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ORIGINAL RESEARCH

PLGA microparticle formulations for tunable delivery of a nano-engineered filamentous bacteriophage-based vaccine: in vitro and in silico-supported approach

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Abstract

Bacteriophages have attracted great attention in the bioengineering field in diverse research areas from tissue engineering to therapeutic and clinical applications. Recombinant filamentous bacteriophage, carrying multiple copies of foreign peptides on protein capsid has been successfully used in the vaccine delivery setting, even if their plasma instability and degradation have limited their use on the pharmaceutical market. Encapsulation techniques in polymeric materials can be applied to preserve bacteriophage activity, extend its half-life, and finely regulate their release in the target environment. The main goal of this study was to provide tunable formulations of the bacteriophage encapsulated in polymeric microparticles (MPs). We used poly (lactic-co-glycolic-acid) as a biocompatible and biodegradable polymer with ammonium bicarbonate as a porogen to encapsulate bacteriophage expressing OVA (257–264) antigenic peptide. We demonstrate that nano-engineered fdOVA bacteriophages encapsulated in MPs preserve their structure and are immunologically active, inducing a strong immune response towards the delivered peptide. Moreover, MP encapsulation prolongs bacteriophage stability over time also at room temperature. Additionally, in this study, we show the ability of in silico-supported approach to predict and tune the release of bacteriophages. These results lay the framework for a versatile bacteriophage-based vaccine delivery system that could successfully generate robust immune responses in a sustained manner, to be used as a platform against cancer and new emerging diseases.

Graphical abstract

Synopsis: administration of recombinant bacteriophage-loaded PLGA microparticles for antigen delivery. PLGA microparticles release the bacteriophages, inducing activation of dendritic cells and enhancing antigen presentation and specific T cell response. Bacteriophage-encapsulated microneedles potentially can be administered into human body and generate robust immune responses.

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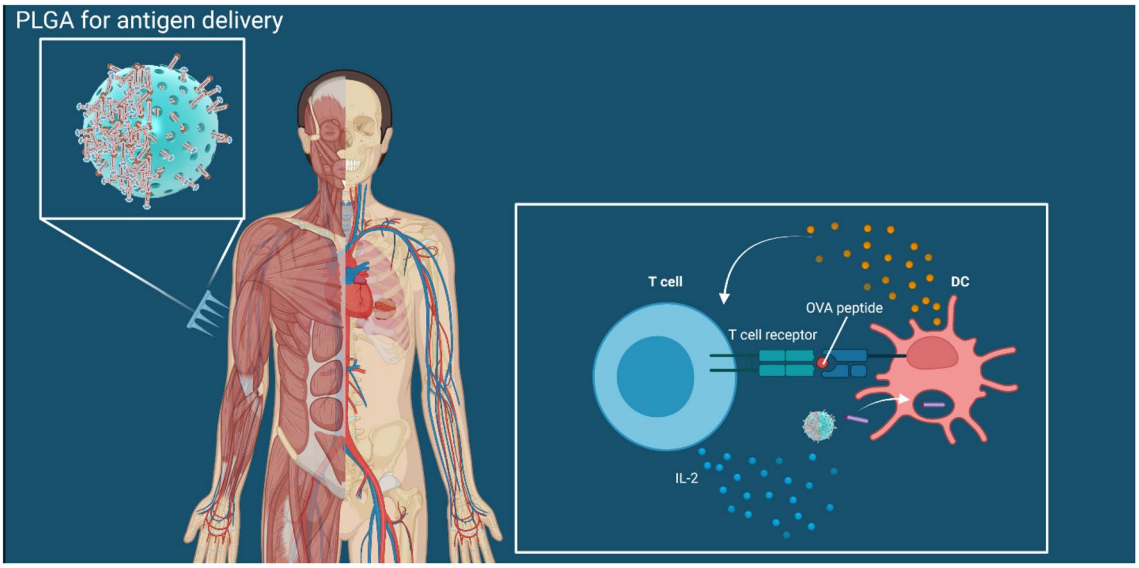

Keywords Controlled release · Immune responses · In silico · Phage display · Vaccination · Nano-engineered bacteriophage

Introduction

Vaccine represents one of the most important inventions in the medical field with promising fatality reduction and population growth. A huge number of previous studies have utilized filamentous bacteriophages, a promising class of nanomaterials, as immunogenic carriers for inducing immune responses against peptides or proteins displayed on their surface [1–7]. Filamentous bacteriophage possesses undoubted superiorities like thermodynamic stability, high biocompatibility, low toxicity, and high antigen density capacity, which make them favorable for vaccine delivery applications [8, 9]. Unmethylated CpG motifs of the bacteriophage genome act as immunostimulatory agents, which subsequently increase immunity [10, 11]. Hence, the fd bacteriophage can stimulate humoral and cell-mediated immune responses without adding any exogenous adjuvants. Bacteriophage-based nano-vaccines have also been demonstrated to induce anti-cancer responses both in therapeutic and prophylactic settings. Bacteriophages expressing epitopes derived from tumor-associated antigen or tumor-targeting peptides were successfully produced and used in vivo in mouse models of tumor engraftment, proving to exert a T cell response protecting animals from tumor growth [4, 12–14].

However, the main issue for vaccine development is designing a platform that stimulates long-term immune responses and preserves the activity of the system even in unfavorable environmental conditions. To meet these goals, the appropriate vaccine delivery system is highly desirable [10, 15, 16]. Particularly, packaging the virions into particles can preserve the integrity of protein structure. MP formulations can extend the half-life of the phage payload and serve as a prolonged-release system, reducing the frequency of administration, and ameliorating the therapeutic index [17]. Up to now, lipid cationic mixture [18] and alginate/$CaCO_3$ microcapsules were applied for entrapment of *Salmonella*-specific bacteriophages [19]. Furthermore, PLGA MPs were utilized to entrap bacteriophage selective for S*taphylococcus aureus* or *Pseudomonas aeruginosa* [20]. However, since the protein structure of the bacteriophage is highly sensitive to environmental conditions, the encapsulation via the double emulsion–solvent evaporation method must proceed with caution [21] or replaced with a post encapsulation strategy [22]. In our previous study, we encapsulated filamentous bacteriophage into PLGA MPs by post encapsulation strategy [10]. The main limitation of this method is the relatively fast release. Indeed, exploiting post encapsulation method, bacteriophages are mainly adsorbed on the external structure of the MPs and exhibited fast release. Here, we demonstrated the ability to make a direct encapsulation thanks to the implementation of the ammonium bicarbonate which makes extremely fast the stripping of the solvent minimizing bacteriophage-solvent contact, thus degradation. Then, we not only compared this method to the post encapsulation in terms of release and immune response, we also combined these two formulations to deliver desired doses of active phages taking advantage from the different kinetic release of the two kinds of MPs which can be coupled by varying their ratio. These therapeutic formulations are stable at room temperature also for a prolonged time and can be used for vaccine administration.

In the present study, we used mathematical models to predict the phage release profile and reach the desired dose of the bacteriophages. Mathematical models represent an outstanding approach to optimize advanced therapeutic agent systems and determine in vitro and in vivo releases [22–26]. They are based on the model fitting of experimental data and enable a quantitative evaluation of the data acquired from a release experiment. In silico models investigate the mechanisms of drug release which decrease the number of experiments. They are specifically promising to evaluate different conditions and methods when the effects of various parameters are included [24]. The quantitative amount and type of the active agent, adjuvants, and polymeric materials can be predicted by mathematical models [27].

Expressed here are the combination and comparison of two different PLGA-MP encapsulation methods to control bacteriophage release and preserve the phage capsid structure. The first method is based on the direct encapsulation of bacteriophage in porous PLGA MPs, while the second one is based on post encapsulation, which allows the elimination of harsh conditions from the act of phage loading. In the end, a non-linear first-order in silico model was applied to predict and adjust their release from PLGA MPs-based formulations which was then verified by the experimental results.

