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## Rational design of rabies vaccine formulation for enhanced stability

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**Background/aim:** Vaccines are often lyophilized in order to retain their stability and efficacy for a longer period of time. However, the same lyophilization process may also cause a major degradation of the vaccine, especially during early phases of manufacturing, leading to a loss of potency of the product. Many viral diseases, such as rabies, are acute and fatal unless the vaccine is administered prior to exposure or the onset of symptoms in the case of postexposure treatment.

**Materials and methods:** We investigated the effect of lyophilization on the stability of the virus structure during rabies vaccine manufacturing using dynamic light scattering and transmission electron microscopy.

**Results:** Our results indicate that some viruses lose their stability and efficacy in the course of lyophilization if the pH of the cell culture medium is controlled by solvated CO<sub>2</sub> because the structure of the rabies virus is very sensitive to the solution pH: the virus either aggregates or its shape is deformed at low solution pH, whereas at high pH empty capsid shells are formed.

**Conclusion:** Based on our findings, we developed a new formulation for the rabies vaccine that is stable in different buffers owing to the prevention of pH upshift upon lyophilization.

**Key words:** Rabies vaccine, rabies virus, vaccine degradation, lyophilization, pH effect, vaccine formulation, rational vaccine design

### 1. Introduction

Rabies is an acute and almost always lethal viral encephalitis affecting only mammals (1) and is one of the major causes of death in many parts of the developing world, killing more than 55,000 individuals, mostly children, each year (2). Some of the reasons for this high death toll are lack of an effective vaccine that works under conditions found in these parts of the world and affordability, which obviates multiple dose administrations.

Rabies is caused by the rabies virus, which belongs to the genus *Lyssavirus* of the family Rhabdoviridae that contains about 80 viruses, often characterized by their bullet shape (3). The rabies virus is approximately 200 nm in length and has a diameter of 80–100 nm (Figure 1). It has nonsegmented, single-stranded, negative-sense genomic RNA (~12 kb) that encodes five proteins (4–11). The RNA is linked with about 1800 nucleoproteins that are tightly bound to a nucleocapsid (12). The matrix proteins combine with lipids to form an envelope surrounding this nucleocapsid.

In order to immunize a person against rabies, it is believed that the structure of the virus is required to

remain intact during administration. Currently, there are three human rabies vaccines: the human diploid cell vaccine (SP), rabies vaccine adsorbed (RVA), and purified chick embryo cell culture vaccine (PCECV) (13). SP was approved in 1980 in the United States and since then it has been widely used globally for general pre- and postexposure immunization of humans (14). Previous vaccines caused adverse side effects and were extremely painful, and in addition they were not 100% effective in preventing fatal infection. In contrast, the SP vaccine provided a rapid immune response with less pain and fewer side effects, required fewer doses, was more effective in preventing infection, and thus was considered to be an advanced vaccine compared to previously offered duck embryo vaccines. Both the liquid form and lyophilized versions are available. SP in its lyophilized form is usually stable and its potency is generally retained for a long time at typical storage temperatures of 2–8 °C (14). However, the lyophilized form sometimes shows degradation after lyophilization, attributed to the drying process. We set out to investigate the reasons for this degradation phenomenon.

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Assuring the stability of viral vaccines is imperative (15,16) and several methods have been developed for this daunting task. The most commonly used excipients for stabilization of formulations are listed in the GRAS list. In fact, this concern is also applicable for other additives and preservatives used in biological drug formulations. Another stabilization method is to lyophilize the vaccine to slow down the degradation process. The major problem with the lyophilization method is the potential degradation of the product during or after the freezing process. In practice, this process may require keeping the virus concentration quite high, so that after lyophilization some whole viruses that are capable of inducing immune response would remain in the vial even if degradation occurs.

The recent increase in awareness of the safety, product constituents, and potential side effects of vaccines has created a new challenge to develop more stable and safe vaccine formulations. Rational drug formulation requires mechanistic insight into molecular interactions, understanding of the mechanism of degradation, and understanding of the efficacy, stability, feasibility, and performance of the vaccine in the host cell (14,17–19). Unfortunately, currently we have a limited understanding of the vaccine degradation process due to the complexity of viral nature, particularly if structurally disintegrated virus particles are involved. Consequently, the stabilization of vaccines is frequently an empirical procedure based on observed changes in their biological activity. Design of experiments, rational design, and orthogonal characterization tools allow supplementing empirical knowledge for the development of new formulations. Nevertheless, in order to avoid degradation of viral vaccines, understanding the underlying mechanism of degradation phenomena at the molecular level is necessary.

