

Evaporative Cooling Hydrogel Packaging for Storing Biologics Outside of the Cold Chain

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Stabilizing thermolabile pharmaceuticals outside of the cold chain has the potential to alleviate some of the logistical and monetary burden of providing health care access in the developing world. Evaporative cooling hydrogel packaging is designed to extend the storage stability of existing pharmaceutical products without the need for reformulation. Hydrogels with high water content and reversible hydrophilicity offer a promising platform for reducing storage temperatures without refrigeration. As a model, poly(*N*-isopropylacrylamide) is selected as a basis for creating a potentially low cost and easy-to-fabricate hydrogels.

The global pharmaceutical market for products that require refrigerated storage and transport is estimated to be \$260 billion, as reported by the Pharmaceutical Commerce's annual Cold Chain Sourcebook. This robust growth is attributed to the treatment shift to using biologics and other sensitive pharmaceuticals. Managing the transportation of temperature-controlled products is an immense economic burden.^[1,2] Biologics mainly include insulin, proteins, antibodies, and vaccines. Vaccines and proteins are highly fragile biologics that can change their molecular structure when subjected to elevated temperatures, thereby reducing their potency and safety.^[3] Several outbreaks have been attributed to inadequate vaccine preservation and handling, including diphtheria in 1990 and whooping cough in 1996.^[4] More than 90% of all vaccines currently in use require cold chain transport.

Advancing technologies have enabled worldwide temperature-controlled transport; however, sustaining a cold chain in developing countries is a persistent challenge. The cold chain is not only a logistical hurdle but also a monetary barrier to emerging markets as it contributes to 20% of the overall vaccine cost as reported by the World Health Organization.^[2] Development of thermostable vaccines and other biologics that can be transported

outside of the cold chain has the potential to dramatically change their availability. Excipient-based stabilization has been investigated for several vaccines and biologics.^[5] However, before these formulations can be commercialized, a clinical trial is required to evaluate their safety and efficacy. Lack of appropriate animal models, the need in large numbers of patients, and ethical issues with using healthy subjects and children are only some of the impediments of new vaccine formulations. New packaging and delivery technologies represent an alternative to new vaccine for-


mulation and are therefore urgently needed to reduce the cold chain burden. Insulated packaging has the potential to maintain an internal temperature within an acceptable range until it reaches its point of use.^[6] Some improvements have been made that combine different aspects of cooling systems have also been patented within the last decade.^[7] The main disadvantage of these systems is the large amount of container material required to maintain refrigeration, which would translate to immense storage space, weight, and therefore increased cost.

To overcome some of these hurdles, we report a simple method of fabricating an evaporative cooling hydrogel packaging system that allows for the direct integration of clinical formulations of vaccines and therapeutics. It occurred to us that hydrogels with high water content and reversible hydrophilicity could offer a promising platform for reducing storage temperatures without refrigeration. As a model, we selected poly(*N*-isopropylacrylamide) (PNIPAM) as a basis for creating a potentially low cost and easy to fabricate hydrogel.

PNIPAM and PNIPAM hydrogels have a lower critical solution temperature (LCST) of around 34 °C.^[8] Below this temperature, materials composed of PNIPAM are generally hydrophilic in character. In the case of hydrogels, this results in the material existing in a swollen state with high water content at temperatures lower than their LCST.^[9] When exposed to temperatures higher than their LCST, these hydrogels become hydrophobic. This leads to a shrinkage, or collapse, and water release. The free water readily evaporates, causing evaporative cooling, which in turn drops the hydrogel to below its LCST (Figure 1A). This process is highly reversible, allowing for repetitive swelling and deswelling with minimal impact to the hydrogel structure. The LCST of PNIPAM, occurring between room and body temperature, has garnered wide interest in many applications. These have included use in drug delivery