Experimental methods

Materials

Poly (lactic-co-glycolic acid) 50:50 (PLGA RESOMER RG 504H, 38,000–54,000 Dalton), was purchased from Ivonik industries. Dichloromethane (DCM) sodium acetate anhydrous, Ammonium bicarbonate (ABC), fluorescein isothiocyanate isomer I (FITC), Polyethylene glycol 6000 (PEG), Poly (vinyl alcohol) (PVA), Sodium chloride (NaCl), were purchased from Sigma Aldrich.

Bacteriophage production and purification

The bacteriophage coat of the hybrid filamentous virions was mainly composed of recombinant subunits of the protein pVIII interspersed with not-engineered pVIII copies. The hybrid bacteriophage fdOVA, delivering the MHC H-2b-restricted SIINFEKL peptide (chicken ovalbumin protein, aminoacids 257–264) fused to pVIII proteins, was generated as previously described [28]

PLGA MP preparation

The microencapsulation of bacteriophage was performed by the double-emulsion ($W_1/O/W_2$) solvent evaporation method as already described [10, 22, 29, 30]. Particularly, for direct encapsulation, 100 mg PLGA was dissolved in 1 mL dichloromethane. Next, 100 µL (7 mg/mL) bacteriophage was added to PLGA solution. 100 µL ammonium bicarbonate (7.5 mg/mL water) as porogen agent was added and homogenized at 20,000 rpm for 30 s, afterward 10 mL of 2% poly(vinyl alcohol) was added and homogenized at 25,000 rpm for 1 min. The solution was then poured into water and agitated in order to completely evaporate the organic solvent. MPs were washed with PBS three times to remove poly(vinyl alcohol) then placed into freeze dryer overnight.

The post-encapsulated bacteriophage as already described were prepared by ($W_1/O/W_2$) (Fig. 1a) [10].

The mean size and scanning electron microscopy

The particle size distribution of the MPs was analyzed using the (Mastersizer2000, Malvern Instruments, Malvern, UK) [10, 22, 31]. Freeze dried MPs (3 mg) were added in water and placed into the tank to evaluate the mean size.

Surface morphology was viewed by scanning electron microscope (LEO1550) after coating the particle surface with gold (10 nm thickness) with a HR208 Cressington sputter coater and analyzed by FESEM ULTRA-PLUS (Zeiss) with the SE2 detector [32, 33].

EDX analysis

To reveal the presence of phages in the samples the Energy Dispersive Xray (EDX) was performed to find sulfur associated to phage proteins by monitoring the absorption band peaking at 2307 keV.

Fluorescein isothiocyanate functionalization and confocal microscopy

100 µL of Hybrid fdOVA virions (7 µg/µL) in carbonate buffer pH 8.2 were conjugated using 20-fold molar excess of Fluorescein isothiocyanate and stirred for 1 h, as previously described [10]. Confocal analysis was carried out applying a Leica SP5 confocal microscope and evaluating the samples at λ_{exc} at 405 nm, λ_{em} 420–500 for a DAPI signal and λ_{exc} 488 and λ_{em} 500–600 for the FITC channel, as already described [34].

Release of bacteriophage from PLGA microparticles and encapsulation efficacy

Lyophilized bacteriophage-encapsulated MPs (5 mg) were resuspended in PBS1X and kept under stirring in a shaker. At defined time points, samples were centrifuged at 3468×*g* and the supernatants were analyzed by UV–Vis assay of

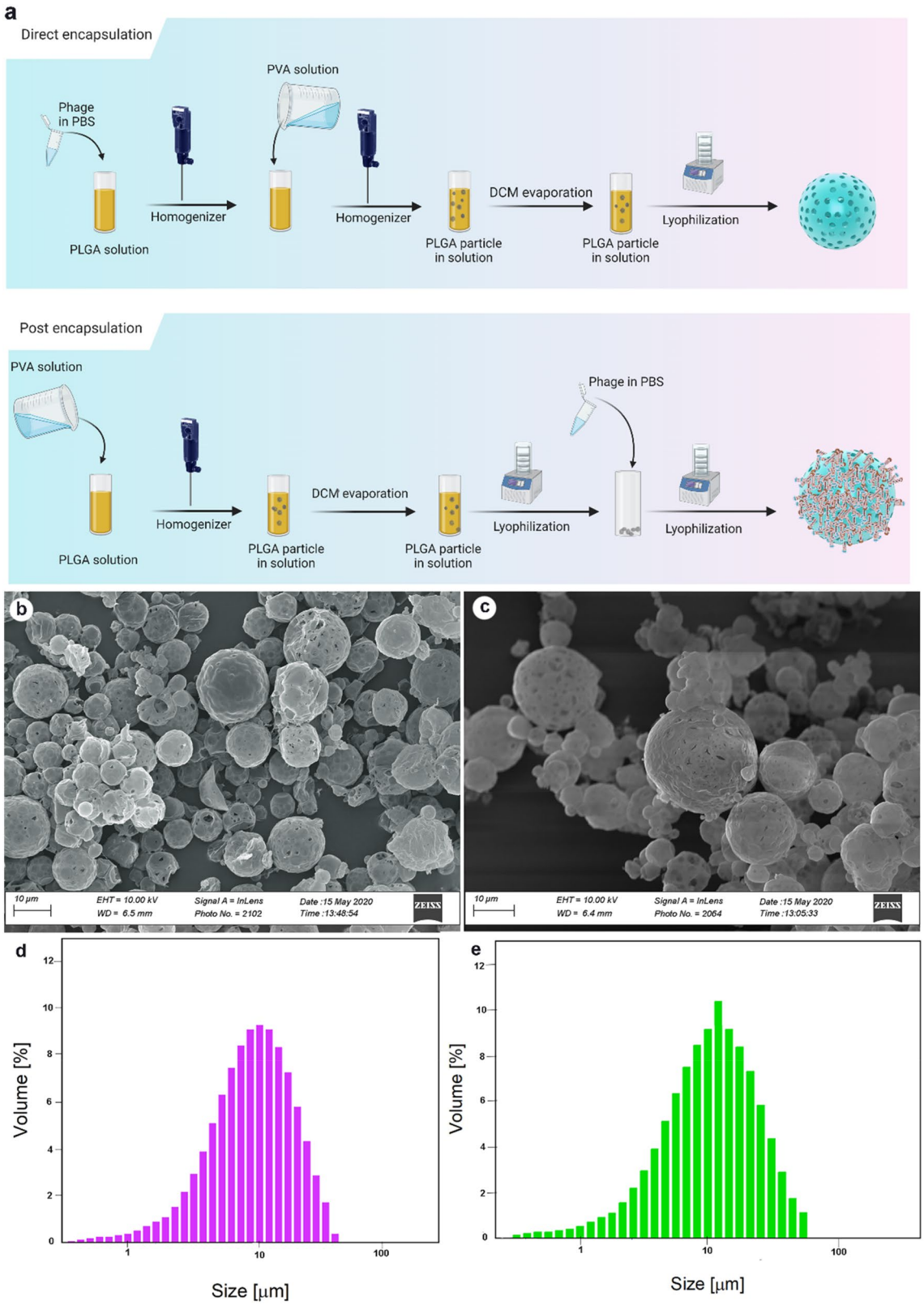

Fig. 1 Microparticle production by double emulsion method and characterization. **a** Direct encapsulation; bacteriophage was added in the water phase. While for post encapsulation empty microparticles were prepared and then resuspended in the bacteriophage solution. **b**, **c** Scanning electron microscopy of post encapsulated and direct encapsulated phage-MPs. **d**, **e** Mean diameter of the post-encapsulated phage is 10 µm and for direct encapsulation is 12 µm. Scale bar represents 10 µm

bacteriophage concentrations, as already described [10]. The release experiments were carried out in triplicate within 8 h for post encapsulation and 72 h for direct encapsulation.