The liquid rabies vaccine SP formulation is an industry standard and a relatively well-established formulation. However, since it is produced with a human cell line, it is not purified, and therefore it contains many nonviral proteins and is susceptible to degradation upon lyophilization. The degradation cannot be detected with a standard ELISA test during quality control after lyophilization, but the lyophilized vaccine fails the NIH test (20).

Presently, knowledge concerning the stability of the rabies virus is relatively limited. Nonetheless, it is known that above 56 °C it has thermolability (21), and that recurring freeze-thaw cycles cause capsid degradation, presumably due to virus disassembly. Below pH 6 its activity drastically decreases due to a conformational change of its glycoprotein G. Divalent cations such as calcium and magnesium tend to stabilize the virus. Reports regarding variation of solution conditions and lyophilization are limited. Although various conditions perturbing the

stability of the rabies virus have been described briefly (14,18,22–24), the underlying mechanism of degradation is not yet understood, as is often the case for viruses.

In order to understand degradation and therefore be able to better take steps to avoid it, we have performed a comparative study of lyophilized and nonlyophilized virus samples that fail the NIH test. We hypothesized that the pH change causes vaccine degradation upon lyophilization by structurally disintegrating virus particles. To test our hypothesis and to determine the reasons for vaccine destruction after lyophilization, we have conducted experiments with three formulations (liquid SP (SP1), lyophilized SP (lyo SP1), and control SP (SP2)) of the rabies vaccine with dynamic light scattering (DLS), transmission electron microscopy (TEM), and optical microscope. DLS and TEM data were correlated in a combinatorial manner in this manuscript. TEM provides both size and shape information on the sample, but only a small fraction of the sample is imaged. There is also a possibility of inducing a large aggregate formation under the vacuum. DLS, on the other hand, requires liquid samples and can be applied to a bulk sample; however, it depends on the model used. Therefore, both methods were correlated to obtain information about the size, shape, and number of particles in vaccines. The SP2 sample was used as the control because it is a purified and reformulated virus and does not degrade noticeably upon lyophilization. Here we show that the structural integrity of the virus particles, and hence in turn vaccine degradation, can be prevented by controlling the solution pH by an alkali at pH 8 during lyophilization. To demonstrate the influence of solution conditions on virus stability, we have lyophilized the virus under different solution conditions such as various buffer types (Tris, PBS, and potassium phosphate). We also show that the shape of the virus structure changes with solution pH, and the degradation mechanism varies depending on whether solution pH is high or low: at high pH, the virus displays only empty capsids, whereas at low pH it splits and aggregates.

## 2. Materials and methods

All vaccines were received from Sanofi-Pasteur in liquid or lyophilized forms. The manufacturing processes and specifications were identical to clinical batches. The inactivation of the virus was done by  $\beta$ -propiolactone. Lyophilization of vaccine samples was done with the same procedure as in the clinical batches in Sanofi-Pasteur facilities. Lyophilization of new formulations was performed with home-built equipment at MIT that included a pump, a vacuum chamber, and a thermostat. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received without further purification. Dialysis tubes (FLT CE 3 ML Spectra/Por

Float-A-Lyzer with 50 kDa) were obtained from VWR (Radnor, PA, USA) for buffer exchange experiments. All experiments were repeated at least three times, and all control studies were conducted where necessary. Furthermore, the background was subtracted from the spectroscopic signals as appropriate. The results have been obtained with controls at similar concentrations to assess the impact of formulation changes.

### 2.1. Dynamic light scattering experiments

We used the Wyatt DynaPro Titan DLS instrument (Santa Barbara, CA, USA) for the DLS experiments. Experiments were conducted with a micro quartz cuvette at room temperature with a volume of 100  $\mu$ L. Results are reported using the Rayleigh Spheres model. We also tried the Coils and Isotropic Spheres models, but found that there was no considerable difference between the results with these models and those using the Rayleigh Spheres model. For each sample, the DLS equipment measures the scattered light for 10 s and repeats the process 10 times. Independently, each DLS experiment was repeated three times with three different sample sets, and the average was taken. DLS data were transferred to data plotting software (Igor Pro) as an ASCII file and replotted as original to display as a higher quality image.

### 2.2. Transmission electron microscope experiments

Imaging was done using the FEI Tecnai Spirit equipped with an AMT digital camera (Eugene, OR, USA). The samples were negatively stained using 2% uranyl acetate (UA), 2% ammonium molybdate (AM), or 1% sodium phosphotungstate (PTA). We found that imaging of the viral structural detail was heavily dependent on the negative staining method; PTA and AM gave better results and higher resolution than UA.

