Review

Single-injection vaccines: Progress, challenges, and opportunities

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A B S T R A C T

Currently, vaccination is the most efficient and cost-effective medical treatment for infectious diseases; however, each year 10 million infants remain underimmunized due to current vaccination schedules that require multiple doses to be administered across months or years. These dosing regimens are especially challenging in the developing world where limited healthcare access poses a major logistical barrier to immunization. Over the past four decades, researchers have attempted to overcome this issue by developing single-administration vaccines based on controlled-release antigen delivery systems. These systems can be administered once, but release antigen over an extended period of time to elicit both primary and secondary immune responses resulting in antigen-specific immunological memory. Unfortunately, unlike controlled release systems for drugs, single-administration vaccines have yet to be commercialized due to poor antigen stability and difficulty in obtaining unconventional release kinetics. This review discusses the current state of single-administration vaccination, challenges delaying the development of these vaccines, and potential strategies for overcoming these challenges.

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1. Background

1.1. Vaccine coverage

Vaccines save an estimated 8 million lives annually and are widely recognized to be the most effective medical treatment for preventing infectious disease [1–3]. While the World Health Organization’s (WHO) Expanded Programme on Immunization (EPI) has been enormously successful in raising worldwide vaccination rates from just 5% in 1974 to 84% today, one in six infants remain underimmunized each year resulting in 1.5 million deaths [4,5]. Infectious disease is among the top killers of young people worldwide and is responsible for nearly one-third of all deaths in children between one month and five years of age [4,5]. Current vaccination schedules, which require multiple visits to a healthcare provider within an infant’s first year of life, represent a significant logistical barrier to immunization — especially in the developing world [6]. Nearly half of underimmunized infants have received at least one dose of vaccine against diphtheria, tetanus, and pertussis (a benchmark for overall infant vaccination rates) but continue to be at risk of contracting these, and potentially other diseases because they did not complete the full three-dose series [4]. Consequently, there are 10 million infants that have some, albeit limited, access to healthcare yet remain underprotected. If a one-step immunization method were established for currently available vaccines with no other changes to patient access or infrastructure, it could save hundreds of thousands of lives annually. Unfortunately despite considerable work towards developing single-administration vaccines, this approach has yet to be successfully commercialized and deployed in the clinic. To date, most research in this field has focused on developing injectable devices that release antigen over the course of months to recapitulate the timescale, if not kinetics, of multiple-injection vaccination regimens with just one administration. This review presents the challenges associated with developing controlled antigen release devices as well as potential opportunities for overcoming these issues.

1.2. Immune memory & vaccine immunology

When the body encounters a foreign antigen it generates a B and T cell-mediated immune response that serves to neutralize the antigen while also preparing for potential future encounters through the formation of immunological memory [7]. In oversimplified form, naïve B cells residing in the spleen or lymphatic system bind to the antigen via a B cell receptor, proliferate in germinal centers, and undergo somatic hypermutation which allows them to become specific to a particular epitope on the antigen. These B cells may then become either plasma B cells, which begin producing antibodies specific for the antigen, or memory B cells which can lay dormant until the antigen is presented again. Over time, the number of short-lived plasma cells drops, as do the corresponding circulating antibody titers [8].

When the antigen is presented a second time, antigen-specific memory B cells quickly proliferate, then differentiate into plasma cells which produce antibodies to prevent full-blown viral or bacterial infection. During this process some B cells retain their memory status and continue to improve their binding affinity for the specific antigen allowing for
even more effective responses in subsequent encounters [9]. B cell progeny that differentiated into plasma cells either die shortly after the pathogen has been eliminated or settle in the bone marrow and become long-lived cells that continually produce antigen-specific antibodies to maintain high circulating antibody titers for years. While the primary antigen response takes 10–28 days to peak, the secondary response peaks in just 2–7 days and is therefore more effective at preventing infection prior to exponential pathogen growth [8,10].

Vaccines leverage the adaptive immune system's immunological memory to provide protection against future encounters with a particular pathogen. Current injectable vaccines come in several forms including live viruses (measles, mumps, rubella), inactivated viruses (influenza, cholera, bubonic plague, polio, hepatitis A, rabies), toxoids (tetanus, diphtheria), protein subunits (hepatitis B, human papillomavirus), and conjugates (meningitis, bacterial influenza, bacterial pneumonia). These antigens are developed to be analogous in terms of epitope structure and immune response, but otherwise innocuous when administered to a healthy individual. This allows vaccines to be administered in a safe manner while still generating immune memory that is effective against harmful environmental pathogens. However, any significant difference in the antigen's structure due to mechanisms including protein cleavage, re-folding, and aggregation may negate the benefit of the vaccine, as the immunological memory developed may not cross-react with (i.e, recognize) the native pathogen [11].

Although the efficacy of a single bolus vaccine dose can lead to seroprotection rates on the order of 75–90% [12,13], multiple doses are scheduled to bring immunity levels up near 100% [12,14]. Further, ambitious goals like the WHO's worldwide polio eradication campaign will likely require multiple doses to ensure complete seroprotection across the entire population. Subsequent dosing, the so-called “boost” doses, are required to rechallenge the immune system and form stronger immunological memory against the same antigen. These doses are most effective when administered after circulating antibody titers have decreased to minimize binding competition between B cells and antibodies that would hinder the formation of immune memory [8]. This is especially true for live vaccines, which are administered in small doses and rely on in vivo expansion to induce an immune response [15]. As a result, most vaccines are administered as a series of boluses spaced months apart in a prime–boost–boost dosing schedule.

1.3. Benefits of single-administration vaccination

The concept of single-administration vaccines dates back nearly 40 years to an epoch when controlled release devices were still in their infancy [16,17]. Nevertheless, this approach has drawn major interest at the crossroads of immunology and drug delivery due to its potentially transformative effect on the field of infectious disease [3]. Single-administration vaccines present a major opportunity for improving healthcare while simultaneously reducing cost. Although traditional vaccines are on the whole incredibly effective when administered on schedule, these multi-bolus regimens are impractical for many in the developing world with limited healthcare access. As a result, the health benefits of single-administration vaccines would have the greatest impact on the developing world where full immunization regimens could be completed despite scarce healthcare infrastructure. This approach is especially exciting because it reduces the number one risk factor in infectious disease — failure to vaccinate. All other risks associated with vaccines such as allergic reactions are minimal with respect to the enormous benefits derived from immunization. In fact the main (albeit very minor) risk associated with vaccines today is related to improper use rather than poor quality control in manufacturing. For example, syringe re-use can lead to the transmission of bloodborne pathogens such as HIV, Hepatitis B, and Hepatitis C [18]. While the advent of specialized single-use syringes has lowered the occurrence of this unsafe practice, single-injection vaccines could further reduce transmission by minimizing the number of both injections and syringes required.

