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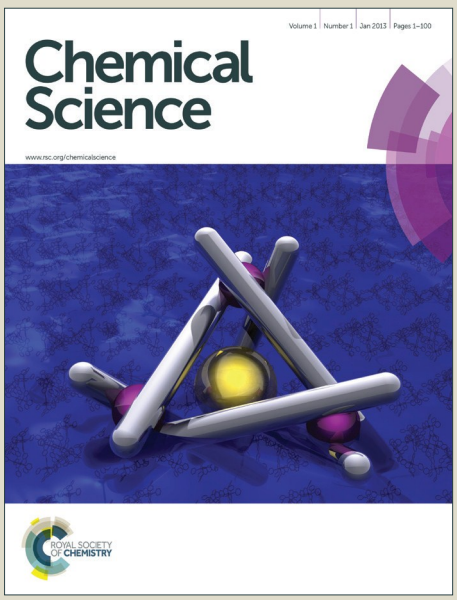

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ARTICLE

Robust vaccine formulation produced by assembling hybrid coating of polyethyleneimine-silica

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Exploring formulation that can improve thermostability and immunogenicity of vaccine holds great promise in advancing the efficacy of vaccination to combat infectious diseases. Inspired by biomineralized core-shell structure in nature, we suggest a polyethyleneimine (PEI)-silica-PEI hybrid coated vaccine formulation to improve both thermostability and immunogenicity. Through electrostatic adsorption, *in situ* silicification and capping treatment, the hybrid coating of silica and PEI was assembled around vaccines to produce vaccine@PEI-silica structure. Both *in vitro* and *in vivo* experiments demonstrated that the thermostability and immunogenicity of the modified vaccine were significantly improved. They could be used efficiently after long-term exposure at room temperature, which would facilitate vaccine transport and storage without cold chain. Furthermore, mechanistic studies revealed that PEI-silica-PEI coating acted as physiochemical anchors as well as mobility-restricting hydration layer to stabilize the enclosed vaccine. This achievement implies a biomimetic surface-modification-based strategy to confer desired properties on biological products.

Introduction

Vaccines, especially live-attenuated vaccines for pathogens, are highly sensitive to temperature shift, thus requiring continuous refrigeration to maintain their potency.[1] Unfortunately, poor regions suffering from present vaccine-preventable infectious diseases are lacking extensive and reliable refrigeration facilities,[2, 3] and the deviation of vaccines from cold chain during the storage and delivery is unavoidable, resulting in the waste of a large proportion of vaccines.[4] Therefore, thermal instability of vaccines has become a significant obstacle to global vaccination programs and viral-based therapy.[5] Robust vaccine formulations with improved thermostability and immunogenicity hold great promise to address these limitations. Several approaches, such as the preparation of carbohydrate glasses, addition of silk protein, and biomimetic mineralization have been used to prepare thermostable and efficient formulations.[6-10] However, there are still no satisfactory formulations for most vaccines.

Natural organisms including ancient phages and some plants use biomolecules to deposit amorphous silica hybrid coatings to survive from environmental stresses.[11-14] Our recent studies also demonstrated that exterior silica nanoclusters could improve the thermostability of yeast cells and picornaviruses.[15-17] In addition, amorphous silica is commonly used as food additive, and generally recognized as safe by US Food and Drug Administration.[18] These achievements indicate that exterior silica layer can be used to develop thermostable vaccine formulations. However, the direct deposition of silica on enveloped viral vaccines without hampering their original efficacy is difficult to be realized, because they lack silica-accumulating sites and are highly sensitive to pH-tuned modification. In nature, organisms use biomolecules that are rich in cationic amino acids or polyamines to direct biosilica deposition process under physiological conditions.[19-21] Polyethyleneimine (PEI), a polyamine that can be synthesized in linear or branched form with varied molecular weight, may represent an ideal nucleating and stabilizing agent to control the deposition of silica nanoclusters on vaccine, as it has been widely used as a gene transfection agent and mucosal adjuvant.[22, 23] Due to the ability of PEI to facilitate cargo delivery into cells expressing heparan sulphate proteoglycans (HSPG) such as antigen-presenting cells (APC), we herein hypothesize that a vaccine formulation with PEI-silica hybrid coating may exhibit improved immune-stimulating ability.

