Development of a Thermostabilization Platform for Point-of-Care Diagnostics, Food Safety, and Vaccine Deployment

Development of a Thermostabilization Platform for Point-of-Care Diagnostics, Food Safety, and Vaccine Deployment

By

Vincent Leung, B.Eng Biosci., M.A.Sc

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AUTHOR: Vincent Leung

B.Eng. Biosci. (McMaster University)

M.A.Sc. (McMaster University)

SUPERVISOR: Professor Carlos Filipe

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Abstract

Thermostabilization of labile biomolecules is crucial for the advancement of global health. The World Health Organization has identified accessible quality-assured diagnostics, prevention of antibiotic resistance, and universal access to vaccination as three of the most important issues for global health today. One major challenge is that many biomolecules, such as enzymes and vaccines, are thermally unstable and require refrigeration at all times. Maintaining refrigeration is a major logistical problem, since many developing countries and rural areas do not have access to reliable electricity. Moreover, the cost to maintain the cold chain infrastructure presents a substantial financial barrier. Therefore, there is a great need for the thermostabilization of labile biomolecules.

This work presents a simple thermostabilization method that uses pullulan and trehalose to encapsulate labile biomolecules within a sugar glass matrix. To address the problem of accessible quality-assured diagnostics, we thermally stabilized molecular biology reagents in ready-to-use mastermixes. Thermally stabilized polymerase chain reaction (PCR) and rolling circle amplification (RCA) mastermixes were shown to be stable for at least 12 weeks at room temperature. This can significantly improve the accessibility of diagnostic tools since, DNA amplification techniques such as PCR and RCA can be used as diagnostic tools to provide rapid detection of diseases with high specificity and sensitivity.

To address the problem of antibiotic resistance, this work demonstrated a method to stabilize bacteriophages as a coating on surfaces for food safety applications. Bacteriophages, are viruses that kill bacteria with high specificity while having a low risk of developing antibiotic resistance. The bacteriophages encapsulated in pullulan-trehalose films were able to retain infectivity for up to 3 months at ambient storage conditions. We further optimized the stability of bacteriophage in pullulan-trehalose films performing a four factor, two level design of experiment (DOE). It was shown that vacuum drying and storing the films in a closed container with low-humidity can increased the long-term viability of bacteriophage by over 1000-fold.

To address the need for universal immunization, we stabilized enveloped DNA and RNA viral vaccines by drying them in a pullulan and trehalose mixture. The thermally stabilized live-attenuated HSV-2 vaccine retained immunogenicity for at least 2 months when stored at 40 °C. Inactivated influenza vaccine stabilized in pullulan and trehalose retained immunogenicity for at least 3 months at 40 °C.

Overall, this thesis presents a versatile thermostabilization method using pullulan and trehalose as stabilizers. This method was used to thermally stabilize molecular biology reagents, bacteriophages, and vaccines. The achievements from this work have the potential to significantly improve global health by creating thermally stable molecular diagnostic kits that are accessible and reliable, by preventing antibiotic resistance with stabilized bacteriophage coatings, and by deploying thermally stable vaccine for universal immunization.

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Chapter 1: Introduction

Significant advances in healthcare technology have been made over the past century. Despite these advances, there are still numerous pressing global health challenges, especially in developing countries where there is inadequate infrastructure and a lack of skilled healthcare workers. To this end, the World Health Organization and the Bill and Melinda Gates Foundation have worked together to identify a number of key developments that must be pursued if global health is to be improved. These developments include widespread access to affordable diagnostic tools, methods of treating infections that do not result in antibiotic resistance, and universal access to vaccination.[1–7]

One major challenge in realizing these key developments is that many vaccines and molecular-diagnostic reagents are thermally labile and require a refrigerated supply chain, called a cold chain, at all times. This poses a problem as, not only does the maintenance of a cold chain significantly increase costs and limit accessibility, it is simply not feasible in developing countries due to limited access to reliable electricity. Moreover, in many areas of the developing world that do have reliable access to electricity, cold-chain equipment is often poorly maintained and unreliable. Furthermore, the costs associated with maintaining cold-chain infrastructure pose a significant financial barrier. For example, cold-chain-related costs can account for more than 50% of the total costs of a vaccination program. Thus, the development of a method for thermally stabilizing labile biomolecules would greatly improve global health, as it would help to vastly expand the availability of vaccines and diagnostic tools.

In this work, we develop a simple thermal-stabilization method for molecular biology reagents, bacteriophages, and vaccines. This method, which can be packaged in ready-to-use kits, provides

a solution to one of the pressing needs related to global health: improved access to affordable diagnostic tools. Furthermore, bacteriophage stabilization is a new approach that is capable of eliminating pathogens without contributing to further resistance to antibiotics. Lastly, the development of thermally stable vaccines represents a key milestone in the realization of universal vaccination.

1.1 Thermally Stable Reagents for Molecular Biology

Molecular biology techniques can be used in a wide variety of applications, including genome sequencing, gene manipulation, and disease detection and prevention. These techniques also play a critical role in global health, especially the use of molecular diagnostics. Molecular diagnostics is widely considered to be one of the top biotechnologies for improving global health due to its ability to rapidly detect diseases with high sensitivity, and it is currently used to identify, treat, and prevent various diseases.[1,8] From a global health perspective, molecular diagnostics can be used to detect diseases such as HIV/AIDS, malaria, tuberculosis, and acute respiratory infections; indeed, it is estimated that the reliable detection of such diseases via molecular diagnostics has the potential to save more than 2 million lives each year.[1] However, if this potential is to be realized, molecular diagnostic tools must be accessible to developing countries.

Molecular diagnostic techniques such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) use thermally unstable reagents that require a cold chain to maintain their activity. Furthermore, these techniques usually require a number of pipetting steps, which can lead to a higher chance of error.[1,9,10] These issues are significant barriers to developing point-of-care (POC) molecular diagnostics for use in areas with limited resources and trained personnel. Thus, the development of thermally stable mastermixes that require minimal pipetting

and that can be stored outside the cold chain would greatly enhance the development of POC molecular diagnostic tools for use in developing countries.

1.1.1 Thermally Stable PCR Mastermixes

Many researchers have documented the development of thermally stable PCR mastermixes, with lyophilization being the most commonly employed approach. For example, Ahlford *et al.*[11] reported a freeze-dried PCR mastermix consisting of primers, dNTPs, buffer, and SmartTaq DNA polymerase, which is thermally stable. They were able to preserve the stability of their mastermix for up to 6 months at room temperature by adding a mixture of stabilizing agents comprised of trehalose, mannitol, polyethylene glycol, and dextran to it. In addition, Ahlford *et al.* also freeze dried exonuclease I and shrimp alkaline phosphatase using the same combination of stabilizing agents, with no significant loss in activity being observed after 5 days at 37 °C.[11] Furthermore, a number of freeze-dried PCR mixes for the detection of specific diseases have also been reported. In one such study, Chua *et al.*[12] utilized trehalose as a cryoprotectant to develop a freeze-dried triplex PCR mix that could detect *Vibrio cholera* while maintaining its stability for up to 60 days at 37 °C. Another group detailed the development of a thermostabilized PCR mastermix for the detection of *Klebsiella pneumoniae* and *Haemophilus influenzae*. This group's mastermix, which was freeze-dried with trehalose as a stabilizer, was found to be stable for 30 days at 37 °C.[10] Likewise, Nagaraj *et al.*[13] also used trehalose as a cryoprotecant in order to lyophilize a PCR mastermix designed to detect *Staphylococcus Aureus*; however, their freeze-dried mastermix was only stable for 15 days at 37 °C. The varying success of freeze-drying reported in the literature may be due to the fact that many commercially available liquid mastermixes contain different amounts of compounds that are not suitable for lyophilization, for example, glycerol and dimethyl sulfoxide.[1,14] Recently, a number of freeze-dried PCR mastermixes have become commercially

available, such as illustra PuReTaq Ready-to-Go PCR Beads from GE Healthcare and PCR Master Mix DRY from Chai. These lyophilized PCR mastermixes are stable at room temperature and only require the DNA template and primers.

Conversely, other researchers have explored alternate drying methods, such as vacuum drying and air drying. For instance, Qu *et al.*[15] vacuum dried primers, Taq polymerase, DNA template, trehalose, and dextran in order to create a PCR mastermix for the detection of *Yersina pestis* that was able to retain its activity for 49 days at 37 °C. Seise *et al.*[9] developed air-dried PCR mastermix to detect *Clostridium* spp. The PCR reagents, which included bovine serum albumin, buffer, $MgCl_2$, DNA primers, and dNTPs, were air dried on polyolefin surfaces and proved to be stable for up to 10 months at room temperature. However, innuTaq DNA polymerase was not included in the dried reagent and was added only when the test was performed, and there was also a significant decrease in sensitivity even though amplification was still successful 10 months following fabrication.

1.1.2 Thermally Stable Isothermal Amplification Mastermixes

Although PCR is still the most commonly used molecular diagnostic technique, the need for thermocycling makes it a sub-optimal choice for POC application. Thus, researchers have increasingly turned to isothermal DNA amplification methods for POC diagnostics.[2,16] Developed by Notomi *et al*.[17] at Eiken Chemical Company, Loop-mediated isothermal amplification (LAMP) has become one of the most widely used and researched isothermal amplification methods. LAMP uses Bst DNA polymerase and 4-6 primers at 60-65 °C for DNA amplification, and it is capable of detecting various diseases with a high degree of specificity and sensitivity.[16] Eiken has developed a number of different LAMP kits, and it has recently documented the development of

dried LAMP mastermixes for the detection of influenza,[18] sleeping sickness,[19] and human herpesvirus 6B.[20] The LAMP reagents in these kits are dried on the cap of a reaction tube, and the sample and primers are added at the time of testing. Once the sample and primers have been added, the tube is inverted to reconstitute the dried reagents and initiate the LAMP reaction, which produces a fluorescent signal that can be detected visually. Although Eiken's dried LAMP kits can be stored at room temperature, their shelf-life and drying methods have not been reported. The procedure for Eiken's dried LAMP kits is shown in Figure 1.1.

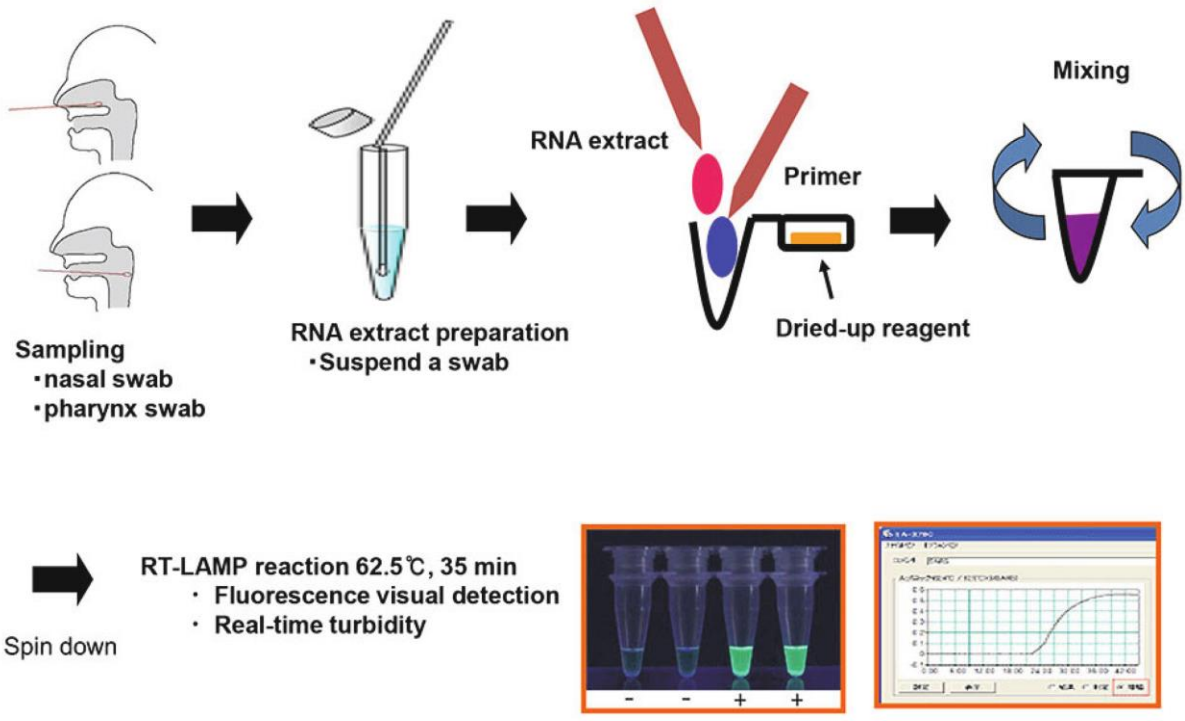

Figure 1.1. Schematic of disease detection using a dried LAMP kit from Eiken Chemical Company. Reproduced from Notomi *et al.*[18]

In addition to the kits developed by Eiken, there have also been several reports documenting the development of thermostable LAMP mastermixes. For example, Borysiak *et al.* [21] developed a

nucleic acid detection method using Isotachophoresis and Loop-mediated isothermal amplification (NAIL), which enables the extraction and amplification of nucleic acids inside of an integrated chip within one hour. By using sucrose to stabilize the LAMP reagents during vacuum drying, Borysiak *et al.* were able to produce quality amplification for up to two weeks at ambient temperature. In contrast, another group opted to lyophilize LAMP reagents in order to detect *Vibrio cholerae*. In this method, glycerol was removed from the Bst 2.0 DNA polymerase prior to lyophilization, and trehalose was added as a cryoprotecant to improve the stability of the lyophilized reagents. Furthermore, the LAMP buffer was not lyophilized and was added just before amplification. This group's dried LAMP mastermix was stable for up to 1 month at 37 °C.[22]

In a different study, Hayashida *et al.*[23] developed a dried LAMP kit for the detection of sleeping sickness in direct blood samples. This procedure required the LAMP reagents to be air-dried on the cap of the reaction tube via a three steps process. Figure 1.2 shows the picture of the dried reagents. The reaction buffer, $MgSO_4$, and Triton-X were not dried and were added as a solution prior to amplification. While the dried reagents remained active at ambient conditions for up to 7 months, there was also a decrease in sensitivity. Finally, Chander *et al.*[24] developed a freeze-dried LAMP mastermix that replaced Bst polymerase with a thermally stable polymerase called OmniAmp. Their results showed that their LAMP mastermix containing OmniAmp was able to maintain its stability for up to 180 days at 37 °C.

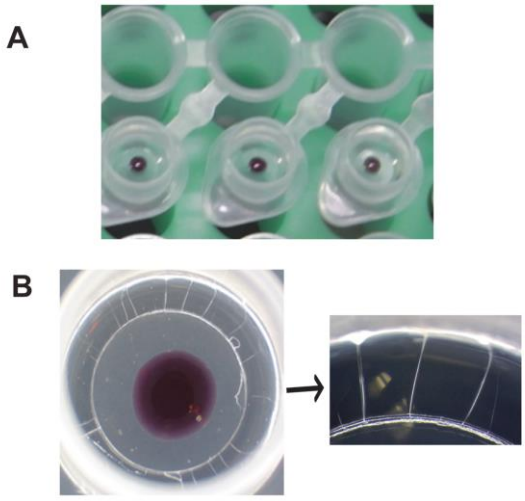

Figure 1.2. A) Picture of LAMP reagents dried on caps of reaction tubes. The primer/indicator mix (purple color) is dried in the center of the cap, while the enzyme and dNTPs mix was dried at the periphery of the cap. **B)** Close-up of the dried LAMP reagents showing the cracks in the dried enzyme/dNTPs solution.

Rolling circle amplification (RCA) is another isothermal amplification method that can be used to detect viral, bacterial, and fungal diseases with high sensitivity and specificity.[16,25–27] RCA uses Phi29 DNA polymerase and a circular ssDNA for amplification at a relatively low temperature of 30 °C, which makes it ideal for POC diagnostic applications. Furthermore, since RCA uses a circular template, it is an ideal method for *in vivo* detecting viruses, phages, and bacteria that possess circular DNA. However, unlike PCR and LAMP, there are currently no commercially available thermally stable RCA mastermix kits. Moreover, the literature contains no reports of thermally stable RCA mastermixes at all. Therefore, developing a method of stabilizing RCA mastermixes would be highly beneficial to the creation of accessible POC molecular diagnostics. *Chapter 2* will discuss the development of a method for thermally stabilizing PCR, LAMP, RCA, and other enzymes used in molecular biology.

1.2 Stabilization of Bacteriophages

Another pressing issue regarding global health is the prevention and control of antibiotic resistance. The World Health Organization (WHO) views the misuse of antibiotics and the attendant rise of antibiotic resistance as one of the biggest contemporary threats to global health and food security.[28] One potential solution to antibiotic resistance is to replace antibiotics with bacteriophages (phage) as antimicrobial agents.[29–35] Unlike broad-spectrum antibiotics, which kill a large range of bacteria including commensal flora, bacteriophages are able to infect bacteria with high specificity: while some are capable of targeting a single bacterial species, others are able to target a broader range of hosts, including multiple species within a genus.[36] Bacteriophages can be used for rapid pathogen detection in POC settings,[37–41] and they can also be used as a treatment for infections in clinical settings, especially for antibiotic-resistant bacteria.[42–46] Phages are also used as a biocontrol agent in the food and agriculture setting.[47–55] Another advantage of using bacteriophages in place of antibiotics is that they do not harm the normal flora, which reduces the risk of secondary infection. Furthermore, phages are generally safer than antibiotics, as there have been no prior reports of negative side effect or allergic reactions. Moreover, bacteriophages are cost effective; since they are ubiquitous in nature, it is much cheaper to isolate them than it is to develop new antibiotics.[30] Phages can also self-replicate in the presence of a host, which means they can be auto-dosed at low dosages. The ability of phages to mutate and evolve can counter phage-resistant bacteria, and phage cocktails can be used to significantly reduce the risk of phage resistance. Lastly, it has been shown that there is a synergistic effect when phage therapy is used alongside antibiotic therapy.[30]

Phages are generally prepared as liquid formulations. Liquid phage formulations need to be stored at 4 °C and usually have a shelf-life of about one year.[56] Since liquid formulations are susceptible

to different environmental conditions, such as temperature and pH, researchers have explored different phage formulation formats. One common method of protecting phages is through encapsulation, which is often used during oral therapy to protect the phages from the harsh conditions of the gastrointestinal system. Some of the more common materials used for encapsulation include alginate,[41,57–61] pectin,[44,57] chitosan,[58,59] whey protein,[34,62] and liposome.[63,64] Long-term stability is often not reported for encapsulated phages; rather, the focus in these instances tends to be on phage viability against low pH and proteases. Kim *et al.*[59] encapsulated *E. coli* phage in chitosan-alginate microspheres. They found that the microspheres were able to protect the phage at pH 2.0 and observed a 1.3 log reduction in titre after 10 hours of incubation at 37 °C. Encapsulated phages are also able to remain active longer *in vivo* than non-encapsulated phages. One report showed that liposome-encapsulated salmonella phages were still detectable 72 hours after administration in 38.1% of chickens, whereas only 9.5% of chickens treated with non-encapsulated phage showed traces of the administered phage following the same time period.[65] Another report showed that phages encapsulated in alginate/$CaCO_3$ were detectable in 71.4% of chickens after 72 hours compared with only 9.5% of chickens that had been treated with non-encapsulated phages.[60]

The use of lyophilization or spray drying to produce dried powder bacteriophages is another common formulation format. Powder phages are desirable because they can be easily aerosolized to treat respiratory infections.[42,66,67] Puapermpoonsiri *et al.*[67] prepared a lyophilized phage formulation that was encapsulated in biodegradable polyester microspheres using phages that are selective for *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Although their freeze-dried formulation was able to retain infectivity immediately after freeze-drying, it showed a complete loss of infectivity within 7 days, even when stored at 4 °C. As a result, a follow-up study was

conducted to determine the effectiveness of gelatin, sucrose, and polyethylene glycol (PEG) as stabilizers for phage lyophilization. It was found that gelatin had no effect on phage stability, while PEG and sucrose both slightly improved the stability of the freeze-dried phage. Moreover, it was observed that phages that underwent two freeze-dry cycles had a longer shelf life. Nonetheless, all of the tested formulations showed significant decreases in phage infectivity after 30 days of storage at 4 °C.[68] Merabishvili *et al.*[56] studied the effects of six stabilizers on the stability of lyophilized *Staphylococcus aureus* phage and found that high concentrations of sucrose and trehalose resulted in the greatest stability. They observed that the lyophilized phages made with these stabilizers exhibited only a minor decrease in infectivity after 27 months of storage at 4 °C. In a different study, Golshahi *et al.*[66] used a lactose/lactoferrin mixture as a stabilizer for the lyophilization of *Burkholderia cepacia* and *Pseudomonas aeruginosa* phages. Their findings showed no significant loss in infectivity in the lyophilized phages after 3 months of storage at 22 °C. In contrast, Vandenheuvel *et al.*[42] demonstrated that the stability of spray-dried phages is highly dependent on both the phage species and the stabilizers used. Spray-dried formulations were generated for the LUZ19 phage, which is in the *podoviridae* family, and the Romulus phage, which is in the *myoviridae* family. LUZ19 showed insignificant titre loss after spray drying, while Romulus showed a titre log reduction of more than 2.5. In addition, they also found that trehalose was the only effective stabilizer, as dextran and lactose were unable to stabilize the spray-dried formulation.

The immobilization of phages onto a surface is another method of phage stabilization. These bacteriophage-functionalized surfaces can be used as biosensors for the detection of bacteria, as biocontrol surfaces in food safety, and as antimicrobial surfaces for medical applications.[69] Tolba *et al.* immobilized T4 phage onto streptavidin-coated cellulose beads. Their immobilized T4 phage

was able to capture *E. Coli* and allowed as few as 800 cells to be detected within 2 hours using real-time PCR. Lone *et al*.[52] encapsulated bacteriophage cocktails in alginate beads, which were immobilized onto positively charged cellulose membranes for the purpose of biocontrolling *Escherichia coli* in food. While the encapsulated phages showed a reduction of 3.5 logs in phage titre after 1 day of storage at 25 °C, no further loss was observed after 7 days. Similarly, Anany *et al.* used electrostatic interactions to immobilize Listeria and *E. Coli* phages on cellulose membranes, which were then air dried. After drying, the phages in the *myoviridae* family showed a significant loss in titre (>3 log), while the phages in the *siphoviridae* family showed only a small loss (< 1 log).

From the above-discussed research, it is clear that bacteriophage formulation formatting is dependent on the desired application. In addition, it is also evident that sensitivity to environmental conditions, such as temperature and desiccation, varies significantly between bacteriophage strains.[70] For example, phages from the *myoviridae* family appear to be especially susceptible to inactivation by desiccation; this may be due to its relatively more complex structure, which includes the presence of a contractile tail. In *Chapter 3*, we detail a method for stabilizing *myoviridae* bacteriophages in pullulan and trehalose films that can be used to coat surfaces for biocontrol in food safety applications. In *Chapter 4*, we optimize phage stability in the pullulan and trehalose film by exploring different storage and manufacturing conditions.

1.3 Thermally Stable Vaccines

Universal immunization is another key contemporary concern for global health. Indeed, although the WHO estimates that vaccines save 2-3 million lives each year, there are still 19.9 million children under the age of 1 who have not received basic life-saving vaccines. It is estimated that

1.5 million more lives can be saved if global immunization coverage improves.[71] Currently, one of the most significant obstacles for universal immunization is the need for a cold chain, as nearly all available vaccines are thermally labile and must be kept between 2-8 ºC at all times in order to retain full potency.[4] For areas that do have access to cold chain equipment, there are considerable financial challenges since the operation and maintenance of the equipment can often account for more than 50% of a vaccination program's total costs. Furthermore, any disruption in the cold chain will result in ineffective vaccines and vaccine wastage, which further increases costs.[72] The necessity of cold chain equipment also poses significant logistic obstacles for developing countries and remote areas because they often lack of access to reliable electricity and cold chain equipment.[5–7]

Given these challenges, researchers have been attempting to increase vaccine accessibility by focusing on developing thermally stable vaccines that can be stored and transported without the need of a cold chain. One area that researchers have focused on is the engineering of thermostable vaccines without the need for adjuvants. For example, Sun *et al.*[73] developed a novel thermostable tuberculosis vaccine by attaching an M. tuberculosis epitope to a self-assembling fibril-forming peptide. The engineered vaccine displayed no conformational change and retained its immunogenicity after 7 days at 45 °C. In another study, Konar *et al.*[74] introduced two mutations to Factor H binding protein (FHbp), which is commonly used as a meningococcal vaccine. When introduced together, the two mutations increased the transition midpoint (T_m) for the N-terminal domain by 21 °C. Rossi *et al.*[75] conducted a follow-up study wherein they engineered a triple FHbp mutant; compared to the wild-type FHbp, their triple mutant strain's immunogenicity was 15 times higher, and its thermostability was increased by 21 °C. Similarly, Campeotto *et al.*[76] engineered a thermally stable protein vaccine by modifying the

malaria invasion protein, RH5, via the introduction of 18 mutations. Their modified RH5 protein showed a 10-15 °C increase in thermal tolerance while maintaining similar immunogenic properties to the wild type.

Other researchers have developed thermally stable vaccines by modifying viral vectors. In one such study, Stobart *et al.*[77] created a live-attenuated vaccine candidate for respiratory syncytial virus (RSV) that featured enhanced immunogenicity and significantly greater thermal stability compared to the wild type. However, a significant decrease in titre was observed after 7 days at 37 °C. In contrast, Wang *et al.* engineered a self-biomineralized virus that was modeled on the human enterovirus type 71 vaccine. The modified virus, which would induce calcium phosphate mineralization on its surface, exhibited greater thermal stability than the wild-type and could be stored for 1 week at 37 °C.[78]

Although the engineering of proteins and viral vectors can improve thermal stability, this approach does not usually provide long-term protection. The reported shelf-life of the vaccines developed in the above-discussed studies was usually about 1 week at an elevated temperature. Consequently, many researchers add stabilizing adjuvants and then dry the vaccine to create formulations that have long-term thermal stability. For example, Pelliccia *et al.*[79] reported that the addition of PEG, gold nanoparticles, and sucrose to adenoviral vaccine formulations can help maintain immunogenicity for up to 10 days when stored at 37°C. In contrast, Pausntz's group used microneedle patches to encapsulate inactivated influenza vaccine (Figure 1.3), which was then dried with a combination arginine and heptagluconate. Their results indicated that the vaccine in the microneedle patches was able to retain its activity for up to 24 months at 25 °C and for up to 4 months at 60 °C.[80–82]

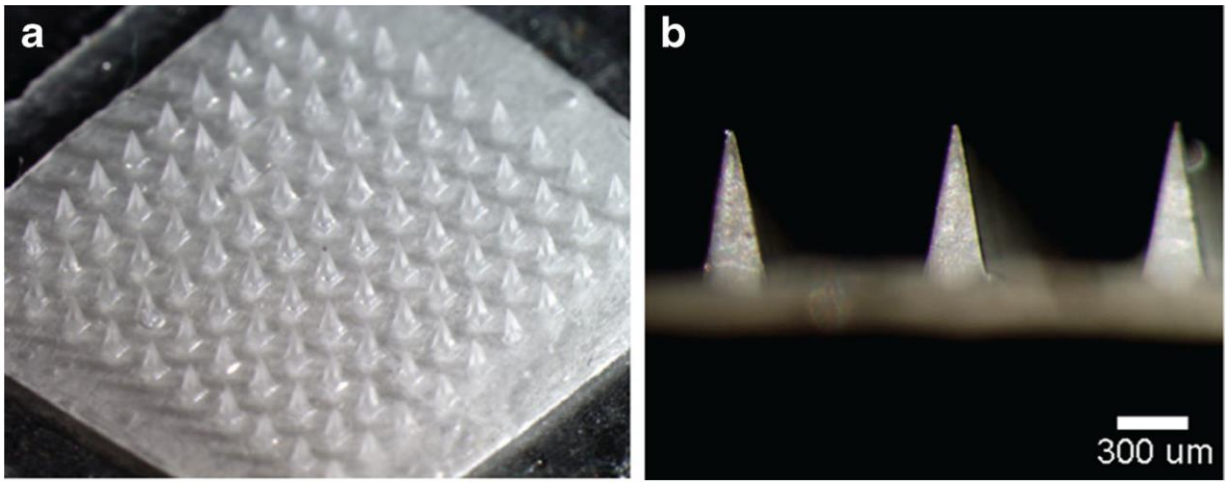

Figure 1.3. a) Top view of microneedle patch encapsulating inactivated influenza vaccine. **b)** Side view of microneedles. Reproduced from Chu *et al.*[80]

Alcock *et al.*[83] air dried adenovirus and modified vaccinia virus onto polypropylene or glass-fiber membranes using trehalose and sucrose as stabilizers. For administration, the membrane was placed into a membrane housing attached to a syringe filled with buffer; the vaccine is reconstituted when the buffer is delivered from the syringe and administered to the patient (Figure 1.4). The dried vaccines retained full viral titre for up to 6 months when stored at 45 °C.

