of ca. 60 μg). The pDNA used in this study was either pEGFP or pCpGfree (Invivogen). pCpGfree is an engineered plasmid DNA free of any CpG Islands. The pEGFP was propagated in DH5α E.coli strain (Invitrogen), whereas pCpGfree was propagated in GT115 E.coli strain (Invivogen). On day zero, blood was collected by submandibular puncture using a 5 mm animal lancet (GoldenRod, NY, USA). Approximately 200 μl of blood was collected in 1.5 ml tubes, centrifuged at 15,000 rpm for 10 min to pellet the cells. The plasma was then removed and transferred to a new tube and kept in −80°C freezer for further analysis. On days 7 and 21, animals were immunized *via* retroorbital injection of the venous sinus using a 27 G needle. Retroorbital injection is a more reliable method for i.v. injection than tail vein (20). On day 28, animals were euthanized, blood was drawn *via* cardiac puncture using a 25 G needle and transferred into 1.5 ml tubes and processed as mentioned above (Fig. 1). ELISA (Bethyl laboratories, Montgomery, TX) was performed to determine IgG and IgM levels in plasma samples according to manufacturer's kit and protocol.

RESULTS

Biopolymer Production

Using standard genetic engineering techniques, the genes encoding RH_3ELP_{AG}, RH_3ELP_{SG}, RH_3ELP_{EG}, and RH_3ELP_{KG} recombinant biopolymers were cloned into a pET vector and expressed in E.coli. This expression method was similar to what we have reported previously for RH_3 biopolymer (17). The fidelity of the sequences to the original designs was verified by DNA sequencing (Table I). Because of their smaller sizes (<40 amino acids), the control peptides R_8ELP_{KG}, R_8ELP_{SG}, RH_3 and RH_3PEG3500 were made cost effectively by using solid phase peptide synthesis method (Table II).

DNA Neutralization and Condensation

A gel retardation assay was performed to examine the ability of the recombinant biopolymers and the control peptides to complex with pDNA and neutralize its negative charges. This experiment helped us identify the amount of biopolymer needed to complex with 1 μg of pDNA and effectively retard its mobility. For example, 12.7 μg of RH_3ELP_{AG}, 12.8 μg of RH_3ELP_{SG}, 18.5 μg of RH_3ELP_{EG}, 10.3 μg of RH_3ELP_{KG}, 8.4 μg of R_8ELP_{KG}, 13.8 μg of R_8ELP_{SG}, 7.2 μg of RH3 and 7.2 μg of RH3-PEG3500 were used to complex and fully neutralize 1 μg of pEGFP. The results demonstrated that all biopolymers were able to effectively complex with pDNA and retard its mobility on agarose gels (Fig. 2a).

To examine whether the neutralized pDNA were not only neutralized but also condensed, all constructs were complexed with pDNA as mentioned above and then characterized in terms of hydrodynamic radius. The results of this study showed all biopolymers were able to complex with pDNA and form nanoparticles with average diameters of 200 nm or less (Fig. 2b). Overall, there was no significant difference in hydrodynamic radius among nanoparticles of RH_3ELP_{AG}, RH_3ELP_{EG}, RH_3ELP_{SG} and R_8ELP_{SG} (ANOVA, $p>0.05$). There was also no significant difference in hydrodynamic radius among nanoparticles of RH_3, RH_3ELP_{KG} and R_8ELP_{KG} (ANOVA, $p>0.05$).

Nanoparticle Surface Charge and Conductivity Analysis

The vector/pDNA complexes were also characterized in terms of surface charge. The surface charge study revealed that as the zeta potential decreased the nanoparticle diameters increased. As expected, RH3-PEG3500 could form small size nanoparticles with 0 mV surface charges. The results also

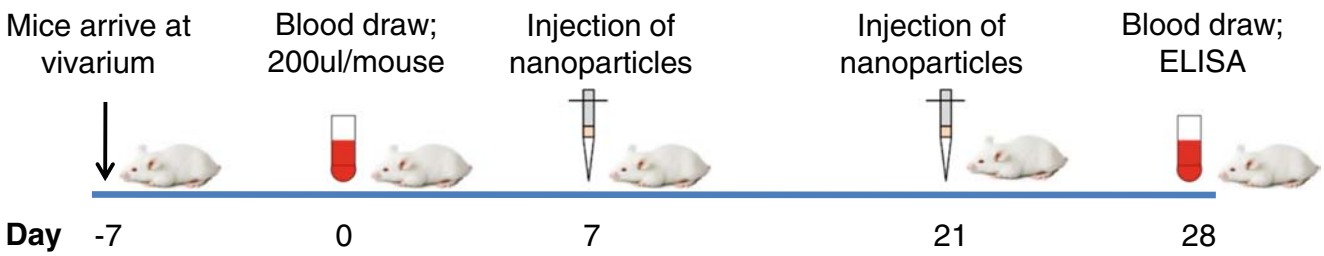

Fig. 1 The timeline and dosing schedule for the evaluation of IgM/IgG response against biopolymer/pDNA complexes.

