Vaccine Stabilization

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Eggshell-Inspired Biomineralization Generates Vaccines that Do Not Require Refrigeration**

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Infectious diseases pose great threats to public health and cause over 17 million deaths each year.[1] Vaccination is the most effective medical intervention,[2] and it has resulted in a dramatic decrease of mortality caused by diseases[3] such as yellow fever, measles, and meningitis, as well as eradication of infectious diseases such as smallpox and polio.[2b] Unfortunately, the use of vaccines is still severely limited in the poorest countries, where more than half of all deaths are caused by infectious diseases.[4] Also, about 50% of lyophilized vaccines are discarded annually;[5] the main reason for this is that live attenuated vaccines are sensitive to heat. Generally, vaccines cannot be stored at room temperature and refrigeration is essential for maintaining their quality.[1,6] Keeping vaccines at low temperatures is difficult and expensive, especially in developing countries lacking extensive and reliable refrigeration infrastructures. Actually, maintaining the cold chain accounts for 80% of the financial cost of vaccination programs,[1] at an estimated 200–300 million U.S. dollars per year.[7] Thermostable vaccines, which are less dependent on cold supply chains, may provide benefits by reducing wastage, ensuring effectiveness and even enabling vaccination when the cold chain breaks down.[8] Various approaches have been developed to make thermostable vaccines. Stabilizers such as gelatin, proteins, deuterium oxide, $MgCl_2$, and non-reducing sugars produce stabilized formulations of vaccines in liquid or dry forms;[9] rational protein engineering is another strategy used to increase the thermal stability of vaccines.[10] However, these methods involve complicated procedures and always lead to a reduction or loss of activity, so that they are rarely applied in the clinic.

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In nature, eggs are one of few biological samples that can be stored under ambient conditions for a long time owing to their mineral shell. Inspired by this biomineralization phenomenon, we proposed that eggshell-like inorganic coatings may improve the thermostability of vaccines in an analogous manner.[11] A challenge is that vaccines cannot generate shell structures spontaneously. In this study, using live Japanese encephalitis vaccine (JEV) SA14-14-2 as an example, we show that in situ biomineralization can enclose the vaccine in a mineral shell. The resulting egg-like vaccine is robust, exhibiting overall increased thermostability without any effect on its original immunogenicity.

Japanese encephalitis is a major cause of viral encephalitis with a high case-fatality rate (30%), resulting in 35000-50000 cases and 10000-15000 deaths each year.[12] Therefore, development of a refrigeration-free JEV could remarkably facilitate vaccination. In situ mineralization of JEV was initiated by an incubation of the vaccine in a calcium-rich Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 2 h (see Supporting Information). Because of the negative surface charge of JEV viral particles,[13] the vaccine particles absorbed and concentrated abundant cationic calcium ions to increase the local supersaturation. Thus, the calcium-rich vaccine surface provided the nucleating sites to induce in situ mineralization of calcium phosphate (CaP),[14] resulting in an ultrathin coating. To optimize the reaction, different calcium concentrations were tested and DMEM with 11.25 mM extra calcium chloride was found to be an appropriate condition (see Supporting Information) because more than 90% of the vaccine particles became biomineralized, termed B-JEV.

The viral solution of JEV in DMEM was clear and the biomineralized JEV was a stable colloid solution (Figure 1a). Usually, viral particles can only be concentrated by ultracentrifugation under complicated conditions; whereas the B-JEV could be concentrated using a normal centrifuge at 16000 g for 10 min (Figure 1b). Furthermore, the obtained viral particles were could be directly observed by transmission electron microscopy (TEM) without any staining (Figure 1b). Under TEM, B-JEV particles were spheres with size distributions of 50–60 nm, which were a little larger than the bare vaccine particles (40–50 nm). Using a negative stain treatment of phosphotungstic acid for the biomineralized particles, the enclosed vaccine was identified (Figure 1b, insert), indicating the eggshell-like structure of the particles. The mineralized vaccines with shells and their dimensions were also confirmed by scanning electron microscopy (SEM, Supporting Information). Energy dispersive X-ray spectroscopy (EDX) found that the coating was composed of Ca, P, and O atoms (Figure 1d), directing to the CaP phase. X-ray

