Stabilization of vaccines and antibiotics in silk and eliminating the cold chain

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Sensitive biological compounds, such as vaccines and antibiotics, traditionally require a time-dependent “cold chain” to maximize therapeutic activity. This flawed process results in billions of dollars worth of viable drug loss during shipping and storage, and severely limits distribution to developing nations with limited infrastructure. To address these major limitations, we demonstrate self-standing silk protein biomaterial matrices capable of stabilizing labile vaccines and antibiotics, even at temperatures up to 60 °C over more than 6 months. Initial insight into the mechanistic basis for these findings is provided. Importantly, these findings suggest a transformative approach to the cold chain to revolutionize the way many labile therapeutic drugs are stored and utilized throughout the world.

measles | mumps | rubella | penicillin

More than 17 million people die every year from infectious diseases, particularly in the developing world (1). Vaccines and antibiotics are important components of an effective infectious disease containment strategy; antibiotics represent a rescue measure while vaccination can be a primary mode of disease prevention. Unfortunately, the use of vaccines and antibiotics is severely limited in the poorest countries where infectious diseases account for more than half of all deaths (2). Due to temperature sensitivity, vaccine and antibiotic formulations must be maintained within a specific refrigeration temperature range. Because ambient temperatures in the developing world deviate significantly from refrigeration temperatures, the successful delivery of active vaccines and antibiotics depends on the “cold chain” system, a distribution network to maintain optimal cold temperatures during transport, storage, and handling. Cold chain requirements represent a major economic and logistical burden, particularly in lower resource settings, where refrigeration and electricity can be limited (3, 4). The cold chain alone can account for 80% of the financial cost of vaccination (1) and is estimated to cost vaccine programs \$200–300 million per year (5). Deficiencies in the process frequently occur even in industrialized countries (6, 7). For temperature sensitive compounds like vaccines and antibiotics, maintaining the cold chain is critical for adequate bioactivity (8, 9). Failures in the cold chain result in costly waste and the loss of nearly half of all global vaccines (10). Such failures can also result in the delivery of ineffective, subtherapeutic doses. For antibiotics, this problem can be associated with the development of antibiotic-resistant strains, a major public health concern.

Silk fibroin is a biologically-derived protein polymer purified from domesticated silkworm (*Bombyx mori*) cocoons that has demonstrated excellent properties for biomedical applications, including biocompatibility (11–14), robust mechanical strength (15), and slow, controlled degradation to nontoxic products in vivo (16). Silk can be prepared in a range of material formats, including films, hydrogels and microspheres (17, 18). Silk can be processed entirely in aqueous systems using mild, ambient conditions of temperature and pressure, allowing the incorporation of labile compounds without loss of bioactivity (18, 19). Silk is composed of a block copolymer structure with large hydrophobic

domains interspersed with small hydrophilic regions that form organized crystalline domains (β -sheets) that stabilize via physical crosslinks. This assembly forms nanoscale pockets that can immobilize bioactive molecules and improve their stability by minimizing water content and reducing protein chain mobility, thus preventing a transition from the native to denatured state (20, 21). Extensive physical crosslinking, the hydrophobic nature of the protein and high glass transition temperature (around 178 °C) render silk highly thermodynamically stable to changes in temperature and moisture, and mechanically robust due to the heavily networked β -sheet structures (21, 22). Due to its unique structure, encapsulation of therapeutic compounds in silk matrices could stabilize labile antibiotics and vaccines, akin to enzyme stabilization in silk (22).

Our model vaccine to investigate stabilization effects of silk matrices was the live measles, mumps and rubella (MMR) vaccine. The commercially available MMR vaccine contains a variety of protein and salt stabilizers; however, even with the stabilizers the vaccine rapidly loses potency at temperatures above the recommended 2–8 °C (23–25). Our model antibiotics were penicillin and tetracycline. Dating back to Fleming’s original 1929 paper on penicillin (26), penicillin is unstable in solution, breaking down within weeks at 25 °C and within 24 h at 37 °C (27). Tetracycline possesses broad specificity and low cost, but undergoes rapid photolysis and hydrolysis in solution (28, 29).

The objective of the present study was to evaluate silk proteins as a matrix for vaccine and antibiotic storage and stabilization. We demonstrate remarkable stabilization of these labile compounds and also provide mechanistic insight. Because silk can be formed into any variety of material formats, including films, hydrogels, microspheres and microneedles (17, 30), the proposed system has potential use ex vivo or in vivo as drug delivery vehicles. Further, the results provide a feasible path forward to revolutionize the cold chain and provide more efficient and widespread distribution of labile therapeutics throughout the world (Fig. S1).