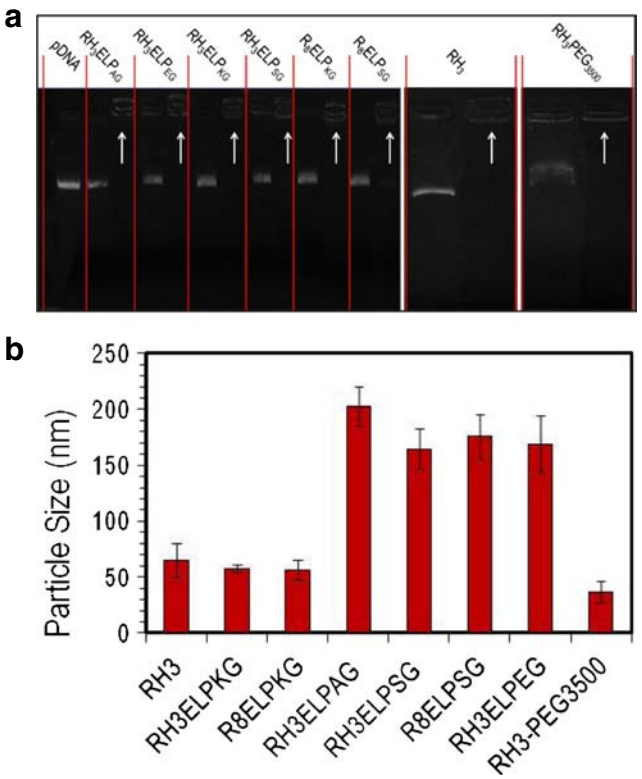

Fig. 2 (**a**) Gel retardation assay demonstrating pDNA charge neutralization. (**b**) Analysis of hydrodynamic radiuses of particles formed through complexation of pDNA with RH_3, RH_3ELP_{KG}, R_8ELP_{KG}, RH_3ELP_{AG}, RH_3ELP_{SG}, R_8ELP_{SG}, RH_3ELP_{EG} and RH3-PEG3500.

showed the prepared nanoparticles had zeta potentials ranging from 0 to 6 mV (Fig. 3a). We did not observe any significant difference in terms of surface charge among nanoparticles of RH_3ELP_{AG}, RH_3ELP_{EG}, RH_3ELP_{SG}, R_8ELP_{SG} and RH3-PEG3500 (ANOVA, $p>0.05$).

The conductivity of the media in which nanoparticles were suspended was also measured by the zetasizer. The results revealed that the conductivities of media for all vector/pDNA complexes were less than 5 ms/cm (Fig. 3b). Based on the plotted standard curve, the 5 ms/cm value corresponds to the conductivity of approximately 50 mM NaCl solution (Fig. 3c). The HEPES buffer showed negligible conductivity at all measured concentrations. For example, the conductivity of HEPES with 100 mM concentration was less than 0.015 ms/cm.

Evaluation of Immune System Response After Repeated Injection of Nanoparticles

Using the injection protocol shown in Fig. 1, the nanoparticles were injected into immunocompetent mice and the IgM/IgG responses were measured by ELISA. First, by using the standard curve for IgM, the IgM response to all genetically engineered biopolymers (*i.e.*, RH_3ELP_{AG}, RH_3ELP_{EG}, RH_3ELP_{SG} and RH_3ELP_{KG}) that were in complex with pEGFP was measured (Fig. 4a and b). Naked pEGFP and