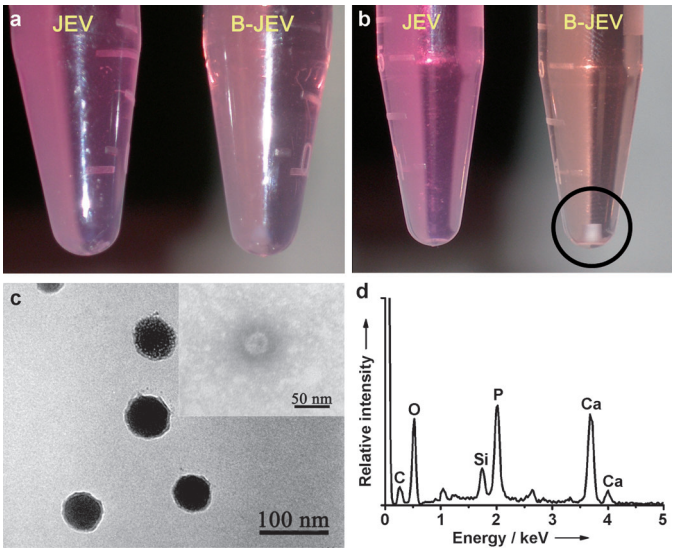

Figure 1. JEV and B-JEV solutions before (a) and after (b) centrifugation; the circle shows the precipitated biomineralized vaccine particles. c) Direct TEM observation of B-JEV particles; insert is negatively stained B-JEV. d) EDX analysis of B-JEV (the Si signal was attributed to the silica substrate).

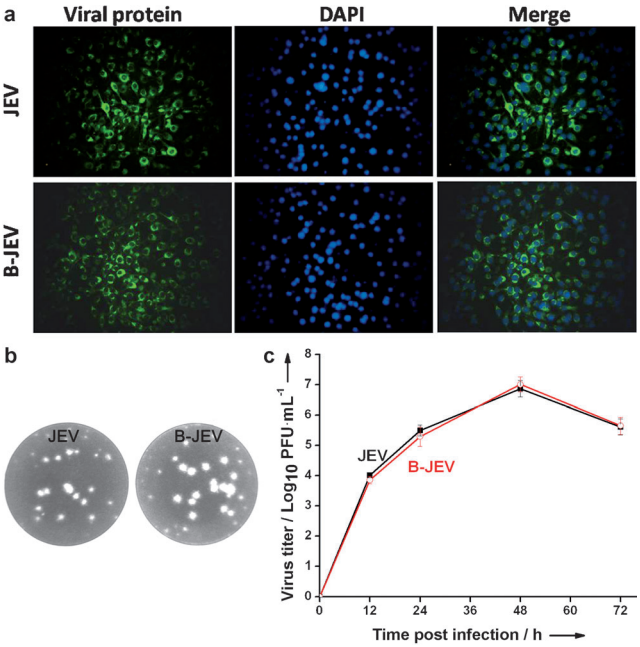

Figure 2. Comparisons of biological activity between JEV and B-JEV. a) IFAs using JEV-specific antibody (green); BHK-21 cell nuclei were stained with DAPI (blue). b) Plaque-morphology test using BHK-21 cells. c) One-step growth curves on BHK-21 cells.

diffraction (XRD) showed that the precipitated CaP phase on the vaccines was poorly crystallized hydroxyapatite (Supporting Information).

Before moving on, it was critical to make sure that the treatment did not affect the biological properties of the vaccine. Indirect immunofluorescence assays (IFA), plaque-formation assays, and one-step growth curves were performed to characterize JEV and B-JEV. Results from IFA with JEV-specific antibody showed that both JEV and B-JEV were infectious in baby-hamster kidney cells (BHK-21); the viral-specific protein (Figure 2a; green) was detected predominantly in the cytoplasm at 12 h post infection. The cell nuclei were stained by 4′,6′-diamidino-2-phenylindole (DAPI; Figure 2a; blue). Merged images (Figure 2a) showed that the viral proteins of JEV were mainly expressed in the cytoplasm, confirming the specific JEV nature of B-JEV. The plaque morphologies on BHK-21 cells of JEV and B-JEV were also similar (Figure 2b). One-step growth curves in BHK-21 cells showed both viral particles peaked at 48 h post infection, and no difference was detected between their growth kinetics (Figure 2c). These phenomena showed that the biological behaviors of B-JEV were identical to JEV and that the biomineralization modification did not alter the properties of the live vaccine.

A key question is the thermostability of B-JEV. Both bare and biomineralized vaccines were incubated at room temperature (26 °C) for days and their remaining infectivities were titrated by quantitative plaque assays. These data are represented by curves of logarithmic infectivity versus time (Figure 3a). As expected, the JEV showed a higher degradation rate in the assay, that is about 1 Log_{10} plaque-forming units (PFU; 90%), 2 Log_{10} PFU (99%) and 4 Log_{10} PFU (99.99%) loss after 2, 4, and 9 days storage, respectively (Figure 3a). According to the World Health Organization (WHO) requirement, effective vaccines require that not more than a 1 Log_{10} reduction of the initial titer has occurred.[15] Therefore, the room temperature storage of JEV should be no more 2 days. However, the degradation rate of B-JEV was much slower than JEV. After 2 days at room temperature, the reduction in titer was less than 0.3 Log_{10} PFU and 1 Log_{10} PFU loss occurred after 7 days, implying an achievement of storage for one week at room temperature.

