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TITLE:

Production of Near-Infrared Sensitive, Core-Shell Vaccine Delivery Platform

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SUMMARY:

This article describes the protocols used to produce a novel vaccine delivery platform, "polybubbles," to enable delayed burst release. Polyesters including poly(lactic-co-glycolic acid) and polycaprolactone were used to form the polybubbles and small molecules and antigen were used as cargo.

ABSTRACT:

Vaccine delivery strategies that can limit the exposure of cargo to organic solvent while enabling novel release profiles are crucial for improving immunization coverage worldwide. Here, a novel injectable, ultraviolet- curable and delayed burst release- enabling vaccine delivery platform called polybubbles is introduced. Cargo was injected into polyester-based polybubbles that were formed in 10% carboxymethycellulose -based aqueous solution. This paper includes protocols to maintain spherical shape of the polybubbles and optimize cargo placement and retention to maximize the amount of cargo within the polybubbles. To ensure safety, chlorinated solvent content within the polybubbles were analyzed using neutron activation analysis. Release studies were conducted with small molecules as cargo within the polybubble to confirm delayed burst release. To further show the potential for on-demand delivery of the cargo, gold nanorods were mixed within the polymer shell to enable near-infrared laser activation.

INTRODUCTION:

Limited immunization coverage results in the death of 3 million people specifically caused by vaccine-preventable diseases[1]. Inadequate storage and transportation conditions lead to wastage of functional vaccines and thus contribute to reduced global immunization. In addition, incomplete vaccination due to not adhering to the required vaccine schedules also causes limited vaccine coverage, specifically in developing countries[2]. Multiple visits to medical personnel are required within the recommended period for receiving booster shots, thus limiting the

percentage of population with complete vaccination. Hence, there is a need for developing novel strategies for controlled vaccine delivery to circumvent these challenges.

Current efforts towards developing vaccine delivery technologies include emulsion-based polymeric systems[3,4]. However, cargo is often exposed to greater quantity of organic solvent that can potentially cause aggregation and denaturation, specifically in the context of protein-based cargo[5,6]. We have developed a novel vaccine delivery platform, "polybubbles", that can potentially house multiple cargo compartments while minimizing the volume of cargo that is exposed to solvent[7]. For example, in our polybubble core-shell platform, one cargo pocket of diameter 0.38 mm (SEM) is injected in the center of a 1 mm polybubble. In this case, surface area of cargo exposed to organic solvent would be approximately 0.453 mm^2. After considering the packing density of spheres (microparticles) within a sphere (cargo depot), the actual volume of microparticles (10 µm in diameter) that could fit in the depot is 0.17 mm^3. The volume of one microparticle is $5.24x10^{-8}$ mm^3 and thus number of particles microparticles that can fit the depot is ~$3.2x10^6$ particles. If each microparticle has 20 cargo pockets (as a result of double-emulsion) of 0.25 µm diameter, then the surface area of cargo exposed to organic solvent is 1274 mm^2. Cargo depot within the polybubble thus would have ~2800-fold less surface area exposed to organic solvent compared to that of organic solvent-exposed cargo in microparticles. Our polyester-based platform can thus potentially reduce the quantity of cargo exposed to organic solvent which can otherwise cause cargo aggregation and instability.

Polybubbles are formed based on phase-separation principle where the polyester in organic phase is injected into an aqueous solution resulting in a spherical bubble. Cargo in the aqueous phase can then be injected in the center of the polybubble. Another cargo compartment can potentially be achieved within the polybubble by mixing a different cargo with the polymer shell. The polybubble at this stage will be malleable and will then be cured to result in a solid polybubble structure with cargo in the middle. Spherical polybubbles were chosen over other geometrical shapes to increase the cargo capacity within the polybubble while minimizing the overall size of the polybubble. Polybubbles with cargo in the center were chosen to demonstrate delayed burst release. Polybubbles were also incorporated with near infrared (NIR)- sensitive (i.e., theranostic-enabling) agent, namely gold nanorods (AuNR), to cause increase in temperature of the polybubbles. This effect could potentially facilitate faster degradation and could be used for controlling kinetics in future applications. In this paper, we describe our approach to form and characterize polybubbles, to achieve delayed burst release from the polybubbles, and to incorporate AuNR within the polybubbles to cause NIR-activation.

PROTOCOL:

1. Polycaprolacyone triacrylate (PCLTA) synthesis

1.1. Dry 3.2 mL of 400 Da polycaprolacyone (PCL) triol overnight at 50 °C in an open 200 mL round bottom flask and K_2CO_3 in a glass vial at 90 °C.

1.2. Mix the triol with 6.4 mL of dichloromethane (DCM) and 4.246 g of potassium carbonate

(K_2CO_3) under argon.

1.3. Mix 2.72 mL of acryloyl chloride in 27.2 mL of DCM and add dropwise to the reaction mixture in the flask over 5 min.

1.4. Cover the reaction mixture with aluminum foil and leave it undisturbed at room temperature for 24 h under argon.

1.5. After 24 h, filter the reaction mixture using a filter paper on a Buchner funnel under vacuum to discard excess reagents.

1.6. Precipitate filtrate from step 1.5 that contains the endcapped polymer in diethyl ether in a 1:3 (vol/vol) and rotovape at 30 °C to remove the diethyl ether.

2. Formation of the polybubble

NOTE: Injecting polymer in the deionized (DI) water would cause the polybubbles to migrate to the bottom of the vial resulting in flattened bottom. Use 10% (wt/vol) carboxymethyl cellulose (CMC) fill the glass vial instead to avoid polybubble flattening.

2.1. Prepare 10% (wt/vol) CMC solution in DI water.

2.2. Fill a 0.92 mL glass vial with 0.8 mL of 10% CMC using a 1 mL transfer pipet.

2.3. Mix 1000 mg/mL of 14 kDa PCL in DCM and synthesize PCLTA in a 1:3 (vol/vol) for a total volume of 200 µL or prepare 200 µL of 1000 mg/mL of 5 kDa poly (lactic-co-glycolic acid) diacrylate (PLGADA) in chloroform.

2.4. Mix the 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (photoinitiator) with the polymer (PLGADA or PCL/PCLTA) mixture in 0.005:1 (vol/vol).