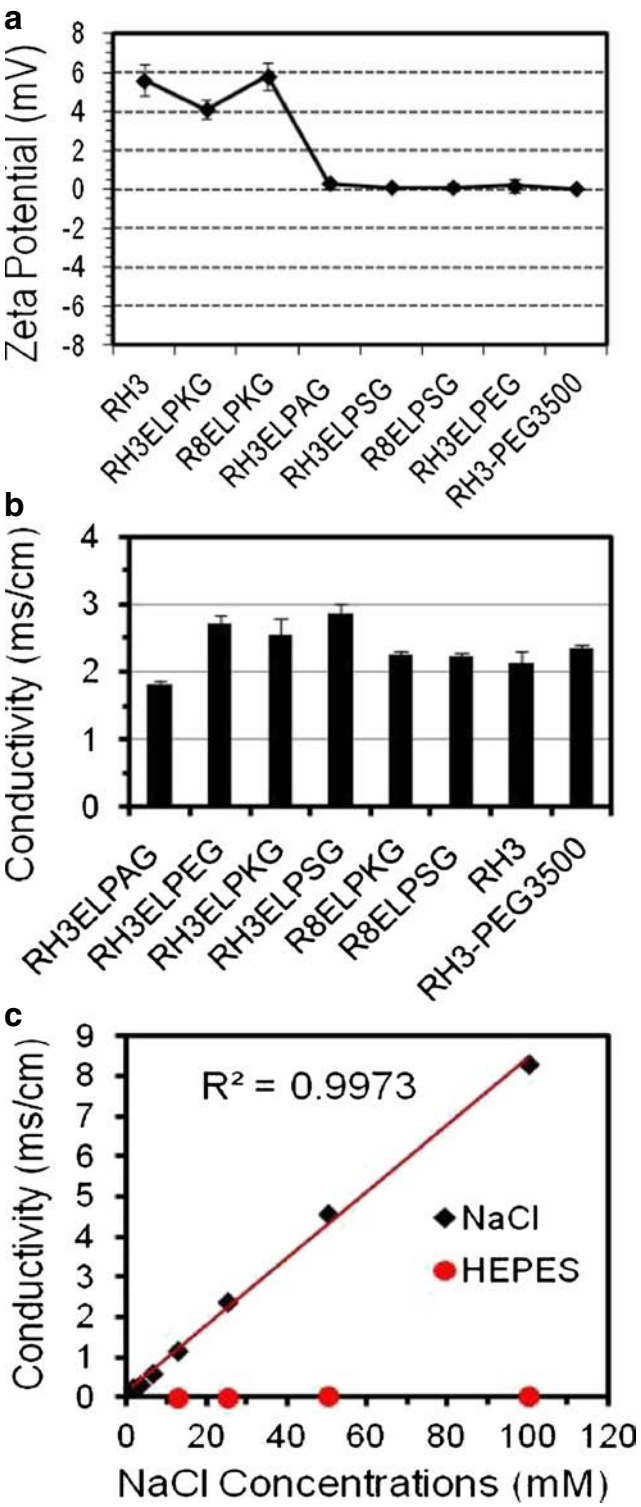

Fig. 3 (**a**) Particle charge analysis of pDNA in complexation with RH_3, RH_3ELP_{KG}, R_8ELP_{KG}, RH_3ELP_{AG}, RH_3ELP_{SG}, R_8ELP_{SG}, RH_3ELP_{EG} and RH3-PEG3500. (**b**) Conductivity of samples containing peptide/pDNA complexes. (**c**) Standard curve of conductivity *versus* NaCl and HEPES concentrations.

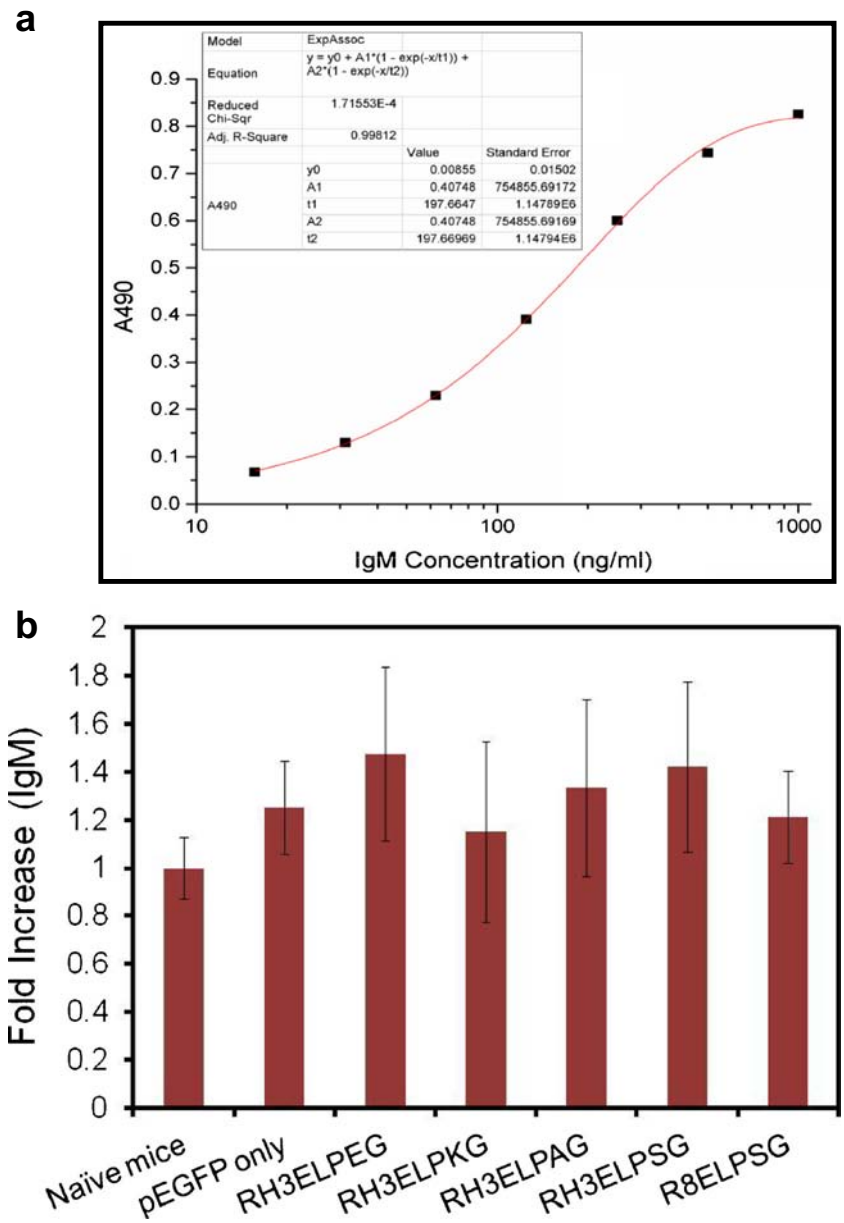

Fig. 4 (**a**) The standard curve fit that was generated to measure the IgM concentrations in mice blood. (**b**) Analysis of IgM production against biopolymer/pDNA complexes. The panel shows the number of folds increase in IgM levels after injecting mice with repeated doses of pEGFP alone or pEGFP in complex with biopolymers.

R_8ELP_{SG}/pEGFP complexes were used as controls. The results of this study showed no significant increase in IgM levels with any of the biopolymer/pDNA complexes or controls (ANOVA, $p>0.05$) (Fig. 4b).

