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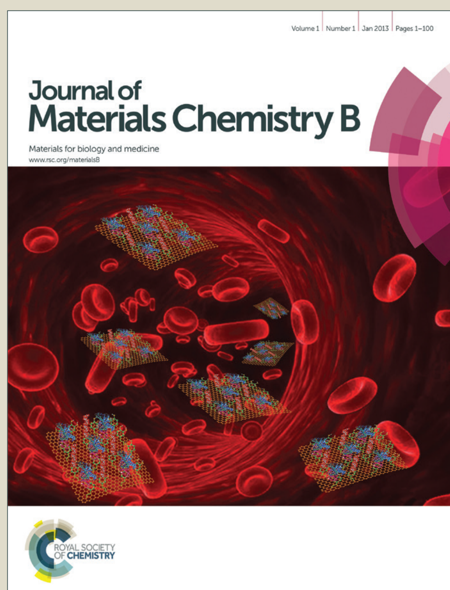

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COMMUNICATION

In silico programming of degradable microparticles to hide and then reveal immunogenic payloads in vivo

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The ability to deliver but hide immunogenic payloads and then reveal them at predetermined times could lead to autonomously boosting vaccine formulations or improved antigen-adjuvant vaccine designs. We used in silico modeling to determine the appropriate formulation and materials properties for poly(lactic-co-glycolic) acid (PLGA) microparticles such that they would delay the *in vitro* "unmasking" of an ovalbumin-alum payload for precise and predetermined intervals[1, 2]. A preferred formulation was then tested *in vivo*. *In vivo* T cell proliferation data confirmed presentation of antigen released through the programmed delayed burst while antibody subclass data demonstrated immunogenicity comparable to that observed with established multiple injection prime-boost regimins.

Adaptive immune responses are strongly influenced by temporal molecular signaling that orchestrates the balance between immune induction and restraint. This is evident in many vaccines in use today, in which boosting at intervals of weeks or months with a "resting" period is essential for optimal immune responses[3]. At the other extreme, consistent delivery of small doses of antigen in a non-immunogenic setting can induce immune ignorance or tolerance[4]. The kinetics of antigen availability can also effect immune skewing that influences cytokine expression patterns critical to the quality of the immune response[5-7]. Precise replication of adaptive immune signalling patterns can control key events such as APC activation, T cell proliferation and B cell recruitment, potentially enabling the development of intelligent adjuvant strategies[8, 9]. Prefered features of a delivery platform that could replicate the on-off patterns of prime-boost vaccines or natural immune activation by timing the bioavailability or release of injected antigens, adjuvants, or other immunomodulatory molecules would include the capacity to:

1. Sequester (or "hide") administered antigen and/or adjuvant, limiting immediate or "leaky" availability to APC, while preserving the payload for subsequent release[10].
2. Precisely tune the "hiding" period prior to boosting release, which could range from days to months for the autonomous replication of prime-boost intervals[5, 11].
3. Avoid inherently immunostimulatory formulation materials, sizes and geometries that could effect innate immune activation and immune skewing[12, 13].

Meeting these requirements necessitates formulations engineered for specific *in vitro* release profiles using a limited range of materials and physical forms.

A wealth of *in vitro* data exists on the design of vaccine formulations that intend to simulate the release of priming and boosting doses of antigen from a single injection. As early as 1993, O'Hagan et al. created ovalbumin (OVA) loaded poly(lactic-co-glycolic) acid (PLGA) microparticles with burst-lag-burst dissolution profiles that seemed to replicate the priming and boosting doses of a vaccine[14]. Empirically varying the molecular weight or L:G ratio of similar PLGA copolymer formulations resulted in changes to the intervals between initial and secondary bursts observed in *in vitro* dissolution testing[15]. A more recent review of additional PLGA microparticle vaccines reports intervals between primary and secondary bursts ranging from just 3 weeks to multiple months depending on both polymer and payload chemistries[16]. Most

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recently, the coupled roles of these variables in setting this interval have even been described quantitatively by mathematical models, a relation confirmed by validation studies on published data[1, 2, 17]. Simulations from these models suggest that formulations (such as injectable and dissolvable PLGA microparticles) could be "programmed" for specific intervals lasting from days to months between their injection and the payload's secondary release.