2.5. Load 200 µL of polymer mixture into a 1 mL glass syringe mounted on a syringe pump that is connected to a dispensing stainless-steel tube with an inner diameter of 0.016 inch.

2.6. Use a micromotor to control the forward and backward motion of the polymer tube to inject polymer into the 10% CMC in the glass vial to form the polybubble.

2.7. Cure the polybubbles under ultraviolet (UV) at 254 nm wavelength for 60 s at 2 W/cm^2.

2.8. Flash freeze the polybubbles in liquid nitrogen and lyophilize overnight at 0.010 mBar vacuum and at -85 °C.

2.9. Separate the polybubbles from the dried CMC using forceps and wash the polybubbles with DI water to remove any residual CMC. Note that other polymers can be used likely with

modifications to alter the release kinetics.

3. Modulation of polybubble diameter

3.1. Fill a 0.92 mL glass vial with 10% CMC using a 1 mL transfer pipet.

3.2. Mix PCL/PCLTA in a 1:3 (vol/vol) with 1000mg/mL 14kDa PCL and synthesize PCLTA. Mix the photoinitiator with polymer mixture in a 0.005:1 (vol/vol).

3.3. Load the polymer mixture into a 1 mL glass syringe mounted on a syringe pump that is connected to a dispensing stainless-steel tube with an inner diameter of 0.016 inch.

3.4. Use a micromotor to control the forward and backward motion of the polymer tube to inject polymer into the 10% CMC in the glass vial to form the polybubble.

3.5. To obtain polybubbles with various diameters, vary dispensing rate from 0.0005 to 1 µL/s.

3.6. Take images of the vial with the polybubbles with varying diameter.

3.7. Use ImageJ to quantify the diameter of the polybubbles and use the size of the vial as scale.

4. Centering cargo within polybubble

4.1. Modulation of PCL/PCLTA viscosity using K_2CO3:

NOTE: Viscosity of PLGADA does not have to be modified using K_2CO_3 because the viscosity of 5 kDa PLAGDA at 1000 mg/mL is sufficient for centering the cargo.

4.1.1. Add K_2CO_3 (that was isolated after the PCLTA reaction) to the PCLTA at varying concentrations including 0 mg/mL, 10 mg/mL, 20 mg/mL, 40 mg/mL, and 60 mg/mL.

4.1.2. Measure the dynamic viscosities of the solutions by changing the shear rate from 0 to 1000 1/s using rheometry.

4.1.3. Manually inject the cargo in the middle (refer to step 4.2 to prepare the cargo mixture) of the polybubbles that were formed using the PCL/PCLTA solutions with different concentrations of K_2CO_3 (step 4.1.1). Determine the optimal concentration of K_2CO_3 by observing which solution from step 4.1.1 can result in retention of the cargo in the middle.

4.2. Centering of the cargo (already shown feasibility with small molecules) with CMC

4.2.1. Mix the cargo with 5% (wt/vol) CMC in a rotator overnight to increase the viscosity of the cargo.

4.2.2. Manually inject 2 µL of cargo mixture in the polybubble and proceed with UV curing at 254 nm wavelength for 60 s at 2 W/cm^2.

4.2.3. Flash freeze the polybubbles in liquid nitrogen for 30 s and lyophilize overnight at 0.010 mBar vacuum and at -85 °C.

4.2.4. Separate the polybubbles from the dried CMC using forceps and wash with DI water to remove any residual CMC.

4.2.5. Cut the polybubble in half and image the halves using confocal microscopy to ensure that the cargo is centered (refer to step 6 for excitation and emission wavelengths used).

5. Cargo Formulation

NOTE: Polybubble formulation can house various cargo types, including small molecules, proteins, and nucleic acids.

5.1. Based on previous studies, in the case of protein cargo, use excipients including polyethylene glycol (PEG)[6], polyvinylpyrrolidone (PVP), and glycopolymers[6] to improve the stability of protein during polybubble formulation.

5.2. Form polybubbles based on the protocol in step 2.

5.3. Prepare the antigen solution by adding 17.11 g of trehalose to 625 µL of HIV gp120/41 antigen.

5.4. Manually inject 1 µL of antigen solution in the middle of the polybubble.

5.5. Open polybubbles on days 0, 7, 14, and 21, and record the fluorescence of antigen with excitation and emission wavelengths 497 nm and 520 nm, respectively.

5.6. Determine the functionality of the antigen using enzyme-linked immunosorbent assay (ELISA) and use 5% nonfat milk as a blocking buffer.

6. Release of cargo

NOTE: Small molecule or antigen can be used as the cargo type

6.1. Small molecule

6.1.1. Incubate polybubbles with centered acriflavine in 400 µL of phosphate buffer saline (PBS) at 37 °C, 50 °C for PLGADA polybubbles and at 37 °C, 50 °C, 70 °C for PCL/PCLTA polybubbles.

NOTE: The reason why we recommend testing above body temperatures is to a) simulate the temperature (50 °C) at which the polybubble reaches while lasering the gold nanorods (AuNRs) within PCL and PLGA; and b) accelerate the degradation process of PCL (50 °C, 70 °C).

6.1.2. At each time point, collect the supernatants and replace with 400 µL of fresh PBS.

6.1.3. Use a plate reader to quantify the fluorescence intensities in the collected supernatants.

NOTE: Use ex/em of 416 nm/514 nm for acriflavine.

6.2. Antigen

6.2.1. Incubate polybubbles with centered bovine albumin serum (BSA)-488 in 400 µL of PBS at 37 °C, 50 °C for PLGADA polybubbles and at 37 °C, 50 °C for PCL/PCLTA polybubbles.

6.2.2. At each time point, collect the supernatants and replace with 400 µL fresh PBS.

6.2.3. Use a plate reader to quantify the fluorescence intensities in the collected supernatants. Use ex/em of 497 nm/520 nm for BSA-488.

NOTE: Release study at 70 °C for PCL/PCLTA polybubbles should not be conducted to avoid exposing the antigen to extreme temperature.