In the present study, using a clinical approved Japanese encephalitis vaccine (JEV) as a model, we propose a concept of designing thermostable vaccine formulation, in which the vaccine surface is modified by PEI-silica-PEI sandwich coatings (Fig. 1A). As our expectation, both *in vitro* and *in vivo* assessment results demonstrate that this hybrid material coated vaccine formulation not only exhibits significantly improved thermostability at a wide

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temperature range, but also enhances the potency of vaccine to elicit humoral and cellular immune responses.

Results

Assembly of PEI-silica-PEI hybrid coatings on viral vaccine.

As a typical enveloped viral vaccine, JEV SA-14-14-2 is highly acid labile. They are inactivated even when the solution pH is slightly below neutrality. For example, treatment at pH=5.6 for 30 min resulted in >95% loss in JEV vaccine titers (Fig. S1A†). However, the previously suggested pH-programmed silicification always needs an acidic procedure at pH range of 5.5-6.0 to silicify viral particles. Since this acidic treatment unavoidably led to a significant decrease in JEV infectivity, the pH-controlled silicification could not be directly used to silicify JEV without greatly losing the original potency (Fig. 1B). Therefore, an alternative approach should be suggested to ensure the silification of this enveloped viral vaccine. Inspired by previous achievements of biosilicification, silica-nucleating molecules such as PEI and protamine, were introduced on the vaccine surface to realize the efficient silicification under physiological conditions.[15, 24-28] Due to the negative zeta potential of JEV vaccine particles, polycationic PEI molecules were adsorbed on virion surface and acted as the nucleation sites for *in situ* silica deposition in near-physiological environment (Fig. 1C). With these adsorbed cationic PEI molecules, JEV vaccine could be efficient silicification under neutral conditions without severely impairing its original potency (Fig. S1B†). When silica nanoclusters were attached on PEI-modified vaccine particles, the resulted hybrid of vaccine and mineral materials could readily be separated from solution by normal-speed centrifugation (16,000 g 10 min) due to the relative high gravity of silica. This feature could enable us to estimate the silicification efficacies of vaccine by quantifying viral particles in the supernatant and precipitate using plaque assays. It was found that more than 90% PEI coated JEV vaccines (JEV@PEI) were efficiently silicified at neutral pH, whereas only 20% native JEV vaccines could be silicified (Fig. 1D). The detrimental effect on vaccine infectivity caused by this PEI-mediated silicification was also evaluated and the results showed that >70% of initial vaccine titers were preserved in the presence of PEI-silica hybrid coating (Fig. S1B†). Finally, 20 μg/mL PEI molecules were added to the solution as the capping agent to stabilize the formed PEI-silica hybrid coating.

Under transmission electron microscopy (TEM), the silicified JEV@PEI vaccines could be directly observed and these dispersive nanoparticles had typical diameter of about 100 nm (Fig. 1E). The inner 50-60 nm virion was identified by negatively staining silicified JEV@PEI using phosphotungstic acid, which was surrounded by an uneven inorganic layer (Fig. 1F and 1G). Further scanning electron