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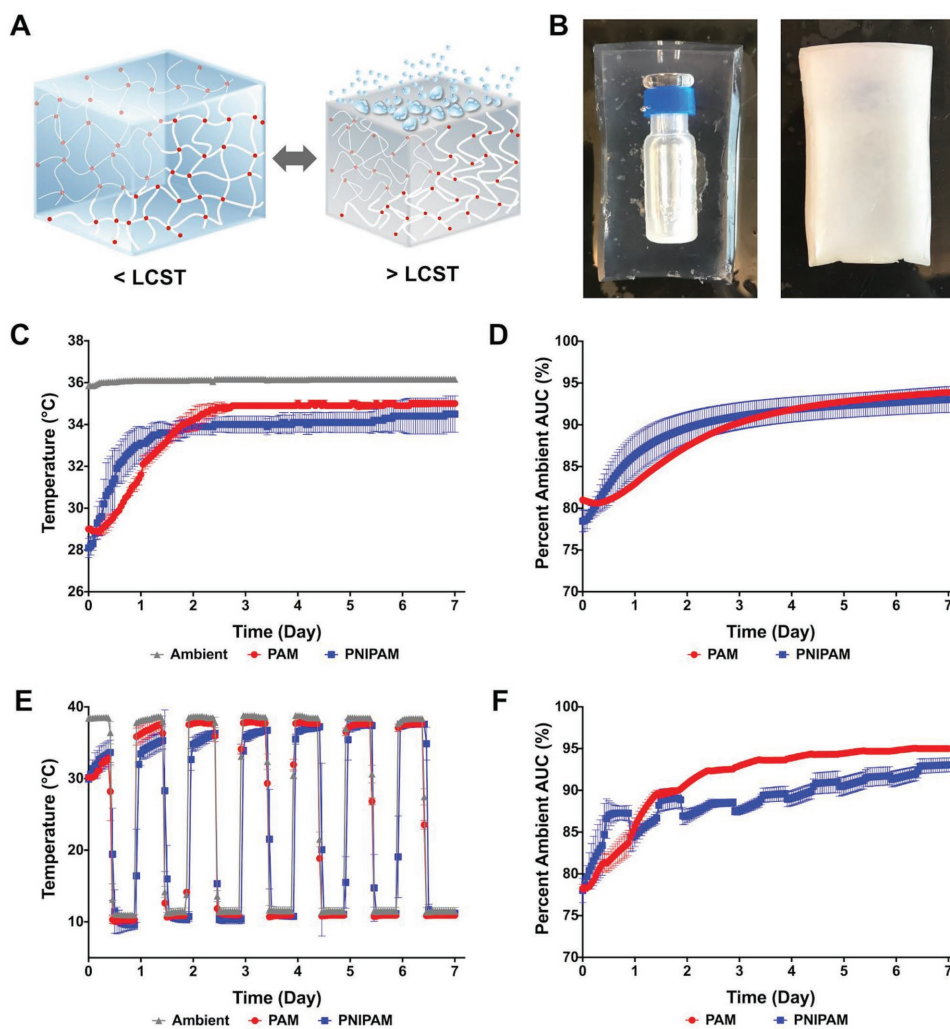


Figure 1. A) Schematic illustrating the cooling mechanism of PNIPAM hydrogels. B) Images of a PNIPAM hydrogel containing an easily fillable vial for storage of biologics. Left, at a temperature lower than its LCST (room temperature; RT) and right, above its LCST (36 °C). After arriving to patient, the vial can be simply removed from the hydrogel and administered. C,E) Temperature data for ambient conditions inside a (C) 36 °C and (E) 39/12 °C cycles environmental chamber and inside hydrogels composed of PAM or PNIPAM hydrogels that were placed inside of the same environmental chamber. D,F) Total temperature burden as assessed by percent of the AUC in ambient conditions for both (D) constant temperature and (F) temperature cycling experiments. Error bars represent standard deviation ($n = 3$).

platforms,^[10] tissue engineering scaffolds,^[11] in vitro 3D cell culture models,^[12] microfluidic systems,^[13] temperature sensors,^[14] and passive temperature regulation.^[15]

To evaluate the concept of evaporative cooling packaging, PNIPAM hydrogels were synthesized around capped vials containing a septum (Figure 1B). Direct encapsulation of the vial allows for any thermosensitive biologic to be easily filled after hydrogel formation, avoiding heat and/or UV exposure during radical polymerization. A variety of synthesis methods have been described in literature for the preparation of PNIPAM hydrogels for different applications, mainly based on either atom transfer radical polymerization^[16] or layer-by-layer assembly.^[17,18] UV initiation was chosen for its applicability to a wide range of comonomers. Nonthermosensitive hydrogels will also undergo evaporative cooling, poly(acrylamide) (PAM) hydrogels were therefore included to evaluate the impact of PNIPAM's LCST on its cooling efficacy.