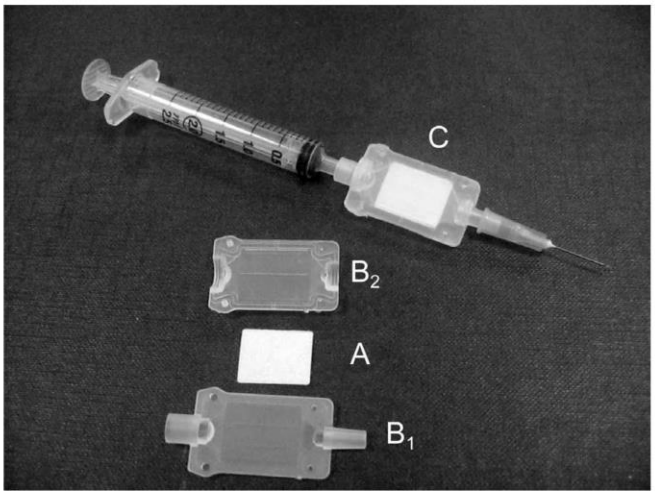

Figure 1.4. A) Vaccine-impregnated membrane. **B)** Lower (B_1) and upper (B_2) membrane housing. **C)** Full assembly of the syringe, membrane, and needle unit.

Lyophilization is also a common method used to generate thermally stable dried vaccine formulations. Hassett *et al*. reported a lyophilized recombinant ricin toxin A vaccine with colloidal aluminum hydroxide adjuvant. When freeze-dried with trehalose as a stabilizing agent, their vaccine was able to protect mice from lethal doses of ricin after being stored for 4 weeks at 40 °C.[84] In addition, Hassett *et al.* also reported that their lyophilized recombinant protein vaccine for anthrax was able to maintain thermal stability after 16 weeks of storage at 40 °C. [85] Lovalenti *et al.* [86] explored the effectiveness of three drying methods—freeze drying, spray drying, and foam drying—for preparing dried live-attenuated influenza vaccine formulations. Their results showed that foam drying produced formulations with the longest shelf life. In addition, they also examined a number of stabilizers and found that gelatin and arginine were optimal for foam drying. In the end, the best formulation had a shelf life of 4.5 months at 37 °C.

Chen *et al.* spray dried a recombinant vaccine for hepatitis B and a protein-polysacharide conjugate vaccine for meningitis A, with both vaccines retraining their immunogenicity after 24 months of storage at 37 °C.[87] In another study, Ohtake *et al*. spray dried a formulation of live-attenuated measles vaccine[88] in addition to foam drying live-attenuated salmonella vaccine[89]. They found that the foam-dried salmonella vaccine was able to maintain its stability for 12 weeks at 37 °C, while the spray-dried measles vaccine was able to retain its potency for up to 8 weeks at the same temperature.

Several conclusions can be drawn from the research summarized above. First, dried vaccines typically exhibit the best thermostability. Second, the addition of stabilizers is crucial for dried formulations, and the effect of the stabilizer can differ for each vaccine and drying method. Third, live-attenuated vaccines are typically less stable than other types of vaccines. Lastly, most drying methods, such as lyophilization, spray drying, and foam drying, require specialized

equipment and expose the vaccine to extreme conditions (low pressure, freezing). In *Chapter 5*, we present a simple method that can be used to thermally stabilize live-attenuated vaccines and inactivated viral vaccines without the use of specialized equipment.

1.4 Stabilizers

The previous sections have highlighted the current research relating to the stabilization of molecular biology mastermixes, bacteriophages, and vaccines. These three areas of research are capable of addressing some of the pressing needs in global health, as they can make key contributions to creating accessible and reliable POC diagnostics, controlling antibiotic resistance, and deploying thermostable vaccines for universal immunization. In this work, we present a versatile stabilization method that is compatible with these three applications through its use of pullulan and trehalose as stabilizers during the drying of reagents.

1.4.1 Pullulan

Pullulan is a polysaccharide that is produced by the fungus-like yeast, *Aureobasidium pullulans*.[90] It is comprised of repeating units of maltotrose, which are connected by α-1,6-glycosidic linkages (Figure 1.5),[91] and its molecular weight usually ranges between 5 kDa to 900 kDa. In addition, Pullulan is a very flexible and linear unbranched biopolymer that is also biodegradable, water soluble, edible, odorless, tasteless, and non-toxic. As such, it is ideal for use in the food and pharmaceutical industries. For pharmaceutical applications, pullulan derivatives have good carrier properties and are used as vehicles for drug delivery and gene delivery.[90–93] In the food industry, on the other hand, pullulan is often use as a filler and food protectant due to its ability to form oxygen-impermeable films.[93]

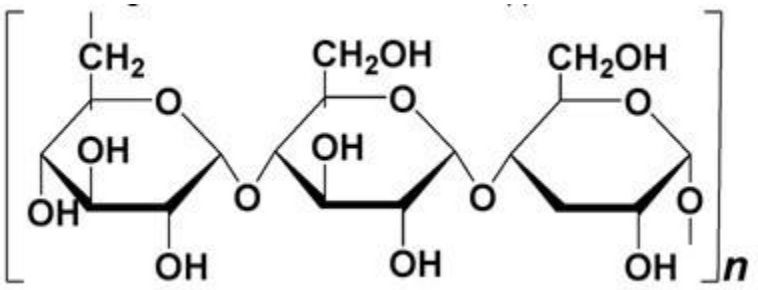

Figure 1.5. Chemical structure of pullulan.

Pullulan film has been shown to have good stabilizing properties. As one study showed, pullulan-based edible coatings were able to slow down browning and extend the shelf life of apples.[94] Similar studies have shown that pullulan coatings can preserve the firmness of strawberries for 15 days[95] and the moisture content in blueberries for 28 days.[96] Pullulan coatings have also been shown to extend the shelf life of eggs by an additional 3 weeks.[97] Krumnow *et al.*[98] preserved *E. coli* and *B. subtilis* by drying the bacteria in pullulan. Although the bacteria dried in pullulan exhibited about a 1–2 log decease in CFU count after drying, their viability was significantly better at elevated temperatures (> 25 °C) than bacteria that had not been dried in pullulan.

Previous studies conducted by our group have shown that pullulan films are able to stabilize and protect various labile biomolecules, such as luciferase, acetylcholine esterase, and indoxyl acetate, from heat and oxidation.[99,100] Pullulan affords thermal stability by restricting the mobility of labile molecules. When pullulan is dried, it forms a sugar-glass through a process called vitrification, which also serves to entrap labile molecules, such as proteins. As shown in a previous study, human serum albumin entrapped in pullulan sugar-glass was unable to unfold at 60 °C,[99] thus demonstrating how molecule mobility is highly restricted in pullulan sugar glasses, even at

elevated temperatures. Moreover, molecules entrapped in pullulan sugar glass are protected from oxidation due to the high oxygen impermeability property of pullulan.

1.4.2 Trehalose

Another stabilizer used in this work is trehalose, which is a non-reducing disaccharide consisting of two glucose units joined by a α-1,1-glycosidic linkage (Figure 1.6). In nature, trehalose can be found in plants, algae, fungi, bacteria, insects, and yeasts. Most commonly, these organisms tend to exhibit elevated levels of trehalose in response to environmental stress,[101] as the accumulation of trehalose can offer protection against osmotic stress, desiccation, and freezing.

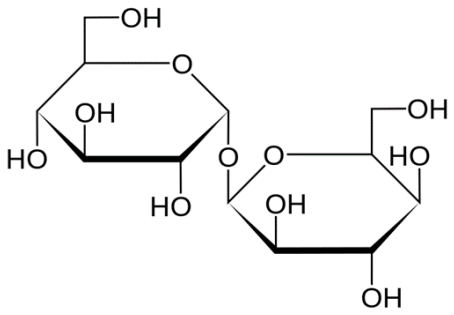

Figure 1.6. Chemical structure of trehalose.

The addition of trehalose has also been shown to offer desiccation protection for cells that do not naturally produce it. For example, Guo *et al.*[102] engineered a human primary fibroblast that expressed trehalose. The trehalose-expressing fibroblast exhibited increased desiccation tolerance and was viable for up to five days in a dried state. Trehalose is a disaccharide that is commonly used as a cryoprotectant and stabilizing agent, especially for lyophilization, and it has been shown to offer protection against freeze-drying for proteins, enzymes, DNA, cells, viruses, and bacteria.[101,103–106]

There several theories that explain trehalose's stabilizing properties. The vitrification theory proposes that trehalose forms a glassy matrix as it dries, which encapsulates labile biomolecules, such as proteins or DNA. This glassy matrix protects the biomolecule from external stresses and provides structural stability by restricting the biomolecule's mobility.[103] The preferential exclusion theory posits that trehalose structures water around itself rather than around the biomolecule. This causes water to be excluded from the solvation layer and restricts the mobility of the biomolecule. Moreover, trehalose's water-exclusion properties result in a reduction in freezable water around the biomolecule, thus providing it with protection against freezing.[103] The water replacement theory suggests that trehalose replaces water by forming hydrogen bonds with the biomolecule, which in turn restricts the biomolecule's mobility and preserves its structure. As in the preferential exclusion theory, the water replacement theory posits that trehalose removes freezable water from the solvation layer, thus protecting the biomolecule from freezing.[103] To date, no single theory has been proposed that fully explains trehalose's protective properties, and there is still much debate surrounding this question.[107–109] Nonetheless, it is likely that the actual mechanism responsible for trehalose's stabilizing properties is a combination of the theories discussed above.

1.5 Objectives and Report Outline

The main objective of this work is to develop a versatile method that can stabilize and prolong the shelf life of labile biomolecules for various applications.

To accomplish the goal, we developed a method of thermally stabilizing molecular biology mastermixes for PCR, RCA, LAMP, Klenow, PNK, and ligase that involves drying the reagents in a mixture of pullulan and trehalose (Chapter 2). This work has led to a manuscript that is pending submission. Secondly, we developed a method for stabilizing bacteriophages, which can then be coated onto surfaces and food packaging for food safety applications (Chapter 3).

Furthermore, we optimized the stability of bacteriophages in pullulan and trehalose film by exploring different manufacturing and storage conditions through a four-factor two-level design-of-experiment (Chapter 4). This work has led to a publication in *ACS Biomaterials Science and Engineering,*[110] as well as another manuscript that is pending submission. Third, we were able to thermally stabilize a live-attenuated vaccine and an inactivated vaccine by drying them in pullulan and trehalose (Chapter 5). This work has led to a pending submission to *Nature Communications.* Finally, the conclusions and impact of this work, as well as suggestions for future research, are discussed in Chapter 6.

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Chapter 2: Ready-to-use Thermally Stable Mastermix Pills for Molecular Biology Applications

Preface

In Chapter 2, all experiment were conducted by me with the help of Meredith Brooks and Sophia Emerson. Meredith Brooks worked with me to complete the RCA, Klenow, PNK, and Ligase experiments. Sophia Emerson worked with me on the real-time PCR and RCA experiments. I initiated the first draft of the paper. Dr. Filipe and Dr. Ali provided many helpful suggestions and aided in revising the draft to the final paper. This work is submitted to *Biotechnology and Bioengineering*.

Ready-to-use Thermally Stable Mastermix Pills for Molecular Biology Applications

Vincent Leung,[†] *Meredith Brooks,*[†] *Sophia Emerson,*[†] *M. Monsur Ali**[,‡] *and Carlos D. M. Filipe**[,†]

[†]Department of Chemical Engineering, 1280 Main Street West, McMaster University, Hamilton, Ontario, Canada L8S 4L7
[‡]Biointerfaces Institute, McMaster University, 1280 Main Street West, Hamilton, Ontario Canada L8S 4L7

Abstract

Rolling circle amplification (RCA), polymerase chain reaction (PCR), and loop-mediated isothermal amplification (LAMP), are powerful tools that can be used for gene manipulation, pathogen detection, and infectious disease diagnostics. However, these techniques often require highly trained personnel, as the numerous pipetting steps involved can easily lead to contamination and, consequently, erroneous results. Furthermore, many of the reagents used in molecular biology are thermally labile and must be kept within a cold-chain. In this paper, we present a simple and cost-effective method that allows molecular biology reagents to be thermally stabilized into ready-to-use mastermixes via drying in pullulan and trehalose films. Our experimental results demonstrate that this method is capable of preserving the activity of RCA, PCR, LAMP, ligase, polynucleotide kinase, and Klenow fragment mastermixes for at least 3 months at ambient conditions. Thus, stabilizing reagents via drying in pullulan and trehalose film may allow for a drastic reduction in the number of pipetting steps and the elimination of the need for a cold chain.

2.1 Introduction

Molecular biology techniques, especially nucleic acid amplification tests, are essential for a wide variety of applications, including pathogen detection, gene analysis, cell manipulation, and point-of-care (POC) diagnostics. Indeed, techniques such as rolling circle amplification (RCA), polymerase chain reaction (PCR), and loop-mediated isothermal amplification (LAMP) can rapidly detect infectious diseases and food-borne illness with high degrees of sensitivity and specificity, while also providing low limits of detection. However, such tests are typically confined to laboratory settings due to the need for complicated sample preparation processes and specialized equipment.[1,2]

In recent years, researchers have increasingly focused their efforts on developing portable, on-site systems for nucleic-acid-amplification-based POC testing.[3,4] Unfortunately, these techniques are limited as they require numerous pipetting steps, which can lead to a higher chance of error and contamination, and the reagents must be stored and transported in refrigerated conditions due to being thermally unstable[1,3,5,6] Such inherent limitations make it difficult, if not impossible, to use these POC diagnostic techniques in settings that lack reliable cold-chain equipment and trained personnel. Therefore, the development of methods for thermally stabilizing molecular biology reagents is highly desirable, as this would allow the reagents to be stored outside of the cold chain and to be conveniently applied in laboratory settings and POC diagnostic procedures.

A number of studies have attempted to address this problem by developing methods of stabilizing mastermixes. The most common approach for stabilizing mastermixes is to lyophilize the reagents via the introduction of stabilizers,[7] as lyophilisation has proven capable of producing dried mastermixes with varying degrees of thermal stability for PCR[3,4,8] and LAMP.[9] Despite the relative success of freeze drying, many commercially available mixes contain compounds that are not suitable for lyophilisation.[6] Other documented approaches include drying PCR reagents on a polyolefin matrix,[1] vacuum drying,[10] and replacing a thermally sensitive enzyme with a thermally stable enzyme for LAMP.[11]

In this paper, we detail a simple method that uses either pullulan on its own, or a combination of pullulan and trehalose to achieve the long-term stabilization of PCR, RCA, and LAMP mastermixes. In addition, we also demonstrate our approach's versatility for use as a stabilizer in

other molecular biology applications by using it to stabilize polynucleotide kinase (PNK), ligase, and Klenow fragments. Pullulan is a polysaccharide with great film-forming properties that is used in the food industry to extend food shelf life due to its ability to act as an oxygen barrier.[12–16] Our group has previously shown that pullulan can provide protection against oxidation and thermal stability for labile compounds.[17–19] However, it was not comprehensively studied for its effects on various reagents used in various methods for DNA amplification and other molecular biology techniques. We demonstrate that pullulan provides thermal stability for PCR mastermixes, and that thermal stability in techniques such as RCA and LAMP, can be achieved using a combination of pullulan and trehalose, which is a disaccharide that is often used as a stabilizing agent during lyophilisation.[20–25] Furthermore, we demonstrate that stability can be significantly increased by simply separating the reaction buffer from other components of the mastermix.

2.2 Materials and Methods

Materials

Pullulan (M_n = ~200 kDa) was purchased from Polysciences Inc., and D-(+)-trehalose dihydrate was obtained from Sigma-Aldrich. Phi29 DNA polymerase, phi29 buffer, DreamTaq DNA polymerase, DreamTaq buffer, T4 Polynucleotide Kinase (PNK), PNK buffers, T4 DNA ligase, ligase buffer, adenosine triphosphate (ATP), and dNTP mix were purchased from Thermo Fisher Scientific. The Klenow fragment, BamHI-HF restriction endonuclease, and buffers were purchased from New England Biolabs. All DNA sequences were acquired from Integrated DNA Technologies and are listed in **Table S1**. Finally, all of the reagents for loop-mediated isothermal amplification (LAMP) were generously provided by Dr. James Mahoney from McMaster University.

RCA Mastermix Pills

Rolling circle amplification (RCA) mastermix pills were made using a procedure that was similar to the one used to make the PCR pills. For the single-pill method 7.5 µL pullulan (10 wt%) and 0.5 M trehalose was mixed with 1 µL Phi29 polymerase, 5 µL Phi29 10x reaction buffer, 2.5 µL dNTPs mix (10 mM), and 1 µL circular DNA template (1 µM). The mixture was then pipetted into a 1.5 mL microcentrifuge tube and air dried overnight. For the two-pill method, the first pill was made by mixing 3.75 µL pullulan (10 wt%) and 0.5 M trehalose with 5 µL Phi29 10x reaction

buffer in a 1.5 mL microcentrifuge tube and allowing it to air dry. The second pill was produced by mixing 3.75 µL pullulan 10 (wt%)/0.5 M trehalose with 1 µL Phi29 polymerase, 2.5 µL dNTPs mix (10 mM), and 1 µL circular DNA template (1 µM) and then placing the mixture into a 1.5 mL microcentrifuge tube to air dry. The obtained pills were then stored at ambient conditions.

For the reaction, the pills were reconstituted in a tube containing 49 µL water and 1 µL primer (10 µM) before being placed in a heating block for one hour at 30 °C. After one hour had elapsed, the tube was heated to 90 °C for one minute in order to terminate the reaction. The resultant RCA products were then visualized using gel electrophoresis. ImageJ was used to further analyze the band intensities on the gel image in order to determine the stability of the RCA mastermix over time.

For the real-time RCA experiments, the pills were reconstituted in 46.5 µL water, 2.5 µL 20x EvaGreen, and 1 µL primer (10 µM). The reconstituted pills were then transferred to a 96-well plate, which was placed in a plate reader (Tecan M1000) for one hour at 30 °C. While in the plate reader, fluorescence was recorded every 30 seconds for one hour.

PCR Mastermix Pills

Two methods were used to create polymerase chain reaction (PCR) mastermix pills: the single-pill method, and the two-pill method. In the single-pill method, 7.5 µL pullulan (10 wt%), 0.5 µL DreamTaq Polymerase (5 U/µL), 5 µL DreamTaq 10x Buffer, 1 µL dNTPs mix (10 mM), 0.5 µL forward primer (50 µM), and 0.5 µL reverse primer (50 µM) were mixed together, pipetted into a PCR tube, and air dried into a “pill”. In the two-pill method, 5 µL DreamTaq 10x Buffer and 3.75 µL pullulan (10 wt%) were mixed together and placed into a PCR tube to dry into “pill” form. In a separate tube, 3.75 µL pullulan (10 wt%), 0.5 µL DreamTaq Polymerase (5 U/µL), 1 µL dNTPs mix (10 mM), 0.5 µL forward primer (50 µM), and 0.5 µL reverse primer (50 µM) were also mixed and left to dry into “pill” form. Once dried, the pills were stored at ambient conditions.

For the reaction, the pills were reconstituted in 49 µL water and 1 µL DNA template (0.1 µM) was added, resulting in a total reaction volume of 50 µL. Next, the tube was placed in a thermocycler (Bio-Rad CFX96) in order to begin the PCR, which was completed after 20 cycles. The resultant PCR products were then visualized via gel electrophoresis. ImageJ was used to further analyze the

band intensities on the gel image in order to determine the stability of the PCR mastermix over time.

For the real-time PCR experiments, the pills were reconstituted in 46.5 µL water, 2.5 µL 20x EvaGreen, and 1 µL DNA template (0.1 µM). The samples were then placed in a thermocycler for 20 cycles, and the fluorescence of the samples was recorded after each cycle.

Loop-Mediated Isothermal Amplification (LAMP) Pills

Loop-mediated isothermal amplification pills were made by mixing a solution of 3 µL (10 wt%) pullulan and 0.5 M trehalose with 1.4 µL dNTP mix (25 mM), 0.05 µL F3 primer (100 µM), 0.05 µL B3 primer (100 µM), 0.2 µL FIP primer (100 µM), 0.2 µL BIP primer (100 µM), 0.1 µL LF primer (100 µM), 0.1 µL LB primer (100 µM), and 1 µL Bst 3.0. The resultant mixture was then air dried in a microcentrifuge tube. The second pill was obtained by mixing 3.75 µL pullulan (10 wt%) and 0.5 M trehalose with 2.5 µL 10x AMP buffer, and 1.5 µL $MgSO_4$ (100 mM) and air drying the resultant mixture in a microcentrifuge tube.

For the reaction, 1.25 µL 20x EvaGreen, 5 µL template, and 18.75 µL water was added to the pills. The mixture was then heated for 30 minutes at 68 °C, with the reaction products being visualized via gel electrophoresis.

Phosphorylation and Ligation Pills

Phosphorylation pills were made using the two-pill method. The first pill was made by mixing 1 µL polynucleotide kinase (PNK) buffer A with 1 µL (10 wt%) pullulan and 0.5 M trehalose in a microcentrifuge tube and allowing it to air dry. The second pill was fabricated by mixing 0.5 µL PNK and 1 µL ATP (10 mM) with 1 µL 10 wt% pullulan and 0.5 M trehalose in a microcentrifuge tube, where it was allowed to dry.

Similarly, the ligation pills were also made using the two-pill method. In this case, the first pill was obtained by mixing 1 µL (10 wt%) pullulan and 0.5 M trehalose with 1 µL T4 ligase buffer, while the second pill was obtained by mixing 1 µL T4 ligase with 2 µL (10 wt%) pullulan/0.5 M trehalose. Both pills were stored at room temperature.

For the phosphorylation reaction, 1 µL of linear DNA (10 µM) and 9 µL of water were added to the pills to obtain a total reaction volume of 10 µL. The reagents were then mixed and heated for 30 minutes at 37 °C. Once this 30 minute period had elapsed, the reagents were heated for an additional 5 minutes at 90 °C before being allowed to cool to room temperature for 10 minutes.

After the reaction had cooled to room temperature, 1 µL ligation template (10 µM) and 1 µL fluorescent DNA (10 µM) were added to the phosphorylation pill mixture. All reagents were mixed and heated for 1 minute at 90 °C and then cooled to room temperature for 20 minutes.

The final mixture for the phosphorylation pills was then added to the ligation pills and 8 µL of water was added to the mixture. The entire mixture was then left to react at room temperature for 1 hour before DNA precipitation was conducted using NaOAc and ethanol.
Following precipitation, the ligated DNA was visualized using 10% dPAGE, and ImageJ was used to further analyze the band intensities on the gel image in order to determine the stability of the phosphorylation and ligation reagents over time.

Klenow Fragment Pills

The Klenow fragment pills were made using the two-pill method. The first pill was fabricated by mixing 5 µL Klenow buffer with 3 µL (10 wt%) pullulan/0.5 M trehalose in a microcentrifuge tube where it was allowed to dry. The second pill was obtained by mixing 1 µL Klenow template DNA (10 µM), 1 µL dNTPs mix (10 mM), 1 µL Klenow fragment, and 4 µL 10 pullulan (10 wt%) and 0.5 M trehalose in a microcentrifuge tube and allowing it to dry. The resultant pills were then stored at room temperature.

For the reaction, 1 µL of fluorescent DNA (10 µM) and 49 µL of water were added to the pills and the tubes were heated for 30 minutes at 37 °C.

The resultant DNA product was visualized using 10% dPAGE, and ImageJ was used to further analyze the band intensities on the gel image in order to determine the stability of the reagents over time.

2.3 Results and Discussion

RCA Mastermix Thermally Stabilized in Pullulan and Trehalose When Dried in Two Pills

Isothermal nucleic acid amplification techniques are important for POC diagnostics because they do not require complex equipment, such as a thermocycler. RCA is a widely used isothermal nucleic acid amplification technique for developing diagnostics and gene analysis.[26,27] Therefore, we investigated the stability of an RCA mastermix in pullulan. The RCA mastermix used in this study consisted of phi29 polymerase, a circular DNA template, dNTPs, and reaction buffer. For the RCA experiments, we first investigated the stability of phi29 polymerase, which, unlike Taq polymerase, is a mesophilic DNA polymerase. **Figure 1A** shows the RCA product using phi29 stored in solution at room temperature. The activity of the enzyme was determined based on the intensity of the band relative to the intensity of the band at Day 0. When the phi29 was stored in solution at room temperature, we observed a 26% activity loss after 1 week, a 72% activity loss after 2 weeks, and a 97% activity loss after 4 weeks. We then tried to stabilize the RCA mastermix by adding pullulan and then drying the mixture into a pill. As **Figure 1B** shows, a very faint band can be observed at the 1 day mark and there was 88% loss in activity after 1 day. Since trehalose is a commonly used stabilizer, we attempted to improve the RCA mastermix's stability by added 0.5 M trehalose and 10 wt% pullulan and then drying the mixture. However, the addition of trehalose only slightly improved the stability of the mastermix. Even after adding trehalose, 63% of the activity was lost after 1 day of storage, and 94% of the activity was lost after 1 week (**Figure 1C**).

These results compelled us to consider the possible causes of this instability. As we had observed in previous studies, the presence of salt in the pullulan film can be detrimental to the stability of molecules that are dried in it.[19] Thus, we separated the reaction buffer and created two pills for the RCA reaction: one with the reaction buffer and pullulan, and another with all of the other reagents and pullulan. We first dried the two pills using pullulan without trehalose. As shown in **Figure 1D**, separating the buffer improved stability and preserved 95% of activity after one day, but only 23% of activity remained after one week. To improve upon this, we again used the two-pill method, only this time we dried the reagents in trehalose instead of pullulan. Although this approach slightly improved the stability of the reagents, 40% of the activity was lost by 4 weeks (**Figure**

1E). Next, we combined the use of both pullulan and trehalose with the two-pill method. As can be seen in **Figure 1F**, this approach yielded significantly improvements in stability, there was no loss of activity even after 12 weeks of storage at ambient conditions (**Figure 2A**).

To further investigate the two-pill method's effectiveness for RCA mastermixes, the real-time fluorescence of RCA was examined using the mastermix pill and the rate of reaction was determined based on the slope of linear portion of the curve (**Figure 2B**). These tests revealed that there was a 25% decrease in reaction rate between the fresh sample and the pills that had been stored for 4 weeks. However, after 4 weeks the reaction rate remained relatively constant, from 4 weeks to 12 weeks of storage there was only a further 5% decrease in the reaction rate.