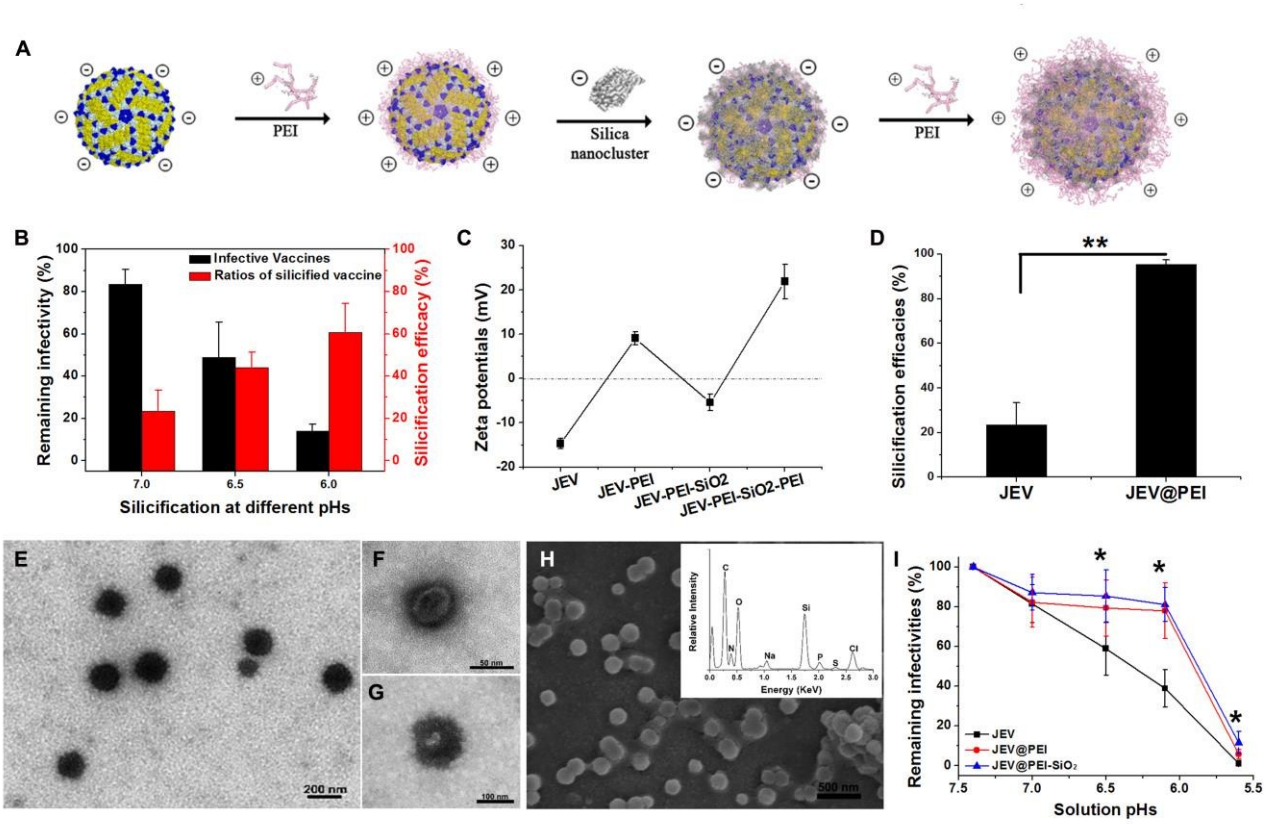

Fig. 1 Polycationic molecule mediated *in situ* silicification of JEV. (A) Schematic illustration of the assembly of PEI-silica hybrid nanocoatings on vaccine. (B) The sensitivity of JEV to pH-tuned silicification by adjusting solution pH to different acidity, with their remaining infectivity and silicification efficacies examined. (C) Zeta-potential of JEV, and JEV coated with PEI, PEI-SiO_2 and PEI-SiO_2-PEI sandwich layers. (D) The silicification efficacies of JEV at pH 7.0 with or without adding PEI as nucleating agent. (E) TEM images of silicified vaccine JEV@PEI-SiO_2 without any staining treatment. (F, G) TEM images of negatively stained JEV@PEI-SiO_2 and image of (G) depicts silicified JEV that was almost totally encased by PEI-silica composites. (H) SEM images of JEV@PEI-SiO_2 nanoparticles, inset represents EDX analysis of JEV@PEI-SiO_2. (I) pH sensitivity of JEV, JEV@PEI and JEV@PEI-SiO_2. (*P<0.05, **P<0.01, n≥3, data represented as means ± SDs).

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microscopy (SEM) confirmed the sphere-like structure of silicified JEV@PEI (Fig. 1H), and energy dispersive X-ray (EDX) diffraction analysis revealed that the modified outmost layers were mainly composed of C, N, O, Si, and S (Fig. 1H, inset), directing to the co-existence of organic and silica components. To verify the sandwich coating of PEI-silica-PEI on virions, we carried out zeta-potential measurements after each treatment. As expected, the surface zeta-potentials changed alternately with silica nanocluster or PEI as the outer layer (Fig. 1C). These results indicated the successful assembly of sandwich PEI-silica-PEI layer on JEV vaccine and the new formulation was named JEV@PEI-SiO_2.