### 2.3. Fluorescence microscope experiments

We used a Nikon Inverted TE300 optical microscope to detect the virus with fluorescein isothiocyanate dye-labeled antirabies monoclonal antibody from Fujirebio Diagnostics, Inc. (Malvern, PA, USA). The virus formulation was dried on a microscope slide and antibody solution was added, and then the slide was washed several times with PBS to remove the unbound antibody prior to recording images. Various virus and antibody concentrations were checked. The best images were obtained with undiluted virus and 10 $\times$  antibody dilution. Standard fluorescein filters were used for both excitation and emission paths.

## 3. Results and discussion

### 3.1. Rabies virus structure

Figure 1 shows the structure of the rabies virus by a TEM experiment, where the surface glycoprotein layer as well as capsid proteins can be seen due to enhanced resolution.

The unstressed but inactivated rabies virus in the control vaccine (SP2) resembles the native virus as it appears in the literature in size and shape, with about 80–100 nm diameter and 100–200 nm length (Figure 1).

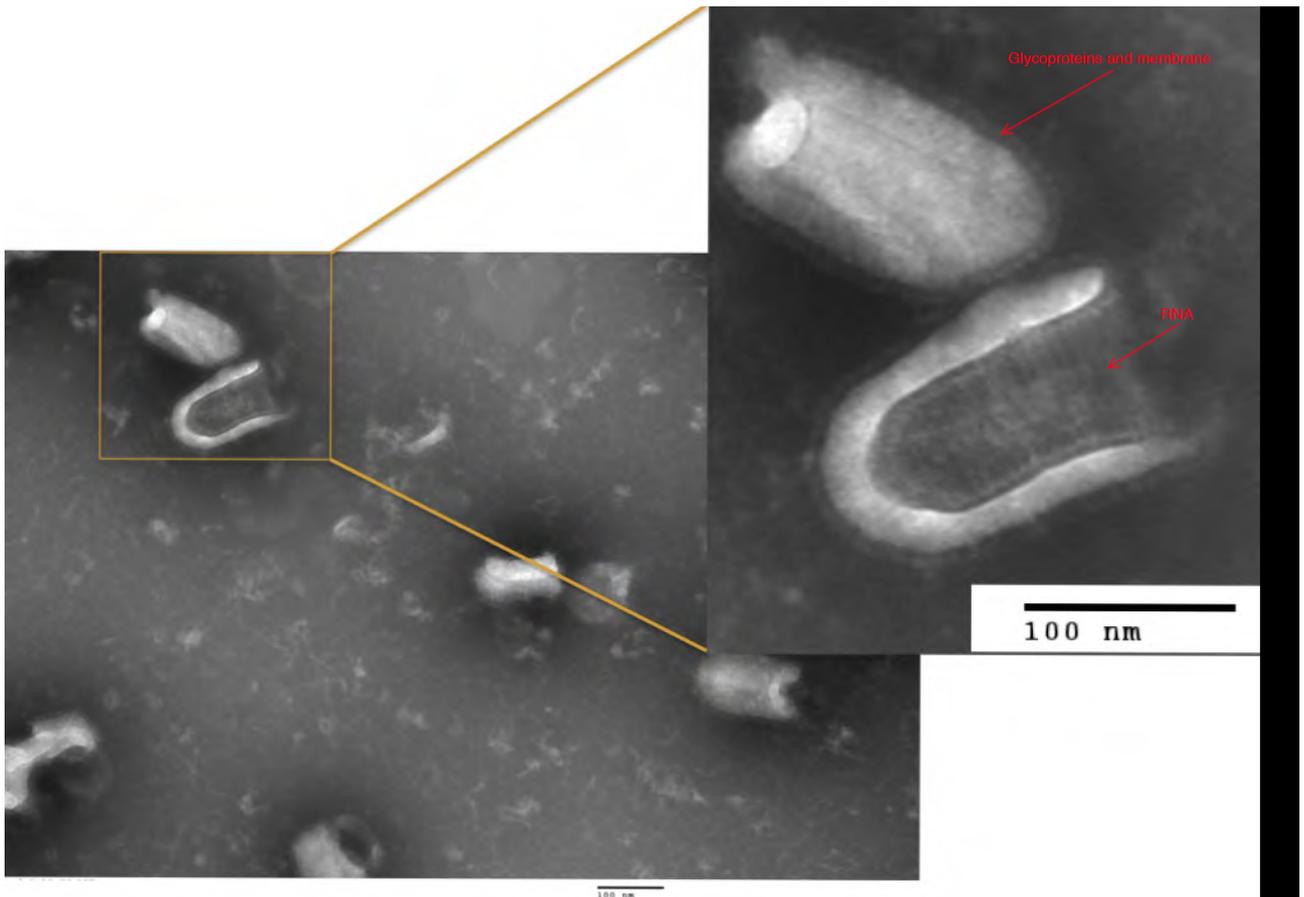
### 3.2. Effect of lyophilization on viral structure in a pH controlled solution