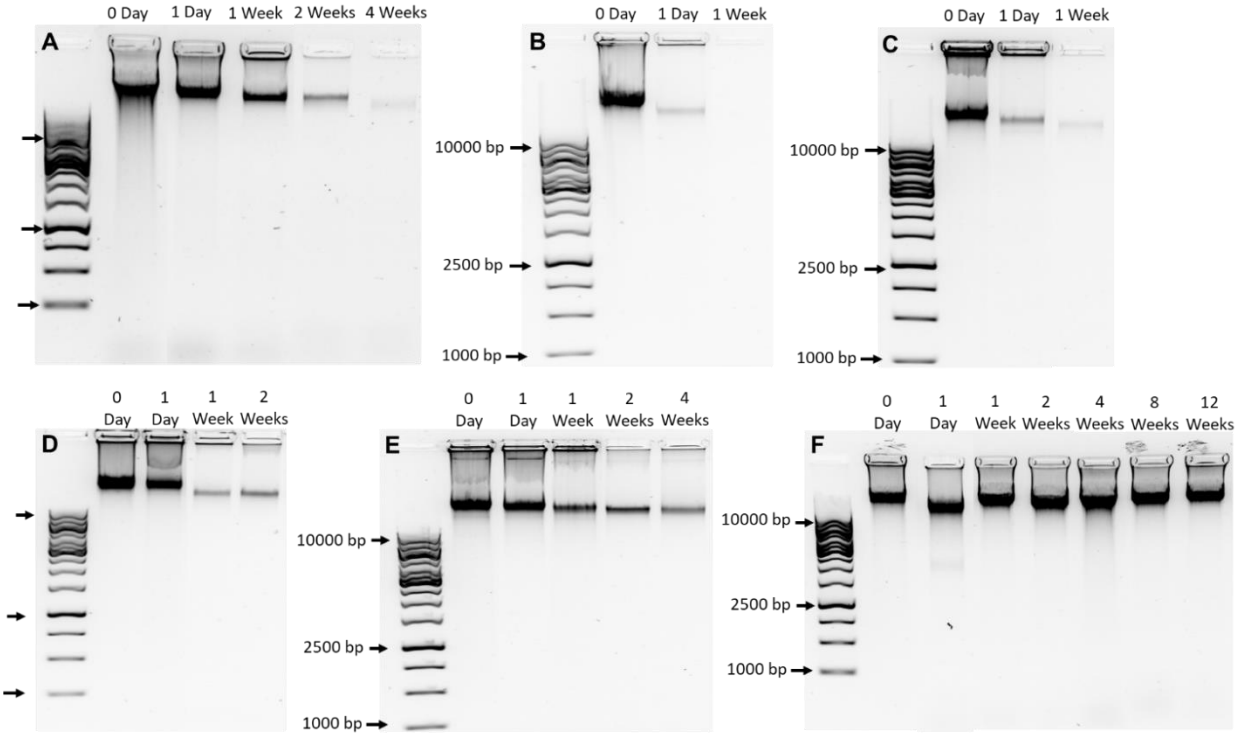


Figure 1. Agarose gel electrophoresis of RCA product at different storage times for different RCA mastermix stabilization methods: **A)** phi29 stock solution stored at room temperature; **B)** RCA mastermix dried in pullulan; **C)** One-pill RCA mastermix dried in pullulan and trehalosee; **D)** Two-pill method: RCA mastermix (except buffer) with pullulan in one pill, buffer alone with pullulan in another pill; **E)** Two-pill method: RCA mastermix (except buffer) with trehalose in one pill, buffer alone with trehalose in another pill; **F)** Two-pill method: RCA mastermix (except buffer) with pullulan and trehalose in one pill, buffer alone with pullulan and trehalose in another pill.

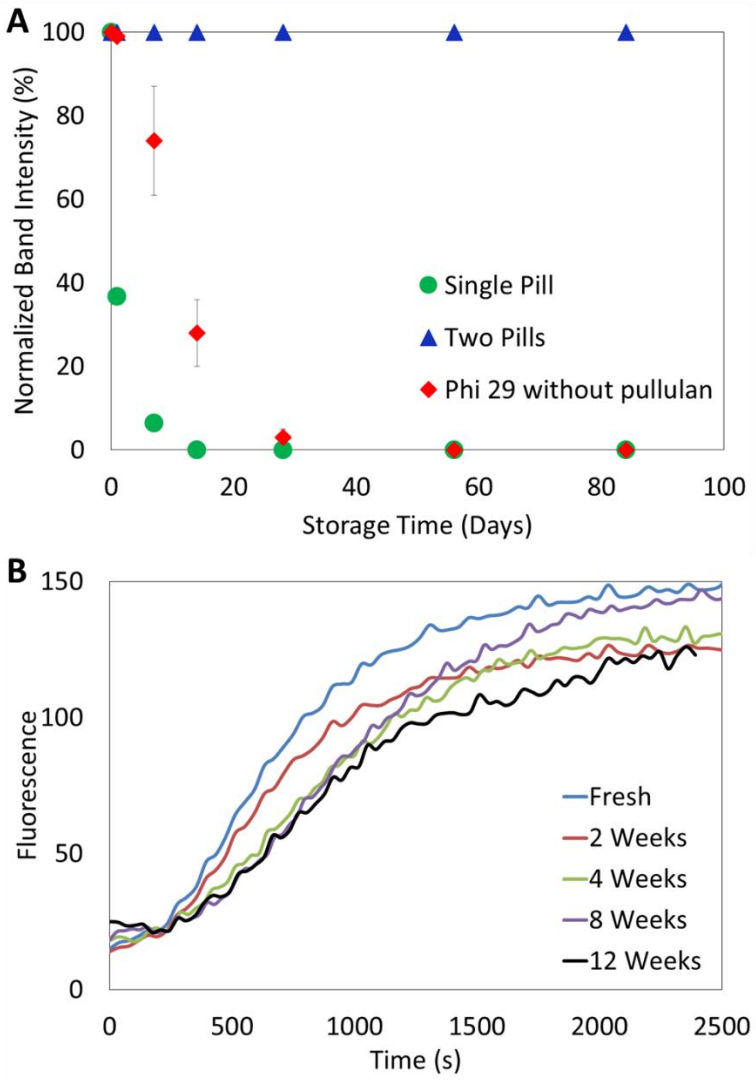


Figure 2 A) Normalized band intensity as a function of storage time for RCA mastermix in single pill with pullulan and trehalose, two pills with pullulan and trehalose, and stock Phi29 solution without pullulan. The band intensities are normalized to the band intensity of the fresh sample (Day 0). Error bars represents the standard deviation **B)** Real-time PCR of two-pill method of experiment. All experiments were conducted in triplicate.

The results provide clear evidence that the presence of buffer in the mastermix greatly decreases stability. We posit that, during the drying process, phi29 polymerase is exposed to a high salt concentration when it is mixed with the buffer, which causes the polymerase to become destabilized. To test this hypothesis, phi29 was incubated overnight at 4 °C under 3 conditions: 1) with reaction buffer in solution; 2) with dried reaction buffer; and 3) with no buffer. After

incubation, the other reagents were added and RCA was performed. Only 3% of the activity remained for the samples consisting of phi29 incubated with dried buffer solution, while 12% of the activity remained for the samples comprised of phi29 incubated with reaction buffer in solution. Significantly, no activity loss was observed for the sample consisting of phi29 that had not been incubated with buffer (**Figure S1)**. This result supports the hypothesis that high salt concentration produces instability in phi29, as the sample containing phi29 incubated with dried buffer had both exposure to the highest salt concentration, as well as the greatest loss in activity. Moreover, it is important to note that the loss of activity was not a result of the addition of pullulan or drying, as the phi29 polymerase was incubated with the buffer in a solution that did not contain pullulan.

To further investigate the effect of the buffer on stability, RCA reagents were dried in pullulan and trehalose with the buffer without phi29. Fresh phi29 was added prior to the reaction. It was found that the buffer destabilized not only phi29 but also the RCA reagents as only 25% of the activity remained after 1 day (**Figure S2**). We then dried the RCA reagents including phi29 with a single component of the buffer. The dried mastermix was then reconstituted with the other buffer components. It was found that the RCA mastermix retained its activity when dried together with only one buffer component (**Figure S3**). This shows that instability cannot be attributed to a single component in the buffer; rather, it is likely a combination of the components. Another experiment was performed where one component of the buffer was removed and dried together with RCA reagents. It was found that the stability of the RCA reagents increased when they were dried without Tris-HCl or $MgCl_2$ in the buffer (**Figure S4**). This may be due to an increase in salt concentration when Tris or $MgCl_2$ are present in the buffer during drying. Also, the presence of Tris may also affect the pH during the drying process which can negatively impact the stability.

Furthermore, since lyophilisation is the most common approach used to stabilize mastermixes, and since we were unable to find any reports on the lyophilisation of RCA reagent, the RCA mastermix was freeze-dried and its stability was compared to the stability of the RCA mastermix pills in pullulan and trehalose. The freeze-dried RCA mastermix exhibited a complete loss of activity after

one day both with and without trehalose. Although the samples that had been freeze-dried with trehalose retained some activity when the buffer was separated from the other reagents, only 26% of the activity remained after freeze drying (**Figure S5 and S6)**. Thus, the two-pill method with pullulan and trehalose is a superior method for ensuring the long-term stability of RCA mastermixes.

Thermostable LAMP Mastermix in Pullulan and Trehalose Pills

Another isothermal amplification method that was studied is LAMP. LAMP mastermix was stabilized using the two-pill method with pullulan and trehalose. There was no decrease in band intensity and no activity loss was observed after 4 weeks of storage at room temperature (**Figure 3**). Similar to the results for the RCA mastermixes, the real-time signaling results for the LAMP mastermix pills shows approximately a 25% drop in reaction rate one week after drying, but no further decrease in reaction over a subsequent 4 week storage period. However, as was the case with the RCA mastermix, gel electrophoresis did not show any noticeable differences in the LAMP products for the reaction mixtures at different storage times.

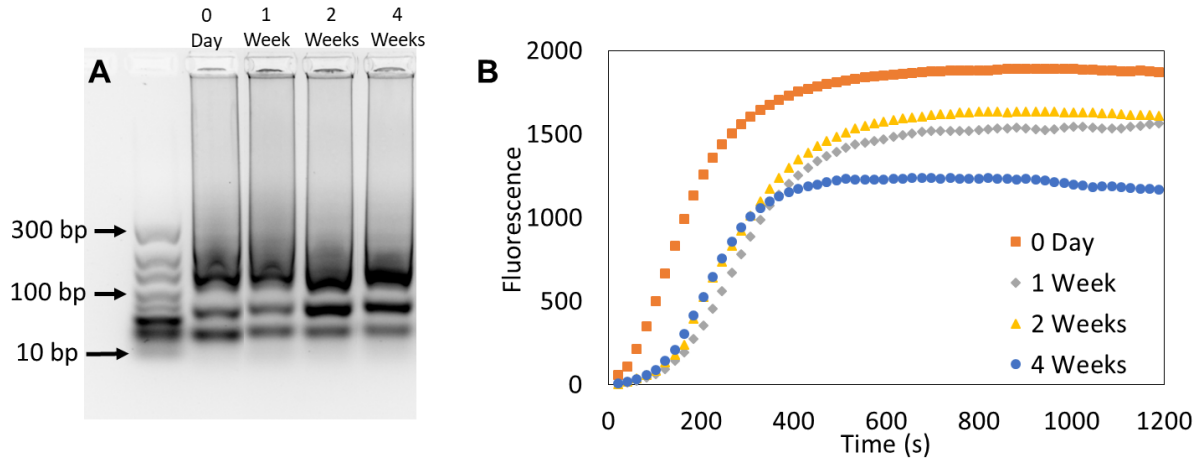
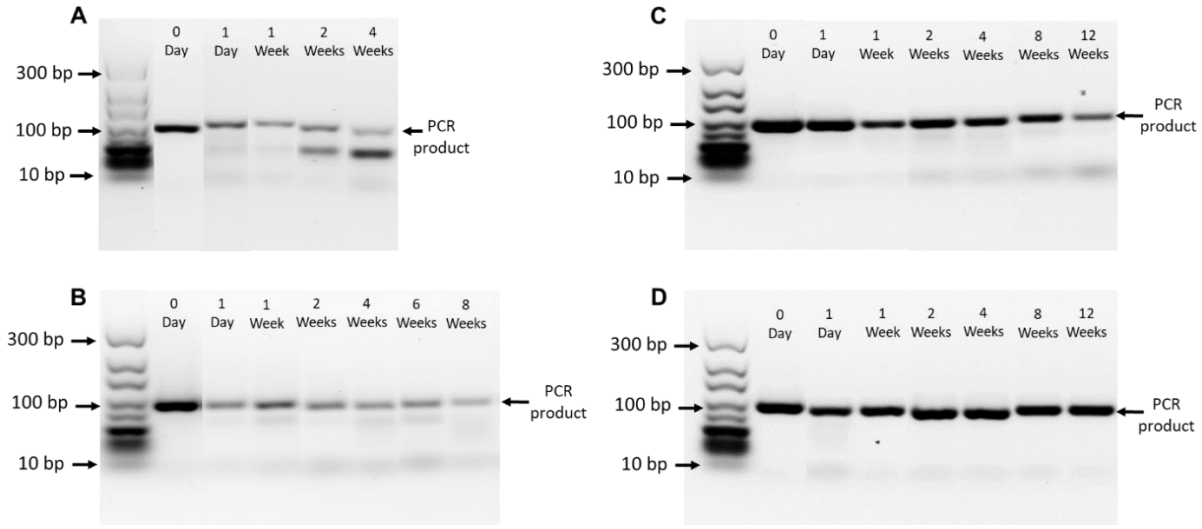


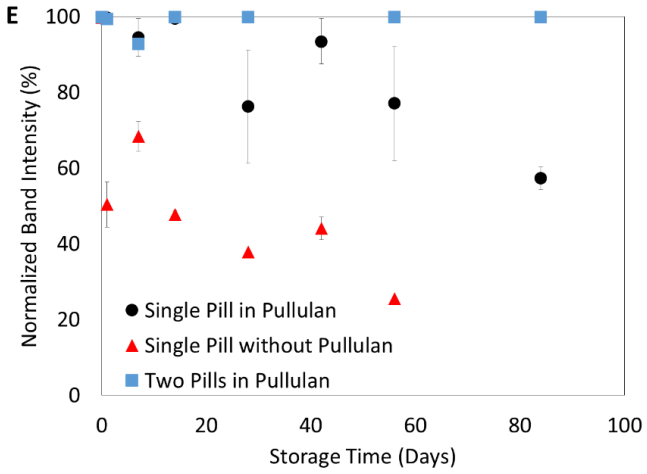
Figure 3. A) LAMP product at different storage times for the two-pill method: dried LAMP pill with pullulan and trehalose + dried buffer pill with pullulan and trehalose; **B)** Fluorescence as a function of time for real-time LAMP pills (two-pill method with pullulan and trehalose) at different storage times. All experiments were conducted in duplicate.

PCR Mastermix Pills Thermally Stabilized in Pullulan

We also thermally stabilized PCR mastermixes by drying the reagents in pullulan without trehalose. With the exception of the target DNA sequence, the PCR mastermix includes all reagents required for the reaction (i.e. Taq polymerase, DNA primers, dNTPs, and the reaction buffer). **Figure 4A** shows the gel electrophoresis images of the PCR products obtained from a PCR mastermix that had been stored at ambient conditions without any stabilizers. The stored PCR mastermix's activity was determined by comparing its product-band intensity to that of a fresh mastermix (0 day) using agarose gel electrophoresis. As can be seen, the stored PCR mastermix exhibited a significant decrease in activity (34%) after only one day of storage. Furthermore, a second band that is not present in the fresh samples becomes clearly visible in the stored sample after two weeks. In an attempt to increase the stability, we tried drying the PCR mastermix without adding any stabilizers. While drying the mastermix successfully increased the fidelity of the reaction (i.e. no second band present), however, there was a 50% decrease inactivity only after one day of storage at room temperature (**Figure 4B**). Next, we added 10 wt% pullulan to the mastermix and air dried the mixture in order to obtain a mastermix pill, which was then stored at ambient conditions. As shown in **Figure 4C,** the PCR mastermix pill retained 93% of the activity after 8 weeks, showing no significant decrease in product-band intensity. Despite the improved band intensity, it should be noted that a faint second band can still be seen at this time interval. Furthermore, after 12 weeks of storage at room temperature the product band becomes noticeably lighter and 43% of the activity was lost. Following our procedure from the RCA experiments, we used the two-pill method to separate the buffer from the rest of the reagents. Separating the buffer significantly improved the stability of the PCR pills. The PCR product-band intensities in **Figure 4D** provide clear evidence that the two-pill method allowed activity to be fully retained after 12 weeks of storage at room temperature. An analysis of the band intensities showed that there was a 75% loss in activity after 8 weeks for PCR mastermix dried without pullulan. Moreover, there was no loss in activity for the two pill-method and 43% loss of activity for single pill after 12 weeks of storage (**Figure 4E)**. However, as **Figure 4F** shows, the two-pill method's real-time PCR exhibited a 35% decrease in reaction rate and also a 25% decrease in signal intensity after the reagents had been dried. Therefore, we can conclude that, aside from some initial activity loss from

the drying process, the dried pills were able to retain their activity over 12 weeks at ambient conditions.





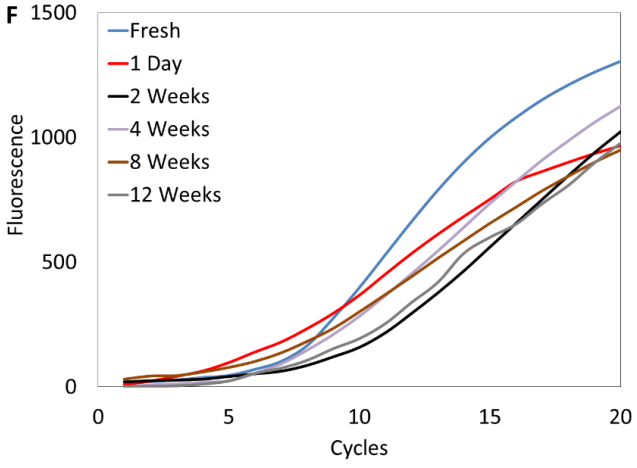


Figure 4. Gel electrophoresis of PCR products for different PCR mastermix stabilization methods as a function of different storage times: **A)** PCR mastermix in solution without pullulan; **B**) Dried PCR mastermix without pullulan; **C)** Dried PCR mastermix pill with pullulan; **D)** Two-pill method, dried PCR pill with pullulan + dried buffer pill with pullulan; **E)** Normalized band intensity as a function of storage time for PCR mastermix in single pill, two pills, and stock PCR mastermix solution without pullulan. The band intensities are normalized to the band intensity of the fresh sample (Day 0). **F)** Fluorescence as a function of the number of cycles for real-time PCR using PCR mastermix pills (two-pill method) stored for different durations. All experiments were performed in duplicate.

Pullulan and Trehalose Pills Offer Thermostability for Other Molecular Biology Reagents

Based on the results obtained for the RCA, LAMP, and PCR mastermix pills, pullulan and trehalose was used to stabilize other common molecular biology reagents via the two-pill method. As **Figure 5A** shows, the use of this method enabled the stabilization of T4 Polynucleotide kinase (PNK) for phosphorylation and T4 DNA ligase for ligation, as both retained their activity after 12 weeks of storage at room temperature and showed no noticeable decrease in intensity of the ligated product. The two-pill method using pullulan and trehalose was also able to stabilize the Klenow fragment (**Figure 5B)** and the restriction enzyme, BamHI (**Figure 5C).** No significant difference was observed between the 12 week samples and the fresh (0 day) samples. Therefore, the two-pill method was capable of preserving the full activity of the Klenow fragment and the BamHI after 12 weeks of storage at ambient temperature. These results demonstrate that the two-pill method using pullulan and trehalose is both versatile and capable of creating ready-to-use molecular biology kits for a laboratory setting and for POC diagnostics applications.

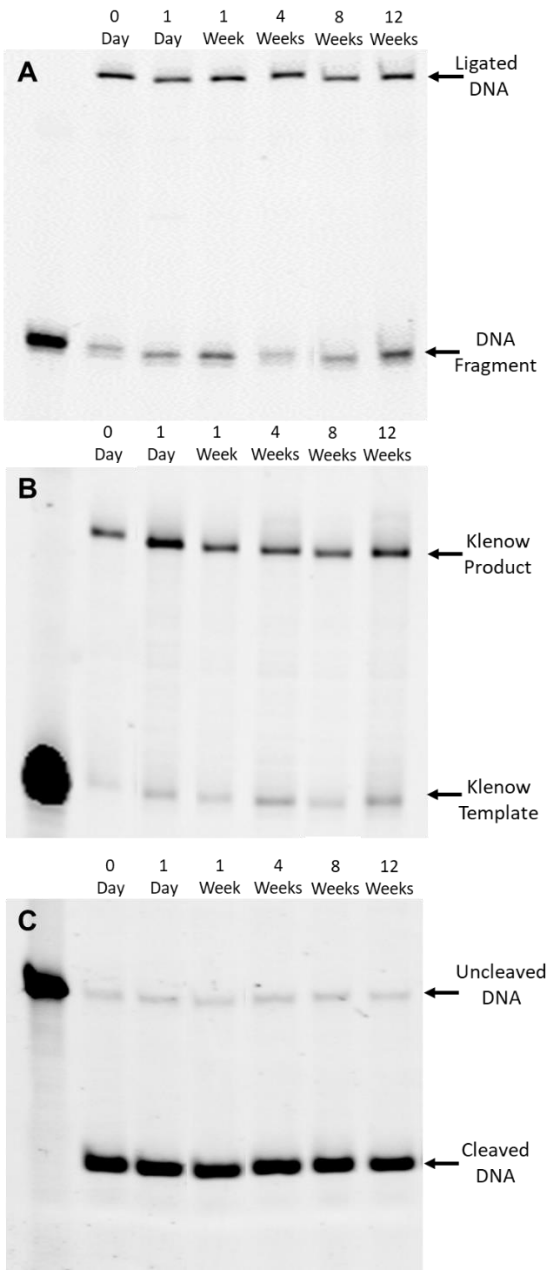


Figure 5. Gel electrophoresis of DNA products from different molecular biology techniques: **A)** Ligation using T4 Polynucleotide kinase and T4 ligase pills; **B)** Klenow fragment pills; **C)** Restriction using BamHI pills.

2.4 Conclusion

We have demonstrated that mastermixes dried in pullulan, or mixture of pullulan and trehalose, have excellent thermal stability at ambient temperature, which is always a challenge with molecular biology reagents, especially when the reaction buffer is dried in a separate pill.

Furthermore, we have shown this method's versatility by stabilizing various molecular biology techniques, such as PCR, RCA, LAMP, phosphorylation, ligation and restriction. Finally, we posit that this method provides a thermal stabilization technology that can be applied to other molecular biology techniques, as it has the potential to overcome some limitations of molecular biology techniques and to revolutionize POC diagnostics by creating ready-to-use thermally stable test kits that can be made accessible to developing countries.

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2.5 SUPPLEMENTARY INFORMATION

Table S1. DNA name and sequences used for all experiments.

Name	Sequence	Note
PCR-T	ATGCCATCCTACCAACCCATGTGGTTTGTTGAGATGGTCTTTGGTATGTGGGGTCCGAGGGTAGAGCTCTGAACTCG	Used as PCR template
PCR-FP	ATGCCATCCTACCAAC	PCR forward primer
PCR-RP	CGAGTTCAGAGCTC	PCR reverse primer
RCA-CT	TGTCTTCGCCTTCTTGTTTCCTTTCCTTGAAACTTCTTCCTTTCTTTCTTTCGACTAAGCACC	RCA template
RCA-LTP	GGCGAAGACAGGTGCTTAGTC	Ligation template to make circle and primer for RCA
KLT	GTAGCCATCGATCGATGCATGCATCTTGGTAGTGAGGTCATAGTCAGTTCATAG	Klenow reaction template
KLP	FTTTTTCTATGAACTGAC**TAT**GACCTCACTACCAAG	Klenow reaction primer, F: fluorescein
BamS	TGAACACTGCAGACAG*GATCC*AGCATGTCGAGTA	BamH sense sequence
BamAS	FTACTCGACATGCTG*GATCC*TGTCTGCAGTGTTCA (Arrow denotes the restriction digestion sites)	BamH antisense sequence, F: Fluorescein
PL-DNA1	FATGCCATCCTACCAACCCATGTGGTTTGTTGAGATGGTC	Acceptor DNA fragment in ligation
PL-DNA2	TTTGGTATGTGGGGTCCGAGGGTAGAGCTCTGAACTCG	Used for phosphorylation and then ligation with PL-DNA1 (donor DNA)
PL-DNA-LT	CCCCACATACCAAAGACCATCTCAACA	Used a ligation template to ligate PL-DNA1 na PL-DNA2

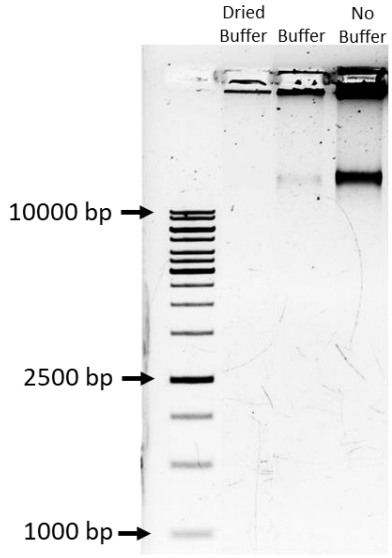

Figure S1. Gel electrophoresis of RCA product for phi29 incubated overnight with dried reaction buffer (left lane), reaction buffer (middle lane), and no buffer (right lane).

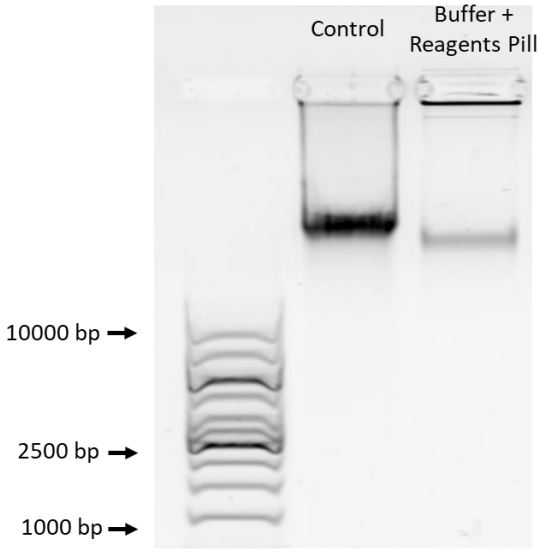

Figure S2. Gel electrophoresis of RCA product for control RCA (All fresh reagents, left lane) and for buffer + all reagent in pullulan and trehalose pill after 1 day (right lane), fresh phi29 was added prior to the reaction.

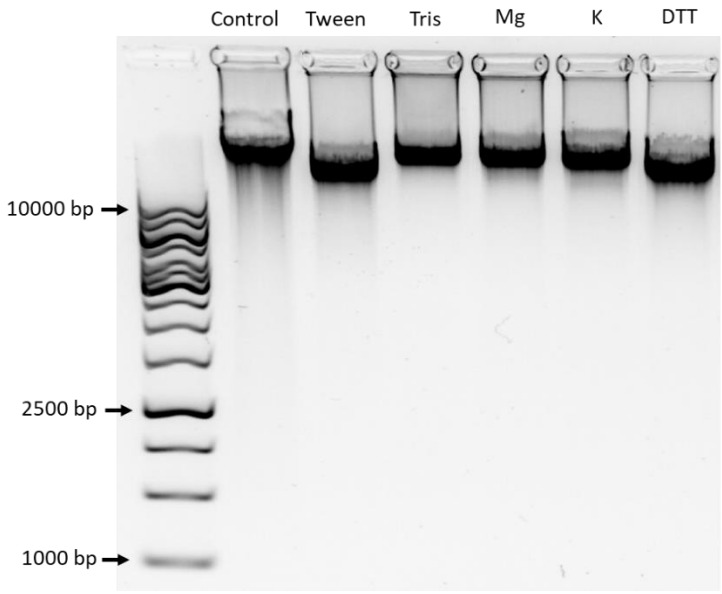

Figure S3. Gel electrophoresis of RCA product for RCA reagents dried in pullulan and trehalose with: Tween, Tris-HCl, $MgCl_2$, KCl, or DTT. The dried samples were reconstituted in water and all other buffer components prior to reaction.

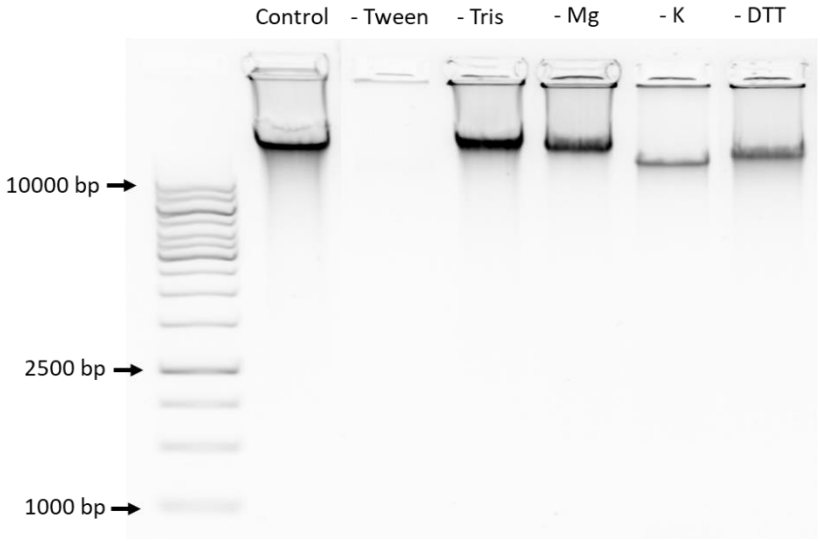

Figure S4. Gel electrophoresis of RCA product for RCA reagents dried in pullulan and trehalose with all reaction buffer components except for: Tween, Tris-HCl, $MgCl_2$, KCl, or DTT. The dried samples were reconstituted in water with the missing buffer component prior to reaction.