Although the advent of single-administration vaccines would certainly have a greater impact on the developing world, this approach would also benefit the developed world. From a patient's perspective, single-injection vaccines would help reduce pain and improve convenience as fewer injections and healthcare visits are needed to provide immunity [3]. This is also advantageous from a reimbursement perspective as healthcare payers (e.g., insurance, government) would need to pay for fewer vaccine-related visits to the doctor [15]. Since the cost of vaccines is typically far lower than the cost to administer them, even a significant increase in the cost of a single-administration vaccine would likely be outweighed by the cost savings from fewer healthcare visits. This would also be true in the developing world where the vaccines themselves constitute a minority of the cost associated with national immunization programs whereas the majority is used for personnel and management [19,20]. While improving worldwide immunization rates is the main goal of single-administration vaccines, cost savings will provide additional impetus for the adoption of this technology since the inclusion of vaccines in national immunization programs is almost entirely based on cost effectiveness [21,22].

1.4. Single-administration vaccines

A vast majority of work towards single-administration vaccines has focused on monolithic systems in which antigen is dispersed throughout a polymer matrix and then released upon polymer biodegradation [23]. This approach allows a proportion of the antigen to be sequestered from the immune system until sufficient degradation has occurred, at which point the antigen is released long after the initial administration (Fig. 1). Although monolithic devices have demonstrated clinical utility for drug and hormone release with products like Gliadel® wafers [24] and the Lupron Depot® [25], there has yet to be a commercialized controlled release device for vaccines. The delay in single-administration vaccines can likely be attributed to the poor stability of vaccine antigens compared to small molecules and hormones as well as the potential need for non-traditional release kinetics. Unlike small molecules and polypeptides that have limited, if any higher-order structure, vaccines typically involve proteins or multi-protein assemblies for which structure is critical for function [11]. Further, while most drug-releasing devices target near zero-order release in order to stay within the therapeutic window, controlled vaccine delivery devices might be more effective if they release antigen as discrete pulses [8,23,26].

The most common methods for fabricating these devices are single- and double-emulsion techniques which are attractive due to their simplicity, ability to produce microspheres small enough to be injected with a traditional syringe, and potential scalability [27,28]. These particles can be injected intramuscularly or subcutaneously at one time point, but deliver their contents with a variety of release profiles over days, weeks, months, or even years depending on formulation and material properties [26,29–34]. While an oral formulation would be ideal for ease of administration [35], vaccines administered via this method may only provide mucosal immunity (rather than systemic) and can be more difficult to reliably administer to infants [36]. Therefore a single-administration injectable vaccine that provides systemic immunity and fits into current vaccine program infrastructure is likely a more realistic goal despite the need for trained healthcare workers. In order to minimize the number of injections needed to receive a full complement of vaccines, one group has begun co-encapsulating multiple antigens in the same particles. While this group has shown the benefit of this approach for antigen loading, potential immunological interference must be considered prior to implementation [37].

Other fabrication methods have also been employed to create particles for single-administration vaccines with some success, but similarly remain at the pre-clinical stage [28]. Non-monolithic strategies to obtain the desired antigen release kinetics such as osmotic pumping
have also been explored [38], but are likely too expensive for global use given the low cost of current vaccines [3]. Therefore, unless otherwise noted, this review will focus on monolithic biodegradable microparticles since these systems have the greatest potential for widespread adoption as single-administration vaccines in the immediate future. The goals of this manuscript are to review the current progress in the field, identify existing challenges, and present potential solutions that may allow these challenges to be overcome.

2. Controlled vaccine release device composition

2.1. Poly(lactic-co-glycolic acid) (PLGA) microspheres

Emulsion-based poly(lactic-co-glycolic acid) (PLGA) microspheres have been the single most-studied devices for both drug and vaccine delivery due to their biocompatibility, extensive history of use, and easily controlled degradation/release kinetics [28,39,40]. PLGA is widely considered “generally biocompatible” and is well-tolerated in both the intramuscular and subcutaneous spaces, which are the preferred injection sites for vaccination [41,42]. PLGA degradation occurs in vivo mainly by the hydrolysis of ester linkages in the polymer backbone resulting in a loss of molecular weight and eventual decomposition into lactic and glycolic acid [43,44]. When polymer chains are cleaved down to a critical size (approximately 15 monomer units in length), they become soluble and can diffuse out of the bulk material resulting in the release of encapsulated macromolecules and loss of structural integrity [45]. Because PLGA is a synthetic polymer, small variations in polymer processing can be used to achieve the desired release rate. Three of the most common ways to tune release the release kinetics from PLGA are to change the molecular weight, alter the relative composition of lactic versus glycolic acid, and add an end cap [43,46,47]. Microspheres fabricated using PLGA with 50:50 lactic-to-glycolic acid ratio degraded more quickly in vitro than 75:25 PLGA at a similar molecular weight [48]. In our experience, the copolymer ratio of lactic-to-glycolic acid was observed to have a greater relative effect on degradation and release kinetics than polymer molecular weight [48,49]. The addition of a carboxylic acid end group also increases PLGA degradation rate by autocatalyzing polymer degradation [50]. Antigen release from PLGA microspheres may also be further modulated by copolymerizing PLGA with an additional polymer such as polyethylene glycol (PEG) to tune degradation properties [51]. Numerous studies using PLGA as well as the homopolymer poly(lactic acid) (PLA) are discussed in the antigen sections below.

2.2. Natural materials

Naturally-derived materials have also been evaluated as potential controlled release devices, but not to the extent that synthetic polymers...
have. As with all natural products, there is inherent batch-to-batch variability and fewer ways to tune material characteristics in order to achieve the desired release kinetics [52]. However, these materials, which can be extracted rather than synthesized, may be far less expensive than synthetic materials like PLGA and still demonstrate the beneficial effects on immune response due to extended antigen release. As a result, several groups have proposed the use of chitosan, alginate, and collagen as potential replacements for PLGA in microsphere-based vaccine delivery systems. Chitosan is a particularly attractive material due to its biocompatibility, biodegradation, and potential adjuvancy properties [53]. Jaganathan and colleagues encapsulated tetanus toxoid (TT) in either chitosan or PLGA microspheres and found that both were able to achieve non-inferior immune responses compared to conventional alum-adsorbed TT bolus injections administered at 0 and 4 weeks [31]. Other groups have reported a similar improvement in immune response using chitosan nanoparticles containing hepatitis B surface antigen (HBsAg) or TT that were administered subcutaneously or intranaturally [54,55]. Chitosan has also been complexed with PLGA, or other natural materials such as alginate, to form delivery systems such as alginate–chitosan–PLGA microspheres that deliver HBsAg and produce comparable immune responses to a two-injection alum-adsorbed HBsAg schedule [56].

Alginate-only particles have been studied for subcutaneous or oral administration. [57–59]. Sarei and colleagues showed that alginate nanoparticles could release diphtheria toxoid (DT) in vitro over the course of 15 days without substantial antigen degradation. They then proceeded to demonstrate that subcutaneous injection of these particles at 0 and 4 weeks results in a 10-fold improvement in immune response in guinea pigs at 8 weeks compared to alum-adsorbed DT administered at those same time points [59]. While this study does not directly study a prototypical single-injection vaccine, it still serves to show that alginate may be well-suited for controlled antigen release. Unlike solid polymeric antigen release devices, antigen release from these hydrated particles may be diffusion-dependent rather than degradation-dependent. Several groups have also explored collagen for controlled vaccine release. These groups have demonstrated that TT or DT encapsulated in that 1 mm × 10 mm collagen minipellets evoke a non-inferior or even superior immune response compared to one or two bolus injections of alum-adsorbed antigen due to prolonged antigen delivery (Fig. 2). However, this particular approach has not yet been able to attain antibody titers on par with three bolus injections [60,61].