In the next step, we plotted the standard curve for IgG and from that measured the IgG levels in all animal groups (Fig. 5a and b). In comparison to the HEPES buffer and pEGFP control groups, the results indicated a significant increase in IgG response to nanoparticles that were formed through complexation of pEGFP with RH_3ELP_{KG}, R_8ELP_{KG} and RH_3ELP_{EG} but not with RH_3, RH_3ELP_{AG}, RH_3ELP_{SG}, R_8ELP_{SG} (*t*-test, $p<0.05$) (Fig. 5b). In comparison to HEPES group, mice that were treated with naked pEGFP showed mild increase in IgG levels although not statistically significant (*t*-test, $p=0.1$). Furthermore, we did not observe any statistically significant difference between the IgG responses to R_8ELP_{KG} vs RH_3ELP_{KG} and R_8ELP_{SG} vs RH_3ELP_{SG} (*t*-test, $p>0.05$) (Fig. 5b). Among all peptides tested, RH_3ELP_{SG} was the least immunogenic (*$p<0.05$).

To remove the potential immune response against CpG islands in pEGFP, the RH_3ELP_{SG} biopolymer was complexed with pCpGfree and the IgG response was measured as described above. Naked pCpGfree, RH_3/pCpGfree and RH_3PEG3500/pCpGfree complexes were used as controls. The results of this study illustrated that RH_3ELP_{SG} sequence did not induce any significant IgG response (*t*-test, $p>0.05$), whereas the RH_3PEG3500 was mildly immunogenic (*t*-test, *$p<0.05$) (Fig. 6).

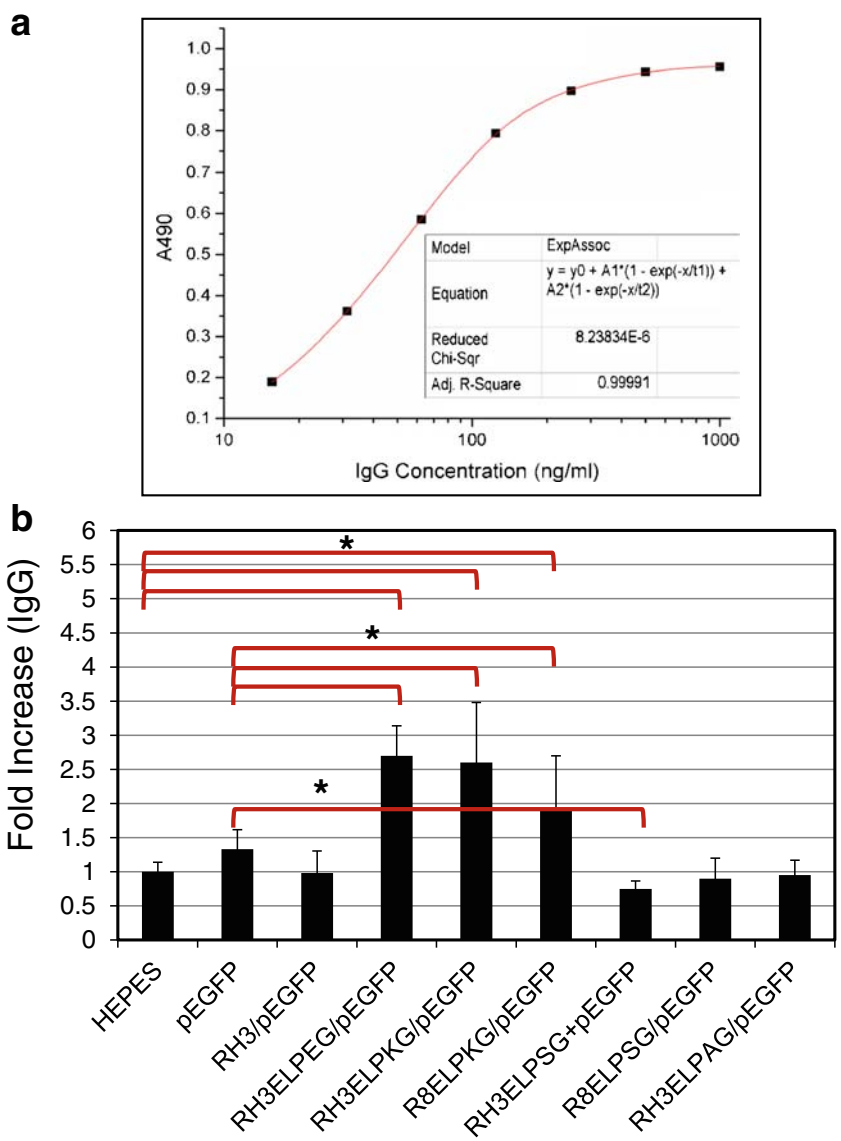

Fig. 5 (**a**) The standard curve fit that was generated to measure the IgG concentrations in mice blood. (**b**) Analysis of IgG production against biopolymer/pEGFP complexes and control groups. The panel shows the number of folds increase in IgG levels after repeated injections. * indicates statistical significance.