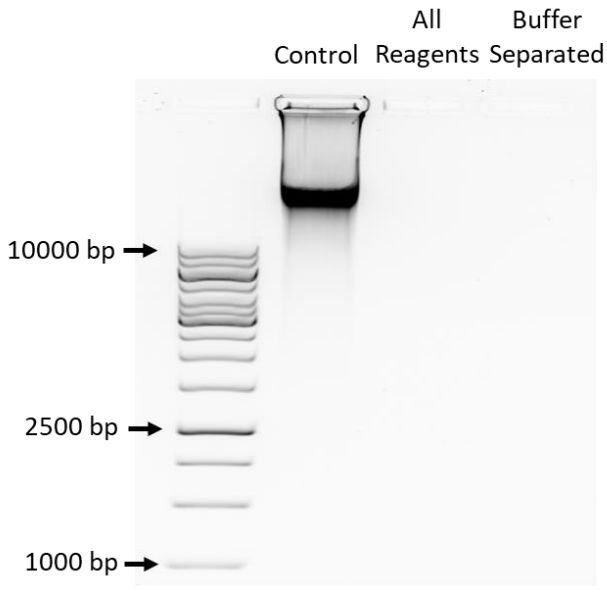

Figure S5. Gel electrophoresis of RCA product for RCA reagents freeze dried with buffer (middle lane) or with buffer separated (right lane).

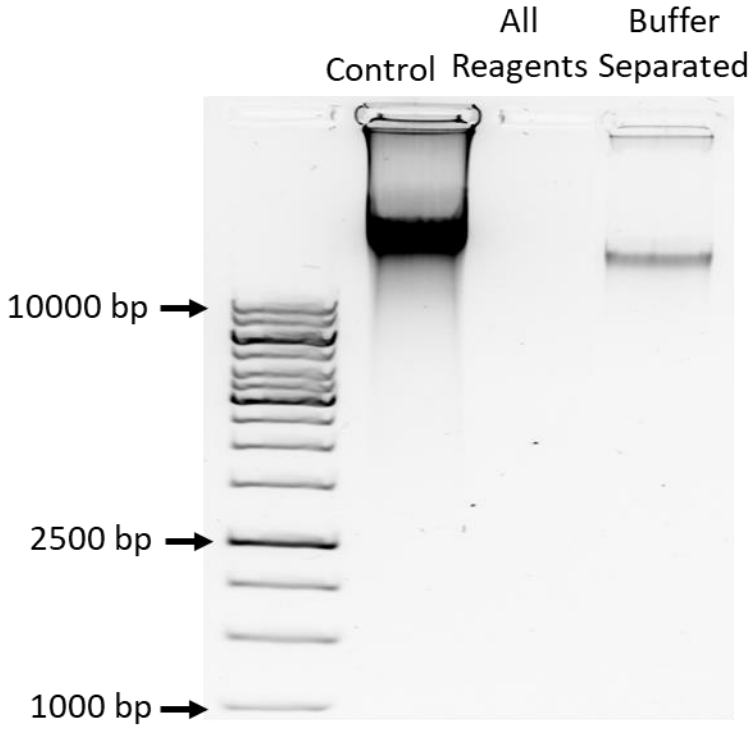

Figure S6. Gel electrophoresis of RCA product for RCA reagents freeze dried with trehalose with buffer (middle lane) or with buffer separated (right lane).

Chapter 3: Long-Term Preservation of Bacteriophage Antimicrobials Using Sugar Glasses

Preface

In Chapter 3, all experiment were conducted by me with the help of Alexandra Szewczyk and Jacqueline Chau. Jacqueline worked with me on the LISTEX experimnets. Alexandra worked with me on the LISTEX, Salmonella, and E. Coli experiments. Logan Grove worked with me on freeze drying experiment found in the supplementary information. Hajar Hawsawi, Hany Anany, and Mansel Griffiths provided the Salmonella and E. Coli phage and provided useful insights for the experiments. I initiated the first draft of the paper. Dr. Filipe, Dr. Ali, and Dr. Hosseinidoust provided many helpful suggestions and aided in revising the draft to the final paper. This work was published in ACS Biomaterials Science and Engineering. V. Leung *et al.* "Long-Term Preservation of Bacteriophage Antimicrobials Using Sugar Glasses," *ACS Biomaterials Science & Engineering Article ASAP* DOI: 10.1021/acsbiomaterials.7b00468. It is reproduced with permission from American Chemical Society.

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Article

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Long-Term Preservation of Bacteriophage Antimicrobials Using Sugar Glasses

Vincent Leung,[†] Alexandra Szewczyk,[†] Jacqueline Chau,[†] Zeinab Hosseinidoust,[†] Logan Groves,[†] Hajar Hawsawi,[‡] Hany Anany,[‡,§] Mansel W. Griffiths,[‡] M. Monsur Ali,*[,†] and Carlos D. M. Filipe*[,†]

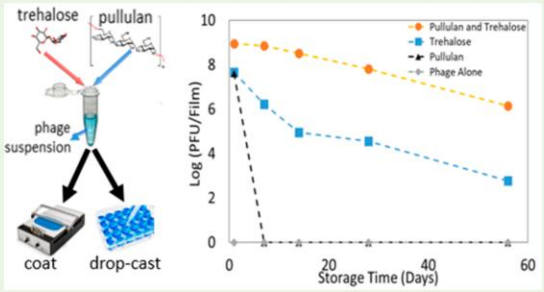
[†]Department of Chemical Engineering, 1280 Main Street West, McMaster University, Hamilton, Ontario, Canada L8S 4L7

[‡]Canadian Research Institute for Food Safety, 50 Stone Road East, University of Guelph, Guelph, Ontario, Canada N1G 2W1

[§]Agriculture and Agri-Food Canada, Guelph Research and Development Center, 93 Stone Road West, Guelph, Ontario, Canada N1G 5C9

Supporting Information

ABSTRACT: The antimicrobial activity of LISTEX P100, *Salmonella* CG4, and *E. coli* AG10 bacteriophages were preserved in pullulan-trehalose mixture as dried films and as coatings on food packaging. The phages encapsulated in pullulan-trehalose films were able to retain infectivity for up to 3 months at ambient storage conditions. Various buffers, disaccharides and disaccharide concentrations were investigated to optimize the long-term stability of the phages in the films. It was found that pullulan and trehalose need to be simultaneously present in the film to provide the stabilizing effect and that the presence of buffers that lead to the formation of crystals in the films must be avoided for phage activity to be maintained. Overall, this study describes a method of preserving bacteriophage activity in a dried format that has great potential for use as coatings, which can be used to create antimicrobial surfaces for food preparation and for food preservation.



KEYWORDS: *bacteriophage immobilization, pullulan, stabilization, encapsulation, food safety*

INTRODUCTION

Bacteriophages (phages) are viruses that infect bacteria. From the early days of their discovery in 1917, lytic bacteriophages have been used as potent antimicrobial agents.[1,2] The critical advantage of phage over other antimicrobial agents is its specificity. Whereas most broad-spectrum antimicrobial agents function like a sledgehammer, wiping out any and all bacteria, bacteriophages specifically target only certain species/strains of bacteria within a mixed population. This specificity has made bacteriophage antimicrobials very attractive for food processing/packaging applications, among others.[3−11] The odor, taste, and texture of most food products, particularly fresh produce, is negatively affected by commercial antimicrobial agents. Bacteriophages exist naturally on fruits and vegetables, and adding phage antimicrobials will not affect the appearance, taste, odor, or texture of produce.[12] Bacteriophage antimicrobials are also particularly useful for decontaminating products such as cheese, for which the quality of the product strongly depends on the presence of beneficial bacteria, or honey, which is consumed raw and without additives or antibiotics. In addition to their specificity, phage antimicrobials have garnered significant attention in the past 20 years as a result of the ever-growing crisis of antibiotic resistance.[13−16] Presence of multidrug resistant bacteria in animal products poses a serious threat to public health, especially in countries where antibiotics are used liberally and without constraint as an integral part of animal husbandry.[17,18]

Using phage-impregnated coatings on food preparation surfaces, surfaces in food processing plants, and for food packaging is a promising way to ensure food safety.[5,8,14,19] However, to effectively incorporate phage as part of a functional antimicrobial coating, certain challenges must be addressed, the most outstanding of which is the issue of phage stability.[7] As with most viruses, bacteriophages are generally resilient to most environmental conditions such as temperature, pH, and salt concentration, although their sensitivity varies significantly among strains.[20] Desiccation, however, is not a hardship that many phages can endure and thus one of the most significant challenges in developing phage-functionalized coatings is finding methods to protect phage against the effects of desiccation.

Among methods proposed to date for long-term stabilization of bacteriophage preparations, freeze-drying[21,22] (also known as

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lyophilization) and freezing in liquid nitrogen are the most prevalent. Neither method can preserve phage stability unless a protectant, such as glycerol, alginate,[8,23−28] pectin,[24,29] chitosan,[25,26] whey protein,[16,30] liposome,[31,32] and poly(ethylene oxide)/cellulose diacetate,[33] is present. Besides, the lyophilized samples must be maintained in vacuum ampules for effective phage stabilization. Both methods require access to specialized equipment for sample preparation (freeze-dryer, vacuum pumps) and sample storage (liquid nitrogen storage).

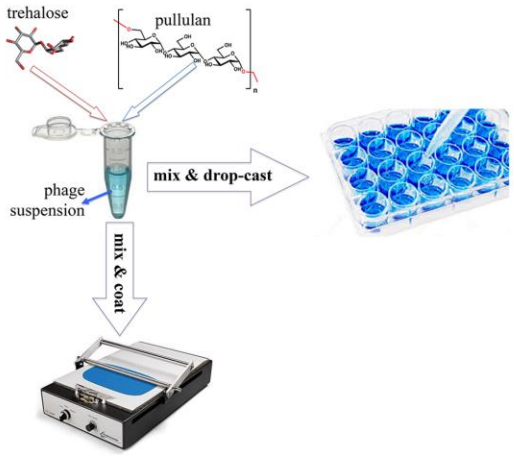
We report an effective and simple method for long-term stabilization of bacteriophages by encapsulating them in sugar glasses composed of a mixture of pullulan and trehalose and demonstrate the feasibility of using this sugar mixture as a surface coating for stabilization of phage. The proposed method is based on drop-casting and does not require access to specialized equipment, controlled storage conditions, or the need of a cold chain distribution system to maintain the coating's activity. Pullulan is a polysaccharide that has excellent film forming properties and has been used in the food industry as an oxygen barrier to prolong the shelf life of foods such as apples,[34] strawberries,[35] blueberries,[36] and eggs.[37,38] On the basis of previous studies from our group, it was found that pullulan is able to provide outstanding thermal stability and protection against oxidation of various labile biomolecules, such as luciferase, acetylcholine esterase, and indoxyl acetate.[39,40] Herein, we show that stabilizing bacteriophages under ambient conditions for prolonged periods of time cannot be achieved using films solely composed of pullulan. The films also need to contain trehalose, a sugar that has been used extensively as a stabilizing agent during lyophilization.[41−46] It is the combination of pullulan and trehalose in the same film that creates a synergistic effect and leads to long-term stability of the bacteriophages in the film.

EXPERIMENTAL SECTION

Materials. Pullulan (PI20 food grade, 200 kDa) was obtained from Hayashibara Co, Ltd., Okayama, Japan. D-(+)-Trehalose dehydrate, D-(+)-maltose monohydrate, sucrose, calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4{\cdot}7H_2O$), Tris, gelatin, Tryptic Soy Broth (TSB), and Listeria enrichment broth (LEB) were purchased from Sigma-Aldrich. Agar and agarose were purchased from Becton, Dickinson and Company (BD). Phosphate buffered saline (PBS) was purchased from BioShop Canada. *Listeria monocytogenes* serotype 1/2a, *E. coli* O157:H7 were kindly donated from Dr. Yingfu Li's lab at McMaster University, and *Salmonella* Newport was obtained from the Canadian Research Institute for Food Safety (CFRIS) culture collection. Two *Myoviridae* phages, *E. coli* O157:H7 phage, EcoM-AG10 (AG10), and *Salmonella* phage, SnpM-CG4−1 (CG4−1), were obtained from the Canadian Research Institute for Food Safety (CFRIS) culture collection at the University of Guelph. LISTEX P100 was purchased from Micreos Food Safety (Wageningen, The Netherlands). Distilled deionized water was obtained from a Milli-Q Advantage A10 water purification system (EMD Millipore) and was autoclaved.

Film Formulation. *Listeria* phage films were made by mixing 100 μL of LISTEX P100 stock solution (2×10^{11} PFU(plaque forming unit)/mL) with 900 μL of 11.1 wt % pullulan solution so that the final concentration of pullulan in the phage/pullulan mixture is 10 wt % and the phage concentration is 2×10^{10} PFU/mL. For some experiments, a disaccharide (trehalose, maltose, or sucrose) was added to the phage/pullulan mixture at concentration of 0.5 M unless otherwise stated. The solution was mixed by repeated pipetting to ensure uniform distribution of all components. The phage films were formed by pipetting 100 μL of the phage/pullulan/sugar solution into individual wells of a 24-well plate and allowed to air-dry for 24 h at room temperature. The diameters of the dried films were approximately 1.5 cm. The titer of each phage stock was determined and the anticipated phage titer per film is 2×10^9 PFU for LISTEX, 1×10^7 PFU for AG10 and 1×10^8 for CG4. The films were then stored under ambient conditions (~22−25 °C) with no humidity or temperature control for various lengths of time. The process for the preparation of phage films is presented in Scheme 1, the same process was used for all the different phages used in this study.

Scheme 1. Phage Film Formation and Coating Preparation: Stabilizing Films Were Prepared by Mixing Trehalose and Pullulan with Phage; Mixture Was Then Either Drop-Cast (To Form Phage Films) Or Coated on Food Packaging (To Make Antibacterial Coatings)



Quantification of Infectivity for Phage Embedded in the Films. The infectivity of phages encapsulated in dried films was quantified using the overlay technique.[47] The phage-containing film was dissolved in 1 mL of CM buffer (prepared by mixing 2.5 g of $MgSO_4{\cdot}7H_2O$, 0.735 g of $CaCl_2$, 0.05 g of gelatin, and 6 mL of 1 M Tris-HCl at pH 7.5, with water for a final volume of 1 L) through repeated pipetting. The reconstituted film solution was then serially diluted in CM buffer, each dilution was mixed in equal volumes with 100 μL of the bacterial host (10^9 CFU/mL) and then incubated at 30 °C for 10 min to allow for phage adsorption. The phage-host mixture was then added to 4 mL of soft Tryptic Soy Agar (sTSA, prepared by adding 0.5% agarose to TSB) and overlaid onto a TSA plate (1.5% agar to TSB). The plates were incubated at 30 °C overnight. Plaque formation was observed the following day, and the plaques were counted to determine the phage titer of each film. For each dilution, triplicate experiments were conducted. The total number of plaques were averaged and considered as the number of viable phages.

Analysis of Phage Activity Loss over Time. The loss of phage activity in the film was modeled using an exponential decay equation in the form of $A = A_0e^{-kt}$, where A is the phage titer at time t, A_0 is the initial phage titer, and k is the decay rate. The activity loss can be described using the activity loss time-constant, which is calculated as the inverse of the decay rate.

X-ray Diffraction (XRD) Analysis. The crystallinity of the films was determined using 2D X-ray diffraction (XRD^2). XRD data were collected at the MAX Diffraction Facility at McMaster University using the Bruker Smart6000 CCD area detector with Rigaku RU200 Cu Kα rotating anode and Göebel cross-coupled parallel focusing mirrors at a voltage of 50 kV and 90 mA. The samples were scanned using a bisecting angle scan between $2\theta = 8−72°$ with a frame exposure of 300 and a detector distance of 16.74 cm.

Phage Coating on Paper. Commercially available meat butcher paper (GTFrench Paper Limited, Hamilton, Canada) was used for phage coating experiments. The phage/sugar mixture was prepared as

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described earlier. Next, 1 mL of the phage/sugar mixture was spread uniformly onto a 12 cm by 12 cm area of an 21.6 cm by 27.9 cm sheet of paper using an EC-200 Variable Speed Drawdown Coater (ChemInstruments, OH, USA) with a size 40 EC-200 rod. The paper was air-dried overnight before being cut into 1 cm by 1 cm squares. The small pieces of the phage-coated papers were stored at ambient conditions. The process for the preparation of phage coatings is presented in Scheme 1.

Quantification of Phage Leaching from Paper. A 1 × 1 cm square of phage coated paper (from the above step) was placed in 1 mL CM buffer with occasional shaking at room temperature. A 100 μL aliquot of eluent was taken from the tube at different time points and transferred to a fresh tube for phage titer determination using the overlay technique, as described above. The data were used to determine the phage elution time that was then used for the subsequent experiments to test the infectivity of phages embedded in the films after different storage times.

Quantification of Antimicrobial Activity of Phage-Coated Paper. To test the infectivity of immobilized phage on paper, overlays of *Listeria* or *Salmonella* (overnight culture, 100 μL) in 4 mL of soft TSA was prepared on TSA plates and allowed to solidify at room temperature. Next, three 1 × 1 cm pieces of phage-coated paper were placed on the solidified agar surface with the phage layer facing the lawn and the plates were incubated at 30 °C overnight. The following day, the plates were inspected for lysis rings. The paper was then removed from the bacterial lawn to visualize the cleared zone produced by the phages. The dimensions of the clear zones were measured using a ruler.

The clear zones were individually excised, transferred into fresh microcentrifuge tubes, crushed using a spatula, and eluted in 1 mL of PBS in the tube by shaking for 1 h. A 100 μL aliquot of the eluent was then serially diluted in PBS, spread-plated onto Listeria Enrichment Agar plates for *Listeria* or Brilliant Green Agar plates for *Salmonella* and incubated at 30 °C for 48 h, after which the number of colonies on the plate were counted. These experiments were conducted in triplicate.

■ RESULTS AND DISCUSSION

In this work, we developed a coating comprising pullulan and trehalose for long-term stabilization of phage antibacterials on food packaging. The effectiveness of the coating was demonstrated against *E. coli*, *Salmonella*, and *Listeria*. The *Listeria* phage, LISTEX P100, is an FDA approved and commercially available bacteriophage, active against *Listeria monocytogenes*, whereas the other phages have been isolated from environmental samples in previous studies.

Phage Infectivity in Dried Pullulan Film. In our first experiment, LISTEX P100 was air-dried in four different stabilizing matrices: 1) 10 wt % pullulan and 0.5 M trehalose 2) 10 wt % pullulan with no trehalose, 3) 0.5 M trehalose with no pullulan, and 4) water (without any additives). The concentration of pullulan and trehalose was chosen based on previously published literature.[48] As shown in Figure 1, phage dried without pullulan or trehalose (preparation #4), loses all infectivity within 1 day. Pullulan alone (preparation #2) did not succeed in providing long-term phage stabilization; all infectivity was lost within 1 week. Interestingly, trehalose alone (preparation #3) was somewhat effective in stabilizing the phage although a significant loss in titer was observed after 2 weeks of storage at room temperature. After 8 weeks, the reduction in phage titer was 6.23 log PFU/film for phage dried in trehalose alone. Phage dried in 10 wt % pullulan + 0.5 M trehalose (preparation #1) was the most effective at preserving phage infectivity. After 8 weeks, the reduction of phage (i.e., the difference between PFU/film at a certain time point and PFU/film on the first day) preserved in a solid film of pullulan + trehalose was only 2.86 log PFU/film. These results suggest a synergistic effect between trehalose and pullulan in stabilizing phage. We hypothesized that the presence of the trehalose as the pullulan film is formed, provides protection against desiccation during the drying process. Moreover, phage air-dried in 10 wt % pullulan + 0.5 M trehalose retained higher infectivity compared to freeze-dried phage (Figure S1). After 2 weeks of storage at ambient conditions, the freeze-dried phage had a titer loss of 1.9 log PFU/pellet, whereas the phage that was air-dried in 10 wt % pullulan + 0.5 M trehalose had a titer loss of only 0.5 log PFU/film.

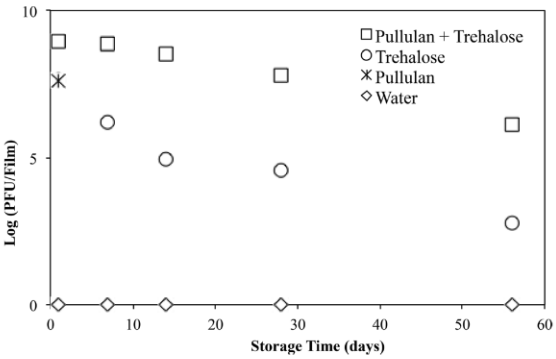

Figure 1. Change in infectivity of dried LISTEX P100 when dried in different stabilizing matrixes as a function of storage time. The samples were stored in ambient conditions. The results shown represent the average and standard error for three samples.

To investigate this synergistic effect further, we quantified the infectivity of LISTEX P100 in the dried films made with 10% pullulan and varying concentrations of trehalose. As shown in Figure 2a, the addition of trehalose, even as low as 0.1 M, had a significant effect on preservation of phage infectivity. After 8 weeks, the reduction of phage titer in pullulan film was 5.24 log PFU/film for 0.1 M trehalose, 4.92 log PFU/film for 0.25 M trehalose, and 2.86 log PFU/film for 0.5 M trehalose. All infectivity was lost after 8 weeks for films with 1 M trehalose. The film with 1 M trehalose showed signs of crystal formation at the center of the film after 4 weeks of storage. After 8 weeks of storage, large crystal structures were clearly visible on the film, which may be the reason for the instability. Crystallization at 1 M of trehalose may be due to saturation of the solute. The solubility of trehalose at 20 °C is 46.6% which is approximately 1.3 M.[49] As the film is being formed (i.e., water is evaporated) the solution may become saturated resulting in crystallization. The loss of activity followed an exponential decay and can be described using the activity loss time-constant, which is calculated as the inverse of the decay rate. The activity loss time-constant is 5.0 days for 0.1 M trehalose, 5.1 days for 0.25 M trehalose, 8.9 days for 0.5 M trehalose, and 2.9 days for 1 M trehalose (see Table S1). Although infectivity declined at all concentrations of trehalose, likely due to the films losing moisture over time, a concentration of 0.5 M was found to be optimal for preserving phage infectivity, exhibiting the lowest activity loss time constant.

The other factor investigated was the effect of salts in the casting solution. For this set of experiments, LISTEX P100 was dried in 10 wt % pullulan + 0.5 M trehalose in three different background casting media: (1) water alone, (2) CM buffer, and (3) PBS (pH 7.5). Figure 2b shows the number of viable phage

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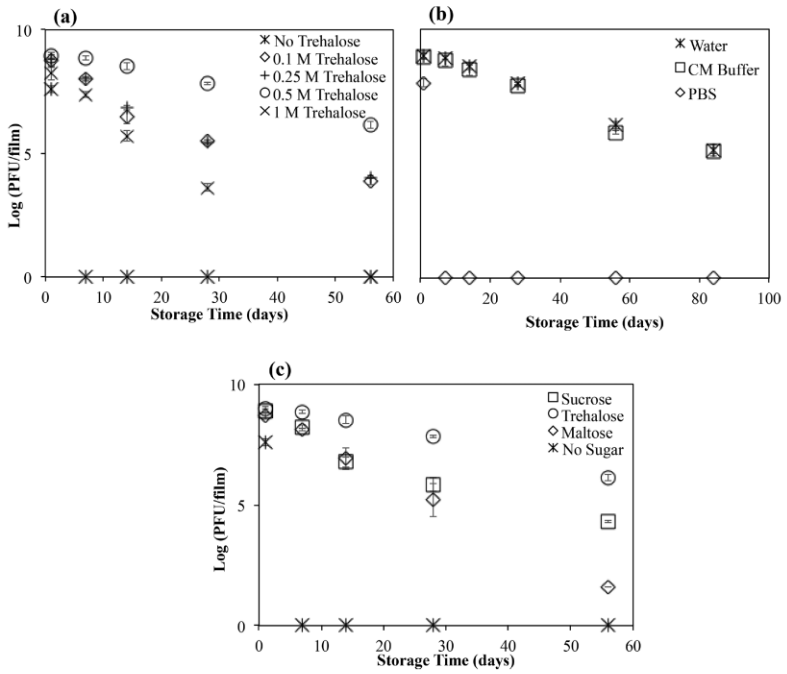

Figure 2. (a) Effect of trehalose concentration on the viability of LISTEX P100 in 10 wt % pullulan films as a function of storage time. (b) Effect of casting buffers on the viability of LISTEX P100 in 10 wt % pullulan + 0.5 M Trehalose films as a function of storage time. (c) Effect of different disaccharides on phage stability as a function of storage time in 10 wt % pullulan. The concentration of each disaccharide used was 0.5 M. All samples were stored under ambient conditions. The results shown represent the average and standard error for three samples.

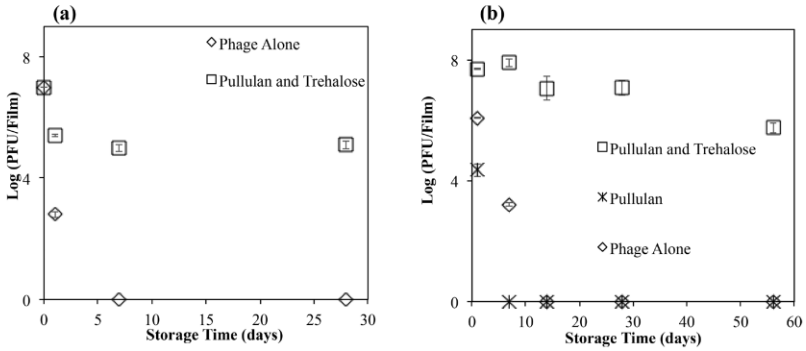

Figure 3. (a) Infectivity of dried *E. coli* phage AG10 in pullulan-trehalose (10 wt % pullulan and 0.5 M trehalose) films compared with phage dried without pullulan and/or trehalose as a function of storage time. (b) Infectivity of dried Salmonella phage CG4 in different stabilizing matrixes as a function of storage time. "Phage alone" indicates phage suspended in Millipore water. The results shown represent the average and standard error for three samples.

in pullulan-trehalose films stored at ambient conditions over a period of 12 weeks. The pullulan films cast with background casting media of water and CM buffer resulted in a reduction in phage titer equal to 3.85 log PFU/film and 3.89 log PFU/film, respectively, after 12 weeks. However, phage in films cast with PBS buffer completely lost their infectivity within 1 day of storage. This loss in infectivity is likely due to crystallization in the pullulan-trehalose film caused by the presence of salts from the PBS buffer. It has been shown in various studies that crystallization in the drying process causes instability and damage to the phage.[22,45,48] In our study, it was found that phage films cast in water and CM buffer were amorphous and did not have any visible signs of crystallization (Figure S2); however, the phage film cast in the PBS buffer exhibited large crystal structures in the center of the film (Figure S2). XRD analysis further confirmed the formation of crystals in the film casted in PBS buffer (Figure S3). Films casted in water and CM buffer showed strong amorphous scattering with no diffraction peaks, which is characteristic of pullulan films (Figure S3a, b).[50] However, films casted in PBS buffer showed strong diffraction peaks that are characteristic of NaCl which indicate the formation of salt crystals in the film (Figure S3c). The phage-dried in films cast using water were the most stable; therefore,

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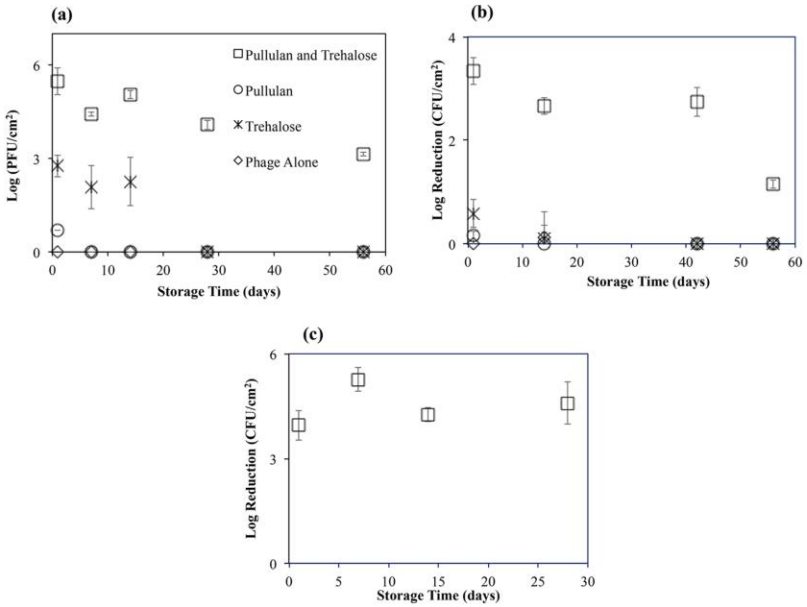

Figure 4. (a) Effect of different coating solutions on viable phage eluted from phage coated papers as a function of storage time. (b) Log reduction of colony forming units of *Listeria monocytogenes* by LISTEX coated paper using different coating solutions as a function of storage time. (c) Log reduction of *Salmonella* Newport colony forming units as a function of storage time by CG4 phage coated paper using 10 wt % pullulan and 0.5 M trehalose. All samples were stored in ambient conditions. The legend is shared between parts a, b, and c. "Phage alone" indicates phage suspended in Millipore water. Results shown represent the average and standard error for three samples.

water was used as the background casting medium in all subsequent experiments.