2.3. Other biodegradable materials

Any material that degrades or disassembles in the in vivo environment has the potential to be used as a controlled delivery device. Cleland has identified a list of the top materials are well-suited for single-injection vaccine delivery systems including poly(caprolactone), polyanhydrides, and poly(ortho esters) [23]. Poly(caprolactone), like PLGA, degrades via hydrolytic ester bond cleavage [62] and is relatively biocompatible. PCL is less expensive, degrades more slowly than most PLGA formulations, and produces degradation products that are comparatively less acidic than PLGA [63]. Although PCL has not been as widely studied for vaccine delivery as other materials described above, studies conducted using BSA as a model antigen have shown extended release over several months in vitro [64].

Tabata and colleagues reported a method for preparing surface-eroding polyanhydride microspheres that achieve near-zero-order release kinetics of ovalbumin (OVA), BSA, lysozyme, hepaparin, trypsin, and immunoglobulin [65,66]. However, due to the rapid polymer degradation rate, these delivery systems release their payload of antigen over a period of a few weeks, which may be too fast to elicit a high quality secondary immune response. Determan and coworkers observed similar antigen release kinetics, but also noted that these microspheres can help to preserve the structural integrity, and thus antigenicity, of encapsulated proteins that are susceptible to moisture-induced aggregation (such as TT), likely due to their hydrophobicity [67–69]. Another group used amphiphilic polyanhydride nanoparticles to deliver Bacillus anthracis, an anthrax antigen, both in vitro and in vivo and found that these delivery systems elicited a substantial, potentially protective immune response [70]. Poly(ortho esters) represent another class of surface-degrading polymers that may be suitable alternatives to PLGA. Depending on formulation and chemical composition, these materials can be tuned to achieve continuous or delayed/pulsatile release kinetics [71,72]. However, to date these materials have only been studied for releasing model proteins or DNA, and not for clinically available vaccine antigens [72–74].

3. Single-administration antigens

3.1. Tetanus toxoid (TT)

TT and other toxoids are among the most thermostable biomolecules used in vaccines today and therefore may not present as difficult of a challenge for single-administration vaccination [75]. As a result, it is not surprising that TT has become one of, if not the most studied antigen for controlled release vaccine delivery systems [76,77]. The current immunization regimen for tetanus begins with children receiving 5 full-strength doses of tetanus toxoid, typically as part of a 5-injection multivalent DTaP vaccine that is administered at the ages of 2, 4, 6, and 15–18 months, and 4–6 years [15].

In a series of papers, Alonso and colleagues studied the in vitro release kinetics and in vivo immune response to TT encapsulated in PLGA microspheres, with the goal of replacing the initial 2 or 3 injections currently used in the current tetanus immunization regimen with one injection of controlled release devices [78,79]. These particles demonstrated pulsatle release of TT over the course of one month in vitro and evoked neutralizing antibody titers that were comparable to a single injection of alum-adsorbed TT at an equivalent dose 6 months after in vivo administration. These results are particularly exciting because only 0.5–1.0% and 2.5–20% of TT remained antigenically active following encapsulation in PLGA and PLGA respectively, suggesting that extended release and microsphere-related adjuvant effects were able to offset a substantial loss of active dose during formulation and the lack of a traditional adjuvant (alum) [79]. In a subsequent paper, this
group showed that TT encapsulated in PLGA microparticles evoked a comparable long-term immune response to both one bolus injection of alum-adsorbed TT or two bolus injections delivered at 0 and 4 weeks [80]. Despite its good stability relative to other antigens, these studies by Alonso and Gupta emphasize the need for long-term TT stabilization in order to retain antigenicity for the duration of the release profile.

More recently papers by Jaganathan, Chang, Tobio, and Schwendeman have reported work towards “second-generation” microsphere formulations containing stabilizing excipients to preserve a higher proportion of TT in its proper antigenic state during both the early burst release phase and the subsequent continuous-release phase [81–83]. In a paper by Jaganathan, a single injection of either PLGA or chitosan microspheres containing TT-stabilizing excipients elicited TT-specific antibody titers in guinea pigs that were comparable or better than those elicited by two bolus injections of alum-adsorbed TT administered at 0 and 4 weeks [31]. These results help to demonstrate the enhanced benefit of controlled release when antigen stability issues are minimized.

3.2. Hepatitis B surface antigen (HBsAg)

The hepatitis B vaccine is a subunit vaccine that uses the HBsAg to stimulate the formation of immune memory. The recommended schedule for hepatitis B vaccination of children involves a primary immunization, a second dose one month later, and a third dose six months later [15]. Similarly to TT, controlled-release formulations for HBsAg have been extensively studied both in vitro and in vivo in an effort to eliminate the need for repeated vaccine administration. HBsAg is an interesting antigen for encapsulation because it is more stable at slightly acidic pH (5–6), which may help it remain stable as the delivery device degrades and generates acidic degradation products [84]. Singh and colleagues encapsulated HBsAg in PLGA or PLA microspheres and studied antibody responses to these formulations in mice. By combining multiple microsphere populations with different release kinetics, they were able to use a single injection of particles to induce non-inferior HBsAg-specific antibody titers after one year compared to an equivalent cumulative dose of alum-adsorbed HBsAg administered in a three-injection sequence at 0, 1, and 6 months [85]. Feng and colleagues also investigated a single injection of HBsAg encapsulated in PLGA microspheres and demonstrated that one injection of these particles was able to elicit antibody titers at the end of the 4-month experiment that were on par with those induced by three injections of alum-adsorbed HBsAg administered at 0, 1, and 2 months [30].

3.3. Diphtheria toxin (DT)

The current immunization regimen for diphtheria is identical to that of TT as both are components of the multivalent DTaP vaccine. However, DT encapsulation and controlled release has not been as extensively studied. Singh and colleagues were the first group to report a controlled-release system for DT in 1991 when they demonstrated continuous release of DT from PLA microspheres over a period of 60 days in vitro. They then assessed the immune response to this formulation and found that it elicited an immune response in mice through 75 days that was comparable to the response evoked by three bolus injections of DT with calcium phosphate administered at 0, 1, and 2 months [86]. In a follow-up study, the same group then compared the immune response to DT encapsulated in PLA microspheres to three bolus injections at 0, 1, and 2 months, but this time the antigen was absorbed to the surface of an alum-based adjuvant. Despite this potentially improved control group, the authors observed that the microparticle group exhibited comparable or even superior DT-specific antibody titers during the 8-month study [87]. In a more recent study, this same group then explored the immune response to DT-containing single injection vaccines that used a combination of polymer properties and formulations in an attempt to recapitulate traditional discrete antigen dosing kinetics. In the end, rats receiving one injection of a formulation containing alum-adsorbed DT and DT encapsulated in microspheres displayed the greatest immune response which was consistently similar or superior to 3 boluses of alum-adsorbed DT at 0, 1, and 2 months over the duration of the 60-week study [32]. This formulation likely benefited from the alum-enhanced primary immune response as well as extended release and particle-related adjuvant effects for an improved secondary response.