DLS experiments give valuable information about the size distribution of molecules in formulations and size inhomogeneity of the samples. We conducted DLS experiments to determine the inhomogeneity of the samples (Figure 2). The DLS results showed that the unstressed virus in the control sample (SP2) has one peak corresponding to a single undegraded virus population, with a diameter of about 100 nm (Figure 2A). The SP formulation, on the other hand, has two different populations (Figure 2B): the peak centered at 200 nm corresponds to undegraded viruses, while the peak at 10 nm corresponds to remaining proteins from culture cells with diameter of less than 10 nm. When SP1 is lyophilized, the virus population is reduced drastically, the virus peak in DLS experiments almost disappears, and larger as well as smaller particles are observed (Figure 2C). The species that have smaller sizes than the whole virus are probably disassembled viruses, and the larger particles are aggregated viruses and proteins.

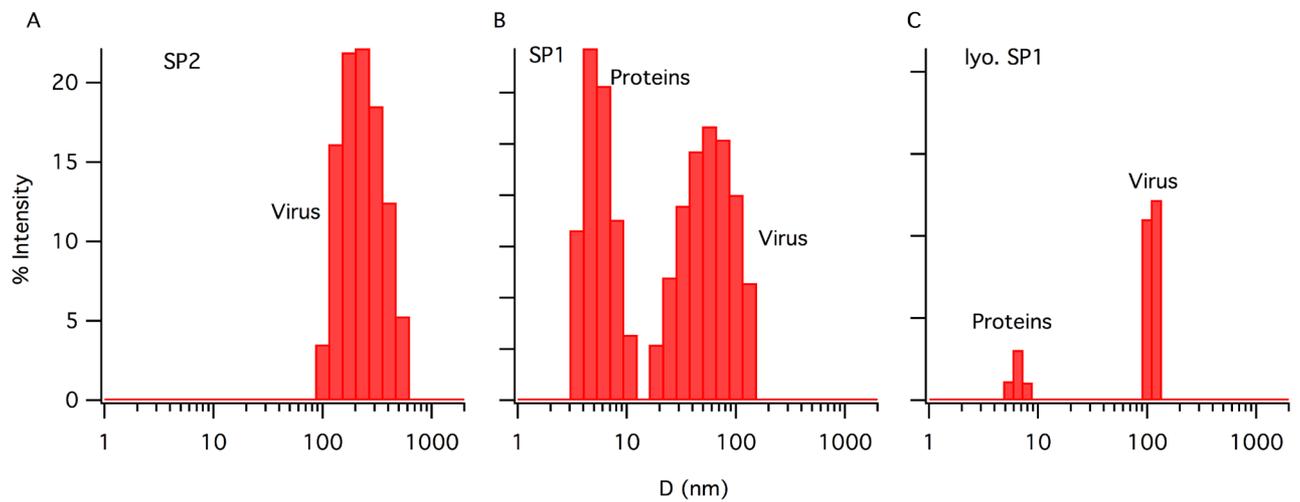
Although there are very little published data on the rabies virus structure and stability, it was reported that the rabies virus has a typical “bullet” shape (25,26). We have found that the observed shape of the virus depends heavily on the method used for negative staining in the TEM experiments. In particular, PTA or AM staining methods were superior to that of UA, which is in agreement with the observations of others (26).

Contrary to TEM, optical microscopy can detect degraded viruses in large-sized clusters that probably contain both viral and protein aggregates. Figure 3 shows the fluorescence of the dye-labeled antirabies antibody. The sample shown in Figure 3A contains the virus sample only, Figure 3B the original liquid sample, and Figure 3C the lyophilized sample. It is expected that each virus would have multiple bound antibodies giving rise to an enhanced detectable signal, assuming that antibody fluorescence can be directly linked to the number of viruses per volume in the sample. The most striking differences between samples are concentration dependencies. Our analysis with the optical microscope indicates the existence of aggregates (large clusters) in the lyophilized sample and shows that the concentration of the whole virus is reduced significantly after lyophilization (Figure 3C).