To investigate the stabilizing effect of trehalose, we replaced it with other disaccharides with a similar chemical structure in the casting medium. Figure 2c shows the infectivity over time for LISTEX P100 in pullulan-sugar films with 0.5 M trehalose, 0.5 M sucrose, or 0.5 M maltose. Although each disaccharide was able to provide some level of stabilization when compared with pullulan films without any added sugar, the phages were most stable in films containing 0.5 M trehalose. The reduction in phage titer after 8 weeks was 2.86 log PFU/film for 0.5 M trehalose, 6.01 log PFU/film for 0.5 M sucrose, and 7.10 log PFU/film for 0.5 M maltose. The activity loss time constant was 8.9 days for 0.5 M trehalose, 5.2 days for 0.5 M sucrose, and 3.47 for 0.5 M maltose (see Table S1).

Stability of two other different phage systems, *E. coli* phage (AG10) and *Salmonella* phage (CG4−1) were also investigated in pullulan-trehalose films. Figure 3a shows the activity of *E. coli* phage over a period of 4 weeks in pullulan and trehalose films. As expected, without pullulan and trehalose, the phages rapidly lost their activity and were completely inactive within 1 week. In films containing both pullulan and trehalose, *E. coli* AG10 phage only suffered a reduction in phage titer of 1.90 log PFU/film in 4 weeks. Similarly, Figure 3b shows the infectivity over time for dried CG4−1 phage in 10 wt % pullulan, 10 wt % pullulan + 0.5 M trehalose, and without pullulan. Similar to LISTEX P100, the phage was unstable in pullulan without trehalose. With the combination of 10 wt % pullulan and 0.5 M trehalose, the phage was stable for 8 weeks in the film with a reduction in titer of 2.25 log PFU/film.

Immobilization of Phage on Food Packaging. One of the potential applications of phages stabilized in soluble sugar films is the development of antibacterial surfaces and coatings for food packaging, food preparation surfaces, and in health care settings. As a proof of concept, we used commercially available meat-butcher paper as the substrate for our antibacterial coating. LISTEX P100 phage embedded in various coating solutions were coated onto the packaging paper. The coating solutions tested were: (1) 10 wt % pullulan + 0.5 M trehalose, (2) 10 wt % pullulan, (3) 0.5 M trehalose, and (4) water (without any additives). Phage infectivity was quantified by phage elution from the coated paper. We found that most of the phages were eluted from the paper surface to the buffer within 2 h (Figure S4). Therefore, the elution time of 2 h was chosen for the subsequent experiments to test the viability of paper-coated phages at different storage times. The results of the infectivity assay are presented in Figure 4a. Phage coated onto the paper without any additives were completely inactive after 1 week on the packaging paper. Similar to the earlier experiment in films, pullulan alone was not able to stabilize the phage on the paper. Using trehalose without pullulan preserved phage infectivity for 4 weeks. However, as expected, the combination of pullulan and trehalose was able to preserve phage infectivity for 8 weeks with a titer reduction of only 3.70 log PFU/cm^2. These results are consistent with the film experiments described above.

To quantify the antibacterial activity of the phage-coated packaging, we placed the packaging in direct contact with a lawn of *L. monocytogenes* and incubated overnight. It was anticipated that, upon contact with host layer, the phages would diffuse from the paper surface to infect the bacterial hosts producing clear zones underneath and at the edges of the paper. As expected, a clear zone appeared due to bacterial cell lysis by the phage, which was coated with pullulan and trehalose

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(Figure S5). Furthermore, the phage coated without any additives or mixed with pullulan alone or trehalose alone before coating did not have clear zones, revealing the lack of adequate preservation by the phage coating. We performed the same experiment using the *Salmonella* phage CG4 and similar results were obtained (data not shown). The log reduction of colony forming units (CFU) of *L. monoytogenes* by the coated papers as a function of paper storage time was calculated by comparing the number of bacteria present on plates in contact with phage-coated paper and number of bacteria present on plates in contact with blank paper (Figure 4b). Only papers coated with phage in pullulan + trehalose resulted in a significant CFU reduction. After 8 weeks of storage under ambient conditions, papers coated with phage in pullulan + trehalose were able to achieve a reduction of more than 2 log CFU/cm^2 for up to 6 weeks. Papers coated with phage and trehalose were only able to achieve a reduction of 0.59 log CFU/cm^2 after 1 day and did not exhibit significant antibacterial activity after 1 week, even though trehalose was effective at preserving phage infectivity, as shown in Figure 4a. The same trend was observed with the *Salmonella* phage CG4, as shown in Figure 4c. CG4 phage paper coated with pullulan + trehalose was able to reduce the *Salmonella* CFU count by 4.59 log/cm^2 after 4 weeks of storage in ambient conditions. Additional studies are still needed to fully assess the stability of different phage in pullulan + trehalose films when stored at different conditions (relative humidity and temperature being the two key parameters) for periods longer than 6 months.

■ CONCLUSIONS

In this study, we developed a coating that effectively preserved the antibacterial activity of three lytic phages against *Listeria monocytogenes, Salmonella* Newport and *E. coli* O157:H7 coated on food packaging. This is a critical factor for the realization of phage antibacterials in the food industry, because many bacteriophages suffer an irreversible loss of infectivity once they dry out. Strategies have been reported for preserving phage infectivity during the freeze-drying process, but our method does not rely on freeze-drying, does not require any specialized equipment for preparation or storage, and allows for preservation of phage antibacterial activity under ambient conditions. The encapsulated phages in the pullulan-trehalose coatings were able to retain their infectivity for up to 3 months when stored at ambient conditions (the effect of different ambient conditions, namely humidity, is the focus of future studies). These films are completely water-soluble and biocompatible, and comprise components that are food-grade and FDA approved. We conclude that the use of pullulan in combination with trehalose is a promising method for preservation of bacteriophage antibacterial activity in a dried format. This technology has the potential to create highly stable phage-coated surfaces that can be used for food protection, food preparation, wound-dressings to prevent/control bacterial infections, and in point-of-use filtration systems for water purification.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.7b00468.

LISTEX P100; kinetics of phage elution from paper; microscope images of pullulan films casted using various buffers; decay constants for LISTEX P100 and Salmonella phage CG4 immobilized in films with various compositions; plaque assay at 6 weeks storage of phage-coated paper with different coating solutions; XRD profiles for pullulan films casted in water, CM buffer, and PBS (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: monsali@mcmaster.ca.
*E-mail: filipec@mcmaster.ca.

ORCID

Carlos D. M. Filipe: 0000-0002-7410-3323

Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Long-Term Preservation of Bacteriophage Antimicrobials Using Sugar Glasses

Vincent Leung,[†] Alexandra Szewczyk,[†] Jacqueline Chau,[†] Zeinab Hosseinidoust,[†] Logan Groves,[†] Hajar Hawsawi,[‡] Hany Anany,[‡,§] Mansel W. Griffiths,[‡] M. Monsur Ali[,†] and Carlos D. M. Filipe*[,†]*

[†]Department of Chemical Engineering, 1280 Main Street West, McMaster University, Hamilton, Ontario, Canada L8S 4L7
[‡]Canadian Research Institute for Food Safety, 50 Stone Road East, University of Guelph, Guelph, Ontario, Canada N1G 2W1

[§]Agriculture and Agri-Food Canada, Guelph Research and Development Center, 93 Stone Road West, Guelph, Ontario, Canada, N1G 5C9

Email: monsali@mcmaster.ca and filipec@mcmaster.ca

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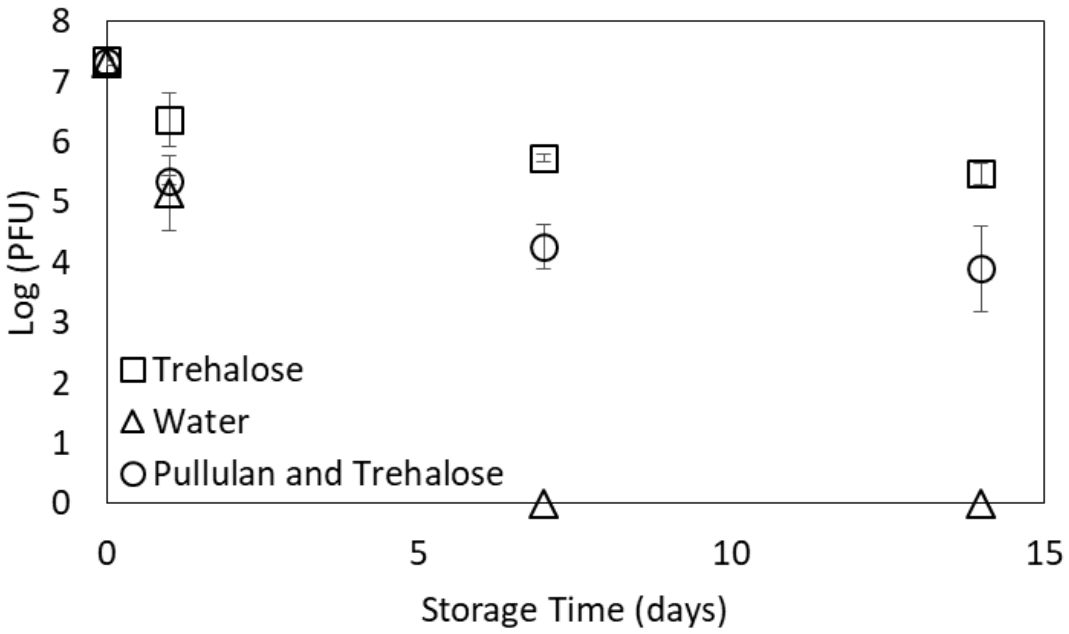


Figure S1. Change in infectivity of LISTEX™ P100 when freeze-dried in different stabilizing matrixes as a function of storage time. The samples were stored in ambient conditions.

Table S1 Decay time constants and log reduction of phage titre for LISTEX™ P100 in films of different compositions.

Film Components	Decay Time Constant (Days)	One Day Decrease (log PFU/film)	Four Weeks Decrease (log PFU/film)	Eight Weeks Decrease (log PFU/film)
0.1 M Trehalose + 10% pullulan	5.00	0.22	3.52	5.24

0.25 M Trehalose + 10% pullulan	5.10	0.19	3.55	4.92
0.5 M Trehalose + 10% pullulan	8.93	0.05	1.19	2.86
1 M Trehalose + 10% pullulan	2.90	0.77	5.39	9.00
0.5 M Sucrose + 10% pullulan	5.18	1.40	4.28	6.01
0.5 M Maltose + 10% pullulan	3.47	0.32	3.80	7.10
0.5 M Trehalose + 10% pullulan in PBS	0.33	1.15	9.00	9.00
0.5 M Trehalose + 10% pullulan in CM	8.85	0.09	1.28	3.15
10% pullulan	0.40	1.42	9.00	9.00
0.5 M Trehalose (no pullulan)	9.09	1.33	4.44	6.23
0.5 M Sucrose (no pullulan)	9.71	2.92	4.19	7.26
0.5 M Maltose (no pullulan)	5.78	1.93	5.15	9.00
Water (no pullulan)	0.05	9.00	9.00	9.00

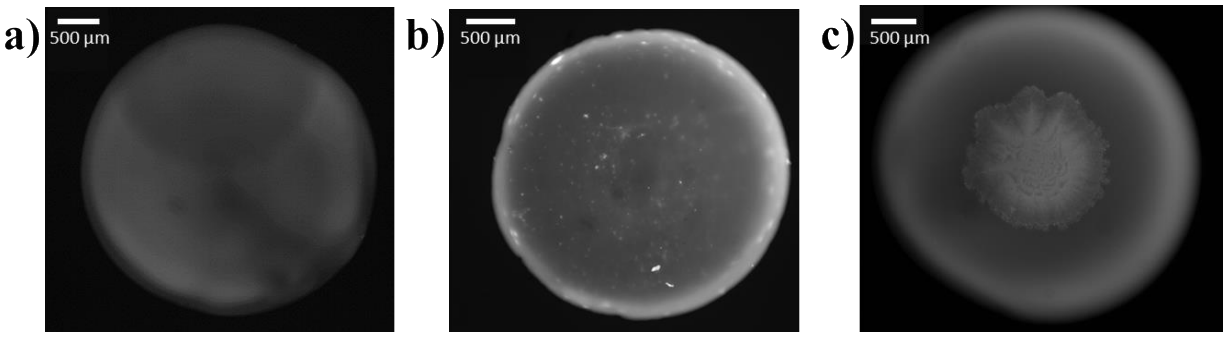


Figure S2. Microscope pictures of pullulan films casted in **a)** water; **b)** CM buffer; **c)** PBS.

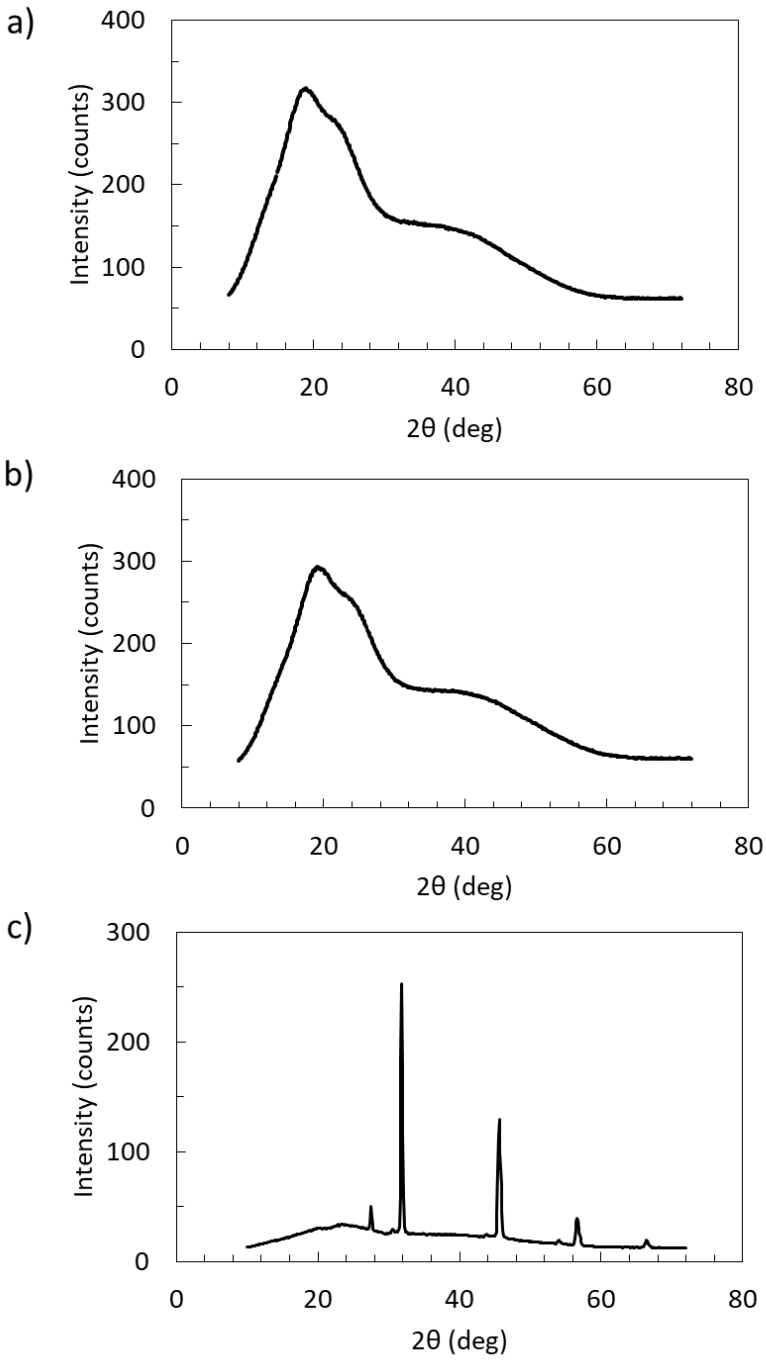


Figure S3. XRD profiles for pullulan films casted in a) water, b) CM buffer, and c) PBS. The films casted in water and CM showed strong amorphous scattering with no diffraction peaks. Whereas the film casted in PBS showed peaks that are characteristic of NaCl.

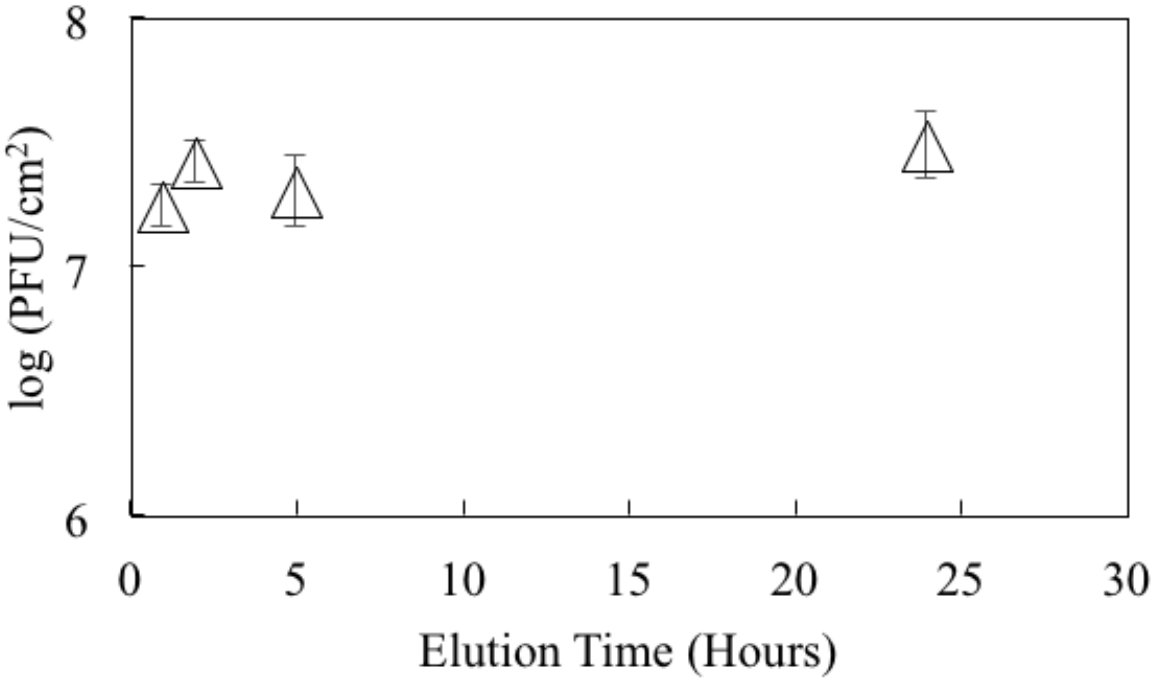


Figure S4. Phage eluted from paper at room temperature as a function of elution time.

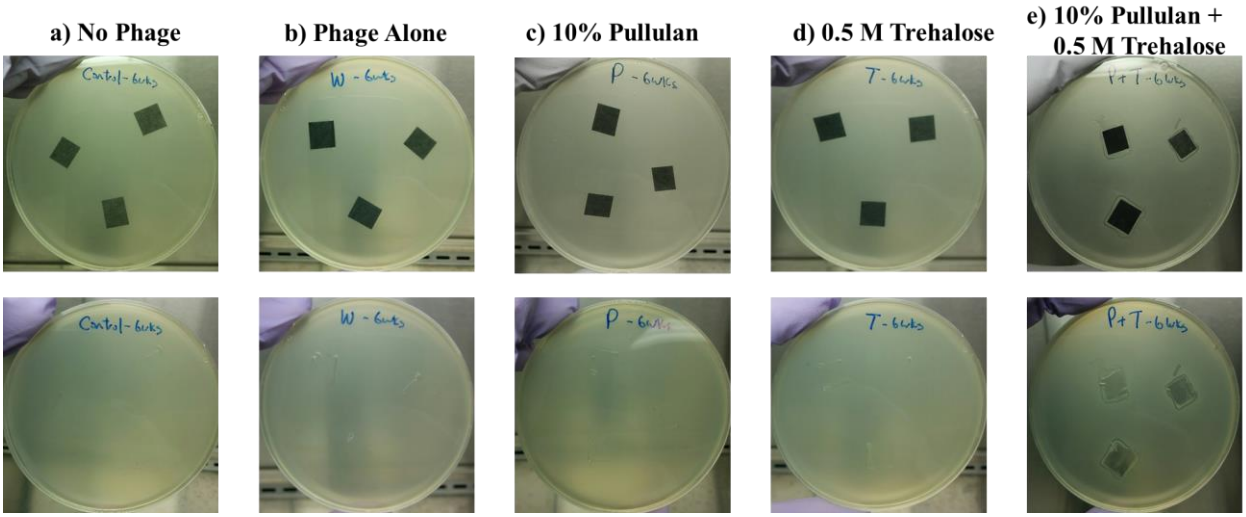


Figure S5. Plaque assay at 6 weeks storage time for phage-coated paper with different coating solutions. a) no phage; b) phage alone; c) 10 wt% pullulan and phage; d) 0.5 M trehalose and

phage; e) 10 wt% pullulan, 0.5 M trehalose and phage. The images shown were obtained with LISTEX™ P100. Similar results were obtained with the Salmonella phage CG4 (not shown).

Chapter 4: Long-Term Antimicrobial Activity of Phage-Sugar Glasses is Closely Tied to Processing Conditions

Preface

In Chapter 4, all experiment were conducted by me with the help Logan Groves. Logan worked with me on the DOE experiments and the data analysis for the DOE. Alexandra Szewczyk wored with me on the preliminary experiments with PEG surface modification and divalent cation. I initiated the first draft of the paper. Dr. Filipe and Dr. Hosseinidoust provided helpful suggestions and aided in revising the draft to the final paper. This work is a manuscript submitted to ACS Omega.

Long-Term Antimicrobial Activity of Phage-Sugar Glasses is Closely Tied to Processing Conditions

Vincent Leung,[†] Logan Groves,[†] Alexandra Szewczyk,[†] Zeinab Hosseinidoust,[,†] and Carlos D. M. Filipe*[,†]*

[†]Department of Chemical Engineering, McMaster University, Hamilton, Ontario, Canada L8S 4L7

Corresponding Authors Email: doust@mcmaster.ca and filipec@mcmaster.ca

Abstract

Bacteriophage-based antimicrobials have proven to be effective in the control of foodborne contaminants at various stages of the food production chain. Incorporating bacteriophages onto food processing surfaces and food packaging can significantly impact food safety. One of the major roadblocks toward using phage as a natural antimicrobial/preservative in food packaging is phage sensitivity to desiccation. We recently developed a coating (film) composed of pullulan and trehalose that proved to be highly effective in preserving the antimicrobial activity of phage for more than 8 weeks. During that investigation, we realized that the conditions under which the films were formed and stored, significantly affected the protective ability of those film towards bacteriophage antimicrobials, leading us to design a more in-depth investigation to identify and evaluate the major factors that affect the efficacy of these antimicrobial films. In this work, we report the outcome of a four factor, two level full factorial randomized design of experiments, in which we examined the effects of four processing/storage factors on the short-term and long-term antimicrobial activity of phage-embedded pullulan/trehalose films. The drying method used to make the films and storage humidity are the two factors that have the most significant impact on the long-term antimicrobial activity of pullulan/trehalose. The long-term viability of the bacteriophage (as measured by loss of titer) can be increased by over 1000-fold by using dry vacuum to form the films and then storing (for 28 days at room temperature) them in a closed container (tube) with low humidity in the headspace. The presence of divalent ions during the drying process and modification of the surface onto which the films were cast did not have a significant effect on long-term phage viability. Coatings, such as pullulan-trehalose film, can help realize the promise of phage in the food industry. Insights in the control and stabilization of active ingredient in pullulan/trehalose films can also be applied to stabilization of viral vaccines and diagnostic bioassays.

4.1 Introduction

Bacteriophages (phages) are viruses that target, infect, and kill bacteria with high specificity.[1,2] This specificity makes phages attractive alternatives to preservatives and broad-spectrum antimicrobial agents that are commonly used in food processing, such as bleach or quaternary ammonium compounds.[3–11] Phages offer comparable antimicrobial effectiveness when compared with common antimicrobial agents; the major difference is that phages act in a highly targeted fashion. This targeted action means that phage antimicrobials can eliminate harmful pathogens from food products without affecting the community of beneficial bacteria responsible for taste, texture and odor of food products.[12–15] The application of phage can be extended further up the food production chain to animal husbandry, where the widespread use of antibiotics has turned agriculture and food animals to a source of antimicrobial-resistant bacteria and thus a major concern for public health.[16,17] Bacteriophages are found naturally in fresh water, on produce, and in the gastrointestinal tract of food animals. Therefore, the use of phage antimicrobials is not expected to disrupt the microbiota of soil, water, or animal GI tracts or cause toxicity to consumers .[18]

Incorporating phage onto food processing surfaces and food packaging holds significant promise for improving food safety. Phages can be embedded in coating materials and applied as antimicrobial coatings to various surfaces and packaging material.[5,8,13,19] The major challenge to this approach is preserving the effectiveness of phage because many bacteriophages are sensitive to desiccation.[20,21] In our previous work, we reported a simple and highly effective coating comprised of pullulan and trehalose that functioned to both encapsulate and provide long-term stabilization in a dried state for bacteriophages and enzymes.[22–24] Trehalose, is a disaccharide commonly used as a stabilizing agent in lyophilization.[25–30] Pullulan (a polysaccharide) has been used as a film forming agent in the food industry to prolong the shelf life of food products.[31–35] It

functions by protecting labile molecules against oxidation and thermal inactivation.[22,23] Although both pullulan and trehalose are common stabilizing agents, neither agent alone offered effective long term stabilization of commercial phage preparations. During the aforementioned investigation, we observed the stabilization effect of the combination to be highly dependent on the storage conditions, suggesting phage antibacterial activity to depend on a complex landscape of physical and chemical parameters. More specifically, we observed a rapid drop in phage activity (manifested as loss of titer) during the drying process as the film is formed, followed by a slow but steady degradation of phage activity during storage of the modified surfaces. These observations suggest that the drying process and storage conditions should be optimized for maximum phage activity after film formation, but other experimental factors might be important as well.

In this work, using a four factor, two level full factorial randomized design of experiment (DOE), we examined the effect of four factors on the short-term and long-term antibacterial activity of a commercially available phage antimicrobial cocktail (LISTEX^{TM} P100), embedded in pullulan/trehalose films. The four factors considered were: the nature of the drying process, the humidity in the air under which the phage/pullulan/trehalose films were stored, modification of the surface onto which the phage/pullulan/trehalose films were cast and the presence/absence of divalent ions in the phage/pullulan/trehalose solution prior to forming the films. The results enable optimization of the processing/storage conditions of the pullulan/trehalose films for maximum phage antibacterial activity. Herein we show that the drying method and storage humidity are the most significant factors that affect the long-term antibacterial activity of bacteriophages in pullulan/trehalose films.