An effective delayed release microparticle design should not only time the boosting release of antigen, but also minimize leakage. Minimizing "antigen leak" would both preserve antigen for subsequent boosting presentation and minimize continuous release of low dose antigen that is known to be tolerogenic. This would be particularily important in a scenario in which the microparticles delivered multiple sequential boosting doses of antigen. Reviews devoted to the topic discuss numerous potential formulation chemistries and production methods for suppressing the initial burst[17, 18]. For instance, Shi et al used a high PLGA to antigen ratio (>100:1, by weight) to create microparticles with a high bulk density that limited the initial burst[19]. These formulations delayed the *in vitro* delivery of recombinant hepatitis surface protein (HSP) for 7 to 8 weeks with only 7% initial burst[19]. More recently, Jhunjhunwala et al reduced initial burst to less than 4% of total release for the delivery of tumour growth factor β (TGFβ), an immunoregulatory cytokine, by fabricating double emulsion microparticles from PLGA with an ester-capped terminus instead of free carboxylic acid[20]. By using ester-capped polymers with new *in silico* models that predict the timing of PLGA controlled release, it should be possible to design microparticles that effectively eliminate initial burst and preserve the payload for delyed release after precise periods of time[1, 2, 17].

Importantly, a formulation engineered for delayed release *in vitro* may or may not provide the same delivery kinetics *in vivo*. Studies on microparticle-based vaccines most often measure *in vivo* performance through "down-stream" indicators, such as antigen specific antibody (IgG) titres. To obviate this extrapolation, we used an OVA-specific CD4+ T-cell transgenic mouse model that enabled us to evaluate antigen presentation in real-time *in vivo*[16, 21, 22]. Microencapsulation of ovalbumin asorbed to the FDA-approved adjuvant alum (OVA-alum) offers a unique opportunity to study the T cell response to large microparticles that incorporate antigen and a clinically approved adjuvant (alum). Alum has been shown to preferentially skew immune responses toward Th2 immunity, with minimal CTL induction[23, 24]. The OVA-alum model therefore enables the real-time, in vivo evaluation of antigen presentation to antigen specific CD4+ T-cells, immune skewing, and antigen specific antibody induction.

We hypothesized that PLGA microparticles engineered to provide delayed *in vitro* release could be programmed to effectively "hide" and then "reveal" (with tunable timing) antigen and adjuvant *in vivo*. Accordingly, here, we describe the design of degradable microparticle formulations for the delayed release of OVA and OVA-alum through the *in silico* optimization of polymer molecular weight (*Mwo*), L-G copolymer ratio (*L:G Ratio*), and surface porosity as defined in recently published models[1, 2]. These simulation results guide the selection of materials and production conditions for new microparticle formulations. The critical quality attributes defined by simulations for these microparticle are evaluated with manufacturer-supplied chromatography data and microscopy studies to confirm successful production . The accuracy of each *in silico* performance predictions is then tested with *in vitro* dissolution testing. The *in vivo* performance of the fully-validated delayed release microparticles is documented in the OVA-alum model through the analysis of OVA-specific CD4+ T-cell stimulation, and the induction of OVA-specific IgG antibodies and IgG subclass responses (IgG1 vs IgG2c).

Table 1: Simulation Inputs and Design Outputs

Inputs		Outputs		
Payload	*Delay*	*L:G Ratio*	Mw_o	*Porosity*
OVA	1 Week	50:50	8kDa	< 5%
OVA-Alum	2 Weeks	50:50	21kDa	< 5%
OVA-Alum	4 Weeks	50:50	53kDa	< 5%

In the model-aided design process, we selected delayed release profiles where either OVA or OVA-alum was delivered at 1, 2 or 4 weeks after hydration as targets for our formulation design simulations. As part of the simulations particle size was restricted between 10μm and 100μm, ensuring that the optimized formulation was too large for phagocytosis by APCs, which would interfere with the delayed release schedule, and too small to cause inflammatory tissue damage, which results from administration of implant-sized formulations[12, 13]. Computations using a non-linear optimization of published models for PLGA controlled release yield the values for polymer molecular weight (Mw_o), copolymer ratio (*L:G Ratio*), and surface porosity defining formulations meeting the desired delivery requirements (Table 1)[1, 2]. These particles formulations require an average surface porosity below 5% in order to minimize initial bursting of antigen. During production by a standard double emulsion process, we tuned microparticle internal microstructure and porosity by selecting ester-capped polymer chain chemistry[20, 25]. We observed that the ester-capped polymers produced microparticles