The release experiments for (75% post + 25% direct), (50% post + 50% direct), and (25% post + 75% direct) were performed.

For encapsulation efficiency, the freeze-dried powders were weighed (5 mg) and dissolved in DMSO, NaOH and SDS as already described [10]. Concentration of bacteriophage was evaluated by UV–Vis spectroscopy. Encapsulation efficiency was calculated applying the following equation.

$$\text{Encapsulation efficiency}(\%) = \frac{\text{quantity of bacteriophage entrapped}}{\text{total quantity of bacteriophage added initially}} \times 100 \quad (1)$$

Circular dichroism

Circular dichroism analysis was carried out with Jasco J-1000 spectropolarimeter (JASCO Corp, Milan, Italy), as already reported [10, 34–39]. Free bacteriophages were evaluated at 0.22 mg/mL while post and direct phage encapsulated were both diluted at 0.14 mg/mL to have the same concentration after 6 h of release.

Colony-forming unit determination

To assess the bacteriophage bioactivity, infective bacteriophages were enumerated by the colony forming unit (CFU) assay using the plating method. Briefly, PLGA MP-released bacteriophages were serially diluted in PBS and aliquots of each dilution (10 µL) was added to of 0.6 OD_{600} *E. coli* TG1 *recO* cells. The mixtures were plated on top of selective LB-agar plates with 100 µg/ml Ampicillin. Positive and negative controls were represented by a bacterial culture infected with a known concentration of purified fdOVA virions, or a bacterial culture without bacteriophage. Plates were incubated at 37 °C, the number of the colonies was counted for each dilution, and phage titer was determined as CFU/ml.

Bone-marrow derived-dendritic cells generation

Bone-marrow-derived dendritic cells [BM-DCs) were differentiated from precursors isolated from tibiae of C57BL/6 mice (Charles River (Lecco, Italy)] as previously described [10]. Briefly, mice were euthanized and the bone-marrow was isolated from the tibiae. Clusters of cells were disrupted, and cells were plated at a density of 250,000/mL in RPMI 1640 complete medium (10% fetal calf serum (FCS), 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin) in presence of 200 U/mL recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF) for 7 days. The immature dendritic cells phenotype was confirmed at day 7 using the monoclonal antibody anti-CD11c-PE-Cy7 (HL3, BD Biosciences) and FACScanto II cytometer (BD Biosciences).

Analysis of IL-6 production

To analyze IL-6 production, BM-DCs (1×10^6/mL) at 7th day of culture were incubated with PLGA-MPs resuspended in PBS at a concentration of 0.1 and 1 mg/mL and cultured in complete RPMI medium. DCs were also left untreated (medium) as the negative control. After 20 h of incubation, cells were centrifuged, supernatants were collected and IL-6 production was measured in supernatants of cultures (0.1 mL/well), according to the manufacturer's instructions, using a commercially available ELISA kit [mouse IL-6 ELISA MAX™ Standard (Biolegend)].

BM-DC cross-presentation assay

fdOVA encapsulated directly or by the post encapsulation method was released from microparticles, and 1×10^6/mL BM-DCs were incubated overnight with 0.06 µg/mL of released bacteriophages (Fig. 6) [10].

Alternatively, BM-DCs (1×10^6/mL) were left to adhere to multiwell plates. Phage-loaded MPs were resuspended in PBS, and a volume of MPs containing 0.06 µg/mL of encapsulated fdOVA was immediately added to adherent BM-DCs.

BM-DCs were then washed twice and plated at 100,000 cells/well, and the antigen cross presentation was detected by adding the OTI hybridoma cell line B3Z (50,000 cells/well) to the culture. The IL-2 released in the culture medium by activated B3Z cells was measured after 40 h using the supernatants of the co-cultures (0.1 mL/well) and a mouse IL-2 ELISA MAX™ Standard (Biolegend).

In silico release study

A mathematical model describing and predicting bacteriophage direct and post encapsulation releases was developed as previously described [29]. First, experimental data were normalized to extract releases for 1 mg of MPs. Then, these data were fitted with an exponential growth model, using Matlab® (v.R2019a). Specifically, the bacteriophage release B_r was defined by:

$$B_r = \boldsymbol{a}(1 - e^{-\boldsymbol{b}t}) \quad (2)$$

a and b were the fit coefficients. $y = 0$ at $t = 0$ was assumed as the initial condition. In the case of MPs with bacteriophage entrapped by post-encapsulation method, Eq. (1) was considered valid for a time t in the interval $0 \le t \le 6h$, being data for $t > 6h$ unavailable having the bacteriophage been already completely released.

Starting from Eq. 1, the release was predicted using a non-linear first-order model:

$$B_r = \frac{\sum_1^n B_n a_n \left(1 - e^{-b_n t}\right)}{\sum_1^n B_n} \text{with} \sum_1^n B_n = B_1 + B_2 + \cdots + B_n = 100 \quad (3)$$

where a_n and b_n were the fit coefficients, B_n was the percentage of weighted bacteriophage MPs, n was the amount of different MP formulations considered; in this specific case we fabricated two MPs encapsulating bacteriophage either directly or with a post-process, then $n = 2$.

Statistical analysis

The statistical analyses were performed using GraphPad Prism v5 (San Diego, CA, USA). The statistical differences between the means were assayed using Student's t test or Analysis of Variance (ANOVA) test, and the differences between groups were analyzed with Tukey's multiple comparison test. Statistically significant differences were set at 0.05 and results were presented as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Results

Scanning electron microscopy and size distribution of fdOVA-loaded MPs

The fdOVA bacteriophage particles were produced and purified in *E. coli* bacterial cells, and then encapsulated in PLGA MPs, by two methods: (1) direct and (2) post encapsulation techniques.

To compare the differences between the direct ad post encapsulated MPs, their morphology was assessed using scanning electron microscopy Fig. 1b, c. The nanostructured porosities regulated the kinetic release of bacteriophage from the polymeric system.

The dimensional distribution of MPs was also assessed with a Malvern Mastersizer. The obtained results demonstrated that phage-encapsulated MPs have a uniform distribution with a mean diameter of 10 µm for post encapsulated bacteriophage-MPs and 12 µm for direct encapsulated bacteriophage-MPs (Fig. 1d, e). The procedure used for PLGA MPs production provide a quite porous inner microstructure as shown in Fig. S3.

Moreover, the presence of phage in both MPs was evaluated by EDX analysis. In detail, the S element was used as a marker, as it is present in phage coat proteins. Both MPs encapsulation displayed the signal for elemental S and other chemicals (C,O) as showed in Fig. S2. The distribution of the 3 main elements found in each sample was reported as EDX map, where the sulfur element was found in overlap with the microparticles.

Assessment of fdOVA encapsulation in the PLGA MPs by confocal microscopy

To evaluate the bacteriophage loading by encapsulation or adsorption technique, we used FITC-conjugated bacteriophages, and the morphology of bacteriophage-loaded MPs was assessed by confocal microscopy. In detail, FITC-functionalized bacteriophage encapsulated PLGA MPs were analyzed using a λ_{exc} of 488 nm and a λ_{emiss} between 500 and 600 nm while the DAPI range was utilized to assess the porosities of the particles by exploiting the autofluorescence of PLGA. As shown in Fig. 2, the correct loading of fd-FITC through adsorption (panel A–C) or direct encapsulation (panel D–F) in PLGA MPs was assessed. In particular, in the first case it was possible to notice how the virions were mainly adsorbed on the external structure of the MPs, while using the direct encapsulation, they were found more uniformly distributed all over the MPs.