4.2 Materials and Methods

Materials

Tryptic Soy Broth (TSB), Tris, gelatin, magnesium sulfate ($MgSO_4 \cdot 7H_2O$), D-(+)-trehalose dehydrate, calcium chloride ($CaCl_2$), gelatin, polyethylene glycol (PEG, M_n = 300 Da), zinc chloride ($ZnCl_2$), lithium chloride (LiCl), magnesium chloride ($MgCl_2$), sodium bromide (NaBr), and sodium chloride (NaCl) were obtained from Sigma-Aldrich. Drierite desiccant was purchased from the McMaster Scientific Store. Pullulan (PI20 food grade, 200 kDa) was purchased from Hayashibara Co, Ltd., Okayama, Japan. Agar and agarose were purchased from Becton, Dickinson and Company (BD). *Listeria monocytogenes* serotype ½a (ATCC Number: BAA-2659) was donated from Dr. Yingfu Li's lab at McMaster University and was routinely cultured and maintained in our lab. LISTEX™ P100 was obtained from Micreos Food Safety (Wageningen, The Netherlands). Milli-Q Advantage A10 water purification system (EMD Millipore) was used for preparing all solutions.

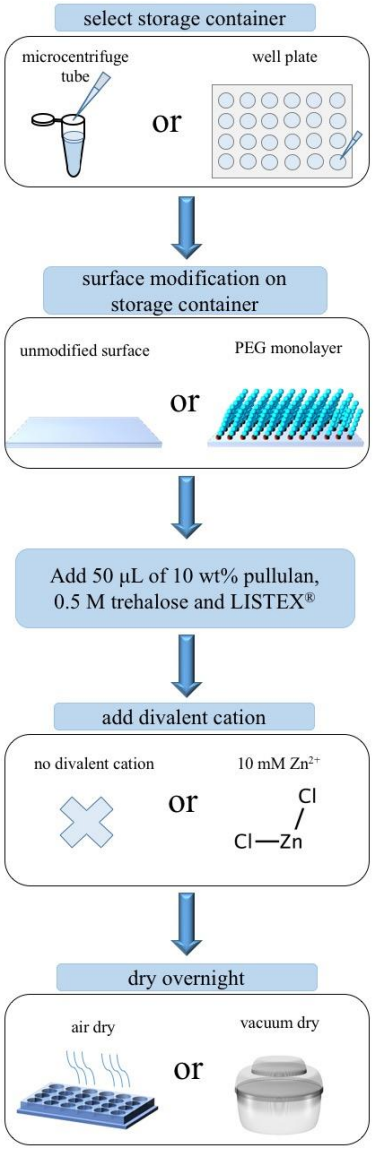
Sample Preparation

Listeria phage films were made by following the procedure as described in our previous study.[24] In brief, 450 µL of 10 wt% pullulan and 0.5 M trehalose solution was mixed together with 50 µL of LISTEX™ P100 (10^9 plaque forming units, PFUs). For some experiments, 10 mM Zinc chloride was added to the solution. The solution was mixed by repeated pipetting and 50 µL of the solution was pipetted into individual wells of a 24-well plate or into a 1.5 mL microcentrifuge tube. The solution was then air-dried or vacuum dried overnight. The air-dried samples were dried by leaving the lid of the 24-well plate or microcentrifuge tube open overnight. The ambient humidity was approximately 50% RH. Samples that were vacuum dried were placed under vacuum (Edwards RV8, P = 2×10^{-3} mbar) in a desiccator overnight with Drierite desiccant. Once dried, the samples in 24-well plates were kept at ambient conditions. The samples in microcentrifuge tubes were capped, wrapped in paraffin wax, and stored in a cabinet at room temperature.

For some experimental conditions, the storage containers (24-well plates or microcentrifuge tubes) were surface-modified with PEG 300. Prior to drying the phage film in the container, 100 µL of PEG 300 was pipetted into the container and air-dried overnight. The containers were then rinsed with Milli-Q water and air-dried. A schematic of the sample preparation procedure including the four DOE factors is shown in Scheme 1.

Determining Concentration of Viable Phage

The titer of the phage encapsulated in dried films was determined by the soft-agar overlay technique.[36] The film was dissolved in 500 µL of CM buffer (2.5 g $MgSO_4 \cdot 7H_20$, 0.735 g of $CaCl_2$, 0.05 g gelatin, and 6 mL 1 M Tris-HCl at pH 7.5, 1 L MilliQ water). The dissolved film was serially diluted in CM buffer. 100 µL of the dilution was incubated with 100 µL of *Listeria* (10^9 CFU/mL) for 10 min at 30 °C. The phage-host mixture was mixed with 4 mL of semi-solid tryptic soy agar (TSA) and overlaid onto a TSA plate. The plate was then incubated at 30 °C overnight. Plaques formed were counted the next day to determine the number of viable phage.



Scheme 1. Schematic of the process for preparation of phage-embedded films. The four blocks represent the four factors investigated in the DOE analysis.

Design of Experiment

A four factor, two level full factorial randomized design of experiment (DOE) with two replicates was conducted to optimize the antibacterial activity of LISTEX^{TM} bacteriophage in

pullulan/trehalose films. Table 1 shows the factors considered for the DOE. Furthermore, Table 2 provides the details of each experiment in the DOE.

Table 1. Factors and levels for the design of experiment.

Variables	Factors	Levels	
		–	+
A	Drying Method	Air-dried	Vacuum-dried
B	Surface Modification	PEG300	Unmodified
C	Divalent Cations	0 mM Zn^{2+}	10 mM Zn^{2+}
D	Storage Container	Tube	Plate

Table 2. Design of the two-level full factorial design of experiment.

Experiment Number	Drying Method (A)	Surface Modification (B)	Divalent Cations (C)	Storage Container (D)
1	+	+	-	-
2	+	+	-	+
3	+	+	+	+
4	+	+	+	-
5	-	+	-	-
6	+	-	+	+
7	-	+	+	-
8	-	-	+	+
9	+	-	-	+
10	+	-	-	-

11	-	+	-	+
12	-	-	-	-
13	-	-	-	+
14	-	+	+	+
15	-	-	+	-
16	+	-	+	-

To identify the significance of each factor in the experiments, a linear model of best fit was produced from the data. The data was made to fit the model:

$$y = A + B + C + D + AB + AC + AD + BC + BD + CD + b \qquad (1)$$

In the model above, y is the log titre decrease, b is the error term, and each capitalized term represents either a factor considered in the experiments or the interaction between pairs of factors. ANOVA analysis was performed using the programming language and environment *R* (version 3.4.0).

Humidity Control

Experiments were conducted to study of effects of storage humidity on phage antibacterial activity in pullulan/trehalose films. The phage films were prepared in microcentrifuge tubes via vacuum drying as described previously. The dried films were stored at room temperature (22-23 °C) at various levels of relative humidity (11%, 33%, 58%, and 75%). The relative humidity was controlled using the following saturated salt solutions: LiCl (11 %RH), $MgCl_2$ (33 %RH), NaBr (58 %RH), NaCl (75 %RH). The samples were stored in air or N_2. The titer of the samples was determined at various storage times using the soft-agar overlay technique described above.

4.3 Results and Discussion

Design of Experiment

In our previous work, we demonstrated the long-term stabilization of phage virions in pullulan and trehalose coatings. We observed that a phage infectivity loss (manifested as loss of titer) was associated with the drying process and storage. The loss in titer due to the drying process could be detected immediately after drying. This was followed by a gradual loss in titer during storage over time. Based on these observations we selected four factors (Table) and investigated their influence on the long-term antibacterial activity of phage in pullulan and trehalose coatings using a two level full-factorial design of experiment.

The drying method was chosen as the first factor to be investigated. The process of air-drying is slower compared to vacuum drying and is expected to cause less desiccation stress. Surface modification (PEG-coating) of the drying containers was the second chosen factor. We postulated binding of phage to the container surface might affect protein conformation and thus irreversibly damage the ability of phage to infect its host bacterium. Bacteriophages have been reported to non-specifically bind to surfaces.[37–39] Furthermore, PEG is commonly used a surface blocking agent to prevent non-specific adsorption of proteins and bacteria onto surfaces.[40] Therefore, the addition of PEG should serve to block the surface and prevent phage from non-specifically binding to the container's surface. The third factor investigated was the addition of Zn^{2+} ions as a stabilizing agent. Our preliminary studies showed that addition of 10 mM Zn^{2+} enhanced phage antibacterial activity in pullulan/trehalose films (Figure S1). Others have also shown that the addition of divalent cations at millimolar levels may prevent the inactivation of bacteriophages.[12,20,41] The last

factor studied in the DOE was the storage method. A number of previously published reports had indicated that pure pullulan films are sensitive to changes in humidity and that the protective and anti-oxidation properties of pullulan are compromised at high humidity.[33,35,42–45] To examine the effect of the environment on the antibacterial activity of phage in pullulan/trehalose films, we compared samples stored in plates, which were exposed to the atmosphere, to samples stored in tubes, which were shielded from the atmosphere.

LISTEX^{TM} P100 bacteriophage was dried in 10 wt% pullulan and 0.5 M trehalose with different combinations of the four factors discussed above. The antibacterial activity (quantified through titer count) of bacteriophage dried in pullulan/trehalose film was determined at two different time points after drying: Day 1 and Day 28. Figure 1 shows the log reduction of phage titer for all 16 experimental conditions outlined in Table 2. It can be seen from Figure 1 that most experiments resulted in a significant titer loss (> 5 logs) after 28 days of storage, except for experiments 1, 4, 10, 15, and 16 that showed less than 2.5 log titer reduction after 28 days of storage. Statistical analysis of the data was performed to gain further insight into the significance of the impact of each factor on phage antibacterial activity in the pullulan/films. The full ANOVA tables are presented in the supplementary information (Tables S1 and S2).

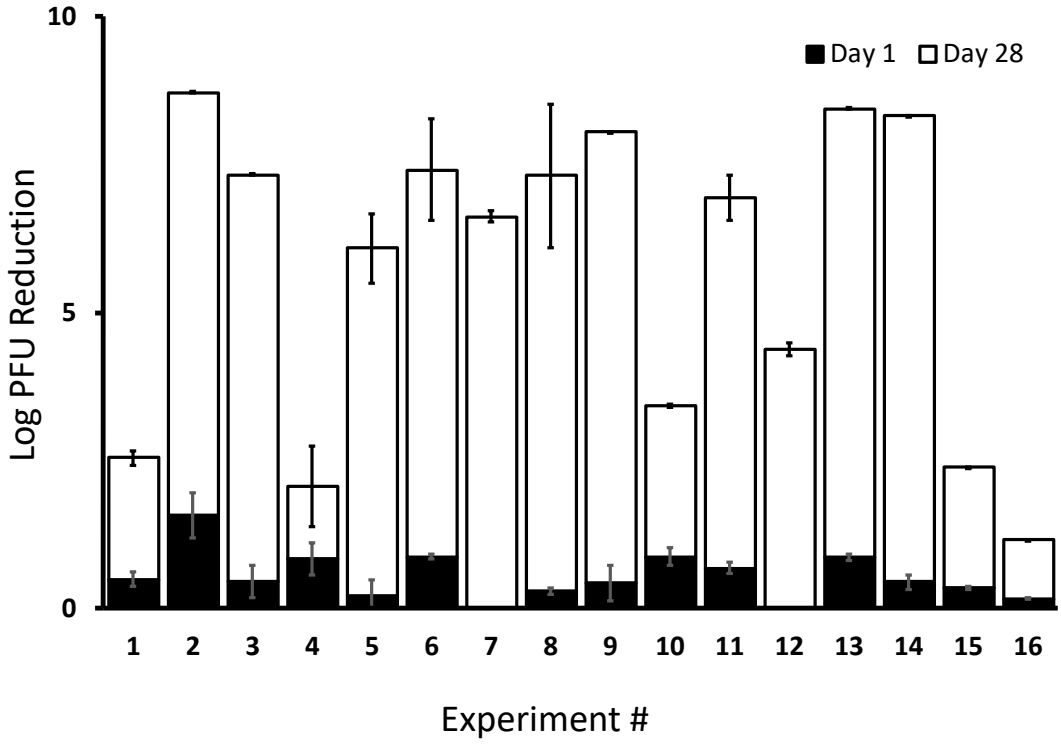


Figure 1. Log reduction of LISTEX^{TM} P100 titer in pullulan/trehalose films for all 16 experiments at day 1 and day 28 of storage. The experiment were performed in duplicates and the error bars indicate the standard deviation of the duplicates.

Drying Method and Storage Container Significantly Impact Antibacterial Activity After 1 Day

The pareto plot shown in Figure 2A highlights that the storage container and the drying method significantly ($p < 0.05$ indicated by *) influenced the phage titer one day after drying. The samples that were air-dried showed greater phage titer than the samples that were vacuum dried (Figure 2B, I). One day after drying, the air-dried samples had an average of 0.34 log PFU reduction (SD = 0.3 log PFU), while the vacuum dried samples had an average of 0.72 log PFU reduction (SD = 0.4 log PFU). This can be attributed to less desiccation stress caused by air-drying compared to vacuum drying. Moreover, it is possible that the air-dried samples were not fully dried after one day. Samples that were air-dried in tubes are especially likely not to be fully dried because of the

smaller surface area for evaporation. Vacuum-dried samples were completely dried after drying overnight. When the samples were weighed after drying overnight, the samples that were air-dried in tubes was 8% heavier than the samples vacuum-dried in tubes (n=4). This difference in weight can be attributed to the fact that more water was retained in air-dried sample during evaporation. Thus, lower desiccation stress can account for the higher phage titer for samples that were air-dried in tubes.

The samples dried in tubes also had a higher titer than samples dried in plates (Figure 2B, IV). Samples dried in tubes had an average of 0.35 log PFU reduction (SD = 0.3 log PFU) while samples dried in plates had an average of 0.71 log PFU reduction (SD = 0.4 log PFU) after one day. Addition of 10 mM Zn^{2+} did not result in a statistically significant ($p > 0.05$) improvement of phage antibacterial activity in the pullulan/trehalose films after one day (Figure 2B, III). Moreover, pretreating the surface with PEG had an insignificant effect ($p > 0.05$) on the antibacterial activity of the phage films (Figure 2B, II). Lastly, two-factor interactions did not have a significant effect on phage titer one day after drying (Figure 2C).

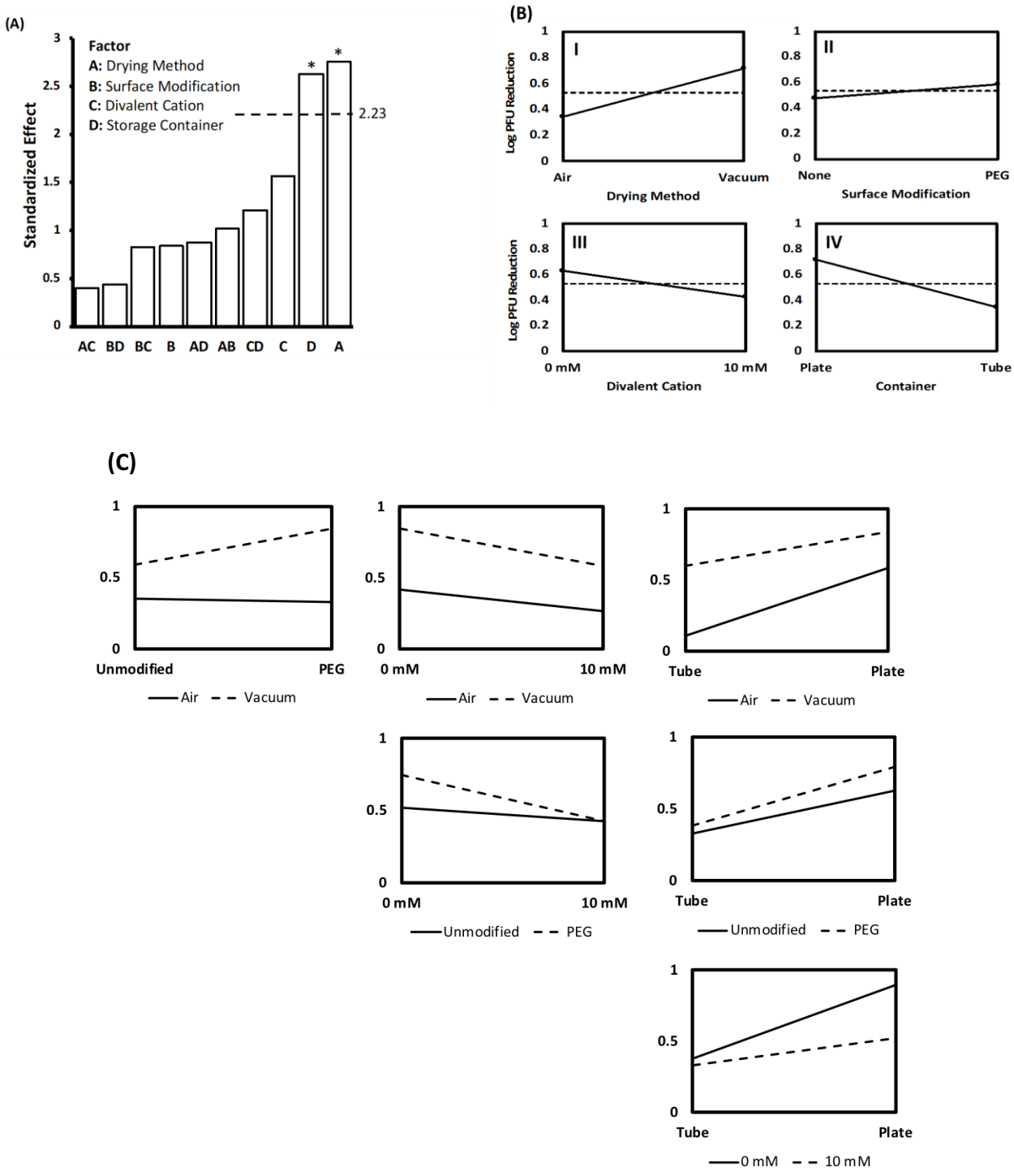


Figure 2. A) Normalized Pareto chart for the main factors and two-factor interactions after 1 day of storage. The horizontal line indicates the t-value at p = 0.05. Asterisk (*) indicates statistically significant factors with $p < 0.05$. **B)** Main factor plot after 1 day of storage. Horizontal dotted line

indicates the average log PFU reduction for all 16 experiments. **C)** Secondary interaction plots between the main factors after 1 day of storage.

Storage Container Significantly Impact Antibacterial Activity After 28 Days

The titer of phage in pullulan/trehalose films were further determined after 28 days of storage to examine the effects of the factors on long-term storage of the phage in pullulan/trehalose films. The results were analyzed using ANOVA (See Table S2). As shown in Figure 3A, the choice of the storage container had the most significant impact ($p < 0.001$) on antibacterial activity of phage films. It was found that storing the phage films in tubes greatly improved the long-term antibacterial activity of the phage films when compared to storing the films in plates. The samples stored in tubes had an average of 3.2 log PFU reduction (SD = 2.1 log PFU) while the samples stored in plates had an average of 7.1 log PFU reduction (SD = 0.5 log PFU) after 28 days (Figure 3B, IV). Moreover, samples that were vacuum-dried had significantly ($p<0.001$) higher phage viability after 28 days than the samples that were air-dried. The vacuum-dried samples had an average of 4.4 log PFU (SD = 2.9 log PFU) reduction and the air-dried samples had an average of 6.0 log PFU reduction (SD = 1.9 log PFU) after 28 days (Figure 3B, I). The interaction between the drying method and the storage container also had a significant ($p<0.001$) impact on long-term phage viability in the pullulan-trehalose films. Samples that were vacuum-dried and stored in tubes had much lower titer reduction when compared to any other combination (Figure 3C).

The results after 28 days of storage clearly show that storing the phage films in a tube is crucial to long-term phage viability. This result suggests that exposure to the environment greatly reduces phage viability in the film. The samples stored in tube were shielded from the environment since the tubes were capped and wrapped in paraffin wax. Exposure to the environment can also explain

why vacuum-dried samples had higher phage titer compared to air-dried samples. Air-dried samples were exposed to the environment during the drying process, which can account for the lower phage titer when compared to vacuum-dried samples. The effects of the environment are especially clear when the interaction between drying methods and storage container is examined. The drying method did not have a significant effect on samples that were stored in plates. Vacuum-dried samples stored in plates had 7.0 log PFU reduction while air-dried samples stored in plates had 7.2 log PFU reduction after 28 days. On the other hand, the drying method had a significant effect on samples that were stored in tubes. Samples vacuum-dried in tubes had 1.7 log PFU reduction (SD = 0.6 log PFU) while samples air-dried in tubes had 4.7 log PFU reduction (SD = 1.7 log PFU). Therefore, it is evident that exposure to the environment during drying and in storage greatly reduces phage antibacterial activity in the film.

Effect of Humidity and Oxygen

Based on the results from the DOE, we postulated that the environmental effects on phage viability may be attributed to humidity, oxidation, or a combination of both factors. To investigate the effects of humidity and oxidation, LISTEX^{TM} P100 phage in films composed of 10 wt% pullulan and 0.5 M trehalose was vacuum-dried in tubes and stored in air and under a N_2 blanket for 28 days at various levels of humidity. Our results show that titer reduction is much lower for samples stored at low humidity (Figure 4). Storing the samples in air or in N_2 for 28 days had insignificant effects on the titer. However, high humidity (> 50%RH) proved detrimental to antibacterial activity of phage-pullulan-trehalose films. Previous studies have shown that O_2 permeability of pullulan films significantly increases at high relative humidity.[44] Also, the finding is consistent with a previous report showing high humidity had a destructive effect on phage powder containing trehalose.[46] We further observed a clear physical change in the appearance of the films at 75 %

relative humidity when the films changed from transparent to opaque within seven days of storage (Figure S2). This change from transparent to opaque was irreversible when the film was returned to lower humidity. It has been shown in previous studies that amorphous trehalose can transform to crystalline trehalose at high humidity.[29,47–49]

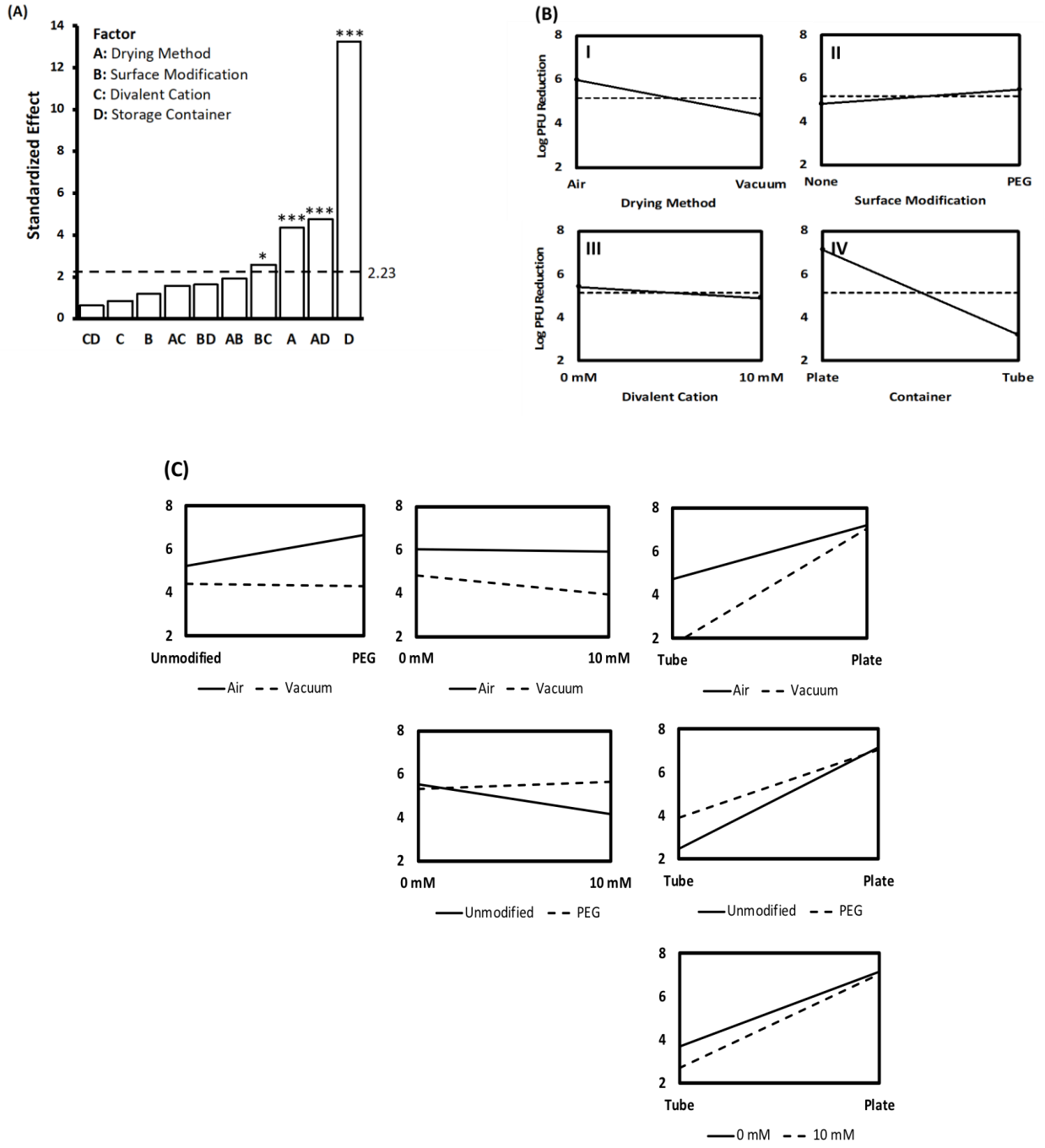


Figure 3. A) Normalized Pareto chart for the main factors and two-factor interactions after 28 Days of storage. The horizontal line indicates the t-value at $p = 0.05$. Asterisk (*) indicates statistically significant factors with $p < 0.05$, *** indicates statistically significant factors with $p < 0.001$ **B)** Main factor plot after 28 Days of storage. Horizontal dotted line indicates the average log PFU reduction for all 16 experiments. **C)** Secondary interaction plots between the main factors after 28 Days of storage.

The absence of O_2 alone did not seem to improve the protective effect of the films, which leads us to hypothesize that the irreversible change in the structure of the film from amorphous to crystalline at high humidity likely affected the phage titer in the film. The observation that the pullulan-trehalose films cause phage deactivation at high humidity is rooted in the film crystalline structure. This finding is important because the major proposed application for phage embedded in pullulan-trehalose films is food packaging, where these films may be exposed to high humidity. Although it is unlikely that the packaging is exposed to high humidity continuously for four weeks prior to being used, it is important to be aware of the limitations imposed on the production, storage and application of such antibacterial films. Furthermore, this observation highlights the importance of conducting similar investigations on other stabilizing material reported in the literature for phage, viral vectors and labile biomolecules to ensure antibacterial activity under conditions prevailing during processing, storage, and application.

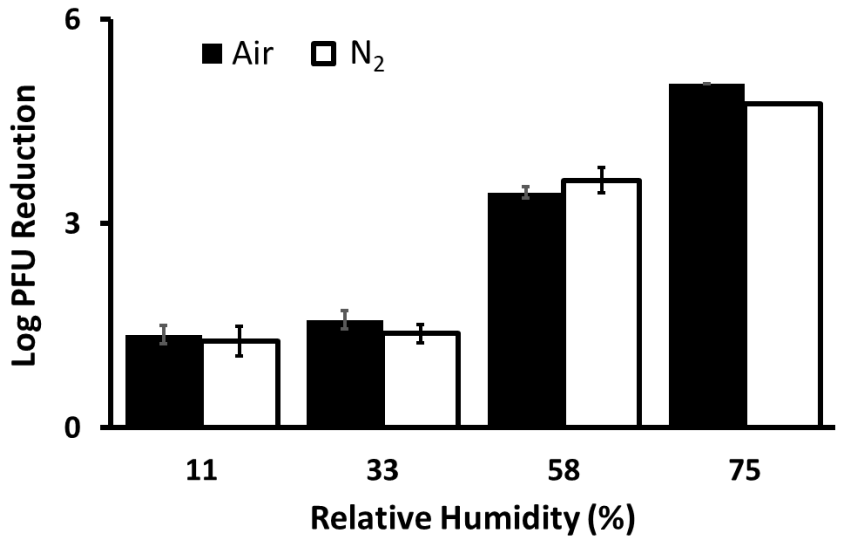


Figure 4. Log PFU reduction of LISTEX^{TM} P100 in pullulan/trehalose films after 28 days of storage at different relative humidity in air and in N_2. The experiments were performed in triplicates and the error bars represent the standard deviation.