3.4. Other antigens

Controlled release devices have been used to encapsulate a large number of antigens against diseases as diverse as the plague [88,89], bacterial pneumonia [90], meningitis [91], tuberculosis [92], brucellosis [93], and anthrax [70]. On the whole, these studies demonstrate a similar adjuvant effect of controlled release devices that results in a stronger immune response, but with additional stability issues. Whereas DT and TT are stable at 37 °C for months and the current hepatitis B vaccine at 37 °C for weeks, other antigens are less stable for as little as two days at elevated temperature [94]. Therefore, the major challenge of developing these vaccines into a single-administration format is the ability to keep them stable for months under physiological conditions. This has been a long-standing goal for conventional vaccines as part of the effort to reduce reliance on the cold chain, but will be even more critical for single-injection vaccines. However, because improving vaccine thermostability has proven exceptionally difficult, the first generation of commercialized single-injection vaccines will likely be limited to antigens that are already moderately stable.

4. Challenges

4.1. Summary

Despite the promising results from a number of pre-clinical studies (Table 1), there are still a number of substantial challenges in the development and commercialization of single-administration vaccines. Some of these challenges relate to logistical feasibility (e.g., cost of implementation, clinical trials, and adoption rate) while others are associated with the technical challenges of this approach. Broadly, these technical challenges fall into four categories: (1) safety, (2) efficacy, (3) antigen stability, and (4) release kinetics. As with any medical treatment, single-injection vaccination must be both safe and efficacious. While these issues are inherently linked for vaccines (i.e., an ineffective vaccine fails to prevent disease), there are also additional considerations that must also be addressed when used in combination with controlled release systems. Antigen stability is already an issue for current vaccines, but will be even more challenging for single-injection vaccines, which require that antigens retain their native conformation during formulation, storage, and for months in a warm, aqueous environment. Achieving optimal release kinetics may also prove difficult because a vast majority of vaccine efficacy data is based on short, bursts of high concentration antigen which may be far from ideal. The following section discusses each of these challenges in detail and, when possible, potential approaches to ameliorate or mitigate specific issues.

4.2. Safety

As with any potential replacement therapy, single-administration vaccines must be at least as safe as the existing alternative in terms of the frequency and severity of adverse reactions. Because vaccine antigens have already been extensively tested for safety in bolus formulations, the major safety issues surrounding single-injection vaccines will likely relate to the microparticles themselves as well as any stabilizing excipients that have been added to improve antigen survival for months in the body. At the most basic level, single-injection vaccines must be prepared under sterile conditions to prevent contamination.
**Table 1**

*In vivo* studies of clinically used vaccine antigens in single-injection vaccines.

<table>
<thead>
<tr>
<th>Release device</th>
<th>Excipients</th>
<th>Release kinetics</th>
<th>Immune response to best formulation</th>
<th>Animal model</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Tetanus toxoid (TT)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PLA &amp; PLGA microspheres</td>
<td>–</td>
<td>Initial burst, then continuous release for 30 days</td>
<td>Superior to one bolus delivered without an adjuvant</td>
<td>Mouse</td>
<td>[78]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>–</td>
<td>Small initial burst, slow continuous release through 10 days, then faster continuous release through 30 days</td>
<td>PLA microspheres generally inferior to one alum-adsorbed antigen bolus, PLGA microspheres were non-inferior throughout the 26-week experiment</td>
<td>Mouse</td>
<td>[79]</td>
</tr>
<tr>
<td>PLA &amp; PLGA microspheres</td>
<td>–</td>
<td>Initial burst, then continuous release for 30 days</td>
<td>Inferior to two alum-adsorbed antigen boluses delivered at 0 &amp; 4 weeks at early time points, non-inferior at 52 weeks and after a subsequent challenge</td>
<td>Guinea pig</td>
<td>[80]</td>
</tr>
<tr>
<td>PLA &amp; PLGA microspheres</td>
<td>–</td>
<td>Initial burst, then continuous release for 30 days</td>
<td>Inferior or statistically similar to two alum-adsorbed antigen boluses delivered at 0 &amp; 4 weeks at early time points, then superior after being challenged at 54 weeks</td>
<td>Mouse</td>
<td>[80]</td>
</tr>
<tr>
<td>PLGA microspheres with gelatin/poloxamer core</td>
<td>–</td>
<td>Burst lasting up to 48 h</td>
<td>Inferior to one alum-adsorbed antigen bolus when delivered alone, but superior when microspheres were co-delivered with a small portion of the dose as alum-adsorbed antigen</td>
<td>Mouse</td>
<td>[82]</td>
</tr>
<tr>
<td>Collagen minipellet</td>
<td>–</td>
<td>Initial burst, then continuous release for 14 days</td>
<td>Superior to two alum-adsorbed antigen boluses administered at 0 &amp; 2 weeks containing twice the cumulative dose</td>
<td>Mouse</td>
<td>[60]</td>
</tr>
<tr>
<td>Chitosan microspheres</td>
<td>Trehalose</td>
<td>Continuous release for 35 days</td>
<td>Superior to two alum-adsorbed antigen boluses administered at 0 &amp; 4 weeks containing twice the cumulative dose</td>
<td>Guinea pig</td>
<td>[31]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>Trehalose, Mg(OH)₂</td>
<td>Continuous release for 35 days</td>
<td>Superior to two alum-adsorbed antigen boluses administered at 0 &amp; 4 weeks containing twice the cumulative dose</td>
<td>Guinea pig</td>
<td>[31]</td>
</tr>
<tr>
<td>PLA microspheres</td>
<td>Albumin, sucrose, NaHCO₃</td>
<td>Initial burst, then continuous release for 120 days</td>
<td>N/A (not compared to a bolus control)</td>
<td>Rat</td>
<td>[199]</td>
</tr>
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**Hepatitis B surface antigen (HBsAg)**