Usually, viral vaccines cannot be separated by using normal centrifugation method. A new characteristic of JEV@PEI-SiO_2 was that the sandwich coating altered surface electrostatic potentials of viral particles from negative to positive (Fig. 1C), and increased their gravity density due to the attachment of PEI-silica nanoclusters; therefore, JEV@PEI-SiO_2 could be effectively separated and concentrated from solution by normal speed centrifugation (Fig. 1D). Besides, we found that the PEI-silica-PEI coating improved the acid resistance of JEV vaccine at pH ranging from 5.6-7.4 (Fig. 1I). These results showed that the assembly of PEI-silica-PEI coating made JEV easier to be separated and more robust.

Biological activity and immunogenicity of PEI-silica coated vaccine.

To study the availability of this formulation, we firstly examined the effect of PEI-silica hybrid coatings on inherent biological properties of vaccine. The results showed that both JEV@PEI and JEV@PEI-SiO_2 still caused typical cytopathic effects in susceptible BHK-21 and Vero cells, and exhibited similar plaque morphologies as the native ones (Fig. 2A and Fig. S2A†) when diluted in non-silica solution. And the infection of JEV@PEI or JEV@PEI-SiO_2 caused efficient viral antigen expression in the cytoplasm of host cells (Fig.2B and Fig. S2B†). Further examinations of their growth curves showed that JEV@PEI-SiO_2 had similar growth patterns to JEV in BHK-21 cells (Fig. 2C) and Vero cells (Fig. 2D), but the vaccine titers produced in JEV@PEI-SiO_2 infected cells were slightly higher than that of native JEV at the early stage (Fig. 2C). The conferred enhancement of infection can be explained by the interaction of positively charged PEI with cell surface HSPG, which increased efficiency of viral attachment and entry into host cells.

Then, mice were subcutaneously immunized with JEV and JEV@PEI-SiO_2 to evaluate and compare the immunization potency. The *in vivo* examinations showed that JEV@PEI-SiO_2 induced 2-fold higher serum IgG antibody titers (Fig. 2E) and slightly higher levels of neutralization antibody (Fig. 2F), in comparison with those elicited by native JEV. These results indicated that the assembled PEI-silica hybrid coatings of JEV@PEI-SiO_2 moderately improved the immunogenicity of vaccine.

Thermal stability of PEI-silica-PEI coated vaccine.

We evaluated the thermal degradation rates of silicified JEV in different formulations, and found that vaccines in formulation of JEV@PEI-SiO_2 exhibited most desirable thermostability (Fig. S3†). Further examinations revealed that the PEI-silica-PEI coatings significantly improved thermostability of JEV at wide range of temperatures (Fig. 3A-C). At temperature of 25°C, native JEV lost approximately 1 Log_{10} PFU titer within 2 days and they were almost totally inactivated after 1 week. The thermal stability of JEV was effectively improved by PEI-silica-PEI coatings. It took 12 days that JEV@PEI-SiO_2 lost approximately 1 log_{10} PFU titer at 25°C. Their activity loss tendency at room temperature was only slightly higher