DISCUSSION

The purpose of this study was to examine the potential application of elastin-like peptides in masking the surface of nanoparticles and minimizing the risk of evoking the immune response. As shown in Table I, the ELP sequences in all constructs were composed of eight repeating units of VPGXG. Our experience with ELPs in the past has shown that more than eight repeating units of ELP tend to produce peptide aggregates making the nanoparticle assembly process non-reproducible. Therefore, in this study we limited the VPGXG repeats to eight. The recombinant biopolymers were then expressed in E. coli expression system, purified and desalted. The RH_3, RH_3-PEG3500, R_8ELP_{SG} and R_8ELP_{KG} peptides were also synthesized, desalted and used as controls in subsequent studies. The desalting step before nanoparticle formation is important due to the following three reasons. First, high salt concentration induces aggregation among ELP chains which is not suitable for nanoparticle assembly process (16). Second, it helps remove the excess ions from the system and stabilize the particles' diameters by minimizing the possibility of inter-particle salt bridge formation and ensuing aggregation. Third, excess salt (>50 mM) interferes with electrophorectic mobility of the nanoparticles resulting in inconsistent zeta potential measurements.

To examine the ability of the biopolymers in complexing with pDNA and neutralizing the negative charges, we performed a gel retardation assay. This experiment was performed to identify the amount of biopolymer that is needed to complex with all pDNA in solution and ensure that no pDNA is remained free (uncomplexed). The results of this assay showed that all biopolymers were able to bind efficiently to pDNA and neutralize its charges and the presence of ELP did not interfere with the complexation process. These results

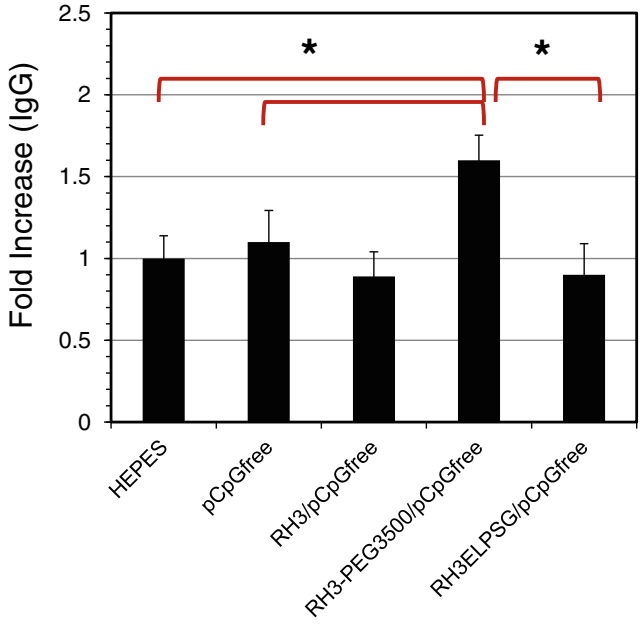

Fig. 6 Analysis of IgG production against biopolymers in complex with pCpGfree plasmid. The panel shows the number of folds increase in IgG levels after injecting mice with control and treatment groups. * indicates statistical significance.

also indicated that the short ELP sequences (eight repeats of VPGXG) in the structure of fusion biopolymers did not promote self aggregation. As a result, the positively charged residues were not buried and remained available to make pDNA condensation process possible (Fig. 2a). As expected, the amount of biopolymers needed to effectively condense pDNA was proportional to their molecular weights and the number of cationic residues in their sequences. For example, almost equal amounts of RH_3ELP_{AG} and RH_3ELP_{SG} biopolymers (*i.e.*, 12.7 μg and 12.8 μg) were needed to effectively neutralize 1 μg pDNA. Each of these two biopolymers has 21 cationic residues in its sequence, whereas the molecular weight of RH_3ELP_{AG} is ~96 Da less than RH_3ELP_{SG}. Therefore, slightly more RH_3ELP_{SG} (0.1 μg) was used to do the same job as RH_3ELP_{AG}. Since immune system response to foreign materials is dose dependent, in immunogenicity studies equal amounts of pDNA and/or biopolymers were injected into mice in order to eliminate the dose related bias. Using the DNA retardation assay data, we then measured the hydrodynamic radiuses of the biopolymer/pDNA complexes to examine whether the biopolymers were able to effectively condense pDNA into nanosize particles.

The particle size analysis study showed that all biopolymers could condense pDNA into nanoparticles with hydrodynamic radiuses of less than 200 nm which indicates effective condensation (Fig. 2b). In comparison to RH_3ELP_{AG}, RH_3ELP_{EG} and RH_3ELP_{SG} biopolymers, RH_3ELP_{KG} could form complexes with significantly lower hydrodynamic radiuses most likely due to the presence of significantly higher surface positive charge. In general, nanoparticles with higher surface positive charge had smaller hydrodynamic radiuses. As the surface charge decreased the nanoparticles hydrodynamic radiuses increased. This could be attributed to the fact that uncharged nanoparticles could collide with each other and form loose aggregates (floccules), whereas presence of surface positive charge impedes such collisions resulting in a deflocculated system. This phenomenon has previously been described by our group in more details elsewhere (21). Overall, the results of the DNA neutralization and condensation study show that all constructs shown in Tables I and II were effective in binding and condensing pDNA and there were no free pDNA in solution.