In vitro release and encapsulation efficiency

Freeze dried MPs were suspended in PBS and allowed to release bacteriophages. At defined time points, samples were centrifuged and supernatants were analyzed by UV–Vis spectroscopy to quantify released bacteriophage concentration following its characteristic peak at 269 nm.

Both of the bacteriophage-loaded MPs formulations exhibited sustained release in vitro, showing an initial burst release followed by a relatively slow-release (Fig. 3). The phage-loaded MPs by post encapsulation method showed faster release compared with direct encapsulated phage-MPs: after 6 h all the bacteriophages were released by post encapsulated phage MPs, while in the case of direct encapsulated phage-MPs, all the bacteriophages were released after 72 h. Encapsulation efficacy of bacteriophage into PLGA MPs for post and direct encapsulation were calculated 40% and 78% respectively, demonstrating that the post encapsulation method has a lower encapsulation efficiency but a faster release kinetic.

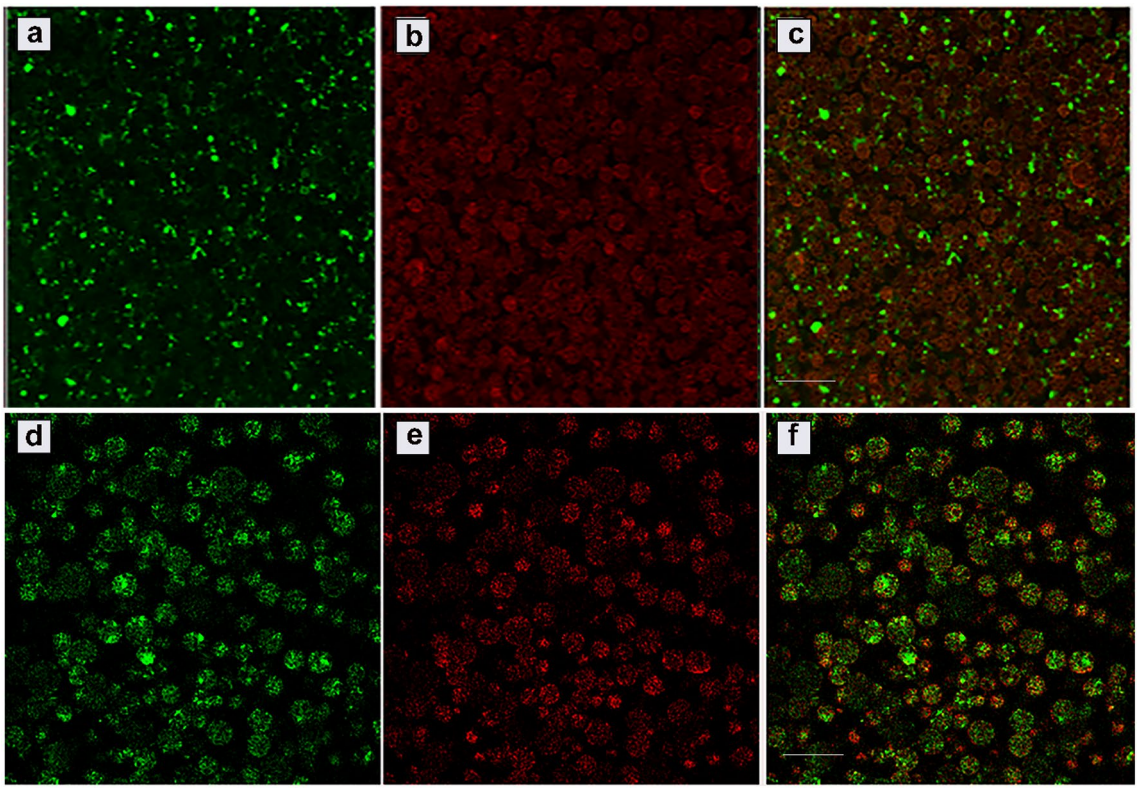

Fig. 2 Confocal microscopy or post (**a**–**c**) and direct (**d**–**f**) encapsulation. Fluorescence images were acquired using a λ_{exc} of 488 nm and a λ_{emiss} between 500 and 600 nm. Red channel is related to PLGA acquired in DAPI range. Scale bars represent 50 µm, 40 µm

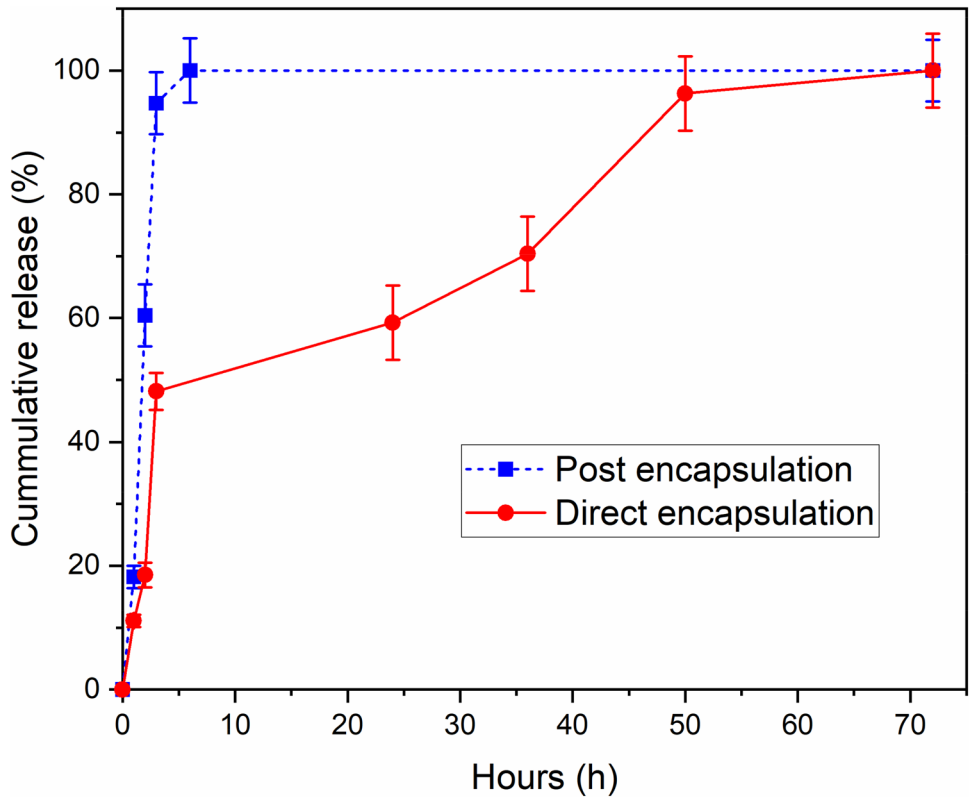

Fig. 3 In vitro release kinetic study of the two bacteriophage-MPs formulations. The phage release versus the time is shown. Cumulative bacteriophage release for direct encapsulation (red) and for post encapsulation (blue) demonstrate that all the bacteriophages were released after 72 h or 6 h, respectively ($n = 3$)

Analysis of the structure of the encapsulated bacteriophages by circular dichroism spectroscopy

Filamentous bacteriophage fd has a proteinaceous coat composed by 2700 copies of the major coat protein pVIII arranged in a closely packet surrounding viral DNA, and 5 copies of several minor proteins [8]. In the intact phage particles, pVIII is mostly alpha helical, with the negative ellipticity at 222 nm and a 205–208 nm minimum [40].