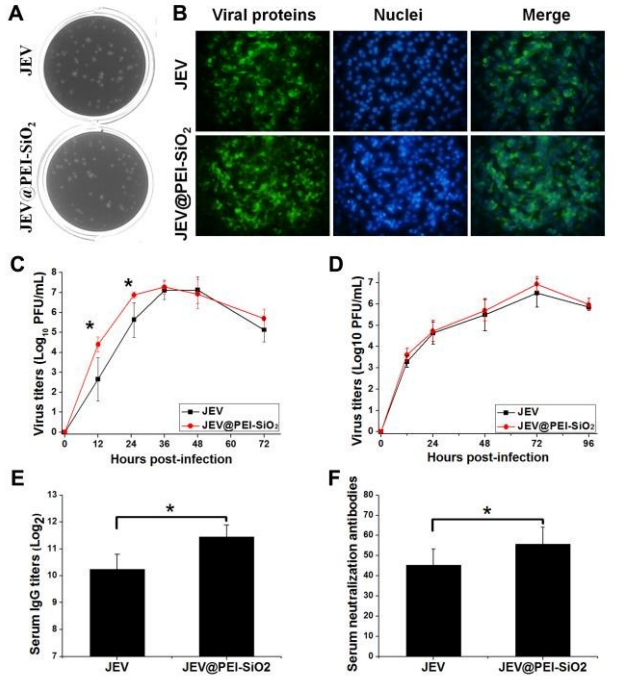

Fig. 2 Biological activity and immunogenicity of JEV and silicified vaccine JEV@PEI-SiO_2. (A) Plaque morphologies in BHK21 cells. (B) The indirect immunofluorescence assays (IFA) of JEV and JEV@PEI-SiO2 in BHK21 cells at 36 h post-infection. (C) Growth curves of JEV and JEV-PEI-SiO_2 in BHK21 cells, and (D) Vero cells (M.O.I.=0.1). (E) The levels of serum IgG antibody and (F) neutralization antibody in mice immunized by the same amount of JEV or JEV@PEI-SiO_2. (*P < 0.05, n≥3, data represented as means ± SDs).

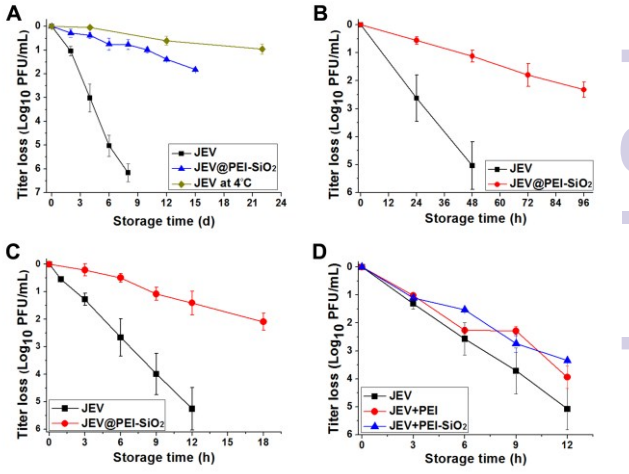

Fig. 3 Thermostabilities of native JEV, JEV@PEI and JEV@PEI-SiO_2 in liquid form. (A) Thermal-inactivation curves of JEV in native and JEV@PEI-SiO_2 formulations at 25°C, or (B) 37°C, or (C) 42°C, with the thermal-inactivation curves of native JEV at 4°C as a reference. (D) Thermal-inactivation curves o JEV, JEV@PEI, and JEV mixed with *ex situ* synthesized PEI-silica composites at 42°C. (n≥4, data represented as means ± SDs).

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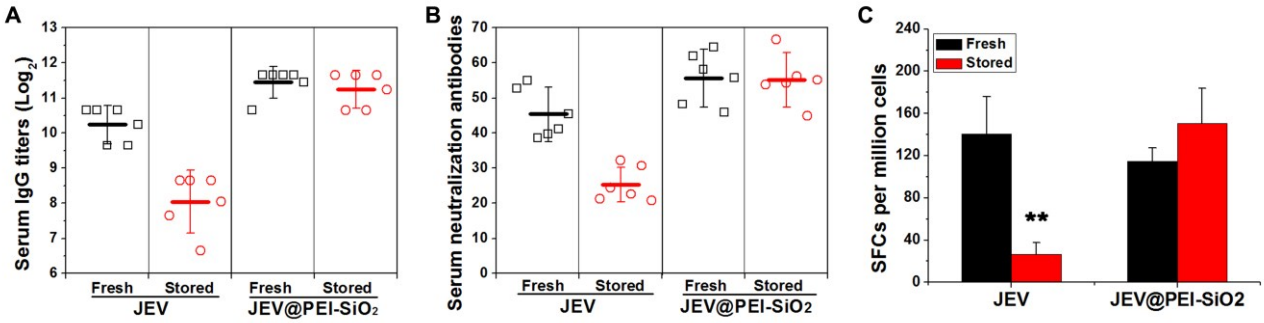