<table>
<thead>
<tr>
<th>Release device</th>
<th>Excipients</th>
<th>Release kinetics</th>
<th>Immune response to best formulation</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA &amp; PLGA microspheres</td>
<td>–</td>
<td>Release over several months (results not shown)</td>
<td>Non-inferior to three alum-adsorbed antigen boluses administered at 0, 1, &amp; 6 months containing an equivalent cumulative dose</td>
<td>Mouse</td>
<td>[85]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>Sucrose</td>
<td>Formulation-dependent: (1) initial burst followed by little release through day 30, then fast continuous release for 100 days or (2) a large initial burst, then continuous release for 20 days</td>
<td>Inferior to two alum-adsorbed antigen boluses when delivered alone, but non-inferior when microspheres were co-delivered with a 25% of the dose as alum-adsorbed antigen</td>
<td>Mouse</td>
<td>[84]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>Trehalose, Mg(OH)₂</td>
<td>Continuous release for 42 days</td>
<td>Superior to one alum-adsorbed antigen bolus delivered at 0 &amp; 2 weeks containing the equivalent cumulative dose</td>
<td>Guinea pig</td>
<td>[176]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>Trehalose, Mg(OH)₂</td>
<td>Continuous release for 42 days</td>
<td>Non-inferior to two doses of alum-adsorbed antigen boluses administered at 0 &amp; 4 weeks containing an equivalent cumulative dose</td>
<td>Guinea pig</td>
<td>[31]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>–</td>
<td>Small initial burst, then continuous release for 60 days</td>
<td>Non-inferior to three alum-adsorbed antigen boluses administered at 0, 4, &amp; 8 weeks containing an equivalent cumulative dose</td>
<td>Mouse</td>
<td>[30]</td>
</tr>
<tr>
<td>Alginate-chitosan-PLGA microspheres</td>
<td>–</td>
<td>Initial burst, then continuous release through 91 days</td>
<td>Non-inferior to two alum-adsorbed antigen boluses delivered at 0 &amp; 4 weeks containing an equivalent cumulative dose</td>
<td>Mouse</td>
<td>[56]</td>
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**Diphtheria toxoid (DT)**

<table>
<thead>
<tr>
<th>Release device</th>
<th>Excipients</th>
<th>Release kinetics</th>
<th>Immune response to best formulation</th>
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<tbody>
<tr>
<td>PLA microspheres</td>
<td>Gelatin</td>
<td>Continuous release for 60 days</td>
<td>Non-inferior to three calcium phosphate-adsorbed antigen boluses administered at 0, 1, &amp; 2 months containing an equivalent cumulative dose</td>
<td>Mouse</td>
<td>[86]</td>
</tr>
<tr>
<td>PLA microspheres</td>
<td>Gelatin</td>
<td>Initial burst, then continuous release</td>
<td>Non-inferior to three alum-adsorbed antigen boluses administered at 0, 1, &amp; 2 months containing an equivalent cumulative dose</td>
<td>Mouse</td>
<td>[87]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>–</td>
<td>Results not reported</td>
<td>Non-inferior to three alum-adsorbed antigen boluses administered at 0, 1, &amp; 2 months containing an equivalent cumulative dose, or superior when microspheres were co-delivered with a portion of the dose as alum-adsorbed antigen</td>
<td>Rat</td>
<td>[32]</td>
</tr>
<tr>
<td>PLGA microspheres</td>
<td>Albumin</td>
<td>Formulation dependent: (1) 50% of antigen released in 2 days or (2) 2% released in 2 days</td>
<td>Non-inferior to one alum-adsorbed antigen bolus</td>
<td>Guinea pig</td>
<td>[200]</td>
</tr>
<tr>
<td>Collagen minipellet</td>
<td>–</td>
<td>Initial burst, then continuous release for 14 days</td>
<td>Inferior to three alum-adsorbed antigen boluses administered at 0, 2, &amp; 4 weeks containing three times the equivalent cumulative dose</td>
<td>Mouse</td>
<td>[60]</td>
</tr>
</tbody>
</table>
while maintaining antigenicity. In addition, most microparticle fabrication techniques use organic solvents to encapsulate antigen in a polymer. Although solvents are subsequently evaporated off, there may be residual amounts of solvent remaining within the particle that could be toxic to the host as well as damaging for the antigen. One group performed a residual solvent analysis on their microspheres and found that they contained 37.8 ppm by mass of dichloromethane (100 ppm is the limit established by the United States Pharmacopeia), which stresses the importance of a thorough drying step [56,95]. A related, and potentially more serious, concern is that the solvents have already had an irreversible, negative effect on the antigen, which will be discussed in greater detail in a subsequent section.

Microparticles, like any implanted material, will be subject to the foreign body reaction [9]. Upon injection, particles trigger a cascade of cellular events that recruit immune cells to the injection site that attempt to degrade the particles. The severity of the foreign body reaction is highly dependent on the material used for encapsulation, anatomical location of the implant, and the size of the release device [90]. If inflammation is severe, the patient can experience redness, pain, swelling, and ultimately loss of function near the injection site [97], though this reaction is also a potential side effect of existing vaccines as well [98]. Surface modifications may be used to minimize inflammation, but their effects on kinetics and antigen stability would need to be studied in parallel [99].

Several additional safety issues must also be considered before single-administration vaccines obtain widespread use. First, several groups have suggested that these systems may induce immune tolerance due to slow and prolonged antigen leakage based on several studies conducted over half a century ago that demonstrated semi-continuous antigen administration could cause “immunological paralysis” [100–102]. However, more recent studies using continuous release microparticles have not observed this effect [80,103]. Second, unless antigen destabilization can be completely overcome, controlled delivery devices will also release some proportion of degraded, potentially toxic antigen [104]. Lastly, from a logistical standpoint, vaccines can only be effective if they reach their intended target, so the administration of these devices must be simple and reliable. For microparticles, this means that all or nearly all particles are injected through a syringe. Although this is a simple enough concept, particle aggregation and nonspecific binding to the inner syringe surface may reduce the amount of load delivered. Therefore, some groups have begun to inject particles in a viscous solution such as carboxymethyl cellulose to mitigate these issues [105–107].

4.3. Vaccine efficacy & adjuvants

The efficacy of a vaccine is just as critical as formulation safety since a failure to induce immunity could have severe consequences. To be seriously considered for widespread adoption, single-injection vaccines will likely need to induce comparable or superior immunity when compared to traditional immunization methods. Even if single-injection vaccines outperform traditional injection regimens in the developing world in “real-world” use due to limited patient access (and thereby poor compliance), this may be insufficient motivation for vaccine manufacturers to develop a product specialized for the developing world. Despite the fact that nearly 90% of vaccines are sold to developing countries, these sales account for only 25% of total vaccine revenue due to tiered pricing [3]. Therefore, to have commercialization potential single injection-vaccines must at the very least match current vaccine efficacy to merit the cost of development which can rise as high as $800 million (though the cost of implementing a novel delivery system using an existing vaccine may be significantly less) [3].