7. Toxicity

7.1. Quantifying chlorine content in polybubbles using neutron activation analysis (NAA)

7.1.1. Use polybubbles that were lyophilized for 2, 4, 6, 20, and 24 h for this study at 0.010 mBar vacuum and at -85 °C.

7.1.2. Measure 5-9 mg of polybubbles and place them on LDPE irradiation vials.

7.1.3. Prepare 1000 g/mL of chlorine calibration solution from national institute of standards and technology (NIST)-traceable calibration solution.

7.1.4. Use 1- megawatts Triga reactor to carry out neutron irradiations on each sample at neutron fluence rate of 9.1×10^{12} /cm^2·s for 600 s.

7.1.5. Transfer the polybubbles to unirradiated vials.

7.1.6. Use HPGe detector to obtain gamma-ray spectra for 500 s after 360 s decay intervals.

7.1.7. Use NAA software by canberra Industries to analyze the data.

7.2. Quantifying chlorine content released from polybubbles using NAA

7.2.1. Incubate polybubbles that were lyophilized overnight (at 0.010 mBar vacuum and at -85 °C) in 400 µL of PBS at 37 °C.

7.2.2. Collect the supernatants at weeks 1, 2, and 3 after incubation.

7.2.3. Analyze the supernatants for chlorine content using NAA using the same method as described above in step 7.1.

8. AuNR Synthesis by Kittler, S., et al.[8]

8.1. Prepare AuNR seeding solution by mixing 250 µL of 10 mM chloroauric acid ($HAuCl_4$), 7.5 mL of 100 mM cetrimonium bromide (CTAB), and 600 µL of 10 mM ice cold sodium borohydride ($NaBH_4$).

8.2. Prepare Growth solution by mixing 40 mL of 100 mM CTAB, 1.7 mL of 10 mM $HAuCl_4$, 250 µL of silver nitrate ($AgNO_3$), and 270 µL of 17.6 mg/mL ascorbic acid to a tube.

8.3. Vigorously mix 420 µL of seed solution with the growth solution at 1200 rpm for 1 min. Then leave the mixture undisturbed to react for 16 h.

8.4. Remove the excess reagents from the mixture by centrifuging at 8000 × *g* for 10 min and discard the supernatant.

9. Hydrophobicization of AuNRs by Soliman, M.G., et al.[9]

9.1. Adjust pH of 1.5 mL of synthesized CTAB-stabilized AuNRs to 10 using 1 mM sodium hydroxide (NaOH).

9.2. Stir the solution with 0.1 mL of 0.3 mM methylated PEG (mPEG) thiol at 400 rpm overnight.

9.3. Mix PEGylated AuNRs with 0.4 M dodecylamine (DDA) in chloroform at 500 rpm for 4 days.

9.4. Pipet out the top organic layer containing hydrophobicized AuNRs and store at 4 °C until future use.

10. NIR-activation of polybubbles

10.1. Mix the polymer (PLGADA or PCL/PCLTA) solution with hydrophobicized AuNRs in a 1:9 (vol/vol).

10.2. Add photoinitiator to the polymer-AuNR mixture in a 0.005:1 (vol/vol).

10.3. Form polybubbles by injecting the polymer-AuNR mixture into a 0.92 mL glass vial with 10% CMC (wt/vol) (refer to step 2).

10.4. Cure the polybubbles at 254 nm wavelength for 60 s at 2 W/cm^2.

10.5. Flash freeze in liquid nitrogen for 30 s and lyophilize overnight at 0.010 mBar vacuum and at -85 °C.

10.6. Separate the dried polybubbles using forceps and wash with DI water to remove any residual CMC.

10.7. Incubate the polybubbles in 400 µL of PBS at 37 °C.

10.8. Activate the polybubbles using 801 nm NIR laser at 8A for 5 min every Monday, Wednesday, and Friday.

10.9. Take forward-looking infrared (FLIR) images of the polybubble before and after laser activation to obtain temperature values.

10.10. Calculate temperature differences between before and after laser activation based on the temperature values from the FLIR images.

REPRESENTATIVE RESULTS:

Polybubbles were extensively characterized using SEM and NAA. Cargo was successfully centered to result in a delayed burst release. Polybubbles were also successfully laser-activated because of the presence of AuNRs within the polybubbles.

Polybubble characterization

Polybubbles injected in an aqueous solution without CMC resulted in a flattened polybubble due to their contact with the bottom of the glass vial (**Figure 1A,B**). Partial flattening was observed when 5% CMC-based aqueous solution was used in place of DI water (**Figure 1C**). Subsequently, 10% CMC-based aqueous solution in the glass vial resulted in polybubble being suspended in the solution and thus successful maintenance of sphericity of the polybubble (**Figure 1D**).

Cargo centering

Cargo injection into the polybubble in the absence of CMC resulted in leakage causing no retention of cargo within the polybubble (**Figure 3**). To counter this challenge, two approaches were used: 1) viscosity of PCLTA was successfully increased using K_2CO_3 that was isolated after endcapping PCL triol with triacrylate (**Figure 2**), and 2) viscosity of the cargo was successfully increased after mixing the cargo with 5% CMC (**Figure 3,4**). Viscosity of the PLGADA polybubbles were sufficient to facilitate centering of the cargo and thus was not modulated using K_2CO_3.

Antigen functionality

HIV gp120/41 antigen was mixed with and without trehalose before injecting into the polybubble (**Figure 5**). Binding efficiency of antibody to the antigen (termed as functionality) with and without trehalose was observed to have no statistically significant difference.

Release studies without laser activation

Delayed burst releases were observed in PLGADA polybubbles with acriflavine in the middle on days 19 and 5 for polybubbles incubated at 37 °C (**Figure 6A**) and 50 °C (**Figure 6B**), respectively. Delayed burst releases were also observed in PCL/PCLTA polybubbles with acriflavine in the middle on days 160 and 60 for polybubbles incubated at 50 °C (**Figure 7A**) and 70 °C (**Figure 7B**), respectively. These release studies were conducted in the absence of laser-activatable AuNRs.