4.4 Conclusion

In this study, we have identified that drying method and humidity are factors that significantly affect the long-term viability of LISTEX^{TM} P100 bacteriophage in pullulan/trehalose films. We further demonstrated that protecting the phage films from the environment by vacuum drying and storing in enclosed containers can enhance the long-term viability of the bacteriophage by over 1000-fold. We also showed that the main cause for titer reduction in the film is exposure to high humidity.

Pullulan-trehalose phage antimicrobial films/coatings hold great potential for long-term stabilization of bacteriophages for use in food, biomedical, and water treatment industries. The present study provides key insights to parameters that affect phage antibacterial activity in these films/coatings. These insights provide simple and practical methods to maximize long-term

antimicrobial activity of the phage films which is critical for manufacturing and commercialization. Based on the results of our study it is clear that phage antimicrobial films/coatings should be manufactured and dried at low humidity conditions or under vacuum. Similarly, once the films/coatings are dried, they should be stored in sealed or vacuum packaging prior to use. The implementation of these simple manufacturing and packaging techniques can greatly increase the long-term activity of phage antimicrobial films/coatings. The reported insights can be directly applied to applications other than phage, such as enzymatic bioassays and viral vectors/vaccines where pullulan or pullulan-trehalose films have demonstrated promising stabilization effect.

4.5 Acknowledgments

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4.6 Supplementary Information

Table S1. ANOVA analysis of design of experiment data from Day 1. ANOVA analysis was performed using R.

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	0.53	0.068	7.878	1.05E-07	***
A	0.19	0.068	2.763	0.0116	*
B	0.056	0.068	0.834	0.4138	
C	-0.11	0.068	-1.569	0.1316	
D	0.18	0.068	2.634	0.0155	*
AB	0.069	0.068	1.017	0.3209	
AC	-0.027	0.068	-0.403	0.6909	
AD	-0.059	0.068	-0.871	0.3935	
BC	-0.056	0.068	-0.832	0.4149	
BD	0.029	0.068	0.434	0.6688	
CD	-0.081	0.068	-1.204	0.2421	

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table S2. ANOVA analysis of design of experiment data from Day 1. ANOVA analysis was performed using R.

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	5.7	0.17	34.248	< 2.00E-16	***
A	-0.73	0.17	-4.392	0.000255	***
B	0.2	0.17	1.209	0.240259	
C	-0.14	0.17	-0.852	0.403698	
D	2.2	0.17	13.281	1.10E-11	***
A:B	-0.31	0.17	-1.897	0.071725	.
A:C	-0.26	0.17	-1.586	0.127752	
A:D	0.79	0.17	4.747	0.000109	***
B:C	0.43	0.17	2.593	0.016965	*
B:D	-0.27	0.17	-1.661	0.11151	
C:D	0.11	0.17	0.643	0.527031	

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

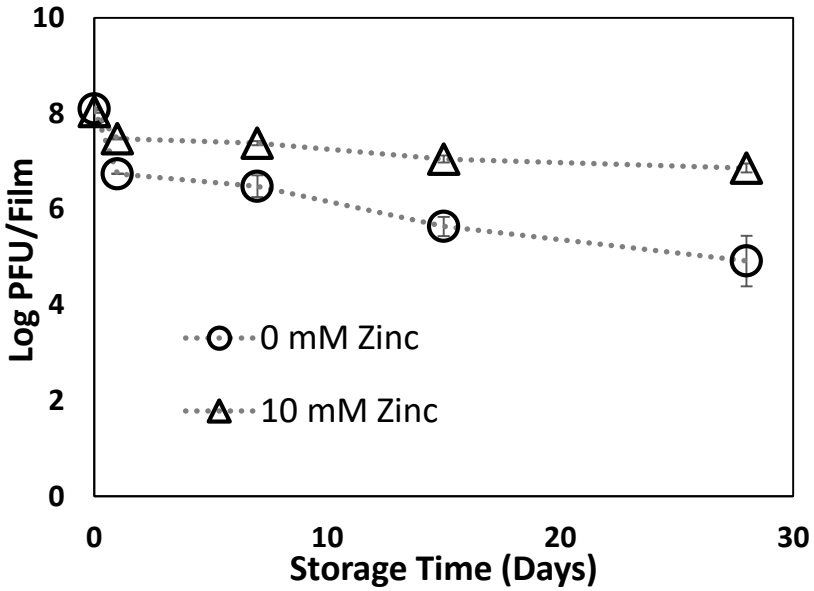


Figure S1. Effect of zinc on the viability of LISTEX P100 in 10 wt % pullulan + 0.5 M Trehalose films as a function of storage time. Dashed lines are added to guide the eye and do not reflect experimental measurements.

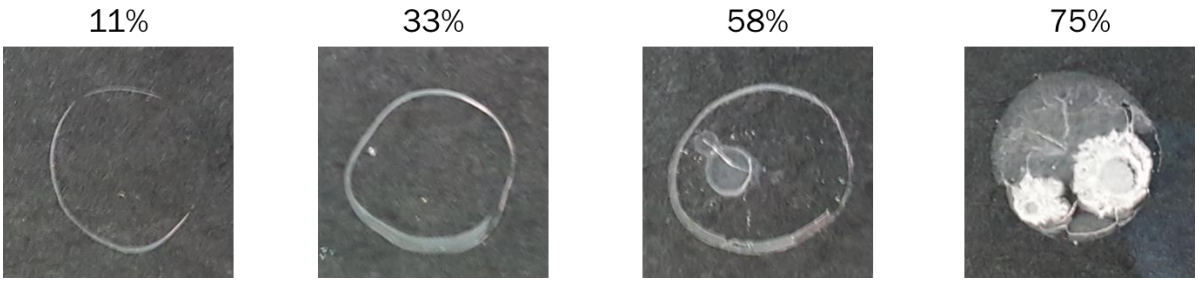


Figure S2. Pullulan/Trehalose films stored for 28 days at different relative humidity.

Chapter 5: Thermal Stabilization of Vaccines in Low-Cost Sugar Films

Preface

In Chapter 5, all HSV-2 experiments were conducted by me with the help of Amanda Lee, Fatemeh Vahedi, Marianne Chew, and Alexandra Szewczyk. All PR8 experiments were conducted by me with the help of Jonathan Mapletoft and Ali Zhang. Braedon Cowbrough and Jann Ang collected and analyzed blood samples for the PR8 *in vivo* expeirments. I initiated the first draft of the manuscript. Dr. Filipe, Dr. Ashkar, and Dr. Miller provided helpful insight of the experiments and edited the paper draft. This manuscript is prepared for submission to Nature Communications.

Thermal Stabilization of Vaccines in Low-Cost Sugar Films

Vincent Leung[1], Jonathan Mapletoft[2], Ali Zhang[2], Amanda Lee[3], Fatemeh Vahedi[3], Marianne Chew[3], Alexandra Szewczyk[1], Jann Ang[2], Braeden Cowbrough[2], Matthew Miller[2], Ali Ashkar[3,*], Carlos D.M. Filipe[1,*]

[1] Department of Chemical Engineering, 1280 Main Street West, McMaster University, Hamilton, Ontario, Canada L8S 4L7

[2] Michael G. DeGroote Institute for Infectious Disease Research, McMaster Immunology Research Centre, Department of Biochemistry and Biomedical Sciences, McMasterUniversity, Hamilton, ON, Canada

[3] Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, McMaster University, Rm 4015 Michael DeGroote Centre for Learning and Discovery, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada

Correspondence and requests for materials should be addressed to A.A. (ashkara@mcmaster.ca) or to C.F. (filipec@mcmaster.ca)

Abstract

Most of the currently available vaccines, particularly live vaccines, require the cold chain to maintain efficacy. Any excursions outside of 2 – 8 °C during storage and transport can lead to significant reduction in the vaccine efficacy. This presents a tremendous financial and logistical burden on vaccination programs, particularly in tropical areas. The development of thermally stable vaccines can greatly alleviate the problem and increase vaccine accessibility worldwide. Here, we demonstrate that a simple and cost-effective method, using FDA approved materials, can stabilize live viral vaccines. Enveloped DNA and RNA viral vaccines were dried in a pullulan and trehalose mixture. Live-attenuated HSV-2 vaccine dried in pullulan and trehalose retained immunogenicity when stored at 40 °C for at least 2 months. Inactivated influenza vaccine was able to retain immunogenicity for at least 3 months at 40 °C. This work presents a simple approach to formulate thermo-sensitive vaccines into thermo stable vaccine that do not require refrigeration and thus enable better vaccine deployment throughout the world.

5.1 Introduction

Vaccines are an essential part of global health. Every year, millions of lives are saved through vaccination. Unfortunately, almost all available vaccines are thermally labile and must be kept between 2-8 ºC at all times to retain their efecacy.[1] Failure to maintain an uninterrupted refrigerated supply chain from production to dispensation, called the "cold chain," leads to vaccine wastage and administration of ineffective vaccines.[2] The need for refrigeration is one of the major causes of under-vaccination globally as the cold chain presents significant economical and logistical problems for vaccination programs. The problem is especially serious for developing countries and remote areas where there are often a lack of dependable cold chain infrastructure and access to reliable electricity is limited.[3–5]

The development of thermally-stable vaccines that can remain active outside of the cold chain can greatly increase the accessibility of vaccination programs and significantly decrease the cost. Therefore, significant efforts have been made in creating thermally-stable vaccines and/or vaccine carriers. One approach is to engineer vaccines that are thermally stable without preservative adjuvants. The engineering of protein-based vaccine had shown some promise. Sun *et al.* demonstrated that by attaching an M. tuberculosis epitope to a self-assembling fibril-forming peptide can produce vaccine that exhibit strong thermal stability, showing no conformational change after being stored for 7 days at 45 °C.[6] Beernink's group engineered a mutant antigen for a recombinant meningococcal vaccine that increased the vaccine's thermal sensitivity by 21 °C. [7,8] Campeotto *et al.* modified a malaria protein vaccine with 18 mutations which increased the thermal tolerance by 10-15 °C when compared to the wild type[9]. Other researchers had modified viral vectors to create thermally stable viral vaccines. Stobart *et al.*

engineered a respiratory syncytial virus (RSV) with enhanced pre-F expression that had greater immunogenicity and demonstrated greater thermal stability compared to the wild type.[10] However, there was still significant loss in titre after 7 days at 37 °C. Wang *et al.* designed a self-biomineralized virus using a human enterovirus type 71 vaccine that can be stored at 37 °C for 1 week.[11] Although designing thermally stable vaccines hold some promise, many of the engineered vaccine still have short shelf-life (~7 days) at elevated temperature (> 37 °C). Moreover, engineering new vaccines is labor intensive and the new vaccines must obtain governmental approval before deployment.

Another common approach to thermally stabilize vaccine is the addition of stabilizing adjuvants. Pelliccia *et al.* created thermally stable adenoviral vaccine formulations that were able to maintain immunogenicity for up to 10 days at 37 °C by adding polyethylene glycol (PEG), gold nanoparticles (AuNP), and sucrose.[12] In addition to stabilizing adjuvants, vaccines are often dried to further increase thermal stability. Prausntz's group encapsulated inactivated influenza vaccine in microneedle patches with different stabilizing adjuvant formulations and the vaccine maintained immunogenicity after 4 months at 60 °C.[13–15] Lyophilized anthrax vaccine was found to have preserved immunogenicity after 16 weeks at 40 °C[16] and lyophilized recombinant ricin toxin A vaccine was stable after 4 weeks at 40 °C.[17] Recombinant hepatitis B vaccine and a protein-polysacharide conjugate vaccine for meningitis A was shown to be stable for 24 months at 37 °C after spray drying.[18] Foam drying of attenuated salmonella enterica vaccine using trehalose methionine and gelatin were able to maintain vaccine potency for 12 weeks at 37 °C.[19] Spray drying formulations using sugars and proteins with live attenuated measles vaccine were shown to be stable for up to 8 weeks at 37 °C.[20] Lovalenti *et al.* stabilized live-attenuated influenza vaccines in a sucrose containing excipient using three drying method, freeze drying,

spray drying, and foam drying. It was found that foam drying with the right excipient composition produced the most thermally stable vaccine that had a shelf life of 4.5 months at 37 °C.[21] Different lyophilized formulations of rotavirus vaccines were able to retain potency for 20 months at 37 °C[22] for up to 20 months.[23]. Alcock *et al.* used sucrose and trehalose to dry adenovirus and modified vaccinia virus Ankara onto polypropylene or glass fiber membranes, the viruses retained titer for up to 6 months at 45 °C.[24] Many of the reports use freeze drying, spray drying, or foam drying which all require specialized equipment for sample preparation (freeze dryer, vacuum pumps) and expose vaccines to extreme temperatures or pressure conditions.[19] Moreover, some formulations require a large number of adjuvants which can increase the cost and complexity of the vaccine product.

In this study, we investigated a simple and low-cost method to thermally stabilize two enveloped viruses: Herpes Himplex Virus (HSV-2), a DNA virus, and influenza virus (PR8), an RNA virus, by drying the viruses in a pullulan and trehalose mixture. Trehalose is a disaccharide that is commonly used as a cryoprotectant and stabilizing agent.[25–30] Pullulan is a polysaccharide with good film forming abilities and is used in the food industry to extend the shelf life of food.[31–35] These two sugars are FDA approved and are readily available and inexpensive. In our previous studies, we have shown that pullulan and trehalose when used together can provide long-term stabilization for enzymes and bacteriophages by protecting them against oxidation and thermal inactivation.[36–38] Live enveloped viruses were chosen for this stable because they are intrinsically more unstable than other types of vaccines, especially RNA viruses [21,24]. Herein, we demonstrate the that the live virus, when dried in a pullulan and trehalose mixture can maintain infectivity *in vitro* and the vaccines were about to maintain immunogenicity *in vivo* for up to 3 months at 40 °C.

5.2 Results

Pullulan and Trehalose (PT) Film Provides Thermal Protection for HSV-2 in vitro

We initially dried HSV-2, 333 strain in three different drying matrices, 1) 10 wt % pullulan, 2) 0.5 M trehalose and 3) a mixture of 10 wt% pullulan with 0.5 M trehalose, to determine the stabilization effectiveness of the three solutions. Each sample had an initial titre of 2×10^4 plaque forming units (PFU) and was stored at room temperature (~23 °C). The titer of the three different dried samples were determined at different storage times and the results are shown in **Fig. 1(A**). HSV-2 dried in 10 wt% pullulan and 0.5 M trehalose was the most effective in maintaining viral titer. After 12 weeks, HSV-2 stored in pullulan/trehalose(PT) film had only a titer loss of 2.3 log PFU/film. In comparison, HSV-2 dried in 0.5 M trehalose had a titer loss of 3.6 log PFU/film after 12 weeks. HSV-2 dried in 10 wt% pullulan was completely inactive 7 days, while HSV-2 stored in PBS buffer had was completely inactive within 28 days.

The results also show that pullulan by itself offers little protection against desiccation. HSV-2 dried in pullulan had a titre loss of 2.2 log PFU/film during the drying process. On the other hand, HSV-2 dried in trehalose alone or in pullulan and trehalose had a titre loss of 0.9 log PFU/film and 0.7 PFU/film respectively during the drying process. Moreover, drying HSV-2 in trehalose alone does not offer long-term stability. After four weeks, HSV-2 dried in trehalose alone had a titre loss of 2.4 log PFU/film while HSV dried in PT films had a titre loss of 1.0 log PFU/film. Furthermore, HSV-2 in PT film demonstrated good stability after two weeks of storage. After two weeks of storage, HSV-2 in PT film had a titre loss of 1.0 log PFU/film. However, between week 2 and week 12, there was only a loss of 0.3 log PFU/film. In comparison, HSV-2 dried in trehalose had a titre loss of 2.0 log PFU/film between week 2 and

week 12. This result shows that pullulan and trehalose have a synergistic effect as a stabilizing matrix and is similar to the our previous work in stabilizing bacteriophages in sugar glasses.[38]

To further study the thermal stabilizing capability of PT films at elevated temperature, we dried live-attenuated HSV-2 TK$^-$ (initial titre, 10^6 PFU) in 10 wt% pullulan and 0.5 M trehalose and stored the samples at room temperature and at 40 °C. The titre were determined at different time points for up to 12 weeks and compared with HSV-2 TK$^-$ stored at the same temperatures. As **Fig. 1(B)** shows, HSV-2 TK$^-$ stored in PT films had a titre loss of 1.6 log PFU/film when stored at room temperature and a titre loss of 3.0 log PFU/film when stored at 40 °C. In contrast, HSV-2 TK$^-$ without pullulan and trehalose was completely inactive within 8 weeks when stored at room temperature and was inactive within 1 week when stored at 40 °C. Moreover, during the first 4 weeks, the storage temperature did not significantly affect the stability of HSV-2 TK$^-$ in PT films. However, at 8 weeks and 12 weeks, HSV-2 TK$^-$ in.PT film was more stable at room temperature than at 40 °C. Overall, these *in vitro* results demonstrate that PT films offer significant thermal protection for HSV-2 and HSV-2 Tk$^-$.

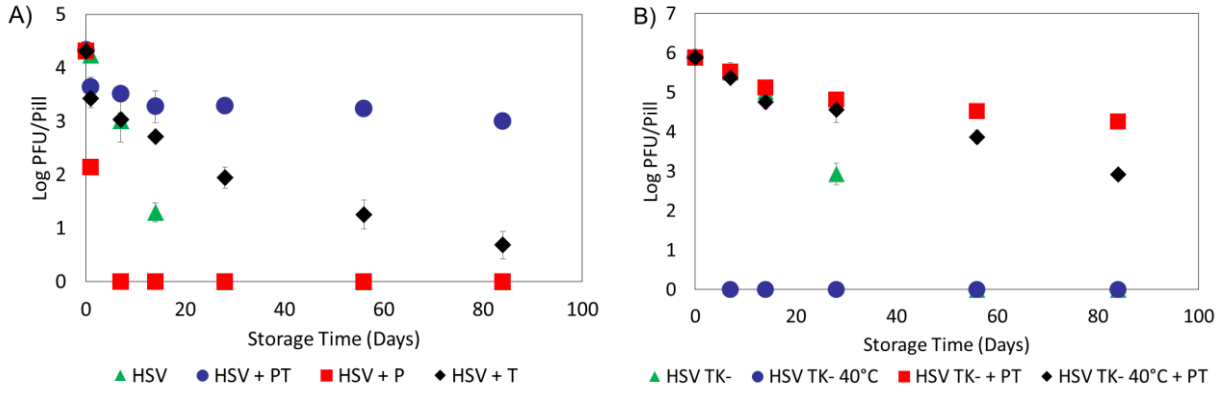
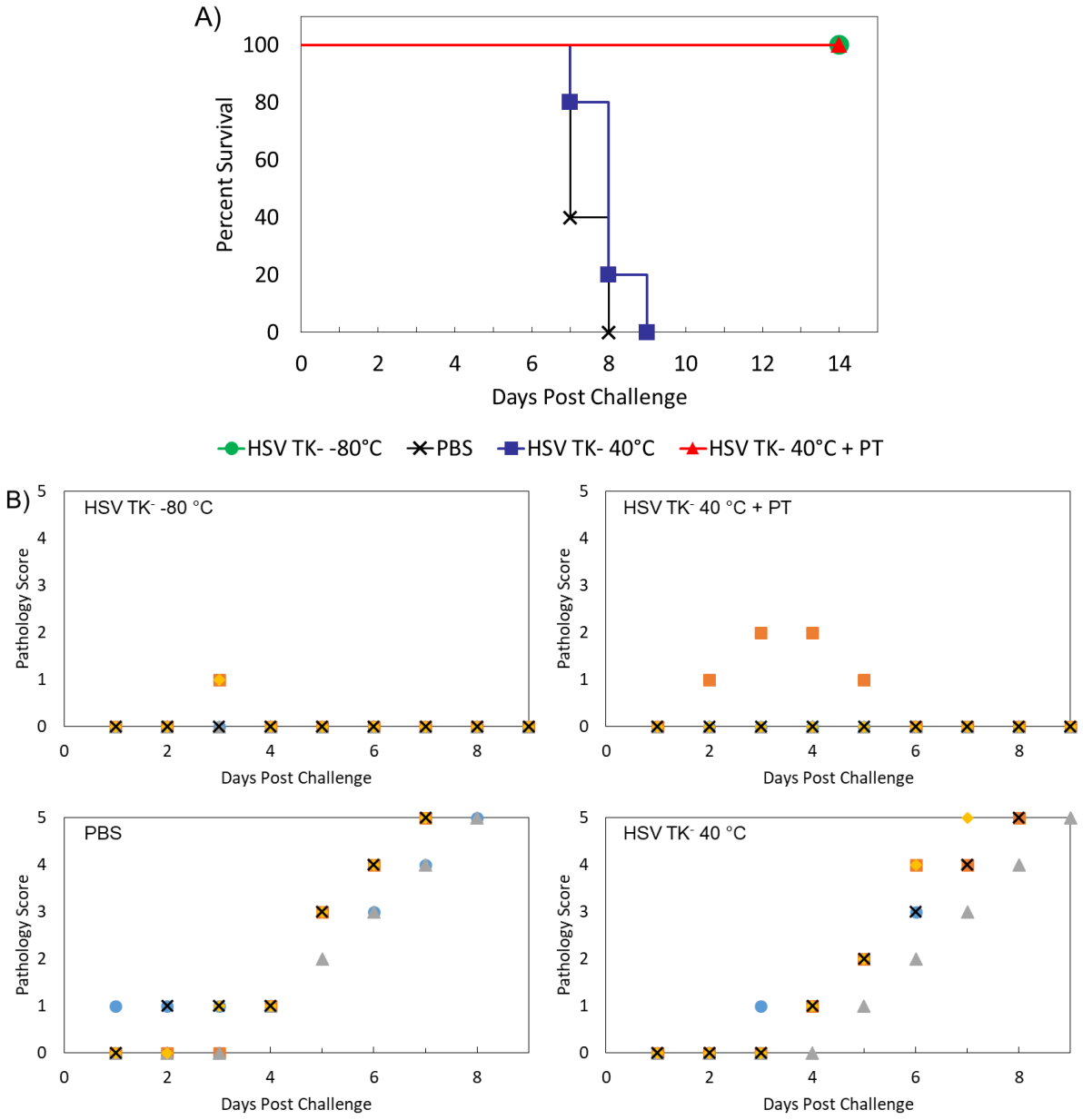


Fig. 1 ***In-vitro*** **Thermal Stabilization of HSV-2 and HSV-2 TK$^-$. (A)** Titre of HSV-2 stored in pullulan (P), trehalose (T), or pullulan and trehalose (PT) as a function of storage time at room temperature. **(B)** TK$^-$ HSV-2 and TK$^-$ HSV-2 + PT titre as a function of storage time at room

temperature and at 40 °C. The *in vitro* experiments were performed in triplicates. The error bars represents the standard deviation.

HSV-2 TK$^-$ Thermostabilized in PT Film Retains Immunogenicity at 40 °C for 8 Weeks

After demonstrating the thermostabilizing ability of PT films on HSV-2 Tk$^-$ *in vitro*, the immunogenicity of HSV-2 TK$^-$ in PT films was determined *in vivo*. C57BL/6 mice were immunized intravaginally with 1) TK$^-$ stored at -80 °C, 2) PBS, 3) TK$^-$ stored at 40 °C for 8 weeks, and 4) TK$^-$ dried in PT stored at 40 °C for 8 weeks. Five mice were immunized for each group. Since the *in vitro* results show that there is a decrease in titer over time in the PT films, the samples were prepared with a higher initial dose than the therapeutic dose. Each sample had an initial dose of 10^6 PFU, whereas the therapeutic dose is 5×10^4 PFU. After the mice were inoculated with TK$^-$, they were challenged with a lethal dose of HSV-2 14 days after immunization. The survival curve is shown in **Fig. 2** (**A**). All mice treated with HSV-2 TK$^-$ in PT films survived the infection showing that it retained immunogenicity after 8 weeks at 40 °C. Four of the five mice showed no visible signs of vaginal pathology. Only one mouse that was immunized with TK$^-$ in PT film showed minor signs of infection prior to recovering **Fig. 2** (**B**). Viral titre of the vaginal washes correlated well with the pathology data **Fig. 2** (**C**). Mice treated with Tk$^-$ in PT film resolved the infection within 5 days and no viral titre from the vaginal washes was detected subsequently. This is comparable to the mice immunized with fresh Tk$^-$. The mice immunized with fresh Tk$^-$ all survived and resolved the infection within 3 days. On the other hand, the mice that were immunized with TK$^-$ stored at 40 °C all showed signs of severe vaginal pathology and reached their clinical endpoint within 9 days. This is similar to the mice immunized with PBS (placebo) which all reached endpoint within 8 days. The results clearly show that PT films was able to maintain the efficacy of live-attenuated HSV-2 Tk$^-$ despite prolonged exposure to elevated temperature.


A)
Percent Survival
Days Post Challenge
HSV TK- -80°C
PBS
HSV TK- 40°C
HSV TK- 40°C + PT
B)
HSV TK- -80 °C
HSV TK- 40 °C + PT
PBS
HSV TK- 40 °C
Pathology Score
Days Post Challenge

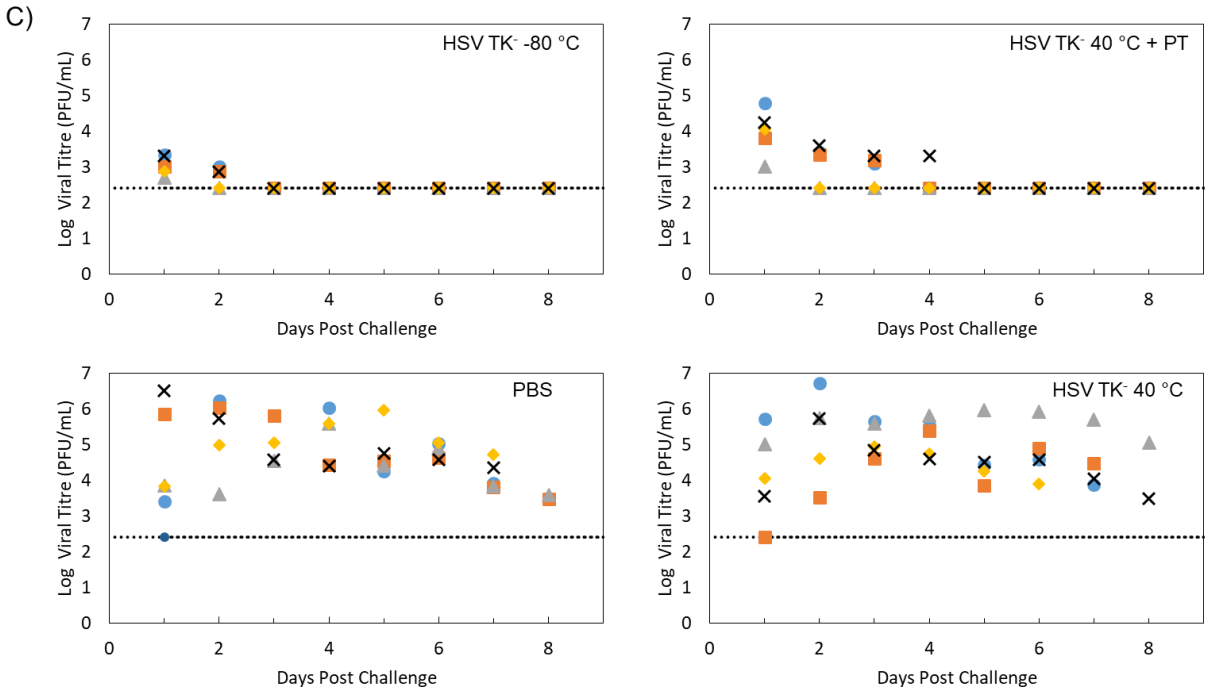


Fig. 2 (A) Survival curve of mice immunized with TK$^-$ HSV-2 and TK$^-$ HSV-2 + PT vaccine after 8 weeks of storage at 40 °C. Five mice were used for each group. **(B)** Pathology scores of mice after infection with HSV-2. Each symbol represent a single mouse. The pathology scores are explained in Methods. **(C)** Viral titre of vaginal washes as a function of days post infection.