Fig. 4 Animal practices with fresh or stored JEV and JEV@PEI-SiO_2. (A) The levels of elicited serum IgG antibody and (B) serum neutralization antibody in mice immunized by fresh or 18-day-stored (25°C) JEV and JEV@PEI-SiO_2. (C) The frequencies of JEV-specific IFN-γ secreting splenocytes of mice 12 days post-immunization were determined by quantifying the numbers of spot-forming cells (SFCs) with ELIspot assay.

than that of JEV at 4°C (Fig. 3A). At higher temperatures of 37°C (Fig. 3B) and 42°C (Fig. 3C), JEV@PEI-SiO_2 could retain 90% of their original infectivity after the storage of >48 hours and ~10 hours, respectively. Generally, the thermal inactivation rate of vaccine in formulation of JEV@PEI-SiO_2 was almost 5-fold slower than that of native ones. Clearly, the thermostability of enveloped JEV vaccine could be significantly improved in formulation of JEV@PEI-SiO_2.

Besides, thermal protective effect of adsorbed PEI and *ex situ* synthesized PEI-silica nanocomposite was also studied. The thermal inactivation assays at 42°C showed that the themostability of JEV@PEI and JEV mixed with PEI-silica nanocomposite was only slightly better than unmodified ones (Fig. 3D), implying that the anchoring of PEI-silica nanoclusters on vaccine initiated by *in situ* silicification was important for exerting their remarkable thermal protective role. Therefore, it was the *in situ* rather than *ex situ* treatment that was the key to guarantee the thermostability improvement by using silica materials.

In vivo examinations of PEI-silica-PEI coated vaccine.

Mice were immunized with native JEV and JEV in formulation of JEV@PEI-SiO_2 after 18 days' storage at 25°C, to confirm the efficacy and thermostability. In one-shot vaccination, the levels of serum IgG antibody (Fig. 4A) and neutralization antibody (Fig. 4B) elicited by stored JEV vaccines were decreased by more than 4-fold and 2-fold respectively. However, there was only a negligible decrease in the levels of JEV@PEI-SiO_2 elicited serum IgG antibody and neutralization antibody after the same treatment, and they were still similar to that elicited by fresh JEV vaccines (Fig. 4A and 4B). In prime/boost vaccination, the levels of IgG antibody and neutralization antibody elicited by 18-day stored JEV decreased by >8-fold, whereas only a slight decrease in JEV@PEI-SiO_2 induced neutralization antibodies after the same storage (Fig. S4†).

Additionally, the potency of JEV@PEI-SiO_2 vaccine formulation to elicit cellular response were evaluated by measuring JEV-specific IFN-γ secreting spleenocytes of immunized mice with ELIspot assays.[29, 30] After the storage of 18 days at room temperature, the frequencies of IFN-γ secreting spleenocytes elicited by stored JEV vaccines decreased significantly, >80% lower than that elicited by fresh JEV (Fig. 4C). However, stored JEV@PEI-SiO_2 still induced high frequencies of IFN-γ secreting cells in mice spleenocytes, similar to that elicited by fresh JEV and JEV@PEI-SiO_2. It followed that long-term storage of enveloped viral vaccines at ambient temperature could be achieved by assembling exterior PEI-silica-PEI coatings.

Protection mechanisms of exterior organics-silica nanocomposite

According to previous study,[16] we speculated that exterior PEI-silica hybrid nanocoatings could confine large amount of water molecules by forming hydrogen bonding,[31] thus acting as molecule mobility buffering layer, as well as physical confining matrix to prevent inner vaccine from conformational changes.[16, 32] We verified this hypothesis by examining the weight loss of samples and heat-flow along temperature increase with thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). Most water molecules in native sample were deprived before 100°C (Fig. 5A), whereas most of the water molecules in silicified vaccines JEV@PEI-SiO_2 were deprived until the temperature reached 150°C according to their weight loss tendency (1.5%) and heat adsorption peak (Fig. 5B). These results indicated that a large amount of water molecules in JEV@PEI-SiO_2 was in hydration form, and the hydrogen bonds with PEI-silica nanocomposites need to be broken before their deprivation. Combining these results, we rationally suggested that the organic-inorganic hybrid nanocoatings and nearby water molecules constituted a protective hydration layer around vaccines, which could relieve the destructive bond exchange between aqueous solution and vaccines by decreasing the thermal motion transfer.