In vitro laser activation of polybubbles

Polybubbles with AuNRs in the shell were successfully laser activated multiple times in PLGADA polybubbles (**Figure 8A**) and PCL/PCLTA polybubbles (**Figure 8B**). Temperature changes from before and after laser activation were 10 ± 1 °C and 5 ± 1 °C in PCL/PCLTA polybubbles with higher and lower AuNR concentration in the shell, respectively. Temperature changes observed before and after laser activation were 11 ± 2 °C and 6 ± 1 °C in PLGADA polybubbles with higher and lower AuNR concentration in the shell, respectively.

FIGURE AND TABLE LEGENDS:

Figure 1: Maintaining sphericity of polybubbles. SEM images of (**A**) 14 kDa PCL/300 Da PCLTA flattened polybubble due to the contact of polybubble with the bottom of the glass vial; (**B**) 14 kDa PCL/300 Da PCLTA polybubble from the top that was not in contact with the glass bottom; (**C**) the PCL/PCLTA polybubbles with lesser degree of flattening when injected into a 5% CMC solution compared to DI water solution, causing the formation of hemisphere-like shape at the point of contact with the vial; (**D**) polybubble that did not reach the bottom of the glass vial when injected into a 10% CMC solution, allowing for the spherical shape to be maintained. All of the scale bars indicated are 500 μm. This figure has been modified from Lee et al.[7].

Figure 2: Modulation of PCLTA viscosity. Concentration of K_2CO_3 was increased from 0 to 80 mg/mL in PCLTA and dynamic viscosity was observed to increase proportionally with the concentration of K_2CO_3.

Figure 3: Cargo injection into the polybubble with and without CMC. Top panel shows frames extracted from the video of cargo leakage during injection in the absence of CMC. Bottom panel shows frames extracted from the video of cargo retention within the polybubble in the presence of 5% CMC. This figure has been modified from Lee et al.[7].

Figure 4: Centered cargo. Fluorescent microscope images of (**A**) PCL/PCLTA polybubble with centered cargo, (**B**) PCL/PCLTA polybubble with cargo in the shell and centered non-fluorescent dye.

Figure 5: Antigen functionality with trehalose. Functionality of HIV gp120/41 with and without trehalose within the polybubble was analyzed using ELISA. The binding efficiency of an antibody to the protein is generally regarded as an indicator for the functionality of the protein. When we discuss the functionality of antigen in this study, we intend it to mean that it aids the antibodies binding the protein of interest (which is an indicator for protein functionality). No statistical significance was observed between the two groups. Confidence intervals are indicated by solid and dotted lines. This figure has been modified from Lee et al.[7].

Figure 6: Delayed burst release from PLGADA polybubbles. Release studies showing delayed burst releases from PLGADA polybubbles with acriflavine in the middle at (**A**) 37 °C, (**B**) 50 °C. Solid line indicates the fitted curve obtained based on the data points.

Figure 7: Delayed burst release from PCL/PCLTA polybubbles. Release studies showing delayed burst releases from PCL/PCLTA polybubbles with acriflavine in the middle at (**A**) 50 °C, (**B**) 70 °C.

Figure 8: NIR laser activation of polybubbles. Temperature change observed before and after NIR laser activation in (**A**) PLGADA polybubbles, (**B**) PCL/PCLTA polybubbles with higher and lower concentration of AuNRs in the polymer shell. This increase in temperature could be leveraged to potentially expedite the polymer degradation leading to earlier release of the cargo.

DISCUSSION:

Current technologies and challenges

Emulsion-based micro- and nanoparticles have been commonly used as drug delivery carriers. Although release kinetics of the cargo from these devices have been extensively studied, controlling burst release kinetics has been a major challenge [10]. Cargo versatility and functionality is also limited in emulsion-based systems owing to the exposure of cargo to excess aqueous and organic solvents. Protein-based cargo are often not compatible with micro-and nanoparticles due to the possibility of cargo denaturation and aggregation[11]. In addition to cargo stability, cargo kinetics is especially important in the context of vaccines because of the need for booster shots leading to seroconversion. Previous efforts to address these challenges in vaccine delivery have not been sufficiently successful, as the notion of single injection vaccine systems have been around for a couple of decades and has not yet been clinically translated.

Our polybubble vaccine delivery platform can potentially overcome the challenges with increased exposure of cargo to organic solvent by minimizing the exposed cargo volume. This technology can potentially accommodate at least two cargo compartments: cargo in the shell and cargo in the center. Polybubbles with centered cargo can be used to control the burst release of the cargo while being compatible to different cargo types, including small molecules and antigen. In this study, we used polyesters with varying degradation times, PLGADA (shorter degradation time) and PCL/PCLTA (longer degradation time), as the polymer carriers and acriflavine (small molecule) as the cargo type to demonstrate delayed burst release. In the following sections we describe the crucial steps in forming polybubbles that are capable of enabling both delayed burst release and NIR activation, especially for future on-demand delivery applications.

Cargo centering within the polybubble

Cargo centering was one of the significant challenges that was encountered during the formulation of the polybubbles. Immediately after injection, cargo would migrate to the surface and the cargo pocket would be stabilized without bursting into the aqueous 10% CMC solution. Polybubbles with such off-centered cargo can result in earlier release due to the non-uniform thickness of the polymer surrounding the cargo. Modulating the viscosity of the polymer and the cargo was thus crucial in resolving issues related to cargo centering. Viscosity of the cargo was increased by mixing the cargo solution with 5% CMC. To increase the viscosity of the polymer, molecular weight of the polymer could have been modified. However, increasing molecular weight often results in slower polymer degradation thus causing further delay in cargo release. Viscosity of the polymer was thus modified by increasing the concentration of the polymer. Higher concentration (1000 mg/mL) was sufficient to increase the viscosity of PLGADA. However, viscosity of PCL/PCLTA was not adequate to retain the cargo in the middle. Thus, K_2CO_3 that was isolated after the endcapping reaction of PCLTA was used to increase the viscosity of PCLTA.

Novel delayed release

Delayed burst release was observed from the release studies conducted using the polybubbles with centered cargo. Small molecule (acriflavine) was used as centered cargo in the polybubbles to study the release profile. Unique release profiles were observed based on the polyester used due to the difference in the degradation time of the polymers. Burst release was observed earlier in PLGADA polybubbles compared to that of PCL/PCLTA polybubbles. Early cargo release was observed in PLGADA polybubbles because PLGA degrades faster compared to PCL[12]. Upon successful modulation of release kinetics with two types of polyesters, we further wanted to engineer the polybubble to potentially enable on-demand release of the cargo.