Circular dichroism (CD) analysis of direct encapsulated bacteriophage as compared to the free and post encapsulated ones was investigated. Experiments were performed in 10 mM phosphate buffer at pH 7.4 at a concentration of 0.14 mg/mL for both formulations and concentration of 0.22 mg/mL for free bacteriophages as we already reported in our previous work [10]. As shown in Fig. 4, the direct encapsulated bacteriophage (red spectrum) revealed a predominant β-sheet conformation underlying as this method causes aggregation processes probably due to the exposure of the protein surface of the bacteriophage to the dichloromethane solvent. By contrast, post encapsulated and free bacteriophages (green spectrum and blue, respectively) represent a typical mixed α-helix and β-sheet conformation with a preponderance of helical conformation corroborated by presence of two negative minimum centered at 222 nm and 205 nm, together with a positive shoulder at 190. This last result certified the hypothesis that the post-encapsulation technique does not affect the secondary structure of proteins expressed by the bacteriophages.

Biological activity of filamentous bacteriophage after encapsulation

Filamentous bacteriophage infects *E. coli* bacteria cells by interaction of the phage protein pIII located at the end of the phage with the tip of the F-pilus of *E. coli*, and subsequent integration of the coat proteins and DNA of the phages into the bacterium [41]. Therefore, the intact structure of the phage is essential to allow it to carry out its biological cycle, and is a hallmark of the integrity of the capsid structure.

Starting from the results obtained by CD spectroscopy, we evaluated the biological activity of filamentous bacteriophages following encapsulation using both strategies. Freeze-dried MPs were resuspended in PBS and phages were allowed to be released. TG1 *E. coli* bacterial cells were then infected with the bacteriophage-containing supernatants. The fdOVA bacteriophage is derived from the modified form of fd phage (fdAMPLAY88), which genome contains the gene encoding for beta-lactamase, conferring the Ampicillin resistance [2, 42]. The bacteriophage titer is then assessed by counting the colony forming units (CFU) of bacteriophage-infected bacteria growing on ampicillin-containing plates. The capability of released bacteriophage to infect bacteria cells by CFU assay demonstrated the good retention

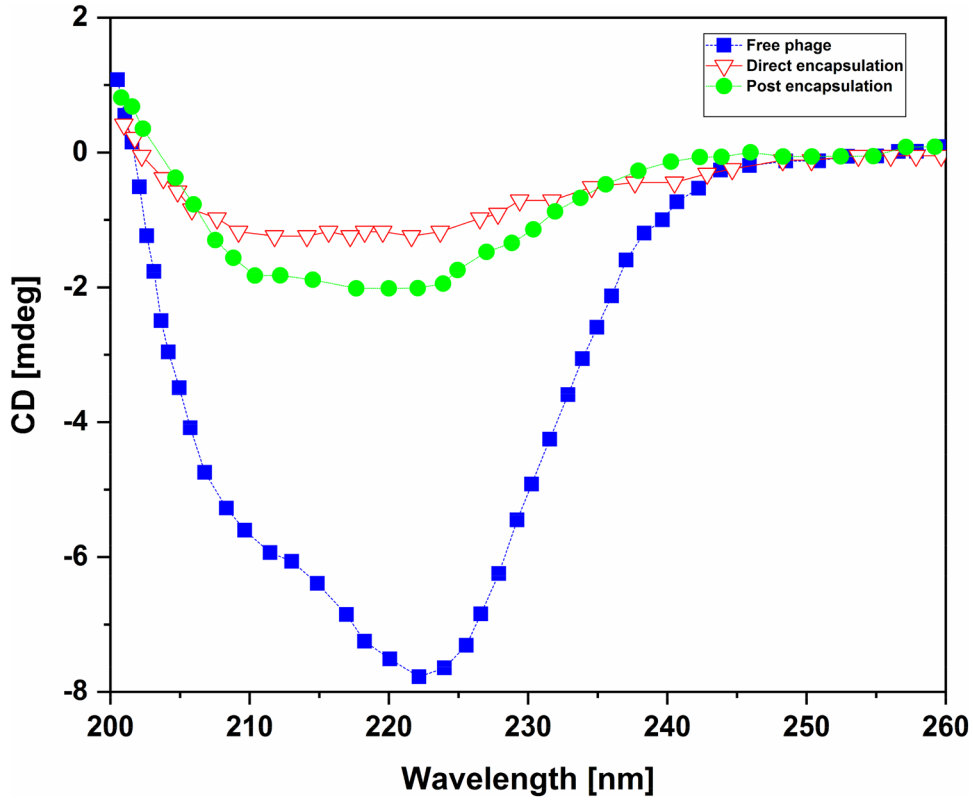

Fig. 4 A Circular dichroism (CD) spectrum of free bacteriophage (blue spectrum), bacteriophage released from post-encapsulated MPs (green spectrum) and bacteriophage released from direct-encapsulated MPs (red spectrum)

of activity of the bacteriophages for both methods of encapsulation (Fig. 5a). A more significant reduction in phage titer was observed for direct encapsulation method. This difference may be due to a greater degree of stress due to the exposure of the bacteriophages to the water-dichloromethane interface.

Moreover, we analyzed the stability of the bacteriophage inside lyophilized MPs prepared by the two encapsulation methods. The stability of vaccines throughout the manufacturing, storage, and shipping process is critical to the success of immunization programs. The temperature sensitivity is one of the crucial requirements to support clinical trial approval of a vaccine, and a major challenge to reach all the world population. The encapsulation of a vaccine based on phage nanoparticles in PLGA MPs can ensure its improved stability as well as provide a controlled release. Lyophilized phage-containing MPs were stored at 4 °C for different times, particles were resuspended in PBS and the released phages were assayed by Colony-forming Unit Determination. As shown in Fig. 5b, the encapsulation of bacteriophages into PLGA MPs was able to successfully protect phage particles. In fact, we observed that the titer of the phages released from MPs remains the same after the different times, without any significant difference ($p > 0.05$ by two-way ANOVA). Previous works have demonstrated that PLGA MPs are able to provide a protective and stable environment to encapsulate drugs and pharmaceutics. Bacteriophage particles encapsulated with the two different methods (direct encapsulation, and post encapsulation) retained the same activity following MPs reconstitution and virion release. The stability of the bacteriophage in the freeze dried MPs was followed over a period of 60 days. We can conclude that both the MPs formulations are able to preserve intact the infectivity of encapsulated phage over time when stored at 4 °C. Although some bacteriophages lost their activity after the lyophilization process by microencapsulation as compared to free fdOVA, but we can take advantage of their prolonged release in terms of generating robust immune responses as it can be seen in Fig. 6c. Additionally, despite the well-known instability of bacteriophages at room temperature here, we assessed the capability of MPs to store phage activity when kept at room temperature (22 °C). fdOVA encapsulated both by post or direct method were stored at 22 °C and the phages released from MPs were analyzed at different times by Colony-forming Unit Determination. As shown in Fig. 5c, we found that direct encapsulation formulation is able to guarantee the same phage activity also after 60 days at 22 °C, while the phage released by post encapsulation method showed a significant loss of the 39% of active particles after the first 30 days of storage at room temperature; after other 30 days, the bacteriophage titer remains almost the same ($p > 0.05$).