Ambient temperature is not always less than or equal to 26 °C, and it occasionally exceeds 35 °C or even 40 °C. To estimate the loss of potency during long periods of storage at different temperatures, an accelerated degradation test (ADT), with samples subjected to elevated temperatures, was adopted. Two commonly used temperatures, 37 °C and 42 °C, were selected. The data revealed that the inactivation rate of B-JEV was always inhibited by at least threefold over JEV. At 37 °C, the bare JEV lost more than 3 Log_{10} PFU at 72 h, while B-JEV lost only 1.2 Log_{10} PFU (Figure 3b). To maintain less than 1 Log_{10} PFU loss of the initial potency, B-JEV could be stored for 60 h at 37 °C and while JEV could only be stored for 20 h. Similarly for the ADT assay at 42 °C, B-JEV activity loss was less than 1 Log PFU at 7 h, while the bare JEV lost more than 2.5 Log_{10} PFU at the same time (Figure 3c). Thus, we concluded that a simple biomineralization treatment can improve the thermal stability of JEV vaccine significantly, because the thermostability of B-JEV was always more than three times higher than that of bare JEV under the same thermal conditions.

To confirm that B-JEV was also effective in vivo, groups of BALB/c mice were immunized subcutaneously with the same dose of JEV and B-JEV. Sera and splenocytes were collected and the neutralization antibody was measured by

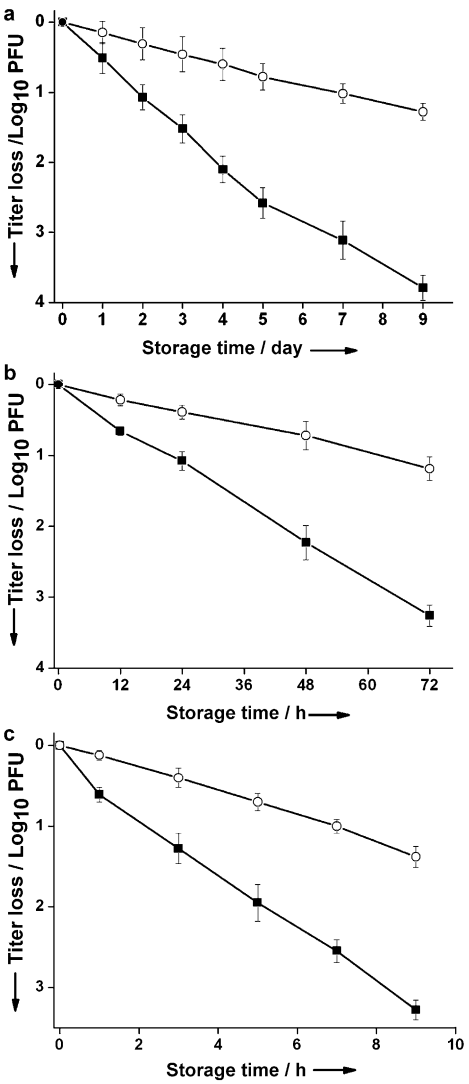

Figure 3. In vitro assessments of thermostability. Remaining infectivity of JEV and B-JEV after the storage at a) 26 °C, b) 37 °C, and c) 42 °C. Squares: JEV; Circles: B-JEV.