The solution pH of the SP1 formulation increases by about 1–1.5 pH units due to lyophilization from pH 8 to pH 9.6, as shown in the Table. The labile buffer system is removed during sublimation and thus the virus is exposed



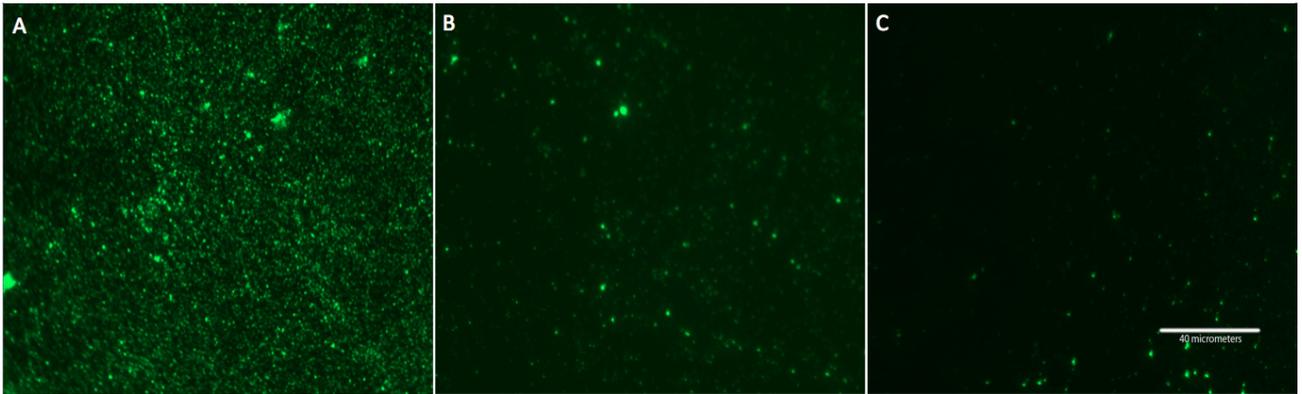
**Figure 1.** TEM image of the rabies virus from the SP2 sample. The sample was negatively stained with PTA. Scale bars are 100 nm.



**Figure 2.** DLS results of the rabies virus vaccine formulations. (A) SP2 sample, (B) SP1 sample, and (C) lyophilized SP1 sample, reconstituted with distilled water.

to about 1–1.5 pH units after rehydration, whereas the SP2 control formulation with higher virus concentration in a different buffer does not show any pH change due to

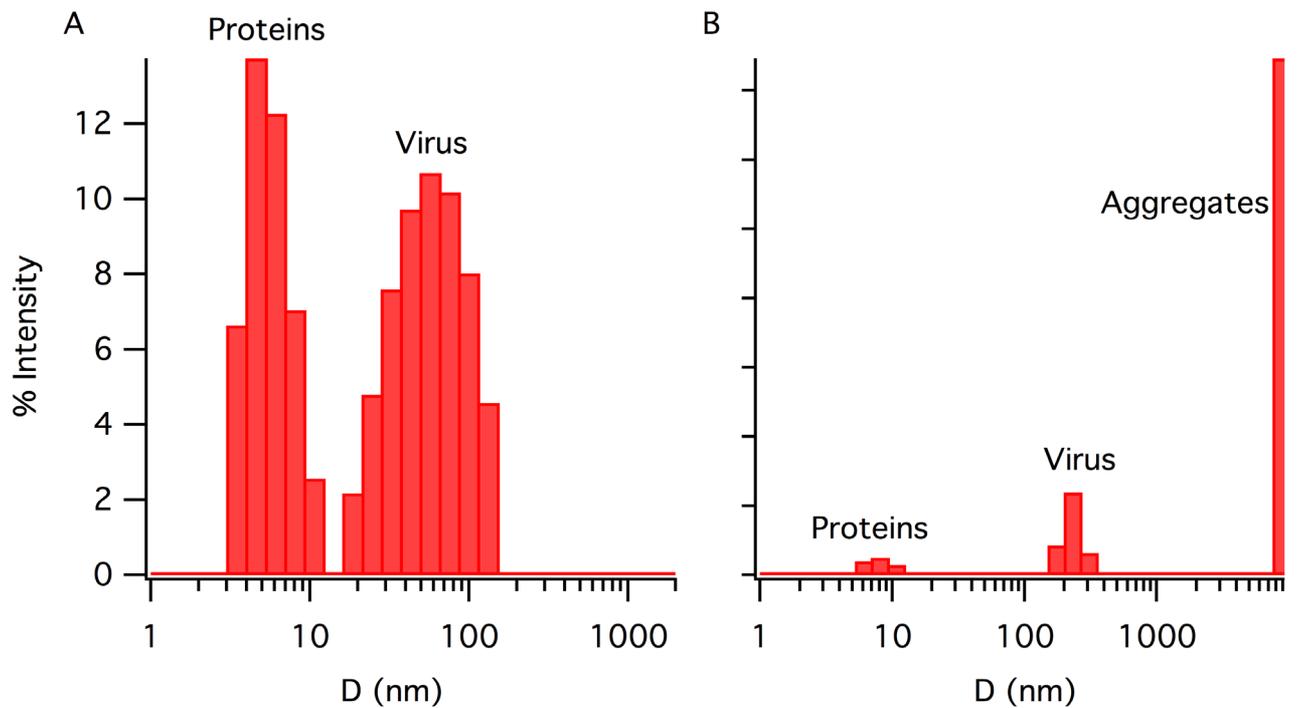
lyophilization. Figures 4–7 show that this pH upshift affects the virus structure considerably. We hypothesize that this sudden pH increase causes degradation of the rabies virus