Adjuvants are immunopotentiators that may be incorporated into vaccine formulations to enhance the efficacy of the antigen. These compounds have been used for both traditional and single-injection vaccines to either improve the immune response with the same quantity of antigen or attain the same magnitude of response using significantly less antigen (i.e. dose-sparing) [108]. Adjuvants function by recruiting antigen presenting cells to the vaccine injection site to enhance the immune response [3]. Therefore, it should not be surprising that adjuvants can increase inflammation at the injection site, especially when implanted subcutaneously [109–111]. Antigen may be adsorbed on the surface of traditional aluminum salt adjuvants such as AlPO₄ and Al(OH)₃ to improve the immune response [39,112–114]. More recently, monophosphoryl lipid A, a less-toxic derivative of a bacterial lipopolysaccharide, has also been used successfully as an oil phase adjuvant in several vaccines licensed internationally for influenza, hepatitis A, and melanoma [115–117]. This compound is currently co-administered as an emulsified oil, but could be easily incorporated into microparticles through co-precipitation with the polymer phase during fabrication using an emulsion/evaporation technique [3,118]. Similarly, water-soluble adjuvants could be incorporated into the water phase during an emulsion/evaporation or other method to co-encapsulate antigen and immunopotentiators [119]. Several groups have used this approach to incorporate alum-adsorbed antigen into microparticles with varying results. Pandit and colleagues demonstrated an approximately 2- to 5-fold increase in antibody titers for the duration of the 400-day experiment when incorporating alum-adsorbed HBsAg into microparticles compared to the encapsulation of HBsAg alone [120]. Alternately, Esparza and Kessel did not observe any difference in immune response between microparticles containing alum-adsorbed or containing TT alone [121]. Polyinosinic acid–polycytidylic acid (poly(I:C)), a mimic of viral double-stranded RNA has also been explored extensively as an adjuvant in both in solution and within polymeric microspheres. While poly(I:C) is an effective immunopotentiator, issues of autoimmunity persist which may limit its use in the immediate future [122]. For a more comprehensive overview of vaccine adjuvants please refer to a review by O’Hagan and Valiente [123].

Controlled release devices themselves may have an even stronger adjuvant effect than traditional immunopotentiators. Whereas classical adjuvants function by stimulating immune cell function at the injection site, particulate-based release devices do this and bring a proportion of the antigen to the immune cell-rich lymph nodes. Macrophages infiltrate the injection site as part of the foreign body reaction and combine with resident dendritic cells to phagocytose small (<10 μm) particles. Macrophages carrying these engulfed particles (and encapsulated antigen) then migrate to the lymph nodes where antigen is in close proximity to lymphocytes and can be maximally effective [44,124–126]. While it is difficult to uncouple this adjuvant effect from the effect of extended release, several groups have shown improved immune responses to bolus and microparticle-based vaccines injected directly into the lymph nodes [127–130]. However, reliably administering vaccines to the lymph nodes in patients worldwide would require an extensive (and costly) training process, so designing particles that target the lymph nodes after administration is likely a more practical approach. Multiple groups have attempted to exploit this routing mechanism using smaller particles, conjugating biological moieties to the particle surface, or by chemically modifying the particle surface to modulate macrophage and dendritic cell behavior [131–133].

Even without specific surface modification, many studies have shown an extreme improvement in antibody titers when antigens are incorporated into small particulate controlled release devices. Eldridge and colleagues encapsulated staphylococcal enterotoxin B toxoid into PLGA microspheres and found that antibody titers were over 4000-fold (225) higher than an equivalent bolus dose 50 days after administration. Interestingly, when a bolus was instead co-injected with empty microspheres this effect was lost suggesting that macrophage routing is the major mechanism of action [44]. Another group demonstrated a similar adjuvant effect with antibody titers increasing 3250-fold at 8 weeks when PLGA-encapsulated OVA was compared to bolus administration [134]. Taken together, these studies suggest that...
single-administration vaccines may be compatible with less potent antigens (such as proteins subunits) or enable dose sparing due to their inherent adjuvant effect [103].

4.4. Antigen degradation during formulation

In order for vaccines to be effective, they must deliver antigen at the correct time and in the correct form. For single-administration vaccines, this means a controlled release system that releases antigen in its native conformation over an extended period of time. Specifically, the antigen must be sufficiently similar to the same epitope on the environmental pathogen. Repeated antigen presentation then helps to establish a more robust immune memory by increasing antibody avidity and the number of long-lived plasma cells [9]. Unfortunately, most vaccine antigens are quite unstable and may undergo a variety of chemical and physical changes including hydrolysis, oxidation, deamidation, unfolding, and aggregation [135]. These processes may occur (1) during microparticle fabrication, (2) while being stored, or (3) after implantation into the body [45]. Antigen degradation after implantation is likely to be the most challenging aspect of single-injection vaccine design because the temperature and hydration state of the formulation cannot be controlled in the way they can during the other two stages. Nevertheless, antigen stability must be optimized for each of these periods in order to immunize patients after just one injection.

Microparticle fabrication methods for single-administration vaccines aim to encapsulate antigens without affecting their structure. A loss of antigenicity at this stage not only reduces the efficacy of the vaccine, but also produces potentially toxic byproducts in the form of denatured or aggregated antigen [104]. The major sources of antigen degradation during fabrication include solvent interaction, physical stressors, drying, and heat. Unfortunately, virtually all methods that are widely used to create microparticles introduce either solvents, heat, or both during some stage of fabrication [28,45]. Emulsion-based methods are attractive for their simplicity, extensive history of use, and potential scalability, but are not necessarily ideal for retaining antigen stability [27,28]. Antigen encapsulated in microspheres formulated by a water-in-oil-in-water (W/O/W) emulsion method may come in contact with organic solvent, be heated, or undergo physical stress as they are sonicated or vortexed — each of which may induce deleterious antigen denaturation or aggregation [136–140]. Antigen encapsulated via a solid-in-oil method would undergo even more stress as the antigen is completely immersed in the solvent rather than just interacting at the phase interface during water-in-oil fabrication [28].

Although not all antigens exhibit the same sensitivity to environmental stressors, some degradation mechanisms can be easily mitigated by changing fabrication parameters. For example, using a more hydrophobic solvent or changing surfactants can help minimize antigen degradation by reducing interaction at the solvent interface [79,141,142]. Similarly, reducing the power and duration of sonication or vortexing when mixing may also lead to greater native antigen recovery by reducing the amount of physical stress and heat generated [138–140]. These changes may also alter the size of the particles and, in turn, the release kinetics. However, increasing particle size may offer a secondary benefit of reducing the surface area-to-volume ratio and thereby relative area for solvent–antigen interaction.

Another stabilization strategy involves the incorporation of additives that reduce stress during microsphere fabrication. These approaches either reduce the interaction between the antigen and its environment or make the antigen’s native conformation more favorable [28,143]. Many groups have added non-reducing sugars such as trehalose or polyols like sorbitol to stabilize secondary antigen structure during device formulation [144–148]. While the mechanisms by which these osmolytes function has yet to be conclusively determined, prevailing theories center on water exclusion and improved hydrogen bond strength that makes conformational changes away from the native state even more energetically unfavorable [149–151]. Most of these compounds appear to minimize antigen damage during both drying and after they are implanted into the body.

Spray drying is an alternative microparticle fabrication method that generates particles with well-defined size and is even more scalable than emulsion-based methods, but presents an additional challenge in terms of temperature [48,152]. Spray drying uses a jet of hot air to quickly evaporate solvent and water during particle formation. Because vaccines are typically heat liable, this is problematic for antigen stability; however, the extremely short duration of heating may limit the amount of damage [28]. As with emulsion-based methods, spray drying parameters can be tuned to alter particle properties and potentially improve antigen recovery after encapsulation. Derivatives of spray drying such as freeze spray drying and coaxial capillary electrospraying have also been developed to produce microparticles with more complex morphology such as core-shell structures with varying wall thickness [153,154].