Results

Antibiotic Stabilization in Silk Films. Tetracycline stability was compared stored in solution (Fig. 1A–B) or entrapped in silk films (Fig. 1C) over 4 wks at 4 °C, 25 °C, 37 °C, and 60 °C with light protection, and 25 °C with ambient light exposure. In solution, loss of tetracycline activity was observed at all storage temperatures, even refrigeration at 4 °C. Entrapped in silk, tetracycline

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Fig. 1. Tetracycline Storage Stability. (A) Aliquots of tetracycline in solution and tetracycline loaded silk films stored at -20°C , 4°C , 25°C , 37°C and 60°C , and 25°C with ambient light exposure at 1 and 4 wks. (B) Residual bioactivity over 4 wks storage of tetracycline stored in solution or (C) in 6% (w/v) silk films at 4°C , 25°C , 37°C , 60°C , and 25°C with ambient light exposure. Bioactivity measured using a zone of inhibition assay in *S. aureus* lawns. $N = 4$, error bars represent standard deviations (D) Comparison of tetracycline stored in silk films and as dry powder at 4°C (refrigeration), (E) 25°C (room temperature), (F) 37°C (body temperature) and (G) 60°C over 9 months. Activity measured using a zone of inhibition assay in *S. aureus* lawns. $N = 3$, error bars represent standard deviations. Data analyzed by one-tailed t-test, $df = 4$; significance levels of individual tests are indicated: $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

activity was lost only for films stored at 60°C , and even at this temperature, activity loss was relatively low; approx. 10% and 20% loss after 2 and 4 wks, respectively, compared to 100% loss after 2 wks for tetracycline stored in solution. Storage at body temperature (37°C) resulted in no activity loss for storage in silk films, compared to 80% lost within 4 wks for storage in solution. Tetracycline storage stability was also investigated in dry powder over 9 mo at various temperatures with light protection: 4°C (Fig. 1D), 25°C (Fig. 1E), 37°C (Fig. 1F) and 60°C (Fig. 1G). With the exception of samples stored for 2 mo at 37°C , retention of tetracycline bioactivity in silk films was equal to or higher than that of tetracycline stored in dry powder format. At the 6 and 9 mo sampling times, residual tetracycline bioactivity was significantly improved for storage in silk films compared with storage in dry powder format for all storage temperatures tested (one tailed t -test, $df = 4$, $p < 0.05$).

Stability studies were also carried out comparing the residual activity of penicillin stored in silk films versus other material formats (including storage in solution, storage as dry powder and entrapped in collagen films) at 4°C , 25°C , 37°C and 60°C (Fig. S2). With a few exceptions the stability of penicillin incorporated into silk films was higher than that of penicillin stored in any other form tested, including dry powder at 4°C . While stability rapidly declined for penicillin solutions stored at 25°C

and 37°C (consistent with previous reports as seen in ref. 27), approximately 50% of the initial penicillin activity was retained after 183 d of storage in silk films for all temperatures tested. For the first 40 d of storage, incubation enhanced penicillin activity above 100% of the initial value, a phenomenon also observed for previously reported enzyme stabilization in silk films and likely related to initial aggregation and subsequent reactivation of the bioactive component (22). Total activity loss was observed within 24 h for penicillin stored in solution at 60°C . Penicillin stored in collagen films or in dry powder exhibited more than 20% activity loss over 30 d. In contrast, no loss of activity was observed for penicillin stored in silk films at 60°C for 30 d.

Vaccine Stabilization in Silk Films. Encapsulation in silk films enhanced stability (expressed as the residual potency remaining post-storage compared with initial potency) of the measles, mumps, and rubella vaccines at elevated temperatures (Table S1). Reconstituted vaccine loses potency rapidly in solution (Fig. S3), a result consistent with manufacturer specifications that any unused MMR vaccine be discarded 8 h post reconstitution. To minimize loss of vaccine potency during the solution stage of silk encapsulation, lyophilized MMR-silk films were prepared. Compared to the initial potency recovered from the air-dried silk

films, the lyophilized films improved the recovery of measles, mumps, and rubella to 94.7%, 87.0%, and 98.4%, respectively.