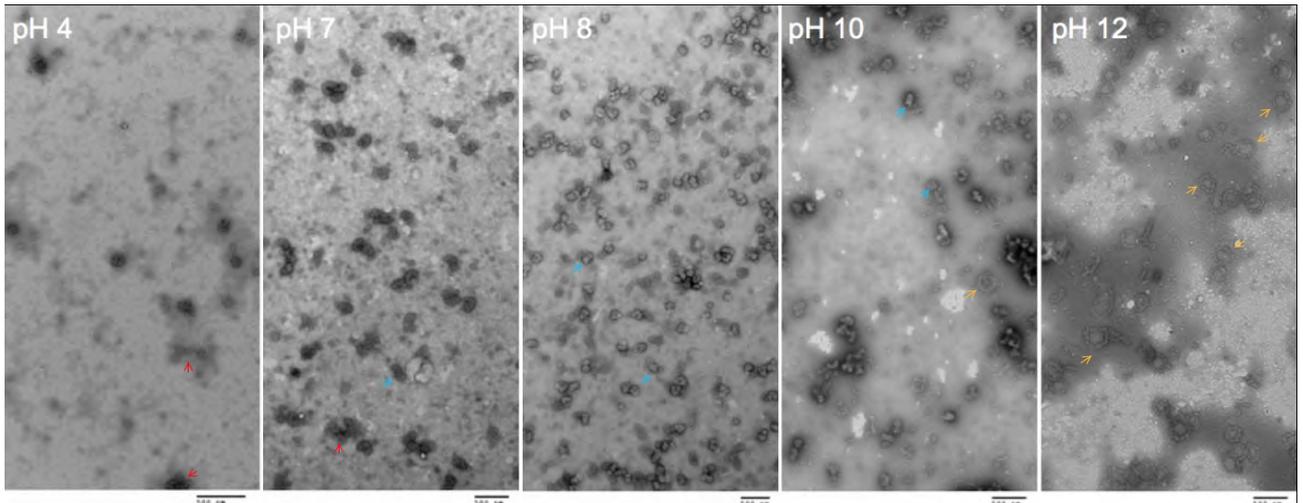
**Figure 3.** Optical microscope results of (A) SP2 sample, (B) liquid SP1 sample, and (C) lyophilized SP1 sample. Green color is due to the fluorescent-labeled antirabies antibodies. Scale bar is 40  $\mu$ m.

**Table.** Effect of lyophilization on solution pH.

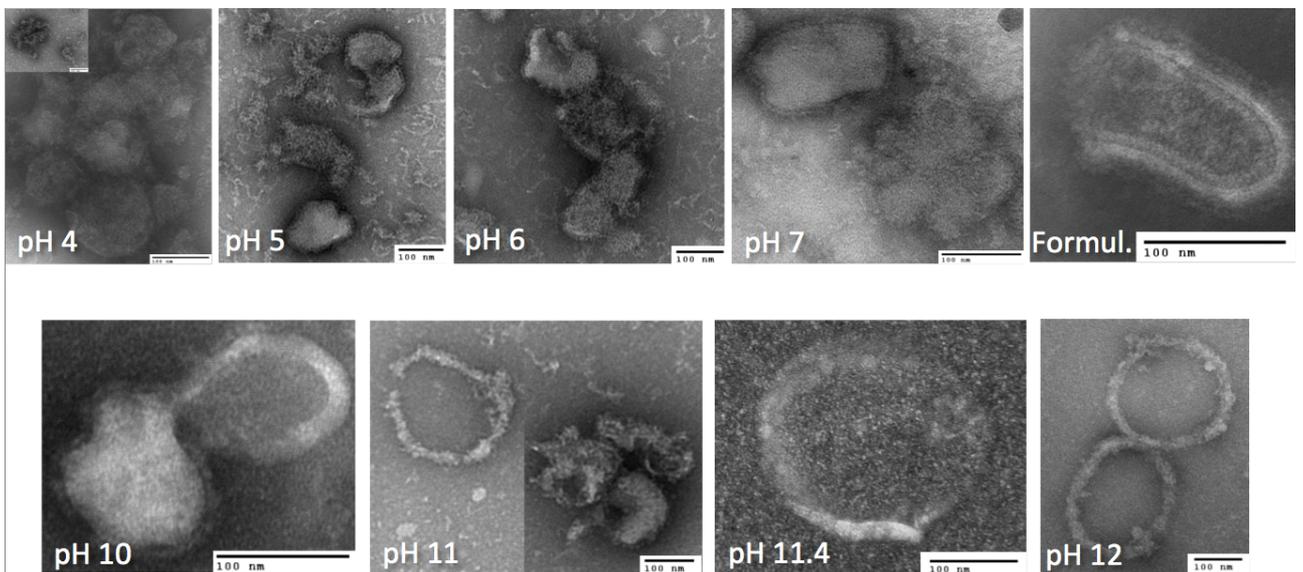
	pH	
	Before lyophilization	After lyophilization
SP2 formulation (control)	8.1	8.1
SP1 formulation	8.5	9.4
Lyophilized SP1 sample after reconstitution with distilled water	-	9.6