NIR-activation of polybubbles

On-demand release of the cargo with respect to timing of the patients' needs has been challenging to achieve using current delivery strategies[13]. We hypothesized that expediting the cargo release on-demand could be possible by accelerating the polymer degradation through the use of NIR- sensitive (i.e., theranostic-enabling) agents. AuNRs have been extensively studied for their ability to be activated using NIR laser that can travel few centimeters through the skin[14]. CTAB-stabilized AuNRs were thus prepared based on the protocol by Kittler, S, et al. and were hydrophobicized based on the methods published by Solimon, M.G., et al. Polybubbles with hydrophobicized AuNRs in the shell were then irradiated with NIR laser at desired time points for 5 min to observe temperature change. Temperatures before and after laser were measured based on the FLIR images. Cured polymer shell helped preserve the shape of AuNRs during the laser activation thus enabling multiple NIR activations of polybubbles. This is an interesting observation because in previous literature, AuNRs have often been known to lose their rod-like shape (crucial for NIR activation) due to laser activation[15]. The successful laser-activation of the polybubbles with AuNRs could pave the way to control on-demand release of the cargo in the next generation of polybubbles.

Significance and future applications

The results obtained from this study thus shows that polybubbles have the potential to be used as a novel vaccine delivery platform. Preparation of polybubbles described in this paper will further enable other researchers to use polybubbles as a delivery platform for other therapeutic applications. For example, in addition to vaccine delivery, polybubbles can also be potentially used to delivery synergistic therapeutic agents with varying release kinetics. Furthermore, polybubbles are made of polyesters that are biodegradable and have been used in many FDA-approved medical devices. We further validated the safety of polybubbles by showing that the chlorine released from polybubbles are well within the safety levels recommended by the EPA[16]. Thus, our novel, injectable, UV-curable polybubble platform has the potential to be used as a safe and effective drug delivery platform for a variety of cargo types.

Limitations of this technology

The polybubble platform technology can be used as a vaccine delivery platform potentially enabling controlled release. Our studies highlight the versatility of this platform capable of delivering different cargo types, including antigens and small molecules. However, one of the current limitations of this technology is that the cargo is currently being injected manually. For scaling purposes, we are currently engineering an automated platform that will enable injection (i.e., as an array) of cargo within the polybubble and will potentially help alleviate the concerns regarding translatability of this technology.

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DISCLOSURES:

Authors have nothing to disclose.

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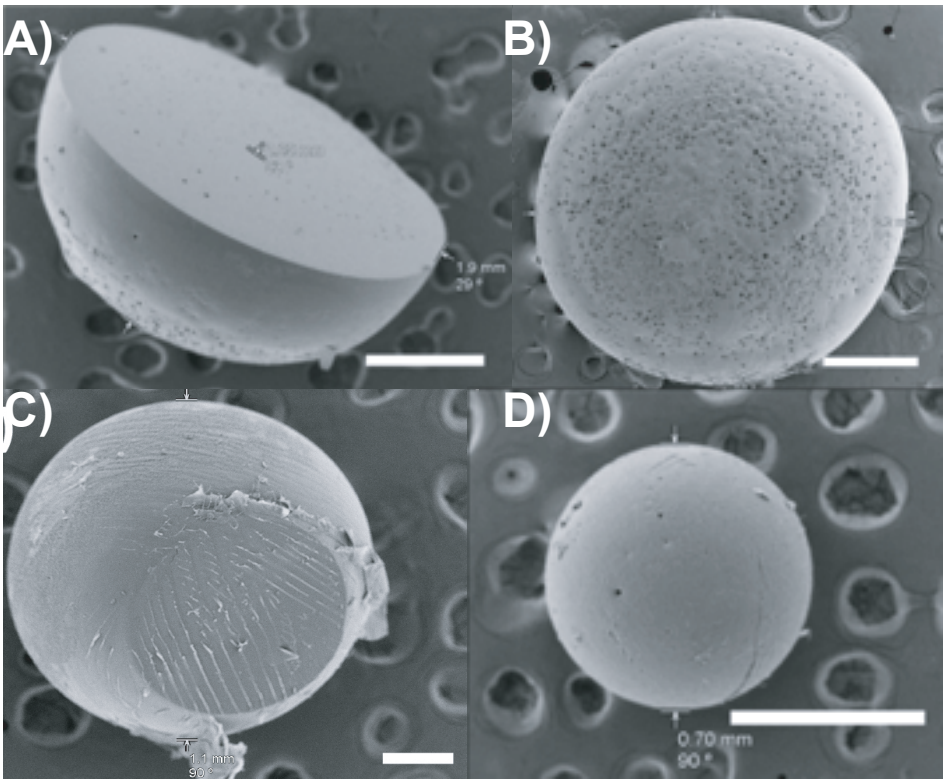

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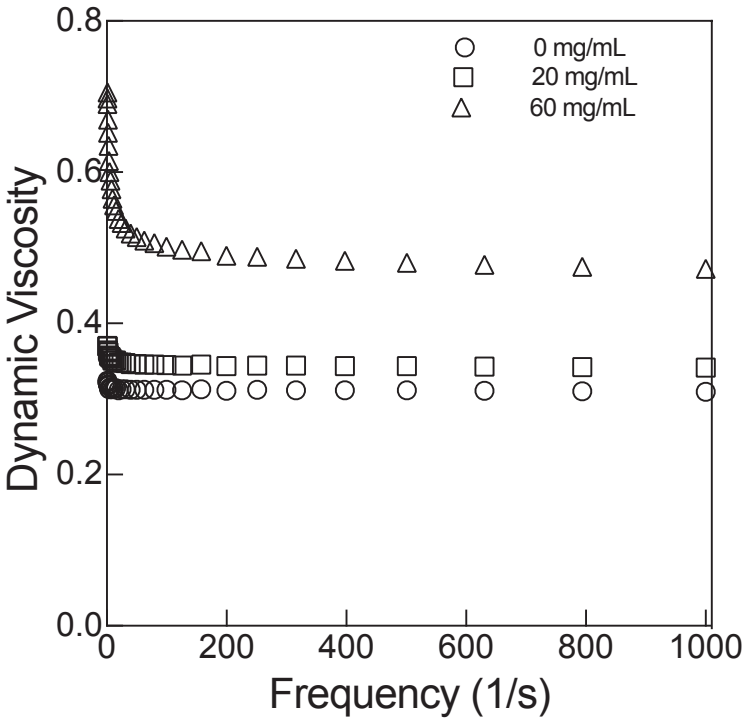