Without active temperature control and refrigeration, temperatures can vary significantly in day/night cycles during transport. Large temperature swings necessitate an adaptable, robust, and reversible system. PNIPAM hydrogels response to thermal stimuli was therefore investigated in both constant temperature and variable temperature studies. In the constant temperature studies, hydrogels were incubated for 7 d at 36 °C with 50% humidity. Cooling performance was evaluated by placing a temperature probe directly into the hydrogel-encapsulated vial. Under these conditions, PAM hydrogels were more effective at cooling over short timescales (up to 3 d), compared to PNIPAM hydrogels (Figure 1C and Figure 2A). The internal hydrogel temperature remains well below ambient as the PAM hydrogels rapidly drop in water content. The PNIPAM hydrogels retain their water content and maintain lower steady state temperature of 34 °C for most of the 7 d period (Figure 1C and Figure 2A). The total temperature burden is easily visualized by

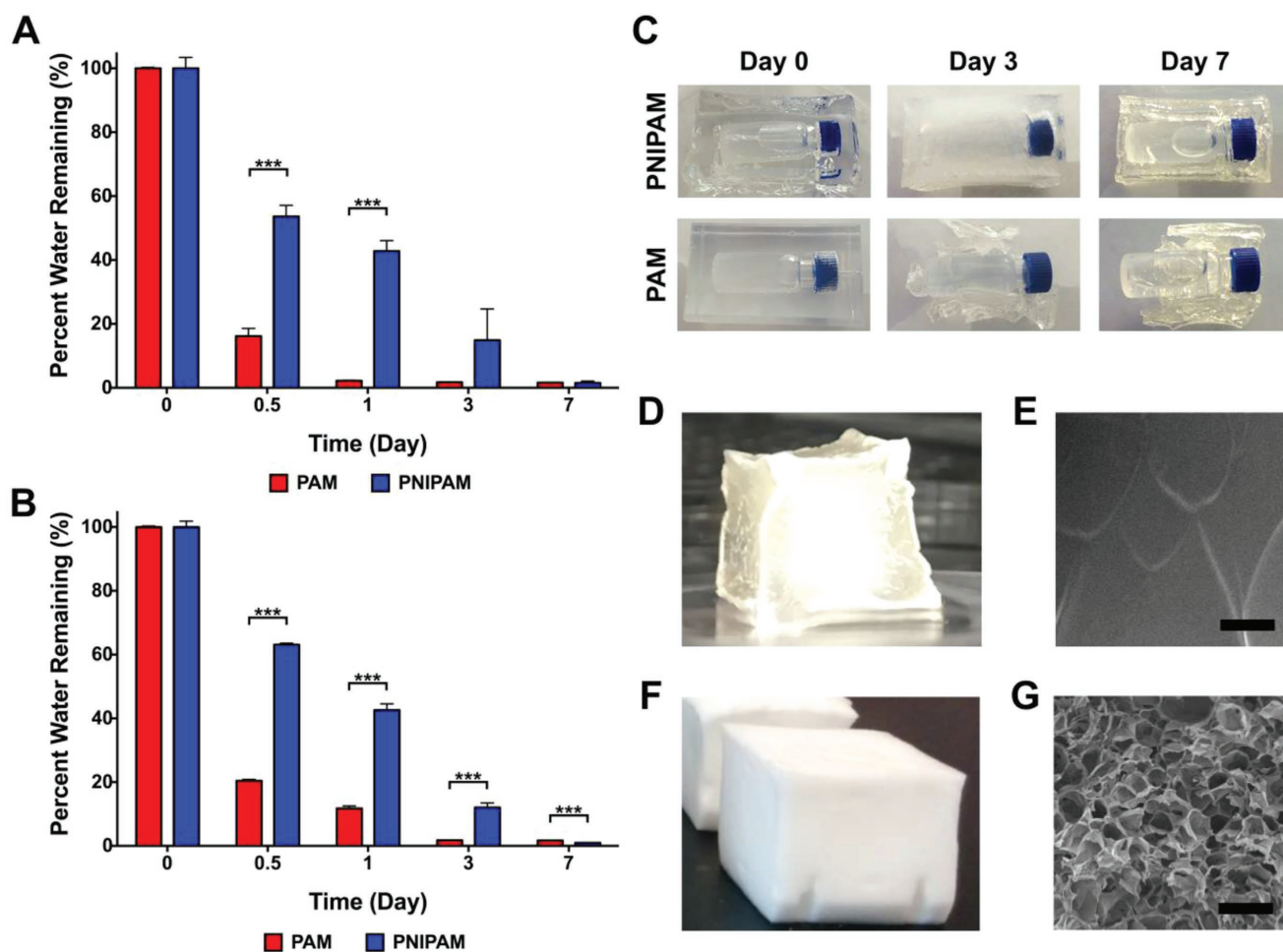


Figure 2. A) Water retention by hydrogels when stored at 36 °C with 50% humidity. B) Water retention by hydrogels when stored at 39/12 °C cycles with 50% humidity. Error bars represent standard deviation ($n = 3$). $***p < 0.01$. C) Representative hydrogels images when incubated at 36 °C with 50% humidity. D,F) Images demonstrating PNIPAM hydrogel structural recovery after drying. After 5 d at 36 °C, (D) PNIPAM hydrogels lose a majority of their water content. After a 2 h incubation in water at room temperature, (F) the hydrogel regains its initial structure. Scanning electron microscopy showing changes in porosity of a PNIPAM hydrogel E) after dehydration and G) after rehydration. Scale bars represent 100 μm .

area under the temperature curve (AUC) as reported as a percent of the ambient (Figure 1D). PAM hydrogels initially exhibit a slight benefit over PNIPAM hydrogels over short timescales; PNIPAM's lower steady state temperature overcomes this difference at around day 3. This indicates that for short-term transport (less than 3 d) at a high constant temperature, simple water evaporation from a high water-content hydrogel is sufficient to maintain lower storage temperature than ambient. However, for longer time periods, PNIPAM's ability to modulate hydrophilicity results in prolonged cooling.