**Figure 4.** DLS results showing the effect of pH on the SP1 vaccine. (A) SP1 formulation. (B) When the solution pH of SP1 was increased to pH 10.



**Figure 5.** TEM images showing the overall effect of pH on viruses. At pH 4 large protein aggregates are observed. As the solution pH is increased empty capsids are formed: at pH 10 there are some empty capsids, but at pH 12 only empty capsids are visible (all scale bars are 500 nm). Arrows indicate some of the particles. Orange: empty capsids, blue: viruses, and red: protein aggregates.



**Figure 6.** TEM images showing the effect of pH on a single virus structure. At pH <7 the virus aggregates as well as disassembles, but at pH >10 it gradually loses its protein layers and eventually only the capsid remains. The scale bars are 100 nm in each picture; “Formul” is the formulation solution at around pH 8; inset images at pH 4 and pH 11 show other types of commonly observed structures.

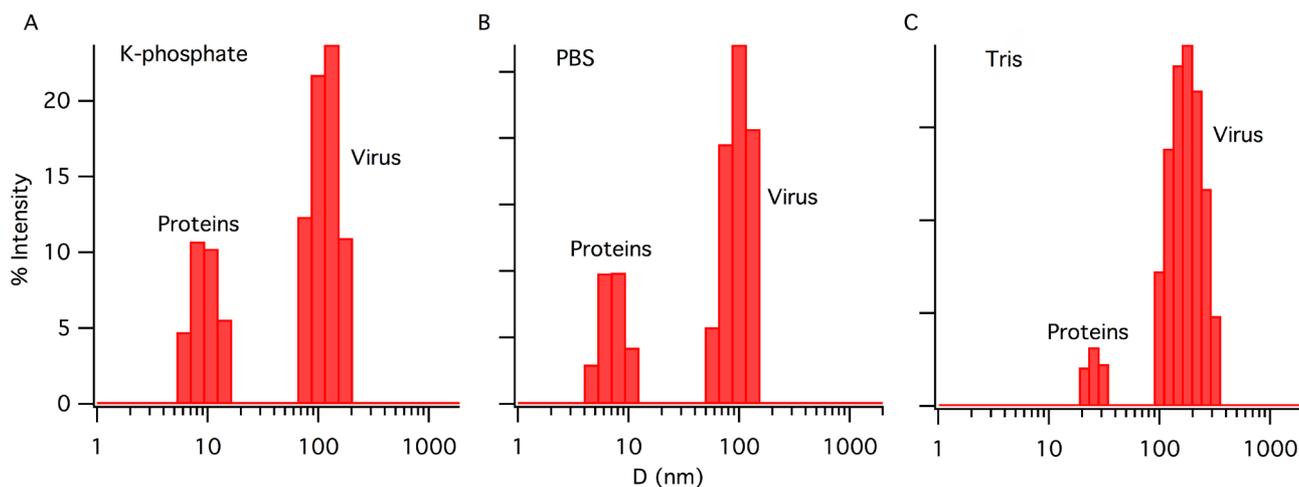
structure. Hence, we have tested the dependence of the virus structure on the solution pH and found that at high pH values, the virus loses its surface protein layers and adopts empty ring structures, probably consisting of the capsid proteins (Figure 6). At low pH values, though, the virus disassembles or aggregates (Figure 6).

The virus structure can be conserved as shown in Figure 7 by using different formulation buffers, or by keeping the solution pH constant. Our method of keeping the solution pH constant by other means than CO<sub>2</sub>, e.g., alkali, preserves

the desired solution pH during lyophilization and this in turn keeps the viral structure intact upon lyophilization.