As it has previously been demonstrated that the highly positively charged nanoparticles (>30 mV) have an adjuvant effect and could induce significant IgG response against even low-immunogenic materials (22–24), in the next step we evaluated the zetapotential of the nanoparticles. Interestingly all constructs after complexation with pDNA formed nanoparticles with surface charges below 10 mV (Fig. 3a). Given that RH_3 and R_8 are highly positively charged peptides, this low surface positive charge of nanoparticles suggests that the cationic residues were effectively neutralized and ELP sequences could mask the charges of the core. To validate the zeta potential data produced by NanoZS zetasizer and ensure that the data are produced by using the same algorithms, we evaluated the conductivity of media in each sample. The NanoZS zetasizer uses different algorithms to calculate nanoparticles' surface charges in a solution of high conductivity (>5 ms/cm) *versus* low conductivity (<5 ms/cm). Since the conductivity of medium in all groups remained below 3 ms/cm, therefore we concluded that the same algorithms were used to determine nanoparticles' surface charges and the measurements were accurate (Fig. 3b and c). Overall, the results of this study show that the zeta potential of nanoparticles in all groups stayed below 6 mV; therefore, the probability of charge-promoted immune response against biopolymer sequences is minimal.

Learning that all biopolymers can efficiently condense pDNA into nanosize particles with low surface charges, we then investigated whether there is a difference among biopolymer sequences in terms of inducing Ig production after repeated injections. In general, introduction of any non-self molecule into the body has the potential to trigger an immune response. After injection into the body, biodegradable particles (nanocomplexes, protein aggregates, viruses, etc.) regardless of their diameter (small or big) and surface charges (cationic, neutral or anionic) could get opsonized, picked up by antigen presenting cells such as macrophages/dendritic cells and digested. The antigen presenting cells then express the epitopes on the cell surfaces and depending on their immunogenicity could induce various levels of IgG responses by B cells. Factors that influence immunogenicity include immunogen's physicochemical properties (*i.e.*, foreignness, size, complexity

of structure, physical form and degradability) and method of administration (*i.e.*, dose and route) (25,26). Therefore, to evaluate the immunogenicity of biopolymers, we set up an experiment where immunocompetent mice were injected twice with nanoparticles carrying equal doses of biopolymer and pDNA in order to evaluate IgG responses (Fig. 1). The dose of the nanoparticles was adjusted so that each mouse would receive approximately 60 μg of biopolymer and/or 3 μg of pDNA after each injection. The exposure protocol is similar to what is reported in literature for the evaluation of the immunogenicity of the nanoparticles (27). Since the blood collection from submandibular vein is an invasive process which could directly affect the Ig levels leading to misinterpretation of data, we collected the blood at the end of the study and analyzed the IgM/IgG levels in the serum on day 28. As expected, we did not observe any significant IgM response to any group as IgM is a short-term low affinity response (Fig. 4).

We then evaluated the IgG response which is an indicative of long-term high affinity immune response against immunogens. Here, we used naked pEGFP as a control because it is known that pDNA by itself could induce immune response due to the presence of CpG islands (28,29). Therefore, potential contribution of pEGFP to immunogenicity could be set as a baseline in order to investigate the IgG response to peptide sequences. The results of this study showed a mild IgG response against pEGFP. One reason for the mild immunogenicity of pEGFP could be due to the presence of methylated cytosines within the CpG islands of pEGFP. In prokaryotes (*e.g.*, E. coli), methylation of cytosines can be observed in diverse locations, especially in dcm motif (30). Given that the pEGFP plasmids in this study were propagated and purified from prokaryotic E. coli DH5α cells; some of cytosine residues within CpG islands could have got methylated during the plasmid replication step. Consequently, methylation of multiple cytosine residues in CpG islands could have circumvented the induction of severe immune response against pEGFP (31,32). Perhaps, more robust response could have been detected if we had exposed the mice to higher doses of pEGFP. In addition to naked pEGFP, we also used RH_3/pEGFP complexes as control in order to eliminate the potential contribution of RH_3 sequence to IgG response. Comparison of the IgG responses to RH_3/pEGFP and HEPES showed that the sequence of this peptide was not immunogenic by itself (*t*-test, $p > 0.05$). This could be related to the surface properties of RH_3/pEGFP complexes that may have preferentially bound to dysopsonizing serum proteins (33). Alternatively, it is possible that the RH3 sequence due to its simple structure is inherently low-immunogenic.

In comparison to uncharged ELP_{SG} and ELP_{AG} which remained invisible to the immune system, the charged sequences of ELP_{KG} and ELP_{EG} triggered significant IgG response. This observation could be attributed to several contributing factors. For example, presence of uncharged S and A residues in VPGXG may not have significantly changed the natural conformation of ELP (*i.e.*, type II β-turn) (34); thereby, remaining invisible to the immune system. Since replacement of S or A uncharged residues with charged K or E induces significant structural changes to elastin conformation (35), it is possible that ELP with charged residues are identified as a foreign antigen by immune system due to the major difference in conformation from the endogenous elastin (VPGVG repeats). It is also noteworthy that foreign antigens have to bind to the major histocompatibility complex (MHC) protein presented on the surface of antigen-presenting cells in order to activate immune system. However, not all foreign peptides could be recognized by MHC molecules. The MHC prefers binding to those peptides with amino acids that can fit into the antigen-binding groove which is either charge sensitive or polar/non-polar sensitive. To recognize VPGXG motif, the charged residues could play a more important role and captured by MHC proteins. Furthermore, the very low immunogenicity of ELP_{SG} could be partially due to the fact that ELP sequence with serine at the fourth position produces the most soluble and neutral (uncharged) design among all other constructs. To investigate further and reconfirm that the ELP sequence truly played a significant role in eliciting IgG response, we evaluated the IgG response to R_8ELP_{KG} and R_8ELP_{SG}. In these two peptides, the ELP sequences were similar to the corresponding RH_3ELP_{KG} and RH_3ELP_{SG} peptides; however, the RH_3 (DNA condensing domain) was replaced with RRRRRRRR (R_8). The R_8 sequence was selected because it is well documented that this sequence is able to efficiently bind and condense pDNA (36,37). The R_8ELP_{KG} and R_8ELP_{SG} polymers were first used to complex with pEGFP to form nanoparticles and then injected into the mice. These results indicate both R_8 and RH_3 were efficiently bound with pEGFP and remained invisible to the immune system and the observed immune response or lack thereof could have attributed to the differences among ELP sequences.