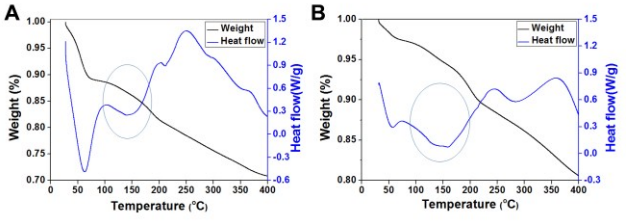

Fig. 5 TGA-DSC analyses of freeze-drying powders of (A) native JEV and (B) JEV@PEI-SiO_2. The results revealed that almost water molecules in native vaccines were deprived at 60°C, whereas most water molecules in JEV@PEI-SiO_2 were lost until the temperature reached 150°C, indicating that the PEI-silica composite had excellent water confining effect.

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Discussion

In our recent study, we have illustrated the potential application of *in situ* silicification in stabilizing non-enveloped picornaviruses.[16] However, pH-tuned silicification approach cannot be directly used to silicify enveloped viral vaccines because they are highly sensitive to the changes of solution pH. Effective nucleating and flocculating agents are required to realize their efficient silicification under physiological conditions. Due to the abundant of amine groups and adjuvanticity of polycationic PEI, it was introduced on the surface of vaccine by electrostatic adsorption to induce the *in situ* deposition of silica nanoclusters. Furthermore, PEI molecules are added as capping agent to stabilize the formed silica nanoculsters, producing a dual functional sandwich coating of PEI-silica-PEI for JEV to fulfil thermal protective and immune-stimulating roles.

It is worth to note that PEI can be replaced by a natural cationic polypeptide, low molecular weight protamine, to facilitate the efficient silicification of JEV vaccine under near-physiological conditions (Fig. S5†) without obviously compromising their native biological activities (Fig. S6†). It means that we can choose either polymers rich in amines or functional polypeptides to assemble desired organics-silica hybrid coating on vaccine, following a general principle of silicification regulation by using cationic amine residues. Similar to the PEI-silica-PEI coating, *in situ* synthesized protamine-silica-protamine coatings also significantly improve the thermostability of vaccines (Fig. S7†). However, the thermostability improvements of vaccine conferred by PEI-silica-PEI sandwich coating are better than that conferred by protamine-silica-protamine coating (Fig.3 and Fig. S7†), which may be due to the better water confining effect of branched PEI macromolecules.

Additionally, the PEI-silica-PEI hybrid coatings also improve the immunogenicity of JEV vaccines, facilitating them to induce a robust humoral and cellular response even after long-term storage. In this vaccine formulation, we suggest that the anchored polycationic PEI molecules can facilitate the interaction between vaccines and APCs, thus promoting their uptake and processing by immune cells. Notably, organics-silica nanocoatings produced by protamine or linear PEI fails to lead so obvious improvement of the vaccine immunogenicity (Fig. S8 and S9†), indicating that the use of branched PEI molecules may be a better choice.

Previously, we have proposed the use of calcium phosphate, an inorganic adjuvant, to coat viral vaccines to improve both their thermostability and immunogenicity, but the thermostability improvements conferred by calcium phosphate is relatively low.[10] And the synthesized calcium phosphate can hardly be stabilized in aqueous solution for a long time due to its biological degradable profiles, which also limit its application. By introducing hydrated silica exterior, we significantly improved the thermostability viral vaccines, with no enhancement of immunogenicity. In the present study, taking advantage of excellent water confining effect of amorphous silica, nucleation ability and adjuvanticity of PEI polymer, we are successful in assembling a thermal protective but immune-stimulating PEI-silica-PEI hybrid coating for viral vaccine through coating vaccine with PEI, *in situ* silicification, and subsequent capping treatment. Some literatures have shown that synthetic silica and PEI have good biocompatibility, and they have been studied as vaccine delivery vehicles and adjuvants.[18, 33] A single dose of vaccination with <20 μg PEI and <1 mg silicates can be considered as safe.[22, 34] In our cases, the introduced both the amounts of PEI and silicates can be controlled around these limits to support the potential use of this vaccine formulation. Therefore, silicified vaccine formulation could be used with a guarantee of material safety.