To mimic harsh day/night weather conditions with large temperature swings, hydrogels were incubated at 39 and 12 °C in alternating 12 h cycles with 50% humidity. The benefit of the swelling to deswelling transition of thermoresponsive hydrogel is more evident under these conditions. After only two cycles of 39/12 °C, PAM hydrogels already fail to maintain a lower temperature, suggesting loss of water content (Figure 1E). PNIPAM hydrogels, in contrast, are able to maintain a core temperature lower than the surrounding environment for 5 d. In this case, PNIPAM hydrogels demonstrate a benefit over PAM hydrogels

after the first 24 h (Figure 1F), indicating that thermoresponsive nature of PNIPAM hydrogels led to better performance in rapidly changing conditions.

Differences in the long-term performance (greater than 1–3 d of storage) of PNIPAM and PAM hydrogels can be largely explained by difference in water retention. PAM hydrogels only retain 16% of their initial water content after 12 h of storage at 36 °C with 50% humidity (Figure 2A). By 24 h, only 4% of the initial water content remains. This results in a rapid cooling effect over the initial 24 h, but failure to regulate temperature over longer time frames. In contrast, PNIPAM hydrogels maintain 50% of their water content after 12 h and maintain 14% of the initial water content at 72 h (Figure 2A). The ability to sustain high water content is attributed to the thermoresponsive behavior of the PNIPAM hydrogels. The improvements in water retention are similarly evident in day/night temperature cycles (Figure 2B).

The structural advantages of PNIPAM hydrogel thermoresponsiveness are also readily apparent when comparing images of the PNIPAM hydrogel packaging to PAM (Figure 2C). PAM

hydrogels dry out and break by day 3 at 36 °C, while PNIPAM hydrogels are able to retain water and maintain complete encapsulation of the vial. Hydrogels are highly porous structures, allowing them to contain a large percentage of water. Therefore, preserving or restoring porosity through harsh changes environmental conditions is crucial for sustained evaporative cooling. After being subjected to 5 d of 36 °C at 50% humidity, PNIPAM hydrogels lose a majority of their water content and porosity (Figure 2D,E). After rehydration, complete recovery of structure is exhibited (Figure 2F,G). PNIPAM's thermoresponsive behavior ultimately allows for the preservation of its ability to retain and absorb water. Future iterations of the packaging will more extensively explore reusability.