Several novel microparticle fabrication techniques are also being developed to minimize stress on the antigen during encapsulation for single-injection vaccines [155–157]. Reinhold and colleagues describe a self-healing method of microencapsulation that eliminates the need for organic solvents. They used an emulsion-based method to encapsulate trehalose in PLGA microspheres and then leached out the sugar to generate pores throughout the particles. These porous particles were then immersed in a lysozyme solution which entered through the pores to occupy some of the internal void space. They then introduced a small amount of heat to bring the polymer just above its glass transition temperature causing the surface “self-heal” (i.e. seal) with lysozyme encapsulated inside [157]. Stenekes and coworkers developed a solvent-free method to fabricate dextran microspheres based on polymerization of methacryloyl groups attached to dextran while emulsified in an aqueous poly(ethylene glycol) solution [156]. Several other groups have also demonstrated the use of phase separation techniques to formulate particles [158]. Although these methods are not as well-established or flexible as emulsion-based techniques, they may be capable of overcoming several antigen stability issues that occur using conventional techniques.

4.5. Antigen degradation during storage

Antigens may also degrade on-the-shelf between vaccine formulation and injection into the body. For a vaccine to be commercially viable, it must retain efficacy (typically defined as $<0.5\log_{10}$ loss of antigenicity) for at least one year [135,159]. Because most traditional vaccines are prone to thermal degradation, there are many facilities across the globe that are already equipped with cold storage [94,160]. This cold chain infrastructure could easily be repurposed for storing single-injection vaccines in the same manner with little, if any, modification. In addition, storage limitations may actually be alleviated by single-injection vaccines which reduce the number of total doses that need to be delivered. Therefore, there is an opportunity for single-injection vaccines to be stored at any temperature down to $-20\,\text{°C}$. However, while freezing will slow biological processes and reduce molecular mobility more than refrigeration, it may also damage the antigen during the freeze–thaw cycle and/or cause particles to aggregate [28]. Just as with traditional vaccines, the storage temperature for a particular single-injection vaccine will require optimization on a case-by-case basis.

The physical state of single-injection delivery systems is also likely to have a major effect on stability. Antigen-containing delivery devices are almost ubiquitously stored in a dry state. This not only eliminates residual solvent and reduces the potential for antigen degradation (via hydrolysis, unfolding, and aggregation), but also prevents the device from degrading and thus “starting the clock” on antigen release prior to administration. This can be done through simple air-drying or vacuum-drying, but is more commonly performed via lyophilization (freeze-drying). Each of these processes can cause extreme stress to the antigen resulting in denaturation and disulfide bond-induced
aggregation [83,161]. As a result, most groups now incorporate cryo- or lyo-protectants into their formulations to vastly improve recovery [94, 145]. Non-reducing disaccharides and surfactants are usually among the best lyo-protectants, but may still need to be used in many-fold excess compared to the antigen they are stabilizing [161].

Trehalose is an especially interesting excipient for lyophilization because it is naturally used by plants and lower animals for protection against both desiccation and heat [162–164]. Jaganathan and colleagues demonstrated that the addition of 0.5 to 2.0% w/v trehalose as an excipient in the encapsulated tetanus toxoid solution improved loading efficiency of native TT into both PLGA and chitosan microspheres from 30–40% to 80–90% [31]. Johansen observed a similar effect with trehalose, showing that the addition of 20% w/w trehalose relative to PLGA mass improved TT recovery after encapsulation from 21% to 31%. Using 15% trehalose with 5% BSA further increased TT recovery to 43% as detected by ELISA [165]. Polyethylene glycol, polyvinylpyrrolidone, sorbitol, mannitol, amino acids, and dextran have also been used to increase antigen recovery after drying, but generally achieve a less pronounced improvement [145,166]. Finally, although these compounds may significantly improve antigen stability during drying and storage, they also substantially increase the amount of material that needs to be encapsulated into microspheres presenting a potential loading issue.

4.6. Antigen degradation after administration

The body itself presents the greatest challenge for retaining antigen effectiveness since it is constantly at an elevated temperature and well-hydrated [167]. Antigens currently stored at −20 °C or 4 °C when administered in multiple doses must retain their antigenicity at 37 °C for weeks or months between primary and secondary antigen release in order to remain effective. Higher temperatures provide energy that increases the probability that the antigen assumes a non-native conformation. Water further increases the potential for antigen degradation by increasing protein mobility and the rates at which hydrolysis, oxidation, and deamidation occur [94]. These processes are discussed in further detail in a book chapter by Schwedeman [45]. The process of rehydration itself has been shown to cause protein aggregation and thus loss of antigen efficacy [160,168]. In addition, pH, although well-maintained by the body, may diverge substantially from neutral within degrading polymeric microspheres resulting in antigen denaturation [169,170]. Although acidification is unlikely to occur quickly enough to affect the initial burst release from the microsphere surface, it may reduce the effective dose delivered during the secondary antigen release phase [171]. Conjugate vaccines may also require additional stabilization to prevent the dissociation of the protein carrier and polysaccharide, which could otherwise lead to ineffective immunization [94].

The most common strategy used to mitigate protein denaturation is to incorporate stabilizing excipients into the formulation. These additives interact with the antigen to make the native conformation more energetically favorable or prevent potentially harmful interactions between the antigen and its environment. Some of the same excipients evaluated for freeze-drying protection also appear to confer protection in a warm, hydrated environment. Non-reducing sugars, buffering agents, osmotic agents, proteins, surfactants, and other small molecules are among the most common therto-stabilizing additives used in existing vaccine formulations and may be beneficial for single-injection vaccines as well [15,75,81,144,172]. Sugars improve thermostability through hydrogen bonding with the antigen [149]. Buffering agents help prevent deamidation, conformational changes, and aggregation by preventing pH change [173,174]. Osmolytes serve to shift protein folding equilibrium in a favorable direction while also preventing aggregation through preferential antigen hydration [148]. Proteins and surfactants also inhibit aggregation while also shielding the antigen of interest from interfacial damage [75]. Lastly, small molecules may specifically interact with proteins to stabilize their structure [175]. Sugars may also be capable of physically entrapping antigen in a glassy matrix that prevents antigen mobility and thus conformational changes [147], though it is unclear if this effect would be lost in hydrated environment. Jaganathan and coworkers demonstrated the beneficial effect of trehalose for HBsAg, as increasing the percentage of trehalose in the encapsulated solution from 0.5% to 2.0% improved the in vitro release of antigenically active HBsAg from PLGA microspheres from 15% up to >90% on day 42 as determined by measuring the amount of ELISA-reactive versus total protein. This study also demonstrated statistically similar antigenicity between HBsAg encapsulated with 1.5% trehalose and 2% Mg(OH)2 compared to alum-adsorbed HBsAg after 16 days in vitro at 37 °C [176]. Shi and colleagues explored sucrose as an additive and observed that adding 2% sucrose into PLGA microspheres increased the in vitro antigenicity of HBsAg from <20% to 65% [84]. Unfortunately, these small molecule excipients may diffuse out of particles more quickly than the larger antigen leading to unstabilized antigen during the latter stages of release [45].