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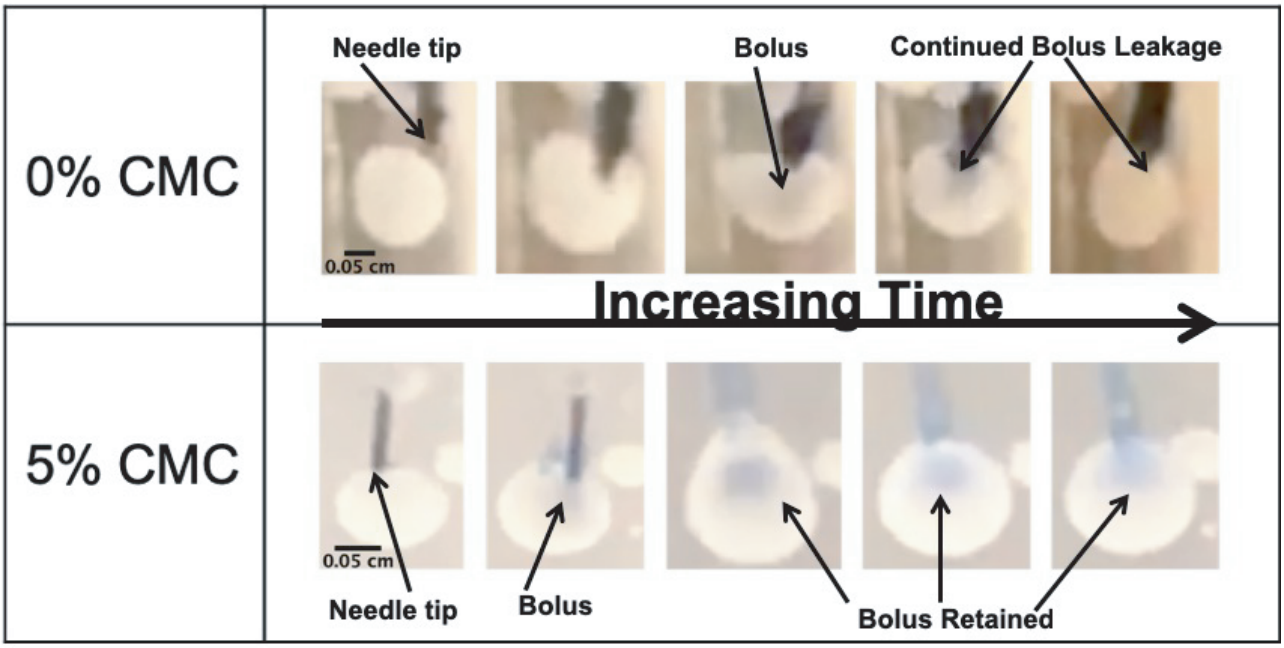

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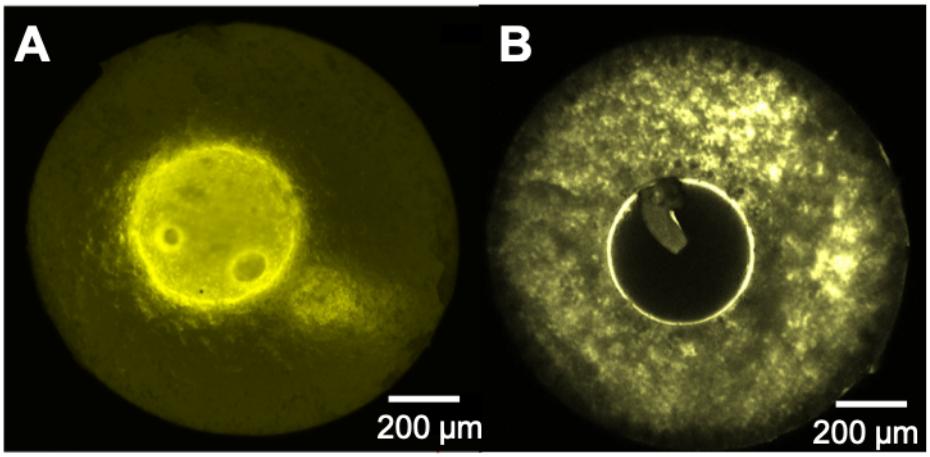

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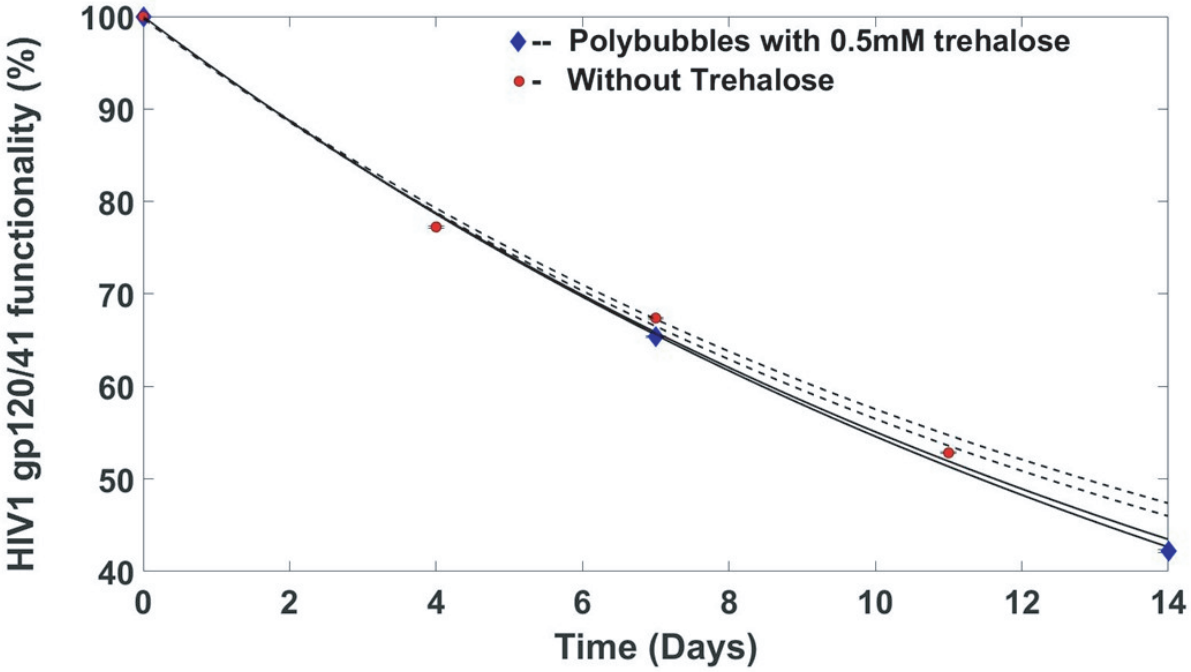