To eliminate potential contribution of CpG islands to immune response and also evaluate the efficacy of the ELP_{SG} in reducing immunogenicity, we performed the next set of studies by comparing the levels of IgG response to pCpGfree plasmid, RH_3-PEG3500/pCpGfree and RH_3ELP_{SG}/pCpGfree complexes. Given that in this study nanoparticles are formed through complexation of several thousand of RH_3-PEG3500 molecules with each pDNA molecule, the assembled nanoparticles have high PEG density on their surfaces. Therefore, the observed significant immune response to PEG coated nanoparticles was expected as PEG density impacts serum protein adsorption and phagocytic uptake (38). Overall, the results of this study also showed no significant IgG response to ELP_{SG} sequence.

CONCLUSIONS

To date, there have been no careful studies of the ELPs that examine their immunological status. This is surprising, especially due to the frequency that investigators in the biopolymer field are asked about the potential immunogenicity of repetitive peptide sequences. This study demonstrates that ELPs with specific length and sequences (*i.e.*, ELP_{SG} and ELP_{AG}) could potentially be used to mask the surfaces of nanotechnology-based drug delivery systems without evoking IgG response. At this stage we have barely scratched the surface and believe that further in depth immunological, toxicological and biodistribution studies are required to understand the underlying mechanisms. Nevertheless, the identification of short ELP sequences that could mask the surface of nanoparticles without eliciting IgG response is an important progress because they could potentially be used in production of safer (non-immunogenic) and more efficient drug and gene delivery systems. Further studies with other types of delivery systems (polymeric, lipidic, metallic, etc.) will help better characterize and examine the broad application of ELPs in surface masking. For example, ELP_{SG} could be conjugated to the surface of liposomes, micelles and nanoparticles to reduce immunogenicity. It could also be expressed on the surface of viruses (*e.g.*, adenovirus) or in fusion with antibodies to increase their half-lives in blood circulation.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was made possible by the Rutgers University Faculty Research Grant (#281663) and animal housing facility at the Center for Cancer Prevention Research at Rutgers University.