Actually, it is the smart of organisms for accumulating silica on their cell wall to improve thermal tolerance.[35] Analogously, the exterior organics-silica hybrid nanocoatings around vaccine can improve its thermostability. Our study may provide a good example of using nature-evolved strategy to produce robust vaccine formulations. This vaccine formulation combined the adjuvant—PEI molecule, thermal protective silica and vaccine together, and will guarantee vaccine efficacy without any access to refrigeration for several days. Such achievement can efficiently advance the terminal distribution of vaccines from local storage centres to immunization clinics or mobile on-site utilization, and extend immunity to world poorest areas against deadly infectious diseases.

Conclusions

By combining layer-by-layer and *in situ* silicification treatment, PEI-silica hybrid nanocoating was assembled on enveloped viral vaccine under near-physiological condition, producing a new robust formulation. Taking advantage of the silica nucleating and cell transfection ability of PEI, and the hydration ability of amorphous silica, the vaccine formulation exhibited significantly improved thermostability and immunogenicity, and it still induced high level of humoral and cellular immune response after long-term storage at ambient temperature. The current biomimetic approach suggests a cost-effective, material-based vaccine formulation to advance the cold chain less-dependent vaccine distribution.

Experimental Section

Biomimetic silicification of JEV vaccines.

Fresh silicic acid (30 mM) was prepared fresh before use as follows: 5.3 μL of sodium silicate (Sigma) was diluted in 1 mL of PBS (pH 7.4); 20 μL of 1.25 M HCl was then added to adjust the solution to pH 7.5~8.0. To obtain *in situ* silicified JEV vaccine, cationic molecules protamine or 600 Da / 10 kD polyethyleneimine (PEI) were added into vaccine solutions (~10^7 PFU/mL) at a final concentration of 100 μg/mL and incubated at 37°C for 5 min, and then 30 mM fresh silicic acid was added to reach a final concentration of 3 mM. The solution pH was adjusted to 6.5~7.0 with HCl to initiate silicification, reacting for 15-30 min. Finally, 20 μg/mL protamine or 600 Da /10 kD PEI was added into silicified vaccine solution as the capping agents.

Thermal stability tests.

Native JEV vaccines, JEV@PTM, JEV@PEI, JEV@PEI-SiO_2, JEV@PTM-SiO_2, and JEV mixtures with *ex situ* synthesized PTM-SiO_2/PEI-SiO_2 were stored at 4°C, 25°C, 37°C, and 42°C, and the samples were collected periodically. Their remaining infectivity was determined by plaque assays. The remaining percentages of infectivity were calculated and represented as a logarithmic scale *vs* incubation time ($n \geq 4$); the data represent as mean ± standard deviations.

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Mouse experiments.

Mice experiments were approved by and performed in strict accordance with the guidelines of the Animal Experiment Committee of Beijing Institute of Microbiology and Epidemiology (China). 4-week old BALB/c mice were subcutaneously immunized with fresh or stored JEV, JEV@PTM-SiO_2, and JEV@PEI-SiO_2 with the same initial titers before storage (200 μL, 10^7 PFU/mL). In prime-boost vaccination, the immunized mice were further boosted with fresh/stored JEV and JEV@PEI-SiO_2 at 4 weeks post 1st inoculation. Mice sera were collected at 2 and 4 weeks post-injection. The levels of serum IgG antibody and neutralization antibody were detected using Enzyme-linked immunosorbent assay (ELISA) and standard plaque reduction neutralization tests (PRNT), respectively.

Standard plaque reduction neutralization tests.

Mouse serum was serially 2-fold diluted in DMEM, starting at 1:8. Virus suspensions (150 μL at 100 PFU) were mixed with 150 μL of diluted sera, and the mixtures were incubated at 37°C for 1-1.5 h. The mixtures were then added to 90-100% confluent BHK-21 cells and incubated for 1 h. Then cells were washed and incubated with DMEM supplemented with 2% FBS and 1% low melting point agarose, and cultured at 37°C, 5% CO_2 for about 3 days before fixation with 4% formaldehyde. Endpoint titers were calculated according to the Karber method, as previously described.[16]

Acknowledgements

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