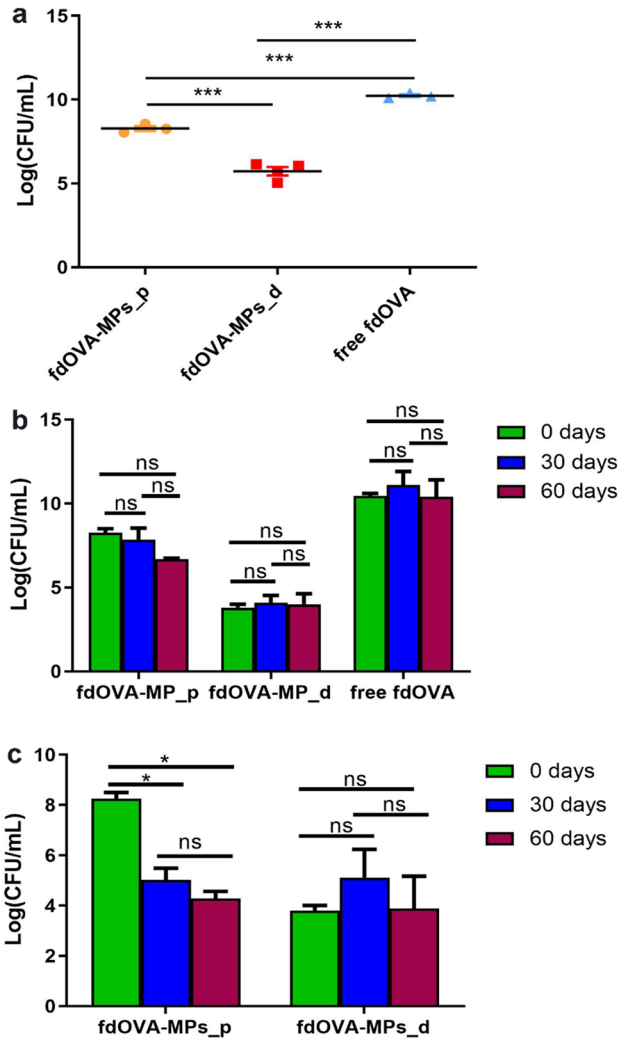

Fig. 5 Biological activity of the encapsulated bacteriophages. **a** Bioactivity of free filamentous bacteriophage fdOVA was compared to the bioactivity of fdOVA released from MPs prepared with the two different methods (direct encapsulation, fdOVA-MPs_d, and post encapsulation fdOVA-MPs_p). The bioactivity was measured as the capability to infect permissive *E.coli* cells and was expressed as the number of colony-forming units (CFU) of TG1 *E. coli* cells infected with a bacteriophage that grew on Ampicillin plates. Each measurement was performed in triplicate, and the median and SEM of three different experiments is reported (black line). Differences of CFU values among fdOVA-MPs_p, fdOVA-MPs_d and free fdOVA groups are statistically significant by one-way ANOVA (***$p < 0.001$). **b–c** Stability of filamentous bacteriophages encapsulated in PLGA MPs using both direct encapsulations, and post encapsulation methods, with free fdOVA bacteriophages used as a control. MPs were stored at 4 °C (**b**) or 22 °C (**c**), and the titer of encapsulated bacteriophages was measured at the reported time points. Each measurement was performed in triplicate, and the mean + SEM is reported. Differences among bars in each group in (**b**) are not statistically significant by two-way ANOVA ($p > 0.05$). Differences among bars in fdOVA-MPs_d group in (**c**) are not statistically significant ($p > 0.05$), while differences among bars in fdOVA-MPs_p group are significant by two-way ANOVA (*$p < 0.05$); (ns = not significant)

PLGA-MPs induction of IL-6 production by DCs

Activation of antigen-presenting cells is important for the induction of an effective adaptive immune response, so we analyze if PLGA MPs can have an adjuvant effect activating the BM-DCs. Immature mouse BM-DCs were generated from bone-marrow precursors, and the effect of PLGA MPs on the production of the pro-inflammatory cytokine IL-6 by DCs was then assayed. To avoid unspecific effects due to endotoxin contamination of the samples, the levels of LPS in MP samples were assayed. The endotoxin levels were < 0.1 EU/mL, under the kit detection limit. BM-DCs were incubated in the absence or presence of empty PLGA MPs at 0.1 or 1 mg/ml for 24 h, and IL-6 concentrations in the supernatants were measured by ELISA. Filamentous bacteriophage is able by itself to induce the production of pro-inflammatory cytokines by BM-DCs (1–2). So, the booster effect exerted by PLGA encapsulation was assayed. PLGA MPs were able to induce the production of IL-6 by bone-marrow-derived DCs in a dose-dependent manner (Fig. 6b, c), demonstrating the adjuvant proprieties of PLGA MPs.

Antigen-specific immune response to encapsulated fdOVA bacteriophage

To assess the immunogenicity of the antigen delivery system based on filamentous phages after direct or post encapsulation in PLGA MPs, we have evaluated the activation of the OVA-specific hybridoma cell line B3Z in response to fdOVA delivering the SIINFEKL peptide.

B3Z is a CD8 + T cell line producing IL-2 exclusively in response to the activation of its SIINFEKL specific T cell receptor [10] and the IL-2 production correlates with uptake and processing of the fdOVA and with $OVA_{257-264}$ cross-presentation by DCs. MPs were resuspended in PBS and allowed to release fdOVA in solution. Thus, the empty MPs were discarded and supernatants containing phages were incubated with BM-DCs. The antigen-specific response to fdOVA released from MPs was compared to the one obtained by directly adding whole fdOVA-MPs to the BM-DC cultures. Phage-loaded MPs were resuspended in PBS and immediately added to adherent BM-DCs. The amount of MPs added to the culture was chosen to obtain the release of an equal amount of fdOVA (about 0.06 µg/mL) after 20 h of DC incubation.

After incubation, B3Z cells were co-cultured with BM-DCs and the IL-2 production by the hybridoma cell line B3Z was thus analyzed by ELISA (Fig. 6a).

As shown in Fig. 6c, we found that both the fdOVA-MPs preparations (produced using the two different encapsulation methods) can stimulate IL-2 release by B3Z cells without significant differences among them. This finding demonstrates that both methods of encapsulating are efficient in preserving intact the antigenic properties of the recombinant fd phages and that after releasing from MPs, fdOVA is correctly presented to T cells. We observed the progressive release of fdOVA from MPs induced a stronger activation of the B3Z cells, compared to the activation obtained using the fdOVA previously released from MPs. In particular, MP_p/fdOVA induced the release of a statistically significant higher amount of IL-2 compared to the one obtained with the free fdOVA-MP_p, in agreement with the expected better response due to the sustained release.

Mathematical prediction of bacteriophage releases

Mathematical modeling is a valuable instrument to provide quantitative information about the mechanisms of release [24] and can be used to regulate, measure, and change the drug dose during therapy [43].

To extract the kinetics of the bacteriophage release, experimental data of the two types of MPs embedding bacteriophage were fitted by a non-linear first-order formula. In (Fig. 7), the data fittings and the corresponding parameters a and b are shown. The mathematical model adequately reproduced the experimental data, as demonstrated from the correlation coefficient R^2 and adjusted R^2 values, representing the feasibility of the non-linear first-order kinetics in describing these release rates.

Once described the time-dependence of the two MP releases, a simple combination of their characteristic equations (Eq. 2) was used to simulate further releases of the encapsulated bacteriophage. In this way, mathematical modeling was employed to meticulously design vaccine delivery devices based on MPs with the aim of releasing, with the desired timing, a precise concentration of phage within the target tissues. Examples of some of such possible MP combinations are shown in Fig. 7. This method can be advantageously adopted to design MPs with specific releases without the necessity of realizing experiments.

Subsequently, bacteriophage releases were extracted in vitro to experimentally validate the accuracy of our mathematical model (Fig. 7). Specifically, three MP combinations were compared with the theoretical studies: (75% post + 25% direct), (50% post + 50% direct), and (25% post + 75% direct) (Fig. 7). In addition, direct and post bacteriophage releases alone were extracted as a control. As shown in Table 1, a good correlation between experimental and hypothetical results has been found, demonstrating the importance of in silico approaches in validly predicting formulations for drug delivery systems.

The amounts of bacteriophage released obtained through in silico models and in vitro experiments after 6 h, are summarized in Table 1.