Encapsulation in silk film (either air-dried or lyophilized) enhanced measles, mumps, and rubella viral particle stability over six months, particularly at elevated storage temperatures (Fig. 2). Both silk films showed improved residual potency of the measles component compared to lyophilized MMR vaccine powder stored at 25 °C, 37 °C and 45 °C. After 6 months stored at 25 °C, measles encapsulated in silk films retained 83.9% potency compared to 74.5% for the lyophilized vaccine powder. After 6 mo stored at 37 °C, silk films showed a dramatic improvement in stability of measles infectivity, resulting in 56.5% potency recovered compared to 9.9% from the powder. At 45 °C, the measles vaccine lost all potency after 20 wks in storage while the silk films retained 53.4% activity after 24 wks. The mumps (Fig. 2B) and rubella (Fig. 2C) components displayed similar trends in potency retention.

Lyophilization of the silk films not only improved vaccine activity recover postencapsulation, but also provided even greater thermostabilization of the vaccines. Stabilization of all vaccine components in lyophilized silk films was found to be independent of storage temperature: After 6 mo of storage at 37 °C and 45 °C in lyophilized silk films, $\geq 85\%$ initial potency was retained for all components of the vaccine (measles, mumps, and rubella). Because activity loss during storage in solution or as lyophilized vaccine powder increased with increasing temperature, the improvement in stabilization resulting from silk film encapsulation is most dramatic at elevated storage temperatures. In plots of predicted half-lives versus storage temperature, slopes for vaccine stored as powder were consistently higher than the slopes for vaccine stored in either silk film systems, indicating that storage in powder results in greater degradation rate increases with increased

storage temperature (Fig. 3B). As seen in the comparison of estimated degradation rates and corresponding half-lives of the three vaccine systems (Table S2), stability improvements observed for silk systems become more dramatic as the storage temperature increases. With the exception of storage at 4 °C, the silk films and lyophilized silk films exhibited a greater predicted half-life of all three viral components of the vaccine. The difference was especially pronounced in the predicted half-life of the virus at 37 °C and 45 °C. For the measles component, entrapment in silk film increased the viral half-life at 37 °C from 9.4 wks for dry powder to 22.0 wks for silk films and 93.8 wks for lyophilized silk films. Storage at 45 °C provided similarly impressive stabilization results: virus half-lives for powder, silk films and lyophilized silk films were 5.0, 19.8, and 107.6 wks, respectively. The Arrhenius plot of vaccine stored as powder has the steepest slope, indicating the greatest increase in rate of degradation from storage in 4 °C to 45 °C and thus a greater temperature-dependence of degradation compared to the silk film systems, which have more shallow slopes (Fig. 3A).

Mechanisms of Stabilization. The shelf life of lyophilized vaccines is dependent on both adherence to the cold chain and maintenance of low residual moisture content (25). The residual moisture of MMR powder, MMR-silk films and lyophilized MMR-silk films was $2.47\% \pm 0.25$, $4.60\% \pm 0.83$, and $1.85\% \pm 0.30$, respectively (Table S3). Due to processing conditions, MMR-silk films had higher residual moisture, but the net increase in moisture at 6 mo of storage at 45 °C was 28.3%, compared to a 59.5% increase for the MMR powder system. Residual moisture content of the vaccine storage systems was found to correspond to residual potency. At elevated temperatures, MMR powder exhibited greater losses

Fig. 2. Measles, Mumps, and Rubella Vaccine Stability. Vaccines prepared in 9% (w/v) silk and lyophilized films over 6 mo at 4 °C, 25 °C, 37 °C, and 45 °C. Residual potency of the (A) measles, (B) mumps, and (C) rubella components of the MMR vaccine. (◆) lyophilized MMR-silk films, (○) MMR-silk films, (■) MMR powder. $N = 3$, error bars represent standard deviations.

Fig. 3. Kinetics of Degradation of MMR-Silk Film Systems. (A) Arrhenius plots of the degradation rates of the measles, mumps, and rubella components of the vaccine as a function of the inverse of the absolute temperature. (B) Predicted half-lives of the measles, mumps and rubella viral components as a function of temperature and the corresponding upper and lower limits of the half-life. The predicted half-lives represent the estimated time required for the viral component to degrade to 50% of the initial value. (◆) lyophilized MMR-silk films, (○) MMR-silk films, (■) MMR powder.

in potency and greater increases in residual moisture than the silk films. Similarly, minimal residual moisture increases for lyophilized MMR-silk films corresponded to greater viral potency retention.