To directly assess the potential of PNIPAM as evaporative cooling packaging for the storage of biologics, oral polio vaccine (OPV) was identified as a thermolabile biologic that is directly relevant in the developing world. OPV contains attenuated live poliovirus. Through mimicking infection by wild-type polio, OPV provides good intestinal immunity with a very low risk of vaccine associated paralytic polio with a very low dose. Poliovirus is a nonenveloped single-stranded ribonucleic acid (RNA) virus with extreme sensitivity to elevated temperatures that leads to losses in infectivity, ultimately resulting in ineffective vaccination.^[19,20] Therefore, we utilized this highly sensitive vaccine to examine the ability of PNIPAM hydrogel packaging to improve biologic stability under extreme temperature variations. Following 7 d incubation at 36 °C and 50% humidity, OPV infectivity was evaluated using a plaque assay. Serotypes 2 and 3 exhibited improved recovery when stored in PNIPAM hydrogels when compared to either PAM hydrogels or without any packaging (i.e., ambient conditions) (Figure 3A). No statistical difference was found in OPV recovery between storing serotypes 2 and 3 in PAM hydrogels and in the absence of packaging. Serotype 1, representing the most thermosensitive serotype, did not exhibit increased recovery with the evaporative cooling packaging. Interestingly, when stored at day/night cycles with 50% humidity, serotype 1 exhibited significantly higher recovery with PNIPAM hydrogel packaging, compared to PAM hydrogel packaging or in the absence of packaging (Figure 3B). No difference in recovery was found between PAM hydrogel packaging and without packaging. The reverse trend is observed with OPV serotype 3. Under day/night cycling conditions, the benefit in storing serotype 3 in PNIPAM hydrogels is diminished. With serotype 2 however, representing the most thermostable serotype, a similar trend in recovery is observed for both storage conditions, with increased recovery when stored in PNIPAM hydrogels. Varying losses of OPV infectivity have been previously reported at different temperatures, possibly resulting from a different inactivation mechanisms.^[20] Additionally, different serotypes demonstrate diverse thermosensitivity.

Mechanical properties of thermoresponsive hydrogels, like elasticity, response rate to external stimuli (i.e., swelling/deswelling transition), water absorbance, and reproducibility are all critical parameters for overall packaging performance. Several strategies to improve the mechanical properties of PNIPAM-based hydrogels have been investigated,^[21] most commonly by generating an interpenetrating networks.^[22] Future studies will focus on the investigation of these strategies as well as packaging parameter optimization.

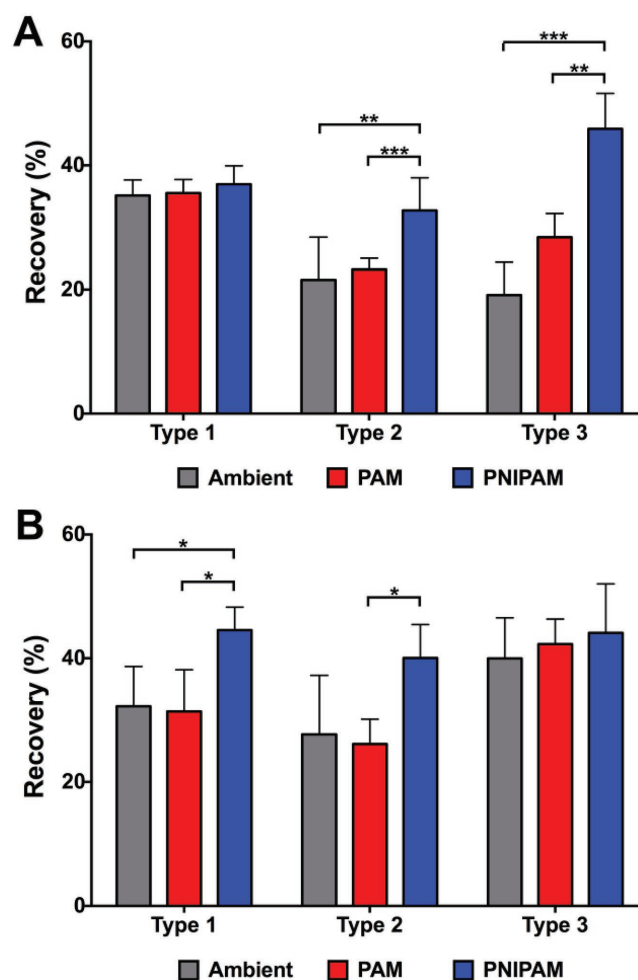


Figure 3. OPV stability at A) 36 °C and B) 39/12 °C cycles for 7 d with and without hydrogel packaging. Data are reported as percent of plaque forming units as compared to a frozen stock of each serotype (percent recovery). Error bars represent standard deviation ($n = 3$). * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$.

In conclusion, a thermoresponsive hydrogel packaging system demonstrated advantages for the storage of thermolabile pharmaceuticals. The use of a thermoresponsive hydrogel allowed for increased water retention and improved ability to regulate temperature as compared to a nonthermoresponsive control. Importantly, when studied in the context of biologic recovery rates of OPV, values were reproducible allowing for doses to be adjusted depending on climate and storage length. Ultimately, evaporative cooling through the use of thermoresponsive hydrogel (based on PNIPAM or other such hydrogels) packaging could provide a promising solution for insulating biologics outside of the cold chain. Hydrogel packaging may be especially relevant in the developing world where maintaining a cold chain is challenging.