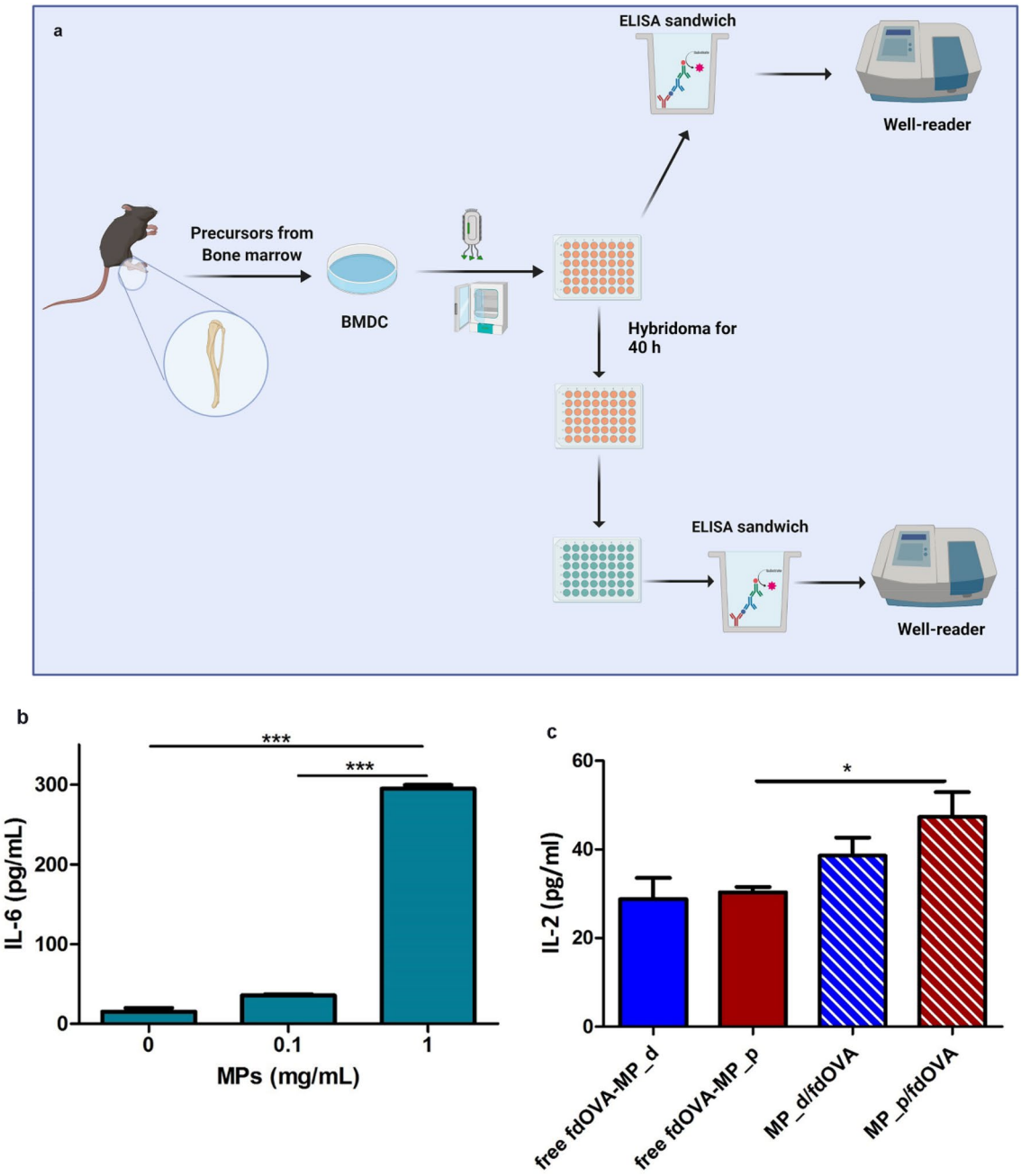

Fig. 6 **a** Schematic representation of the in vitro experiment. BM-DCs were obtained by culturing bone-marrow precursors isolated from C57/BL6 mice in presence of GM-CSF. Cells were incubated with PLGA-MPs or fdOVA bacteriophages overnight. Later, cells were washed and plated. ELISA sandwich was performed to assess IL-6. To evaluate IL-2 secretion, DCs were co-cultured in presence of B3Z hybridoma cells for 40 h. Cytokine release in the culture supernatants was measured by ELISA. **b** IL-6 release of BM-DC pulsed with PLGA MPs. Supernatants were assayed by ELISA in duplicate. Mean + SEM is reported, one representative experiment of two is shown. Differences are significant by one-way ANOVA(***$p < 0.001$). **c** B3Z hybridoma cell line activation in response to $OVA_{257–264}$ SIINFEKL peptide delivered by bacteriophages and presented by DCs. BM-DCs were incubated with fdOVA previously released from MPs (free fdOVA-MP) or with fdOVA-MPs encapsulated using both direct encapsulations, and post encapsulation methods (MP/fdOVA). BM-DCs were co-cultured with the OTI B3Z hybridoma cells and culture supernatants were assayed in duplicate. Mean + SEM is reported, one representative experiment of two is shown. Differences are significant by Student's *t* test (*$p < 0.05$)