The enhanced thermostability provided by silk in contact with the vaccines was assessed with differential scanning calorimetry (DSC) and light scattering. DSC demonstrated that the silk encapsulation increased glass transition (T_g) of the vaccine (Fig. 4B). Solid-state DSC of lyophilized silk films showed a T_g at 178 °C. The DSC thermogram of the MMR powder showed a T_g at 68.9 °C, while the lyophilized MMR-silk films showed a T_g at 89.2 °C as well as two peaks at 116.6 °C and 164.8 °C indicative of a T_m and a T_d (melting and degradation temperatures, respectively) of the complex vaccine system. It was unclear whether the T_g shift was due to structural stabilization by the silk or by the interaction between silk and the various excipients present in the vaccine sample. Also, the DSC curve of MMR powder includes a complicated mixed melting and degradation process. Without the protection of the silk proteins, the degradation of the MMR proteins occurred at a much lower temperature (starting around approximately 110–120 °C) and this fully or partially covered the melting process of MMR powders. Therefore, the vaccine was purified to remove the excipients. As the viral sample is a liquid solution, nano-DSC was run on these samples. The nano-DSC thermogram of the purified viral particles in water showed a transition point (Tp) at 16.8 °C, indicating the viral proteins were undergoing a conformational change (Fig. 4C). The solution of purified viral particles in silk showed an elevated transition point at 68.3 °C. While the range of the nano-DSC does not extend far enough to show the T_g of the silk, a thermogram of silk solution is still shown to illustrate that neither of the transition point values can be attributed to a change in the silk structure alone, indicating a positive impact of silk proteins on the vaccine as reflected in the elevated T_g .

The elevated transition point of the vaccine-encapsulated silk solution is due to structural stabilization provided by the silk to

prevent viral protein denaturation and aggregation (Fig. 4A-ii). The transition point value at 16.8 °C is likely due to the unfolding of the viral surface glycoproteins (F and N of measles and mumps and E1 and E2 of rubella) due to the elevated heat applied to the viral particles. This denaturation most likely leads to the aggregation of the viral particles (Fig. 4A-iv), preventing them from binding and fusing with the animal cells used in testing, thus leading to loss of infectivity of the viral particles and vaccine. The broadness of the transition peaks is likely a result of the combined contribution of the unfolding of several proteins present in the sample. Furthermore, protein aggregation may also contribute to the broad peaks by overlapping with the endotherm of denaturation (31). The infectivity of MMR is dependent on the conformational stability of the viral proteins. The presence of silk molecules increased transition point values, indicating that the silk provided structural stability to the viral proteins, shielding them from thermal denaturation. The interaction between the viral particles and the silk hydrophobic regions, as well as limited chain mobility, might prevent viral protein aggregation, thereby preserving viral and vaccine infectivity.

To further investigate whether transition points corresponded to viral particle denaturation due to aggregation, viral particle size was examined by dynamic light scattering (DLS see Fig. 4D). The average mean effective diameter of a naked viral particle was around 250 nm, consistent with reported diameter ranges of measles and mumps (32, 33). Purified virus solution exhibited an increase in mean effective diameter around 16 °C, indicating the presence of viral protein aggregation due to the increased thermal input. In contrast, viral particles in silk solution did not show signs of aggregation until 70 °C, indicating silk provided structural stability that prevented thermal-induced aggregation of the viral proteins. These DLS results correspond well with the TP values determined by the nano-DSC, as the aggregation detected by light scattering occurred within the temperature range of the protein unfolding measured by DSC.

Discussion

Activity loss at body temperature represents a serious problem for long-term, implantable drug delivery systems, as well as for the cold chain requirements. Formulations which are unstable at temperatures ≥ 25 °C are difficult to transport and store, particularly in developing countries where refrigeration is limited. Immobilization of bioactive molecules leads to an increase in stability by maintaining constant environmental conditions to protect the bioactive molecules against potential degradative variables such as pH, temperature or ionic strength changes and reducing molecular mobility (34). The chemistry, structure, and assembly of silk generates a unique nanoscale environment (35) and makes this protein polymer an attractive candidate for the stabilization of bioactive molecules over extended periods of time. Without chemical processing, aqueous silk solution can be used to entrap bioactive molecules in amphiphilic, self-assembly domains.