Experimental Section

PNIPAM Hydrogel Synthesis: 15% (w/v) *N*-isopropylacrylamide (NIPAM), 5% (w/w, MBA/NIPAM) *N,N'*-methylenebis(acrylamide)

(MBA), and 0.004% (w/w, IRG/NIPAM) Irgacure 2959 (IRG) were dissolved in ethanol under bath sonication for 15–20 min. An appropriate amount of volume was added to a mold, and the mold was put under UV light using a Dymax BlueWave 200 Light-Curing Spot Lamp for 15–20 min. Gels were then transitioned from ethanol to water using dilutions of ethanol over 2–5 d. For hydrogels containing vials, a plastic HPLC vial with septa was placed into the mold before polymerization.

PAM Hydrogel Synthesis: For PAM hydrogels, 15% (w/v) acrylamide (AM) and 5% (w/w, MBA/PAM) MBA were dissolved in water. 0.0125% (v/w, tetramethylethylenediamine (TEMED)/AM) TEMED was then added, followed by the addition of 0.0125% (w/w, ammonium persulfate (APS)/AM) APS, which were subsequently dissolved by vortexing. The solution was then directly poured into a mold and allowed to polymerize. For hydrogels containing vials, a plastic HPLC vial with septa was placed into the mold before polymerization.

Temperature and Stability Studies: All temperature studies were performed in a Caron environmental chamber at 36 °C and 50% humidity or alternating 12 h cycles of 39 and 12 °C. Dickson temperature loggers equipped with probes were used to record temperature values every hour for 7 d. Hydrogel water content loss was measured by mass loss at time points of 0, 0.5, 1, 3, and 7 d.

Scanning Electron Microscopy (SEM): SEM was used to qualitatively assess hydrogel morphology. At specific time points hydrogels were removed from the environmental chamber, flash frozen with liquid nitrogen, and lyophilized. Samples were then mounted onto SEM mounts using carbon tape and sputter coated using a Hummer 6.2 sputter coating system. Samples were imaged using a JEOL 5600LC scanning electron microscope.

Preparation of Vials and Molds for OPV Studies: For the studies containing oral polio vaccine (OPV), 5% (w/v) bovine serum albumin (BSA) was used to block sterile plastic HPLC vials by incubation for 1 h at room temperature. The BSA solution was removed by aspiration. Two sterile needles were also placed through the septa of each vial and sealed with parafilm. The vials were then inserted into the prehydrogel solution before polymerization in a 20 mL histological mold. Hydrogel synthesis then took place as described previously, and OPV stock solution injected into the vial before removing the needles.

OPV Stock Solution: Stock solutions of the three OPV serotypes were prepared by diluting to 1x (serotypes 1 and 3) or 10x (serotype 2) of the human dose, based on the TCID₅₀, in 1 M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer containing 1 M MgCl₂. 1.5 mL of each serotype stock solution was then injected into preblocked HPLC vials.

Plaque Assay: A viral plaque assay was performed to assess OPV recovery. African green monkey kidney cells (VERO) were seeded at 300 000 cells per well in six well plates in Dulbecco's modified Eagle medium (DMEM) cell culture media supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution. Three days later, the media was removed and washed with PBS. 1 mL of OPV sample diluted in P5 (DMEM, 7.4 g L⁻¹ sodium bicarbonate, 16.28 g L⁻¹ magnesium chloride hexahydrate, 2.04 g L⁻¹ bovine serum albumin, 150 mg L⁻¹ DEAE-dextran, 2% pen strep, 2x nonessential amino acids, and 50 × 10⁻³ M HEPES) was then added to each well in technical duplicate and incubated for 30 min at room temperature. The sample was then aspirated and replaced with 3 mL of P5 containing 1.5% (w/v) of low melting temperature sea plaque agarose at 42 °C. After several minutes at room temperature, this solution solidified and was returned to 37 °C incubation. Three days later, the agarose was removed and cells were stained with a solution containing crystal violet (1 g L⁻¹ crystal violet, 20% ethanol, and 80% water). The staining solution was removed by washing twice with excess phosphate buffered saline. Plaques were then manually counted and recorded.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biologics, cold chains, hydrogels, packaging, storage

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