PT Films Thermally Stabilize Live Influenza Virus PR8 in vitro and Retains Infectivity in vivo

The results from the HSV-2 experiments demonstrated the thermal stabilizing ability of PT films on a DNA virus. Further investigation was needed to determine the stabilizing effect of PT films on an RNA virus. Therefore, we dried an influenza virus, PR8, in 10 wt% pullulan and 0.5 M trehalose and stored the samples at 40 °C over a period of 12 weeks. The titre of the samples were determined at different storage times and compared to PR8 stored at 40 °C. **Fig. 3(A)** shows that PR8 stored at 40 °C was inactive within 14 days of storage while PR8 stored in PT only had a titre loss of 2.0 log PFU/film after 2 weeks. Similar to HSV-2, PR8 in PT films had a rapid loss in titre initially followed by a more gradual loss in titre. After 4 weeks of storage at 40 °C there was a titre loss of 2.9 log PFU/film. Between week 4 and week 12 there was only a titre

loss of 0.3 log PFU/film. The total titre loss after 12 weeks of storage at 40 °C, there was a titre loss of 3.2 log PFU/film for PR8 in PT films. The *in vitro* results show that PT films was able to offer some thermal protection for PR8. However, PR8 was less thermally stable than HSV-2 and significant titre loss was observed within the first 4 weeks. .

PR8 in PT films was further test *in vivo* to determine the infectivity of the dried virus. Balb/C mice were infected intranasally with 1)PR8 stored at -80 °C, 2) PBS, 3) PR8 stored at 40 °C for 12 weeks, and 4) PR8 dried in PT and stored at 40 °C for 12 weeks. The initial dose of the samples was 10^5 PFU/mouse. **Fig. 3(B) and (C)** show that the mice that were infected with PR8 in PT films had similar response to the infection when compared with mice infected with fresh PR8. The mice experience significant weight loss and reached clinical endpoint within 5 days for mice infected with PR8 in PT films and within 6 days for mice infected with fresh PR8. In contrast, the mice that were infected with PR8 stored at 40 °C did not experience any weight loss and did not show any clinical signs of infection. This demonstrates that PR8 in PT films were still able to infect even after being stored at 40 °C for 12 weeks. On the other hand, PR8 without PT was completely inactivated and was unable to infect.

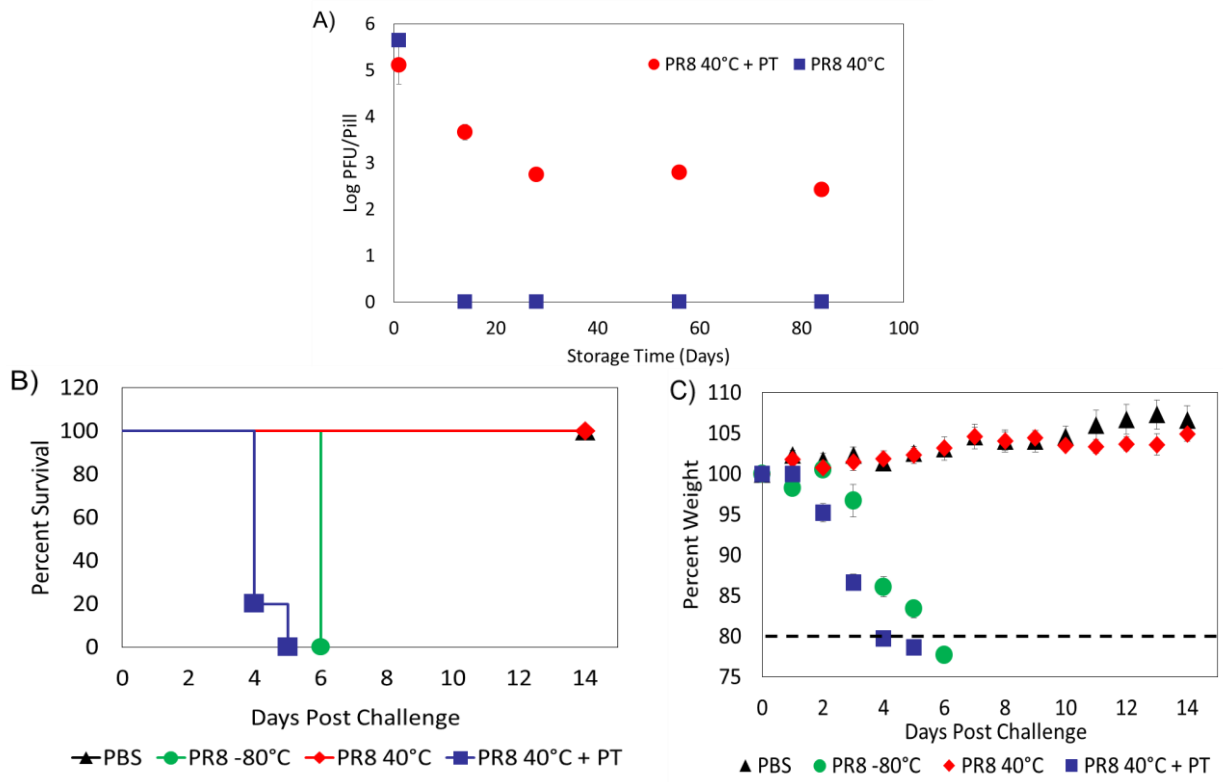


Fig. 3 Thermal Stability of PR8 in PT Films *in vitro and in vivo*. (A) Titre of PR8 and PR8 + PT versus storage time at 40 °C. Experiments were performed in duplicates. Error bars indicate standard deviation. **(B)** Survival curve and **(C)** weight loss curve of mice infected with **i)** PBS; **ii)** PR8 stored at -80 °C; **iii)** PR8 stored at 40 °C for 12 weeks; **iv)** PR8 in PT film stored at 40 °C for 12 weeks.

Inactivated Influenza Vaccine Thermostabilzed in PT Film Retain Immmunogenicity at 40 °C for 12 Weeks

The experiments shown above demonstrate that PT films are able to thermally stabilize live viruses (HSV2 and PR8) while maintaining their infectivity or immunogenicity *in vivo*. We also wanted to demonstrate the ability of PT to thermally stabilize inactivated viral vaccines. Therefore, we dried formalin-inactivated PR8 in PT films and stored the films at 40 °C for 12 week. Balc/C Mice were immunized with 1) fresh vaccine (stored at -80 °C), 2) PBS, 3) inactivated PR8 stored at 40 °C for 12 weeks, and 4) inactivated PR8 dried in PT stored at 40 °C for 12 weeks. The initial dose of each sample was twice the therapeutic dose to account for loss

in activity during the drying process and storage. Mice that were immunized with the vaccine in PT film stored at 40 °C did not show any clinical difference when compared to mice that were immunized with fresh vaccine. All mice in both groups survived 14 days after infection (**Fig. 4(A)**) and did not exhibit any weight loss (**Fig. 4(B)**). In contrast, four out of five mice that were vaccinated with the vaccine stored at 40 °C reached clinical endpoint (> 20% weight loss) within 7 days post infection. The one mouse that did not reach endpoint still experienced significant weight loss (> 15%) before recovering. The mice that were given placebo (PBS) all reached clinical endpoint 8 days after infection.

To further investigate the immunogenicity of the vaccines, blood samples were taken from the mice 14 days after immunization to determine the immune response and antibody production induced by the vaccine. The total IgG and flu-specific IgG were quantified by ELISA. Mice that were immunized with the vaccine in PT film had a significantly higher level of flu-specific IgG when compared with mice that were immunized with the vaccine stored at 40 °C (**Fig. 4(C)**). Moreover, there was no significant difference in the level of flu-specific IgG between the mice immunized with fresh vaccine and the mice immunize with vaccine in PT film. This clearly shows that the vaccine dried in PT films was able to induce the production of flu-specific antibodies in mice and provide protection against influenza infection even after being stored at 40 °C for 12 weeks. In contrast, the vaccine without PT after 12 weeks of storage at 40 °C did not exhibit any immunogenicity. The mice immunized with vaccine without PT had the same level of flu-specific IgG as mice immunized with PBS. This result was further confirmed by hemagglutination inhibition (HAI) assay of the serum. HAI assay showed that mice immunized with vaccine dried in PT had a HAI titre of 32 and mice immunized with fresh vaccine had a HAI titre of 6. On the other hand, mice immunized with the vaccine stored at 40 °C and mice

immunized with PBS both had a HAI titre of 0. The HAI assay shows that vaccine in PT was able to generate a greater immunoresponse in mice compared to the fresh vaccine. This might be due to the fact that the initial dose of the vaccine in PT film was twice the therapeutic does. Nonetheless, this demonstrates that inactivated PR8 in PT films exhibit excellent thermal stability and was able to maintain vaccine potency for 12 weeks at 40 °C.

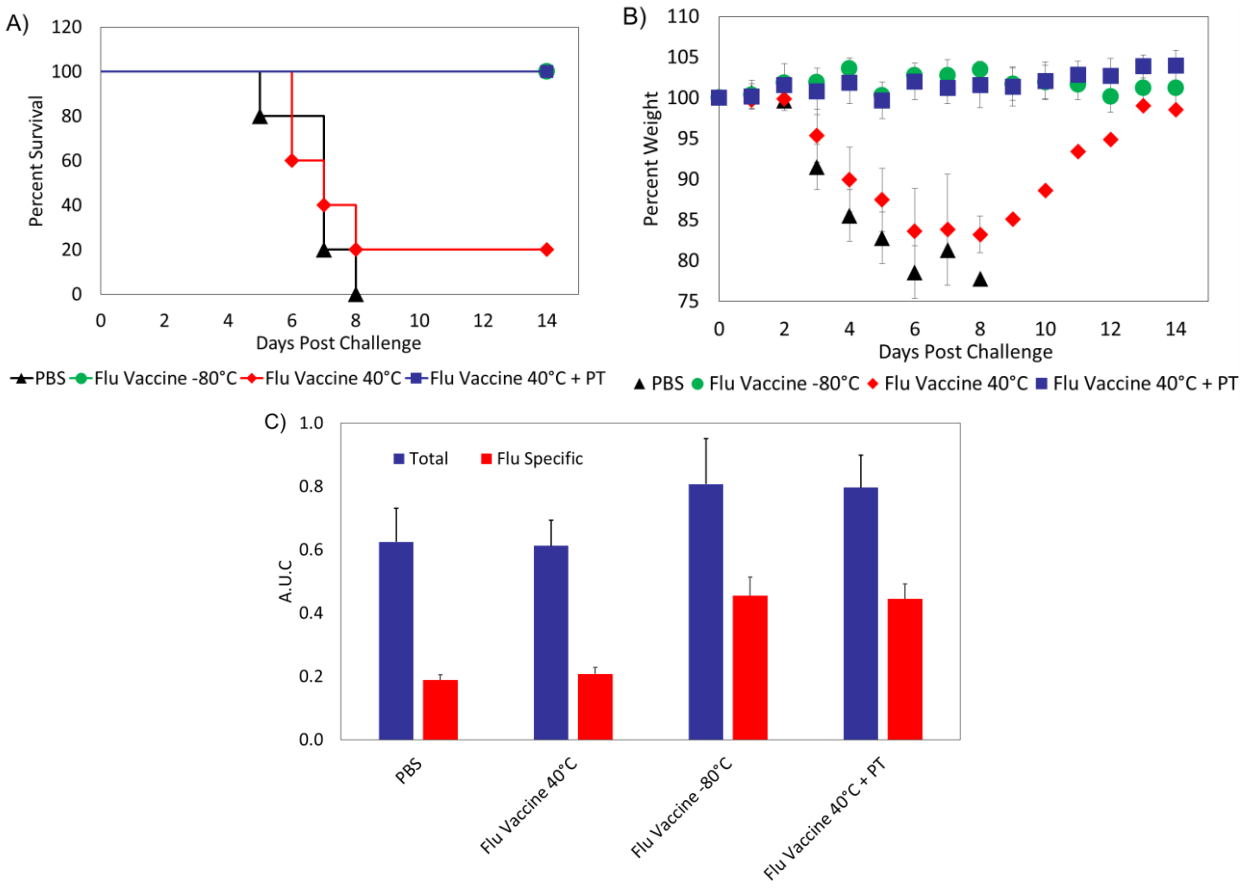


Fig. 4 *in vivo* results of inactivated PR8 vaccine after 12 weeks of storage at 40 °C. (A) Survival curve and **(B)** percent weight versus days post infection for mice immunized with inactivated PR8 and inactivated PR8 + PT after 12 weeks of storage at 40 °C. **(C)** Area under curve of ELISA assay for total IgG and and flu specific IgG for serum samples taken from mice immunized with inactivated PR8 and inactivated PR8 + PT after 12 weeks of storage at 40 °C. Five mice were used for each group.

5.3 Discussion

The development of thermally stable vaccines is crucial for the realization of universal immunization. This study presents a cost-effective and simple platform for thermo-stable vaccines. It has been shown that drying vaccines in pullulan and trehalose, which are inexpensive and FDA approved, significantly extends the shelf-life of the vaccine outside of the cold chain. Specifically we have demonstrated that this method can thermally stabilize a DNA virus (HSV-2), an RNA virus (Influenza PR8), a live-attenuated vaccine (HSV-2 TK^-) and an inactivated viral vaccine (formalin-inactivated PR8) for up to 12 weeks at 40 °C. Moreover, it was shown that PT films do not interfere with the *in vivo* infectivity or immunogenicity of the viruses and vaccines.

The *in vitro* experiments showed that pullulan and trehalose together offered better thermal stability than using pullulan alone or trehalose alone. In was observed that using pullulan alone as the stabilizer was unable to protect the virus during desiccation and using trehalose alone as the stabilizer offered poor long-term stability. From these observations, a possible explanation for the synergistic behavior for the combination of pullulan and trehalose is that trehalose provides protection during desiccation while pullulan offers long-term stability by immobilizing the viruses in a glassy matrix. This conclusion is consistent with previous published results. Trehalose is known to be a desiccation protectant and is often used during lyophilisation. Moreover, in our previous work, we have shown that mobility of enzymes is restricted in a pullulan film even at 60 °C.[37] The limited mobility in pullulan glass matrix results in enhanced thermal stability. Furthermore, pullulan films are oxygen impermeable and offers protection from oxidative degradation. Therefore, the synergistic nature of pullulan and trehalose may be attributed to the combination of desiccation protection from trehalose and immobilization within the pullulan sugar glass.

The *in vitro* experiments also show that a significant decrease in viral titre takes place within the first 4 weeks of storage and then the viral titre remains relatively stable for up to 12 weeks. This may suggests that there are multiple degradation mechanisms within the PT films. Further investigation on the degradation of viruses within the PT film may help further improve thermal stability by limiting the initial loss of viral titre. One drawback of using PT films as a stabilization method is that although the rate of titre loss for samples stored in PT films is much lower than samples stored in solution, there is still a loss in titre over time. Thus, there is a need for higher initial dosing to compensate for the activity loss. One solution to this problem is by optimizing the formulation, preparation and storage conditions of the PT films which we have shown in a previous study to significantly improve the long-term stability of bacteriophages in PT films.[38]

While there was a loss in titre for live viruses in PT films, there was no observable loss in efficacy for inactivated PR8 viral vaccine in PT films. This is likely due to the fact that the inactivated viral vaccine only require the antigenic proteins to be preserved rather than the whole viral vector. Thus, although PT films may need to be further optimized for live-attenuated vaccines, the *in vivo* results show that PT films can afford long-term stability for inactivated vaccines without further optimization. Moreover, Pullulan and trehalose films have been shown in previous studies to provide thermo-stability for other labile biomolecules, we suggest that this technology may be extended to other types of vaccines such as recombinant or conjugate vaccines.

In addition to thermal stabilization, another benefit of drying vaccines in PT film is ease of administration for certain applications. Since pullulan is a good film forming agent that is water soluble, it is possible to administer vaccines in PT films directly in a film format without reconstitution. This is especially useful for vaccines enter the body through mucosal surfaces such

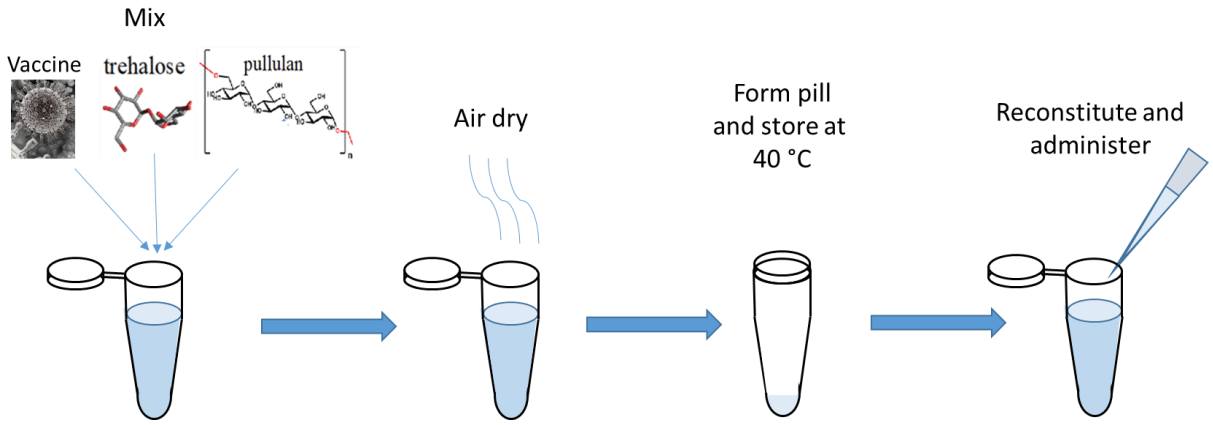
as oral or vaginal administration. The use of vaccine films can minimize storage space required and increase the ease of administration since it does not require reconstitution and needles.

Overall, we have shown a simple and versatile method to thermally protect viral vectors using pullulan and trehalose films. This technology has the potential to solve the cold chain problem for vaccines and greatly improve global health by giving people access to vaccines around the world.

5.4 Methods

Pullulan and Trehalose Pill Preparation, Storage, and Reconstitution

Pills containing HSV-2, HSV-2 TK^-, or PR8 were prepared by mixing 1 μL of solution containing the virus with 9 μL of a solution containing 10 wt% Pullulan (Polysciences, 200 kDa) and 0.5 M trehalose (Sigma). For formalin-inactivated PR8, 50 μL of the inactivated virus was mixed with 50 μL of 10 wt% pullulan and 0.5 M trehalose. The mixture was then air dried in a 1.7 mL microcentrifuge tube overnight in a biological safety cabinet. After drying, the tubes were capped and wrapped in paraffin film. The wrapped tubes were then placed in a heating block and stored at 40 °C for up to 3 months. At each time point, a sample was removed from the heating block and reconstituted in PBS. The reconstituted sample was then used for titration for *in vitro* study or administered to a mouse for *in vivo* study. **Scheme 1** shows a schematic of the process for pill preparation, storage, and reconstitution.



Scheme 1 The preparation, storage, and reconstitution of vaccines dried in pullulan and trehalose pills.

In-vitro HSV-2 viral titration

Vero cells were grown in a monolayer to confluence in 12- well plates in alpha minimum essential medium (α-MEM) supplemented with 1% penicillin and streptomycin, 1% l-glutamine, and 1% Hepes. Samples were resuspended and serially diluted (10^{-1} to 10^{-6}) in PBS and incubated with the monolayer for 2 h at 37 °C. The vero cells were then overlaid with α-MEM supplemented with 0.05% human immune serum and incubated for 48 h at 37 °C. After incubation, the cells were fixed and stained with crystal violet, and plaques were quantified using a light microscope. The PFU per pill was calculated using the plaque count and the corresponding dilution factor.

Genital HSV-2 immunization and infection

C57BL/6 (B6), 6–8-wk-old mice purchased from Charles River were used in the HSV-2 studies. The mice were housed at McMaster's Central Animal Facility (CAF) in pathogen–free conditions with a 12-h day and 12-h night cycle. All experiments were performed in accordance with Canadian Council on Animal Care guidelines and approved by the Animal Research Ethics Board at McMaster University. For the HSV-2 infection study, mice were injected

subcutaneously with 2 mg Depo-Provera (medroxyprogesterone acetate) 5 days before HSV-2 infection. The mice were then infected intravaginally with 10 μL of the reconstituted HSV-2 sample. For the immunization experiments using TK^- HSV-2. Mice were injected subcutaneously with 2 mg Depo-Provera (medroxyprogesterone acetate) 5 days prior to immunization. Mice were then immunized with 10 μL of the reconstituted HSV-2 TK^-. 9 days after immunization, the mice were injected subcutaneously with 2 mg Depo-Provera. 14 days after immunization, the mice were infected with HSV-2, 333 strain, at a dose of 10^5 PFU per mouse. For both the infection study and immunization study the mice were assessed for genital pathology and survival. Genital pathology was scored on a scale of 5 according to severity of redness, swelling, lesion development, hair loss, ulceration, and lower limb paralysis. Ulceration of a lesion and/or lower limb paralysis was considered endpoint.

Influenza Virus Infection

6-8 week old BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) received either PBS, Influenza Virus A/PR/8/1934 (PR8) (initial titre 10^5 PFU) stored at 40 ºC for 12 weeks, PR8 (initial titre 10^5 PFU) in combination with PT at 40 ºC for 12 weeks, or 250 PFU PR8 stored at -80 ºC. Mice were anesthetised with isoflourane and inoculated with 20 uL per nostril, for a total volume of 40 uL per mouse. Weight was monitored for 14 days as a measure of morbidity. Weight loss and survival were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Mice were euthanized after loss of 20 % of initial body weight. All animal procedures were approved by the Animal Research Ethics Board of McMaster University.

Influenza Virus Vaccination

6-8 week old BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) were vaccinated *i.m.* in the left hind limb with PBS, formalin-inactivated PR8 stored at 40 ^{o}C for 12 weeks, formalin-inactivated PR8 stored at 40 ^{o}C in combination with PT for 12 weeks, or formalin-inactivated PR8 stored at -80 ^{o}C. All vaccinations were administered in 100 uL volumes. 14 days post-vaccination mice were bled via the facial vein and blood was stored at 4 ^{o}C overnight. Following incubation overnight, blood was centrifuged at 16,000 x g for 10 min at 4 ^{o}C to separate serum. 30 days post-vaccination mice were challenged with 250 PFU of PR8 per mouse, as described above, and monitored for weight loss as a measure of morbidity. Weight loss and survival were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Mice were euthanized after loss of 20 % of initial body weight. All animal procedures were approved by the Animal Research Ethics Board of McMaster University.

ELISA

Enzyme linked immunosorbent assays (ELISA) were performed in 96 well plates (ThermoFisher Scientific, Mississauga, ON, CA). Plates were coated with IgG capture antibody (ThermoFisher Scientific, Mississauga, ON, CA) or formalin-inactivated PR8 influenza virus at 2 μg/mL for 24 hours at 4^{o}C in bicarbonate/carbonate coating buffer (0.05M Na_2CO_3, 0.05M $NaHCO_3$, pH 9.4). Plates were then blocked using 100μL of 5% non-fat milk in PBS with 0.1% tween (PBS-T) for 1 hour and room temperature (RT). Following blocking, serum samples were added at starting dilutions of 1:800 in blocking buffer for IgG wells and 1:50 for whole-inactivated virus-containing wells, and were diluted 1:2 across the plate 11 times, leaving the last well as a blank control. Samples were incubated for 1 hour at RT. Following the 1-hour incubation period, plates were washed 3 times with PBS-T, after which 100uL of IgG-HRP (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added at 0.1 μg/mL, diluted in PBS-T, and incubated for 1 hour at

room temperature. Following the incubation period, plates were washed 3 times with PBS-T and 100µL of Sigmafast OPD substrate (MilliporeSigma, Oakville, ON, CA) was added for 10 minutes. The reaction was stopped with 50µL of 3M HCl. Plates were then analyzed on the Spectramax i3 plate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance of 490nm, data was then analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Data was transformed into a log(X) scale and a nonlinear fit was performed using the log(agonist) vs. response with a variable slope (four parameters). The area under the curve (A.U.C.) was then graphed and statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test.

Influenza Virus Quantification

PR8 viral titres were determined by plaque assay on MDCK cells. Titres were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

Hemagglutinin Inhibition (HAI) assay

Prior to performing the HAI assay, serum was pooled and subsequently inactivated. 0.5 volumes of 8 mg/mL TPCK-treated trypsin (MilliporeSigma, Oakville, ON, CA) was added to 1 volume of serum and incubated at 56°C for 30 minutes. Following incubation, 3 volumes of 0.011 M metapotassium periodate (MilliporeSigma, Oakville, ON, CA) solution per volume of serum was added and incubated for 15 min at RT. Following incubation, 3 volumes of 1 % glycerol saline solution was added and incubated at RT for another 15 minutes. Finally, 2.5 volumes of 0.85% saline was added to the serum. Inactivated serum samples were serially (2-fold) diluted across a 96 well plate (Fisher Scientific, Ottawa, ON, CA) at 25 uL/well. A dilution of PR8 virus sufficient to produce 3 wells of HA activity was added to all of the wells (25 uL/well) and incubated for 30 mins at RT to allow for antibody-virus neutralization. After incubation, 0.5%

chicken red blood cells (Canadian Food Inspection Agency [CFIA], Nepean, ON, CA) was added to each well at 50 uL/well. The plate was then incubated at 4°C for 45 minutes. HAI titre was graphed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

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Author Contributions

V.L., A.L., F.V, M.C., and A.S. performed stability experiments with HSV-2. V.L, J.M., and A.Z. performed stability experiments with PR8. B.C. and J.A. collected and analyzed blood samples for PR8 experiments. V.L., C.F., A.A. and M.M wrote the paper.

Chapter 6: Concluding Remarks

In summary, this work presented a thermal stabilization method that can be applied to form molecular diagnostic kits, antimicrobial packaging, and thermal stable vaccines. These three applications address three of the most pressing global health issues today namely: the need for accessible and reliable diagnostic tools, the spread of antibiotic resistance, and the need for universal immunization.

The use of pullulan and trehalose pills to thermally stabilize RCA, PCR and LAMP mastermixes was reported in *Chapter 2*. The pills were also able to stabilize PNK, ligase, Klenow fragment, and BamHI restriction enzyme. It was found that by separating the buffer from the other reagents in the "two pills method" greatly improved the stability of the reagents. The mastermix pills using the two pills method retain their activity for at least 3 months at room temperature. This work has the potential to significantly improve point-of-care diagnostics since DNA amplification techniques such as RCA and PCR are powerful diagnostic tools for the detection of diseases and contamination. Thermally stable mastermixes will improve the access and the ease of use of molecular diagnostics. One possible next step for this work is to use the mastermix pills for detection of diseases in real-life samples. To achieve a diagnostic tool that can truly be used in point-of-care applications the development of simple sample preparation and portable incubation will also be required.

The development and optimization of a thermally stable bacteriophage film was presented in *Chapters 3* and *4*. It was shown that bacteriophage dried pullulan and trehalose films maintains antimicrobial activity for at least 3 months at room temperature. Moreover, it was shown that the combination of pullulan and trehalose had a synergistic effect and had greater stabilization effect

than pullulan alone or trehalose alone. Pullulan and trehalose can also be used as a coating material for antimicrobial food packaging. Through optimizing manufacturing and storage conditions, it was found that vacuum drying and storage at low humidity greatly improved the stability of bacteriophage films. The results from *Chapters 3* and *4* provide valuable insights for the manufacturing of antimicrobial surfaces without the use of antibiotics. Moreover, since bacteriophages can be used in biosensors, it is also possible to use these phage films to create smart surfaces that can detect contamination and food borne diseases.

Lastly, it was demonstrated in *Chapter 5* that pullulan and trehalose films can thermally stabilize DNA and RNA viruses as well as inactivated viral vaccines. It was shown that HSV-2 TK^- in pullulan and trehalose films maintained immunogenicity *in vivo* for up to 2 months and 40 °C.Similarly, PR8 maintained infectivity *in vivo* and inactivated PR8 retained immunogenicity for up to 3 months at 40 °C. The results presented are promising although further optimization is needed to improve the stability of live viruses in pullulan and trehalose film. Thermally stable vaccines are crucial for the development of a universal immunization program. Furthermore, drying vaccines in water-soluble films may improve the ease of administration, since the films can be directly administered to mucosal surface without the need for reconstitution. Lastly, since pullulan and trehalose have been shown to stabilize other biomolecules, it is believed this method can be applied to other types of vaccines such as recombinant, conjugate and toxoid vaccines.