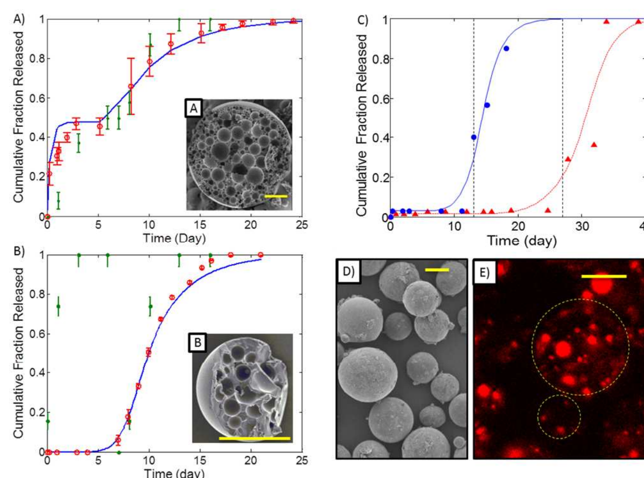

Figure 1: ***In vitro* antigen release from delayed release particles depends on polymer chemistry and internal microstructures.** A) Ovalbumin (OVA) encapsulated in 8kDa, PLGA microparticles with a porous internal morphology (*insert*) releases in a pattern of two bursts (*red circles*) that mimic the dissolution of OVA from a two dose (day 0, day 7) OVA-alum vaccine (doses shown together, *green circles*). B) Ovalbumin (OVA) encapsulated in 8kDa ester-terminated PLGA microparticles with discrete occlusions and a solid morphology (*insert*) releases after a 7 day delay (*red circles*) mimicking the 2nd, boosting dose of a two dose OVA-alum vaccine (doses shown separately, *green dots*). In both, A and B the *in*

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vitro release data closely follows *in silico* predictions (blue lines) from a published math model of PLGA degradation and erosion[1]. C) OVA-alum encapsulated in microparticles made of 21kDa and 53kDa ester-terminated PLGA produce timed bursts with either 2 week or 4 week delays, respectively. D) A representative SEM image reveals spherical particles larger than 10µm in diameter. Analysis with ImageJ software quatified the suface porosity of these particles at <0.5%. E) Imaging by confocal microscopy of particles doped with Texas-red labeled OVA shows the protein antigen in discrete occlusions. Select microparticles in the plane of focus have been ringed with dashed yellow lines under higher contrast to highlight their boundaries. All scale bars are 10µm.

with much less burst release of OVA than acid terminated ones (Fig. 1a,b). This effect corresponds to a change in particle microstructure, as documented by scanning electron microscopy (Fig. 1 insets). It appears that acid terminated polymers form particles with a highly connected internal pore structure, while the ester-capped polymers produce particles with discrete pockets surrounded by dense polymer matrix. We speculate these acid end-groups act as amphiphiles helping to stabilize oil-water interfaces during double emulsion processing better than the more hydrophobic ester-capped polymer chain ends, yielding microparticles with a more interconnected pore structure[26]. This release pattern also seems to hold for the OVA-alum payloads formulated into 21kDa and 53kDa ester-terminated PLGA microspheres. As predicted, these particles displayed minimal (<5%) initial burst as well as the intended 2 and 4 week lag periods before burst release (Fig. 1c). Further, the bulk of release was completed within 4 days of onset, demonstrating the potential for delivery of antigen over relevant timeframes to produce an immune response *in vivo*. Sizing of these delayed release microparticles by the impedance method yielded a volume-averaged diameter of 25.4±7.8µm with 98% of particles being greater than 10µm in diameter, suggesting that the large majority of antigen loaded into particles too large to be internalized by phagocytosis, which is important for limiting unwanted adjuvancy from this effect. Microscopy analysis of the microparticles further supports the sizing results, and revealed a uniform spherical structure with ovalbumin loaded in discrete occlusions associated with a dense polymer matrix and minimal initial burst (Fig. 1d,e). The morphology of these dense microparticles is starkly constrasted by prior scanning electron microscopy (SEM) analyses of porous microparticles[27].