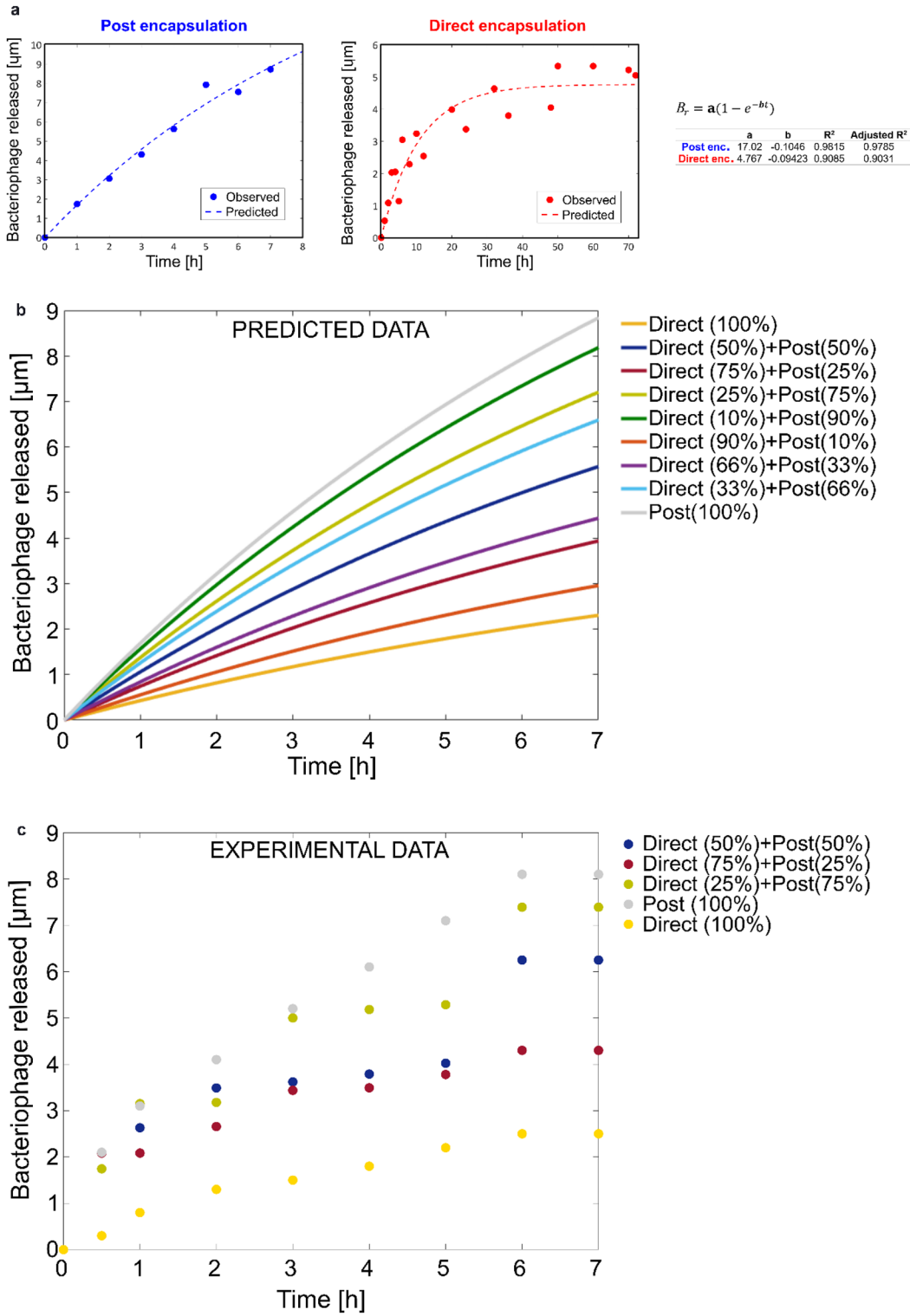

Fig. 7 Bacteriophage releases obtained from experiments were fitted with non-linear first-order equations (dashed lines). In the table are shown the model parameters *a* and *b* and R2 and adjusted R2 values, expressing the goodness of the fitting. Correlation of bacteriophage released from different MP combinations. **b** In silico predictions. **c** In vitro predictions. Data are normalized for 1 mg of MPs

Table 1 The amounts of bacteriophage released obtained through in silico models and in vitro experiments after 6h, are summarized here.

MPs	µg of Bacteriophage released in silico (6 h)	µg of Bacteriophage released in vitro (6 h)
Direct (D)	2.06	2.5 ± 0.20
Post (P)	7.93	8.2 ± 0.15
50% P + 50% D	4.99	6.2 ± 0.16
75% P + 25% D	6.46	7.4 ± 0.21
25% P + 75% D	3.53	4.37 ± 0.09

Experimental data were normalized to extract releases for 1mg of MPs

Discussion

Micro and nanoscale carriers have been used for drug delivery applications [43–49]. PLGA-based particles can be used as protein carriers by encapsulation, chemical binding, or simply adsorption of proteins [49–52]. PLGA-particles possess several characteristics including biodegradability, biocompatibility, high safety profile, sustained and controlled release of the encapsulated drugs, and the possibility of surface functionalization [53]. In this work, we have encapsulated fdOVA bacteriophages, a particulate immunogenic carrier delivering the OVA immunogenic peptide, in PLGA-MPs using two encapsulation methods. The adsorption of proteins or drugs on the MPs by post encapsulation allows to avoid the degradation of the proteins caused by the use of organic solvents in the majority of MPs encapsulation techniques.

Both fabrication methods were demonstrated to be effective in encapsulating phage particles. In vitro release study showed that post-encapsulated-bacteriophages displayed a prolonged release profile as that of the direct encapsulated fdOVA MPs, although there is a faster initial releasing speed, indicating that both methods of encapsulations guarantee a sustained release effect. Post encapsulation resulted in an initial faster release of bacteriophage compared to direct encapsulation, so we can conclude that also post encapsulation is an efficient way to load bacteriophages on the PLGA-MPs. In addition, both methods of production of fdOVA-MPs have been shown to ensure correct conservation of active phages when stored at 4°, without loss of phage titer over time. The stability study conducted at room temperature has instead shown that fdOVA-MPs constructed with the direct encapsulation method are superior in keeping the phage titer active up to 60 days at 22 °C.

According to the previously reported observations [54, 55], PLGA MPs possess adjuvant properties and activate dendritic cells as stated by the release of the proinflammatory cytokine IL-6, a hallmark of DC maturation. The adjuvant effect of PLGA-MPs by post and direct encapsulation of phage particles in the development of an adaptive immune response was evaluated. Both formulations were able to induce activation of T cell-specific for the antigen displayed on bacteriophage carrier and it was shown that the fdOVA prolonged release as compared to the free fdOVA previously released from MPs, can promote a stronger immune-stimulation even in an in vitro model which is not as sensitive as the in vivo one.

The results demonstrated that PLGA MPs can retain the structural integrity of bacteriophage after the encapsulation and freeze-drying, providing a prolonged release after resuspension in PBS and efficient delivery to dendritic cells. Accordingly, the bacteriophage is highly appropriate for peptide delivery, due to the low-cost preparation method, safety, and adjuvanticity. Additionally, the possibility to retain the integrity of bacteriophages in MPs in a freeze-dried form could extend the storage time of the encapsulated vaccine. All in all, this characteristic may exhibit a drastic practical and economic superiority for the storage and administration of phage particles in underdeveloped countries where healthcare is poor. Finally, MPs allow a controlled and sustained release of the entrapped therapeutic, which can ensure continuous exposure to the drug, avoiding or reducing the problem of repeated administration. The different release profiles obtained for the two types of phage-MP preparations (direct and post encapsulation) offer the possibility to program and finely regulate the release of a drug based on the filamentous phage in the target tissues through different combinations of direct or post encapsulation MP preparations,

which may allow the different modulation of immune responses given the impact of the prolonged release as compared to the free phage administration. Additionally, in silico approach can be simply expanded to other drugs besides vaccines and it will be promising to predict the release of drugs to the targeted regions with a controlled timing and quantitative value.

Additionally, phage display technology can be applicable for COVID-19 vaccine. In detail, both B or T antigenic immunodominant epitopes derived from the SARS-CoV-2 Spike protein can be selected and expressed on phage to induce neutralizing antibodies and/or CD4 or CD8 T cell responses. The presence of phage can improve peptide immunogenicity and phage encapsulation in MPs can increase peptide stability in plasma, protecting them from proteases activity.

Polymeric microneedles possess some advantages as compared to other routes of administration. Taking advantages of self-administration, cost-efficient and high efficiency, they have been studied extensively as drug and vaccine delivery platform. Additionally, the dermis is highly populated by dendritic cells which further enhance immune system responses. Incorporating phage microparticles into polymeric microneedles could be a promising route for the

induction of robust humoral and cell-mediated immune responses.

Conclusions

To conclude, here we are presenting the direct encapsulation of active filamentous bacteriophage by using a fast evaporation method which minimizes the bacteriophage-solvent contact as a way to slow down the kinetic release and enhance the encapsulation efficiency. Then, we compared the direct encapsulation with the post encapsulation and we combined these two formulations in different ratios to tune the release of the final formulations. We also showed that the immune response can be enhanced by a prolonged release promoted by the use of MPs. Interestingly, we found that both the MPs formulations are able to activate antigen specific T cells inducing IL-2 production without huge differences between them. The activity study performed at room temperature has shown that fdOVA-MPs constructed with the direct encapsulation technique can preserve phage stability up to 60 days.

Interestingly, we also described the possibility to finely tune and mathematically predict the release of bacteriophages by combining different fractions of the two kinds of MPs to the aim of optimizing the therapeutic efficacy in vivo. The final aim is to use this system for intradermal delivery by microneedle patches. Fd-OVA-encapsulated microneedles promise a bright future for the development of long-term immune response.

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Author contribution RJ, RS, CDN: Performing experiment, formal analysis, writing—original draft, and editing. VO: Mathematical modelling and writing, VM, RM: Performing experiments. PAN: supervision, funding acquisition. RV, PDB: supervision, writing—review and editing.

Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval This study was carried out in accordance with European Union Laws and guidelines (European Directive 2010/63/EU). The study was approved by our institutional review board and the animal procedures (i.e., sacrifice, bone marrow isolation) were performed according to rules approved by the ethics committee (permission no. 551/2020-PR).

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