Prolonged exposure to temperatures above recommended storage conditions can damage vaccines in various ways, most notably by altering the tertiary structure of the viral proteins, reducing viral infectivity and thereby decreasing vaccine potency (36). The main cause of viral inactivation is disruption of viral surface proteins and stresses such as elevated temperature can induce conformational changes in viral proteins (37). These conformational changes may affect the stability of the virus by inducing viral particle aggregation that prevents viral fusion and uptake, thus leading to virus inactivation (Fig. 4A and ref. 38). The silk films may have improved recovery of active vaccine by minimizing exposure to degradative enzymes (39). Generally, storage in either silk film format decreased observed degradation rates for all three virus components. Lyophilized silk systems exhibited the lowest degradation rates and longest half-lives, while lyophilized vaccine powder degraded fastest at all storage temperatures.

Fig. 4. Thermal Analysis of Stabilization of MMR-Silk Films. (A) (i) Measles and mumps structures consist of single-stranded, negative-sense RNA enclosed in nucleocapsids within a lipid bilayer. The viral envelop consist of the matrix (M), haemagglutinin (H) and fusion (F) protein. (ii) By a combination of hydrophobic interaction and limited chain mobility, silk-entrapped viral particles maintain structural activity at elevated temperatures. (iii) Structurally intact F and H proteins bind and fuse to the receptors CD46 and CD150 to gain entry into the cell to initiate viral replication. (iv) Denaturation of the surface proteins can result in aggregation of the viral particles and binding and fusion cannot occur. (B) Solid-state DSC. T_g = glass transition point. (C) Nano-DSC. T_p = protein transition point. The presence of silk increases the T_p of the viral particles, indicating the effect of silk on the encapsulated viral proteins. (D) Comparison of dynamic light scattering of purified viral particles in water and purified viral particles in silk solution.

Humidity can also have a significant effect on vaccine products as the excess water introduced to the system can lead to increase in mobility and corresponding reactivity of the viral proteins (40). The residual moisture analysis of the films revealed increased residual moisture over the course of 6 mo in storage, especially in the high temperature ranges. While MMR powder and lyophilized MMR-silk films were stored in low humidity conditions provided by lyophilization vials and sealing in a nitrogen-rich environment, MMR-silk films stored in Eppendorf tubes allowed greater absorption of humidity. The increase in residual moisture in silk films at elevated temperatures could also be explained by water desorption from the silk, because they still contain traces of intermolecular tubes bound water (41). Though humidity absorption is higher in nonlyophilized silk films, silk films stored in Eppendorf demonstrate the feasibility of silk as a simple, straightforward storage platform that significantly enhances thermostability compared to existing more complicated vaccine formulations. Lyophilized silk films combine the advantages of silk storage and freeze drying, further reducing protein mobility and improving stabilization compared to air-dried films. Though the absolute residual moisture of the silk films is higher than that of the MMR powder, the percent increase of the residual moisture in the powder over the tested temperature range was greater than that observed in the silk films. The increase in temperature appears to have had a greater impact on moisture in the powder than the silk films. This suggests silk inhibited molecular mobility during storage to prevent protein unfolding and subsequent aggregation.

The mechanisms involved in antibiotic stabilization in silk may be attributable to a combination of low water content and silk surface chemistry to reduce aggregation or degradation (42).

Conclusions

We have shown silk as an effective carrier material for enhanced thermostability of both antibiotics and vaccines. Both silk film systems were able to increase the half-lives of the vaccine compared to the manufacturer supplied vaccine formulation at 25°C , 37°C , and 45°C . Silk reduced the temperature-induced protein unfold-

ing and subsequent aggregation by reducing residual moisture during storage at elevated temperatures, and also provided structural stability to the vaccine to elevate the temperature at which the viral proteins denature.

Pronounced stabilization by silk at the elevated temperatures that result in vaccine spoilage or antibiotic activity loss in conventional formulation when the cold chain is broken (37°C and 45°C) suggest silk films would provide sufficient stability over a wide range of storage temperatures. When the stabilization data presented here are combined with the remarkable mechanical features and tunable release kinetics characteristic of silk carriers (30, 43, 44), a robust stabilization and delivery platform for antibiotics can be envisioned, extending even to microneedle formats (30). In total, these findings suggest a new path towards eliminating the cold chain, providing new venues towards improved processing, distribution and use of labile therapeutics such as antibiotics and vaccines.

Materials and Methods

Silk Fibroin Purification. Silk fibroin aqueous solutions were prepared as previously described (16).

Antibiotic Stabilization Studies. The bacteria strains used were *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (American Type Culture Collection, Manassas, VA).