The ability of our delayed release microparticles to sequester then release their antigen-adjuvant payload was tested *in vivo* through the analysis of ovalbumin-specific CD4+ T-cell (OT-II) proliferation. OT-II cells are a clonal population of transgenic T-cells expressing the receptor for an OVA-derived epitope OVA $_{323-339}$ (ISQAVHAAHAEINEAGR) processed and presented in the context of a class II major histocompatibility complex (MHC IA^b). When presented by activated APCs (such as those stimulated by adjuvant) in the context of the MHC IA^b OT-II cells recognize ISQAVHAAHAEINEAGR, become activated, proliferate, and secrete pro-inflmmatory cytokines. In this way OT-II proliferation *in vivo* is indicative of the temporal processing and presentation of antigen by an activated APC, and serves as a surrogate marker of immunogenicity. Bolus OVA-alum injected freely without encapsulation is known to initiate the proliferation of OT-II cells *in vivo* within 3 days, reflecting the processing and presentation of readily available antigen by activated APCs, and the antigen-specific activation of T-cells leading to proliferation[23]. Comparable conditions were used here to assess antigen delivery from *the in silico* designed, delayed release microparticles. As expected, immunization with free OVA-alum induces strong OT-II proliferation by day 4 after injection (Fig. 2a). To test the delayed release microparticles, a formulation designed to produce a 2-week delay was used to ensure an ample window for differentiating between antigen presentation resulting from particle phagocytosis and digestion or initial release of OVA-alum from the particles, vs. the presentation of OVA-alum released by the particles following the programmed delay (day 22). As expected, injection of delayed release microparticles did not induce significant proliferation of OT-II cells at day 4 (Fig. 2b), indicating that the formulation effectively "hides" administered OVA-alum. At day 22, this formulation effectively activated OT-II T-cells within 4 days of the programmed antigen release. Specifically, this time-point corresponds to the point 4 days after the injection of free OVA-alum and resulted in comparable OT-II proliferation (Fig. 2a,c). Results were consistent for 9 mice immunized using three different delayed release formulations (Fig. 2d). These data also are consistent with the kinetics of antigen release observed *in vitro* (Fig. 1).

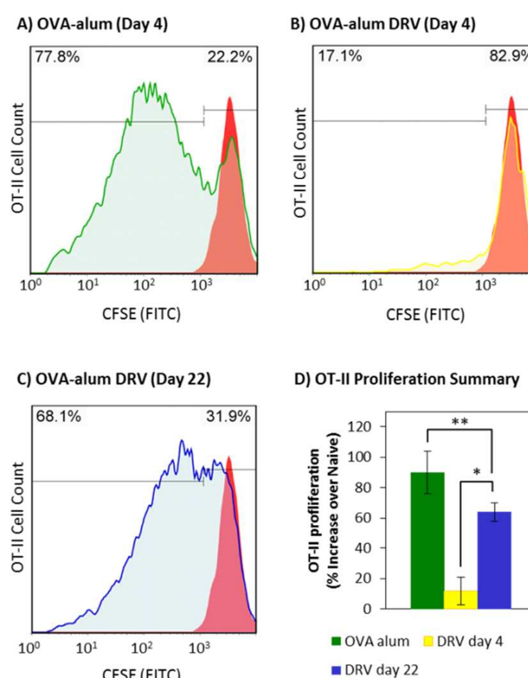

Figure 2: Delayed release microparticles "hide" and then synchronously release OVA-alum in a timed fashion *in vivo*. Proliferation of OT-II cells as determined by reduction in CFSE in activated dividing T-cells in immunized animals was measured to evaluate the kinetics of antigen presentation. The proliferation of OT-II cells in naïve mice (red peak) serves as a basis for comparison across multiple studies. Immunization with OVA-alum results in OT-II proliferation indicative of antigen processing and presentation 4 days following immunization (A). Injection of delayed release miroparticle OVA-alum does not induce proliferation 4 days after immunization (B), but does induce potent OT-11 proliferation 22 days after immunization (C), similar to that induced by injection of free OVA-alum (A), consistent with the early absence of antigen and antigen release at the later time point. Data compiled from groups of 6 mice and normalized by naïve proliferation quantitates the performance of the delayed release microparticles (D). Both the day 4 OVA-alum and day 22 delayed release particles produce significantly more OT-II proliferation than the delayed release particles at day 4 (*T-test, $p < 10^{-4}$, n = 9). The day 4 OT-II response to the OVA alum vaccine was slightly higher than the response to the delayed release particles at day 22 (**T-test, p = 0.05, n = 9).