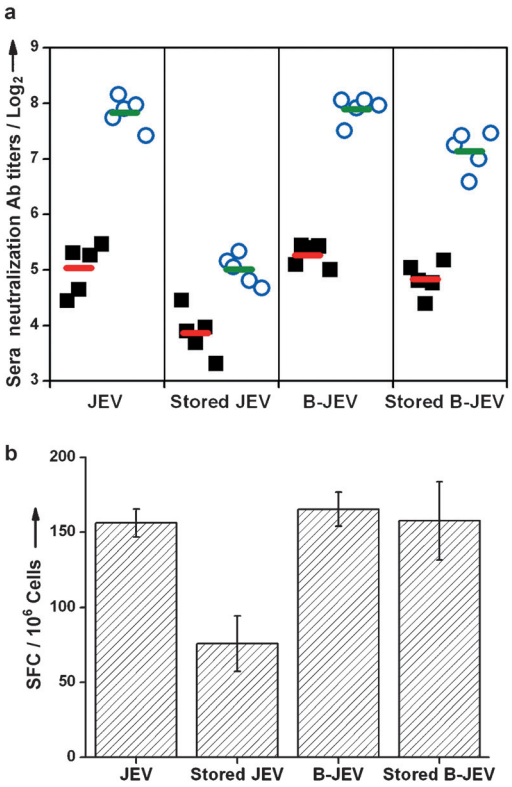

Figure 4. In vivo assessments of JEV and B-JEV before and after storage for one week at room temperature. a) Sera neutralization antibody titers at two (squares) and four (circles) weeks post injection; the bars represented the average values. b) Production of INF-γ by T cells of mice immunized with JEV or B-JEV. Splenocytes collected from groups of mice ($n \geq 5$) were stimulated with natural JEV and the numbers of spot-forming cells (SFC) were quantified in a 20 h ELISPOT assay.

standard plaque reduction neutralization tests (PRNT). Since CD8 + T cells play an important role in host defense so that IFN-γ secretion, which represents the humoral and cellular immune response in vaccine efficacy,[16] was examined by enzyme-linked immunosorbent spot assay (ELISPOT). The results showed that high titer neutralization antibodies and robust INF-γ secretion were successfully induced by both fresh JEV and fresh B-JEV (Figure 4). No obvious difference was observed between the two groups, indicating that B-JEV was as effective as native JEV in inducing an immune response. Subsequently, both JEV and B-JEV were stored at room temperature (26 °C) for 7 days and then tested in animals. The experiments showed that neutralization antibody titers induced by the stored JEV decreased remarkably, by two- to sevenfold, compared with the fresh JEV at the indicated times post immunization (Figure 4). Accordingly, the IFN-γ secretion of the stored JEV was only a half that of the fresh JEV. However, only a slight decrease (less than onefold, only 25–40 %) in neutralization antibody titers for the stored B-JEV was recorded (Figure 4a) and there was no significant reduction (within 5 %) in IFN-γ secretion for B-JEV even after storage for one week at room temperature without refrigeration (Figure 4b). These in vivo results were in accordance with the in vitro findings of the thermal inactivation tests, implying that we realized the goal of a vaccine that is stable "in your pocket" for one week through our biomineralization strategy.

Currently, viral diseases still represent a big challenge for global health. Because the maintenance of vaccine efficacy without a cold chain has the potential to extend immunity against deadly diseases, more robust vaccines could provide significant promise for a wide variety of applications. Different from previous attempts,[10–15] this biomimetic technique is fast, inexpensive, and also easily adapted to large-scale production. In this study, we used JEV as a model virus to demonstrate that an eggshell-inspired biomimetic coating gives vaccines with mineral shells, to help achieve room-temperature stabilized vaccines as a proof-of-concept.

Why does the mineral shell protect a vaccine from thermal inactivation? In nature, creatures such as fungi, archaebacteria, and plants can extend their survival by becoming dehydrated in hostile environments.[17] They can elevate their resistance to heat, cold, and other environmental insults with

this technique. Analogously, the shell introduced to the vaccine can protect the vaccine from direct interaction with aqueous solution, protecting the viral proteins from inactivation or destruction caused by heat. Besides, the shell can form electrostatic interactions with viral proteins through interactions between multivalent calcium or phosphate ions and polar amino acids, which can enhance the stability of the protein structures and interactions between protein subunits. This mechanism may also play an important role in thermostability improvement.

With modified vaccines, the spontaneous release of vaccines from biomineralized particles is required to ensure effective vaccination, this release can be attributed to the pH-sensitivity of CaP. This biomineral is stable at pH values greater than 7.2 but can dissolve readily when the pH value is less than 6.5 (Supporting Information). Physiological body fluid and incubation medium typically have a pH value of around 7.4, so that the biomineralized vaccines would be stable under extracellular conditions. Analogous to most nanoparticles, endocytosis is a likely pathway for the cellular uptake of the B-JEV particles and this is accompanied by acidic conditions, which could induce the degradation of the shell and subsequent recovery of the vaccine under intracellular conditions.[13a] Therefore, the selection of CaP is a key factor in the biomineralization-based vaccine modification. Actually, CaP is the primary component of hard human tissues (bones and teeth) so that this shell has a unique biocompatibility characteristics.[18]

In summary, a vaccine with an eggshell-like coating becomes robust, with a significant gain in thermal stability. Such egg-inspired modification provides a biomimetic strategy towards ambient-temperature-stabilized vaccines that do not need expensive cold chain maintenance anymore. And the current work provides an excellent example to highlight the potential of biomineralization in healthcare applications.

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