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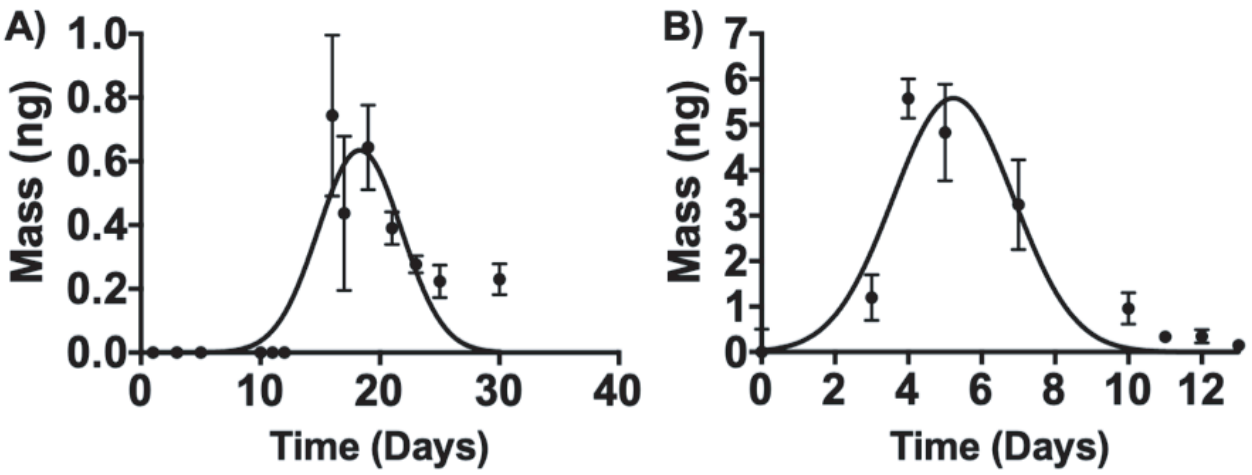

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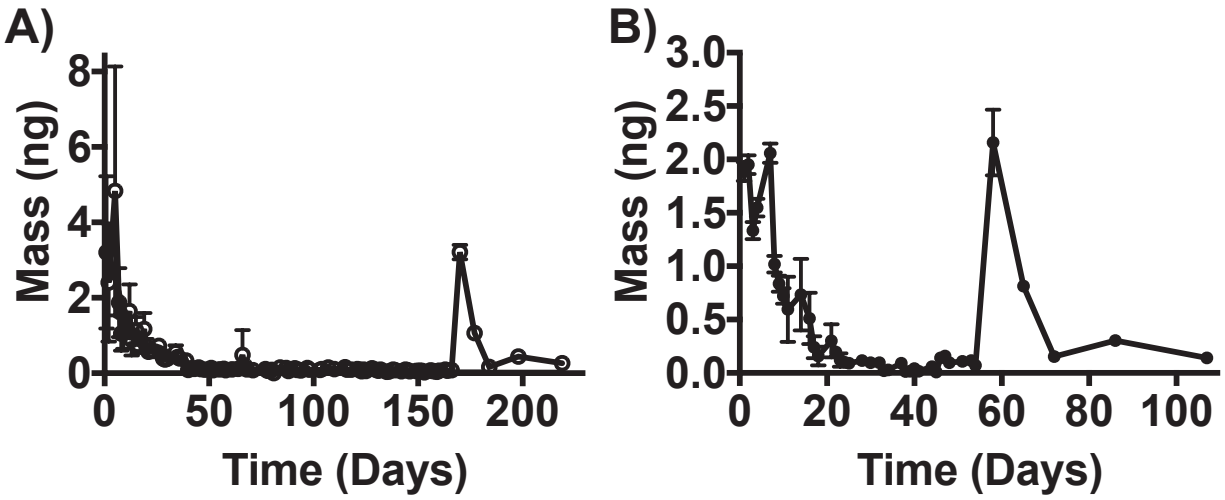