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To evaluate the effect of delayed release microparticles on the induction of antigen-specific antibody responses, we compared their immunogenicity to that of a vaccine with prime and boosting doses of OVA-alum injected at days 14 and 28. This prime and boost format generates high IgG antibody titres that persist in the absence of antigen and can be detected months after of immunization[14]. We attempted to mimic this dosing using delayed release formulations with 2 and 4 week delays, injected at day 0. Accordingly, OVA specific IgG2c (Th1) and IgG1 (Th2) antibody levels in the serum were determined during weeks 4 and 10 by ELISA. The 2 injection prime-boost immunization resulted in high levels of OVA-specific IgG1, but not IgG2c (Fig. 3), which is consistent with literature documenting alum's Th2 skewing adjuvancy[23]. Importantly, delivery of the combined of delayed release microparticles induced a similar OVA-specific antibody titers with similar Th2 skewing (IgG1>IgG2c) (Fig. 3).

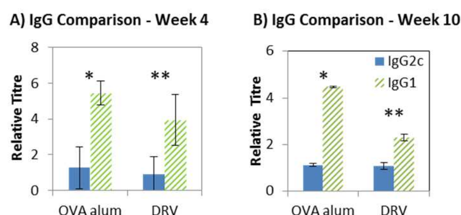

Figure 3: The traditional two injection, two-dose, OVA alum vaccine and the single injection, two-dose delayed release vaccine induce comparable OVA-specific IgG antibody titers (A) 4 weeks and (B) 10 weeks after initial injection. The delayed release microparticles (DRV) are designed to match the 2 week interval prime-boost schedule of the OVA-alum control (Fig. 1c). One tailed T-test of IgG1>IgG2c yield * $p<0.006$, ** $p<0.03$. Testing IgG1 for OVA-alum>DRV yields p values of 0.13 at 4 weeks and 0.07 at 10 weeks. Serum from mice immunized with OVA adsorbed to 1-5µm iron beads (IgG+) was used as an internal control to confirm successful completion of the assay (data not shown).

Conclusions

In summary, degradable microparticles were engineered for delayed synchronous antigen/alum delivery *in vitro*. *In vivo* immunization with these delayed release microparticles elicits strong antigen-specific T-cell proliferation following delayed antigen release, and antigen-specific antibody responses with similar immune skewing as that observed in traditional multiple injection prime-boost regimins. This type of delayed release platform might forward the development of single injection versions of the pneumococcal or human papillomavirus vaccines by autonomously supplying the boosting doses now required as separate injections spaced over multiple months. Future work will address the controlled delivery of antigen and adjuvant, optimizing the sequential delivery of antigen/adjuvant for more efficient antigen uptake and antigen presenting cell activation.

Notes and references

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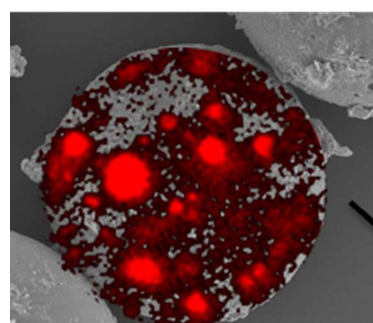
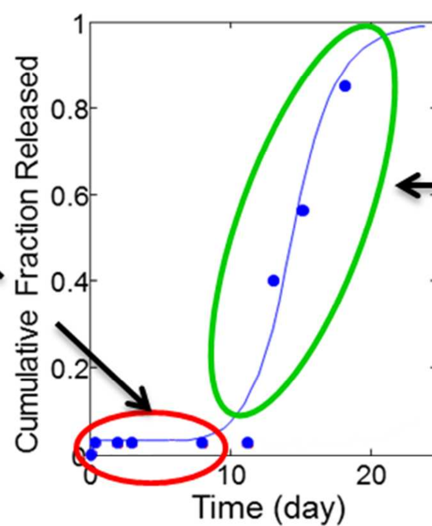
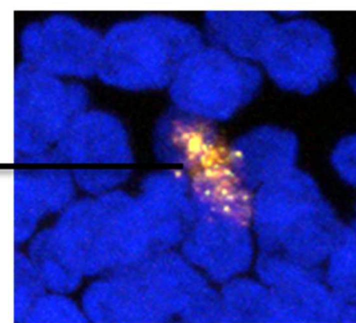
Table of Contents Entry

Poly(lactic-co-glycolic) acid microparticles, mathematically designed for delayed release *in vitro*, hide and then reveal ovalbumin-alum *in vivo* without altering its immunogenicity.







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