REFERENCES

1. Ishida T, Harashima H, Kiwada H. Liposome clearance. Biosci Rep. 2002;22(2):197–224.
2. Chekhonin VP, Zhirkov YA, Gurina OI, Ryabukhin IA, Lebedev SV, Kashparov IA, *et al.* PEGylated immunoliposomes directed against brain astrocytes. Drug Deliv. 2005;12(1):1–6.
3. Funhoff AM, Monge S, Teeuwen R, Koning GA, Schuurmans-Nieuwenbroek NM, Crommelin DJ, *et al.* PEG shielded polymeric double-layered micelles for gene delivery. J Control Release. 2005;102(3):711–24.
4. Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003;42(6):463–78.
5. Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, *et al.* Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J Control Release. 2006;112(1):15–25.
6. Judge A, McClintock K, Phelps JR, Maclachlan I. Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. Mol Ther. 2006;13(2):328–37.
7. Verhoef JJ, Anchordoquy TJ. Questioning the Use of PEGylation for Drug Delivery. Drug Deliv Transl Res. 2013;3(6):499–503.
8. Betre H, Liu W, Zalutsky MR, Chilkoti A, Kraus VB, Setton LA. A thermally responsive biopolymer for intra-articular drug delivery. J Control Release. 2006;115(2):175–82.
9. Heilshorn SC, Liu JC, Tirrell DA. Cell-binding domain context affects cell behavior on engineered proteins. Biomacromolecules. 2005;6(1):318–23.
10. Heilshorn SC, DiZio KA, Welsh ER, Tirrell DA. Endothelial cell adhesion to the fibronectin CS5 domain in artificial extracellular matrix proteins. Biomaterials. 2003;24(23):4245–52.
11. Betre H, Setton LA, Meyer DE, Chilkoti A. Characterization of a genetically engineered elastin-like polypeptide for cartilaginous tissue repair. Biomacromolecules. 2002;3(5):910–6.
12. Urry DW, Parker TM, Reid MC, Gowda DC. Biocompatibility of the bioelastic materials, poly(GVGVP) and its gamma-irridation cross-linked matrix: summary of generic biological test. J Bioact Compat Polym. 1991;6:263–83.
13. Shah M, Hsueh PY, Sun G, Chang HY, Janib SM, MacKay JA. Biodegradation of elastin-like polypeptide nanoparticles. Protein Sci. 2012;21(6):743–50.
14. Sandberg LB, Leslie JG, Leach CT, Alvarez VL, Torres AR, Smith DW. Elastin covalent structure as determined by solid phase amino acid sequencing. Pathol Biol (Paris). 1985;33(4):266–74.
15. Urry DW. Physical chemistry of biological free energy transduction as demonstrated by elastic protein-based polymers. J Phys Chem B. 1997;101(51):11007–28.
16. Urry DW, Urry KD, Szaflarski W, Nowicki M. Elastic-contractile model proteins: physical chemistry, protein function and drug design and delivery. Adv Drug Deliv Rev. 2010;62(15):1404–55.
17. Canine BF, Wang Y, Hatefi A. Biosynthesis and characterization of a novel genetically engineered polymer for targeted gene transfer to cancer cells. J Control Release. 2009;138(3):188–96.
18. Canine BF, Wang Y, Ouyang W, Hatefi A. Development of targeted recombinant polymers that can deliver siRNA to the cytoplasm and plasmid DNA to the cell nucleus. J Control Release. 2011;151(1):95–101.
19. Urry DW, Gowda DC, Parker TM, Luan CH, Reid MC, Harris CM, *et al.* Hydrophobicity scale for proteins based on inverse temperature transitions. Biopolymers. 1992;32(9):1243–50.
20. Yardeni T, Eckhaus M, Morris HD, Huizing M, Hoogstraten-Miller S. Retro-orbital injections in mice. Lab Animal. 2011;40(5):155–60.
21. Karjoo Z, McCarthy HO, Patel P, Nouri FS, Hatefi A. Systematic engineering of uniform, highly efficient, targeted and shielded viral-mimetic nanoparticles. Small. 2013;9(16):2774–83.
22. Ma Y, Zhuang Y, Xie X, Wang C, Wang F, Zhou D, *et al.* The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses. Nanoscale. 2011;3(5):2307–14.
23. Barnier Quer C, Elsharkawy A, Romeijn S, Kros A, Jiskoot W. Cationic liposomes as adjuvants for influenza hemagglutinin: more than charge alone. Eur J Pharm Biopharm. 2012;81(2):294–302.
24. Christensen D, Korsholm KS, Rosenkrands I, Lindenstrom T, Andersen P, Agger EM. Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines. 2007;6(5):785–96.
25. Nechansky A, Kircheis R. Immunogenicity of therapeutics: a matter of efficacy and safety. Expert Opin Drug Discovery. 2010;5(11):1067–79.
26. Hatefi A, Canine BF. Perspectives in vector development for systemic cancer gene therapy. Gene Ther Mol Biol. 2009;13(A):15–9.
27. Jain A, Yan W, Miller KR, O'Carra R, Woodward JG, Mumper RJ. Tresyl-based conjugation of protein antigen to lipid

nanoparticles increases antigen immunogenicity. Int J Pharm. 2010;401(1–2):87–92.
28. Hyde SC, Pringle IA, Abdullah S, Lawton AE, Davies LA, Varathalingam A, *et al.* CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat Biotechnol. 2008;26(5):549–51.
29. Yew NS, Cheng SH. Reducing the immunostimulatory activity of CpG-containing plasmid DNA vectors for non-viral gene therapy. Expert Opin Drug Deliv. 2004;1(1):115–25.
30. Marinus MG, Morris NR. Isolation of deoxyribonucleic acid methylase mutants of Escherichia coli K-12. J Bacteriol. 1973;114(3):1143–50.
31. Reyes-Sandoval A, Ertl HC. CpG methylation of a plasmid vector results in extended transgene product expression by circumventing induction of immune responses. Mol Ther. 2004;9(2):249–61.
32. Feltquate DM, Robinson HL. Effect of CpG methylation on isotype and magnitude of antibody responses to influenza hemagglutinin-expressing plasmid. DNA Cell Biol. 1999;18(9):663–70.
33. Johnstone SA, Masin D, Mayer L, Bally MB. Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages. Biochim Biophys Acta. 2001;1513(1):25–37.
34. Yao XL, Hong M. Structure distribution in an elastin-mimetic peptide (VPGVG)3 investigated by solid-state NMR. J Am Chem Soc. 2004;126(13):4199–210.
35. Reguera J, Fahmi A, Moriarty P, Girotti A, Rodriguez-Cabello JC. Nanopore formation by self-assembly of the model genetically engineered elastin-like polymer [(VPGVG)2(VPGEG)(VPGVG)2]15. J Am Chem Soc. 2004;126(41):13212–3.
36. Ye SF, Tian MM, Wang TX, Ren L, Wang D, Shen LH, *et al.* Synergistic effects of cell-penetrating peptide Tat and fusogenic peptide HA2-enhanced cellular internalization and gene transduction of organosilica nanoparticles. Nanomedicine. 2012;8(6):833–41.
37. Khalil IA, Hayashi Y, Mizuno R, Harashima H. Octaarginine- and pH sensitive fusogenic peptide-modified nanoparticles for liver gene delivery. J Control Release. 2011;156(3):374–80.
38. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, Harnisch S, *et al.* 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids Surf B: Biointerfaces. 2000;18(3–4):301–13.