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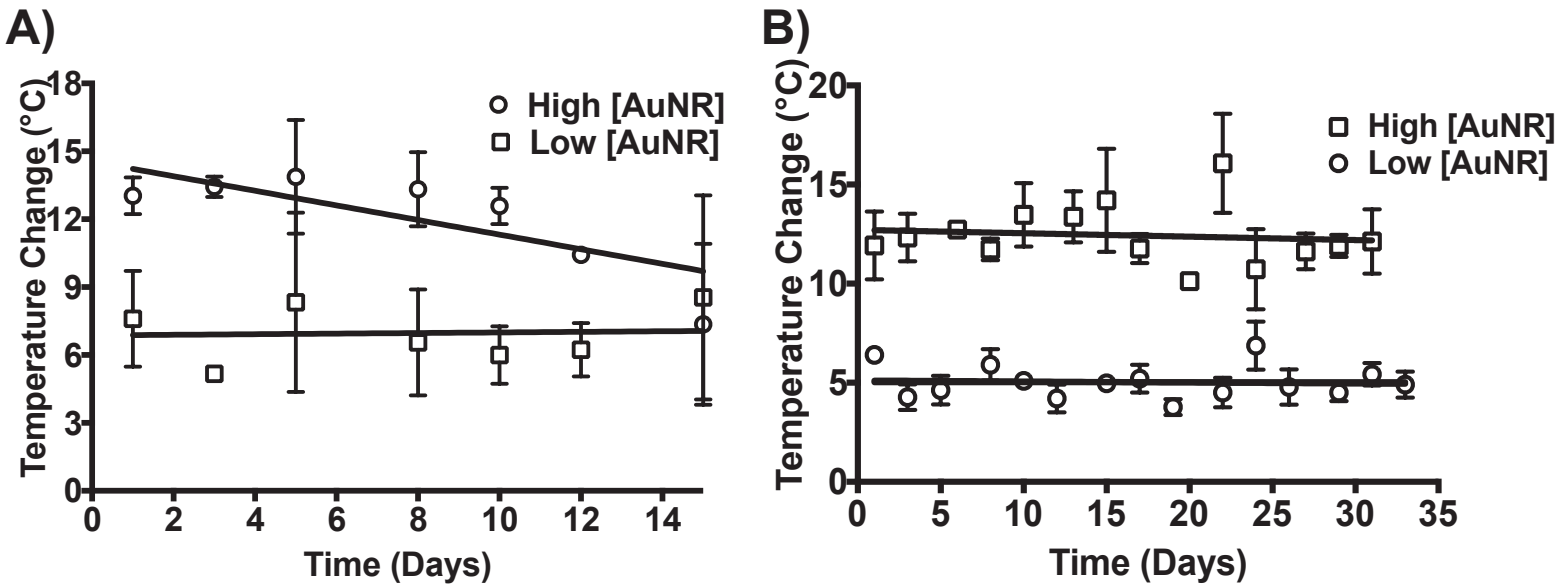

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-Step Ultra Tetramethylbezidine (TMI	Thermo scie	34028	
2-Hydroxy-2-methylpropiophenone	TCI AMERIC/	H0991	
450 nm Stop Solution for TMB Substra	Abcam	ab17152	
Acryloyl chloride	Sigma Aldric	A24109-100G	
Acriflavine	Chem-Impex	22916	
Anhydrous ethyl ether	Fisher Chem	E138-500	
Anti-HIV1 gp120 antibody conjugated to horseradish peroxidase (HRP)			
Bovine serum albumin (BSA)	Fisher BioRe	BP9700100	
BSA-CF488 dye conjugates	Invitrogen	A13100	
Bromosalicylic acid	Acros Organ	AC162142500	
Carboxymethylcellulose (CMC)	Millipore Sig	80502-040	
Centrimonium bromide (CTAB)	MP Biomedi	ICN19400480	
Chloroform	Fisher Chem	C2984	
Coating buffer	Abcam	ab210899	
Dichloromethane (DCM)	Sigma Aldric	270997-1L	
Diethyl ether	Fisher Chem	E1384	
Dodeacyl Amine	Acros Organ	AC117665000	
Doxorubicin hydrochloride	Fisher BioRe	BP251610	
L-ascorbic acid	Acros Organ	A61 100	
Legato 100 Syringe Pump	KD Scientific	14 831 212	
mPEG thiol	Laysan Bio	NC0702454	
Nonfat dry milk	Andwin Scie	NC9022655	
Potassium carbonate	Acros Organ	AC424081000	
Phosphate saline buffer	Fisher BioRe	BP3991	
(Poly(caprolactone)	Sigma Aldric	440744-250G	
(Poly(caprolactone) triol	Acros Organ	AC190730250	
Poly (lactic-co-glycolic acid) diacrylate	CMTec	280050	
Potassium carbonate	Acros Organ	AC424081000	
Recombinant HIV1 gp120 + gp41 prote	Abcam	ab49054	
Silver nitrate	Acros Organ	S181 25	
Sodium borohydride	Fisher Chem	S678 10	

Tetrachloroauric acid	Fisher Chem G54 1
Trehalose	Acros Organ NC9022655
Triethyl amine	Acros Organ AC157910010

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We would like to thank the editor for this suggestion. We have proofread the manuscript ensured that there are no spelling or grammar issues.

2. The highlighted protocol steps are over the 2.75-page limit (including headings and spacing). Please highlight fewer steps for filming.

Thank you for your comment. We modified the highlighted protocol to fit the 2.75 page. Now the highlighted steps are 2 pages long.

3. Reviewer 1's comment: The proposed advantage of this method is decreased exposure of vaccine components to organic solvents compared to conventional microsphere/microcapsule fabrication protocols, leading to better stability of the proteins. However, no data supporting this claim is provided.

We would like to thank the reviewer for their helpful comment. We would like to provide an example to clarify the claim regarding the reduced surface area exposure. We have also added this example to the manuscript for better clarity.

"For example, in our polybubble core-shell platform, one cargo pocket of diameter 0.38 mm (SEM) is injected in the center of a 1 mm polybubble. In this case, surface area of cargo exposed to organic solvent would be approximately 0.453 mm^2. After considering the packing density of spheres (microparticles) within a sphere (cargo depot), the actual volume of microparticles (10 µm in diameter) that could fit in the depot is 0.17 mm^3. The volume of one microparticle is 5.24E-08 mm^3 and thus number of particles microparticles that can fit the depot is ~3.2E6 particles. If each microparticle has 20 cargo pockets (as a result of double-emulsion) of 0.25 µm diameter then the surface area of cargo exposed to organic solvent is 1274 mm^2. Cargo depot within the polybubble thus would have ~2800-fold less surface area exposed to organic solvent compared to that of organic solvent-exposed cargo in microparticles."

We also modified the sentence "Our polyester-based platform can thus potentially alleviate the concerns with cargo aggregation and instability while enabling the release of multiple cargo boluses" to the following to reflect the result from the example used:

"Our polyester-based platform can thus potentially reduce the quantity of cargo exposed to organic solvent which can otherwise cause cargo aggregation and instability"

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BIOMEDICAL ENGINEERING
TEXAS A&M UNIVERSITY

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