### 3.3. Influence of CO<sub>2</sub> on solution pH

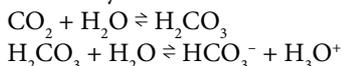
As mentioned, the virus structure is extremely sensitive to sudden pH changes. This is especially true in the SP1 formulations, where only a limited amount of virus is available and the solution pH is controlled by CO<sub>2</sub> during the cell culture stage. Release of the labile buffer system from the sample increases the solution pH, and thus the virus structure is destroyed due to high pH. Dependence



**Figure 7.** DLS results of the lyophilized SP1 sample using different buffers: (A) potassium phosphate buffer, (B) PBS buffer, and (C) Tris buffer. Solution pH was controlled (pH 8) with alkali. The lyophilized virus was reconstituted with distilled water.

of the structure of the virus on solution pH is observed as an enlarged virus in the DLS results (Figure 4), where the virus diameter increases at high pH, forming empty virus structures seen in the TEM results (Figure 5) and probably containing only the virus capsids.

A certain amount of carbon dioxide ( $\text{CO}_2$ ) may be dissolved in the buffer as long as the pressure is maintained at a relatively constant level, and the pH of the solution changes due to dissolved  $\text{CO}_2$ . If the pressure drops significantly in a short period of time,  $\text{CO}_2$  is expected to be released from the environment. During the lyophilization process, both the temperature and the pressure drop drastically, and we believe that this process releases  $\text{CO}_2$  from the system, which in turn causes the pH upshift. When  $\text{CO}_2$  is dissolved in water, it forms carbonic acid ( $\text{H}_2\text{CO}_3$ , a weak acid), which in turn forms a bicarbonate ion ( $\text{HCO}_3^-$ ) and a hydronium cation ( $\text{H}_3\text{O}^+$ ) after reacting with water. Note that both  $\text{CO}_2$  and  $\text{H}_2\text{CO}_3$  react slightly and reversibly with water based on the following reactions:



To circumvent this problem, we developed novel stable formulations that have the same pH before and after lyophilization, as discussed in the following section.

### 3.4. Stable formulation that does not degrade during lyophilization

The stability of SP1 with pH fixed at 8 with NaOH (our new formulation) was compared to the control sample SP2 and to the lyophilized SP1 whose pH during the cell culture process was controlled with dissolved  $\text{CO}_2$ . In order to prepare the new formulation, liquid SP1 samples were subjected to buffer exchange by dialysis for 48 h in 15 mM Tris, 15 mM potassium phosphate, or PBS buffers, all at pH 8. Dialysis buffers were replaced with

freshly prepared buffers every 12 h. After the dialysis, the samples were immediately frozen at  $-80^\circ\text{C}$  and lyophilized, and subsequently the samples were analyzed with DLS (Figure 7). In summary, our biophysical analyses of the reformulated SP1 show an enhanced stability upon lyophilization compared to the original lyophilized sample. The strong correlation between solution pH and structural stability of the virus is a proof-of-concept for our methodology to stabilize the rabies vaccine. Recently we reported the effect of a surfactant on the influenza virus with observations akin to this study, albeit with different excipients and conditions (27). Our current method allows preparing the vaccine in many different buffers (e.g., Tris or potassium phosphate) and can also be applied in other lyophilized vaccines or therapeutic protein formulations where pH is controlled by  $\text{CO}_2$  or other methods in the liquid form but is possible to be shifted upon lyophilization.

To conclude, we have studied the mechanism of the rabies virus disassembly at various solution pH, different buffers, and elevated temperature conditions. We have verified our hypothesis that during lyophilization the vaccine degrades due to destruction of the viral structure. This destruction is caused by an increase of the solution pH during lyophilization and/or upon reconstitution. This pH upshift is due to the substantial loss of dissolved  $\text{CO}_2$  in the labile buffer system that is used to control the solution pH in cell culture medium 199. Since the virus structure is marginally stable in a narrow pH range in solution, the upshift of solution pH during the lyophilization process causes the virus to lose its structural integrity, thus resulting in the observed degradation of the vaccine. We have also conducted accelerated studies to determine the effect of pH on the virus structure further. We showed that the rabies virus in the reference sample (SP2 formulation)

is more stable both at high and low pH conditions than the virus in the SP1 formulation. In addition, we found that the virus shape was dependent on the negative staining method used in the TEM experiments. Lastly, we have developed a new rabies vaccine formulation with enhanced stability by keeping the solution pH constant with NaOH at pH 8. We showed that the new formulation is stable in various buffers including Tris, potassium phosphate, and PBS after lyophilization as opposed to the old rabies vaccine. The next step for our new formulation will be a stability study as a function of solution pH. Our methodology will be of great interest to the pharmaceutical industry for improving vaccine formulations, especially for labile bioproducts to meet strict stability guidelines. In addition, formulations prepared with our approach could contain much less virus, allowing more vaccines to be

manufactured with the same amount of virus produced. The latter would have benefits for patients, such as fewer side effects, since the formulation needs to contain a low amount of biological material. It is also expected to cause less pain associated with vaccine administration, since the solution pH is closer to physiological pH.

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