For long-term stability studies, tetracycline and penicillin loaded silk films were prepared as previously described (preparation, loading and treatment details in *SI Materials*). Collagen films were prepared by dissolving Avitene® Microfibrillar Collagen Flour (Bard Davol, Warwick, RI) in sterile ultrapure water in a weight/volume concentration equal to the silk solution used for film preparation, then mixing with antibiotic solution, casting films and drying overnight at ambient conditions. Storage temperatures tested were 4°C , 25°C , 37°C and 60°C . For tetracycline studies, samples were wrapped in foil to protect from light exposure, except a group of samples stored at 25°C with exposure to ambient light. Residual bioactivity of antibiotic in various storage systems was evaluated using a direct zone of inhibition assay based on the principle of the Kirby-Bauer Susceptibility Test (45, 46) (*SI Materials*).

Vaccine Stabilization Studies. Trivalent vaccine. A commercial source of trivalent measles, mumps, rubella vaccine was used for potency estimation:

MMR® II (Merck & Co., Inc., USA), a sterile lyophilized live virus vaccine containing the Enders' attenuated Edmonston measles, the Jeryl Lynn mumps and Wistar RA 27/3 rubella. Virus was purified by reconstituting lyophilized vaccine powder in sterile water, loading into 0.5 kDa dialysis tubing (Sigma Aldrich) and dialyzing against a 0.15 M NaCl solution to remove excipients. The recovered vaccine solution was then run through a PD-10 desalting column (GE Healthcare) per manufacturer specifications to remove excess salt. Recovered purified viral particle solution was collected and stored in an Eppendorf tube at -80°C until use. Viral infectivity was evaluated using a quantitative real-time RT-PCR viral infectivity assay (*SI Materials*). Degradation behavior was characterized in terms of predicted viral half-life, degradation rate and slope of the Arrhenius plot (*SI Materials*).

Vaccine entrapment in silk films. Standard MMR-silk films were prepared by casting a 1:1 weight ratio mixture of MMR: silk on Teflon-coated molds and drying films at room temperature for 12 h in a sterile hood protected from light. Individual films were placed in Eppendorf tubes, under ambient conditions, and stored at 4°C , 25°C , 37°C , and 45°C for stability studies. Lyophilized MMR-silk films were prepared by aliquoting a 1:1 weight ratio MMR: silk solution into 96-well plates and freeze dried using a VirTis 25L Genesis SQ Super XL-70 Freeze Dryer. The samples were frozen at -45°C for 480 min. The primary drying occurred at -20°C for 2,400 min and secondary drying at 35°C for 620 min. The samples were held at -45°C until they were removed from the lyophilizer. The films were then removed from the well plates and transferred to 5 cc glass serum vials. Five mm lyophilization stoppers were applied to the vials under nitrogen and vacuum conditions in a MBRAUN LABmaster glovebox (Garching, Germany) and a 5-mm crimper was used to tighten the 5-mm alum seal on the vials. The vials were stored at 4°C , 25°C , 37°C , and 45°C for stability studies. The vials, stoppers, seals and crimper were supplied by VWR (Bridgeport, NJ).

Residual moisture determination. Residual moisture of the lyophilized vaccine powder, MMR-silk films, and lyophilized MMR-silk films was measured by the thermo-gravimetric method, modified from Worrall et al. (47)

Differential scanning calorimetry (DSC). Five mg of solid samples were encapsulated in Al pans and heated in a TA Instrument Q100 DSC (New Castle, DE) with a purged dry nitrogen gas flow of 50 mL/min. Tg was recorded as the onset temperature of the discontinuity curve of the heat flow versus temperature. All measurements were made at $10^{\circ}\text{C}/\text{min}$. The samples were initially equilibrated at -20°C for 5 min and then heated to 200°C , held at 200°C for 5 min, followed by cooling to 20°C . Nano-DSC measurements were taken on a CSC Model 6100 Nano II Differential Scanning Calorimeter (London, UT).

Dynamic light scattering (DLS). The size of the measles, mumps and rubella viral particles as a function of temperature was monitored by DLS. A 400 μL aliquot of 2 mg/mL sample solution was filtered through a 0.45 μm syringe filter (GE, Fairfield, CT). DLS was conducted using the Dyna-Pro DLS system (Wyatt Technology, Santa Barbara, CA) with parameters set at 60 s acquisition time, 10 number of acquisition and laser power of 75 mW. A 100 μL aliquot of the sample was transferred into an RNase-free, DNase-free, protein-free UVette Eppendorf cuvette to be inserted into the DLS.

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