Acid-induced aggregation, deamidation, and conformational changes caused by a drop in particle pH due to acidic degradation products can be mitigated by including buffering excipients, altering polymer properties or changing the geometry of the delivery device. Many studies have incorporated basic excipients such as Mg(OH)2, MgCO3, and NaHCO3 to counteract the acidity of degradation products with good results [167,171,177]. Proton sponges and buffers have also been evaluated, but have not been as widely reported [178]. Because the pH-sensitivity of antigens and acidity of polymer degradation products can vary greatly, the best pH-stabilizing excipients will likely be dependent on the particular delivery system studied. More basic excipients can better prevent acidification, but result in an initially alkaline environment that may cause some antigens to denature. Likewise, bases with high solubility will be able to diffuse through pores more easily to neutralize pH, but may diffuse out of the particle quickly reducing the duration of this effect. To date, Mg(OH)2 has been the most widely used excipient for limiting pH change due in part to its poor solubility which allows it to act as a largely solid-state buffering agent to prevent acidification without initially increasing pH [169,177]. Device geometry may also play an equally important role in the pH of the antigen microenvironment as the core of larger particles can become more acidic than small particles [170]. These aspects will be discussed further in the following section as both pH and particle size have significant effects on antigen release kinetics.

4.7. Antigen release kinetics

At the most basic level, controlled release devices have garnered attention for their ability to release a therapeutic over an extended period of time. For most drug delivery applications this consists of steady, continuous release of small molecules to maintain a drug concentration in the therapeutic window, which is a drastic improvement over bolus injections that cause drug levels to spike and then quickly fall. Unfortunately for vaccines, it is not yet clear what antigen release kinetics are optimal for inducing robust immunological memory [8,179,180]. This area is severely understudied due to the efficacy of existing vaccination schedules and prohibitive cost associated with conducting such a study. Further, in some cases it may even be unethical to explore novel alternatives to multi-bolus vaccine regimens due to already high seroconversion rate as the potential benefits do not justify the risks.

Nearly every polymer and device characteristic has the potential to impact degradation and thus release rate including chemical composition, molecular weight, hydrophobicity, glass transition temperature, crystallinity, device dimensions, and porosity [181]. While this certainly complicates the optimization process, it also provides multiple opportunities to improve upon existing designs. For the purposes of single-injection vaccination systems, release kinetics may be generally classified as either continuous or pulsatile (Figs. 3 & 4). Pulsatile antigen release kinetics are ideal from a regulatory approval standpoint because they recapitulate traditional multi-bolus vaccine administration which
has well-known safety and long-term efficacy [103]. As a result, a controlled release device with these kinetics could present the easiest path to clinical use by eliminating concerns over tolerance and chronic inflammation that might otherwise delay regulatory approval [100–102]. However, the current multi-bolus regimen has arisen out of convenience rather than a thorough optimization process, so there are likely different kinetics which elicit superior seroconversion rates. The results of several recent studies suggest that semi-continuous or continuous antigen delivery can elicit a superior immune response with increased antibody affinity and titers [30,54,182,183]. Continuous release may work well for inactivated, conjugate, and subunit vaccines which rely on high antigen doses, but will likely be incompatible with live (attenuated) vaccines. Live vaccines are administered at a low dose and must replicate in the body to evoke an immune response. However, continuous release could result in high antibody titers that neutralize the antigen and prevent in vivo replication during the secondary immunization phase, potentially negating the benefit of extended release [15].

Virtually all release profiles from monolithic controlled release devices begin with an initial burst as antigen is desorbed from the surface of the device. The relative size of the burst compared to the total encapsulated antigen can be affected by a number of parameters including loading. Higher antigen loading as a percentage of total device mass will increase the relative size of the initial burst through a positive feedback loop in which antigen that desorbs from the surface and generates a void that allows water to access more deeply encapsulated antigen and further increase the pore depth [23]. Device size also affects the magnitude of the initial burst through a similar surface accessibility phenomenon. Smaller particles have a higher surface area-to-volume ratio and thus there are more antigens at the particle surface relative to their interior. As a result, these particles release a larger proportion of their total dose during the first burst than do larger, similarly-prepared particles [184]. One study using bolus vaccine administration has shown that a smaller priming dose may aide in the formation of immune memory, so it may be desirable to keep this burst relatively small [185].

Subsequent release kinetics are dependent on a number of factors including the degradation properties of the encapsulating material and device geometry (a more complete list is included in Table 2). Synthetic polymers may be particularly well-suited for obtaining the desired release kinetics as each property can be precisely and reproducibly tuned. Increasing the molecular weight of the encapsulating polymer typically increases the duration of release as can altering the co-polymer ratio. Bulk-degrading materials such as PLGA will also exhibit far different kinetics than surface-degrading materials such as polyanhydrides. PLGA microspheres can be formulated differently to obtain continuous or pulsatile release kinetics (Figs. 3 & 4) [26,48,179], but surface-degrading materials will generally produce an inherently smooth and continuous release profile unless a more complex, layered approach is adopted (Fig. 1) [65,66]. Antigen properties may also greatly affect the release rate from controlled release devices. For example, TT is larger (150 kDa vs. 60 kDa) and more hydrophobic than DT suggesting that it would be released more slowly from an otherwise equivalent device. Further, TT would not be released until the pore diameter was somewhat larger and even then may escape more slowly because the aqueous pore environment is less favorable for TT than the relatively hydrophilic DT [77]. This theory has been verified in practice by Higaki and colleagues who showed that TT releases from collagen minipellets more slowly than DT [60].

In addition to affecting antigen stability, pH change inside degrading polymeric vaccine-delivery devices may also affect release rate. Encapsulating buffering agents, as in the previous section, is therefore likely to have a two-part effect in both stabilizing the antigen and reducing release rate. Adding buffer will help to maintain a near-neutral pH and prevent acid-catalyzed hydrolysis which would otherwise increase release rate as a function of polymer degradation [187]. The pH inside microspheres is also dependent on a number of factors including the polymer type, molecular weight, and particle size [170,188].

### Table 2
Factors affecting antigen release kinetics.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
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<tbody>
<tr>
<td>Antigen encapsulation technique</td>
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<tr>
<td>Antigen loading</td>
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<tr>
<td>Antigen–polymer interactions</td>
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<tr>
<td>Antigen size</td>
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<td>Device size</td>
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<td>Excipient loading</td>
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<td>Injection site</td>
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<tr>
<td>Polymer type</td>
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<td>- Molecular weight</td>
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<td>- Copolymer ratio (if applicable)</td>
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<td>- End cap group</td>
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<tr>
<td>- Degradation products</td>
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<td>- Hydrophobicity</td>
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<td>- Crystalinity</td>
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properties will determine not only the speed at which these particles degrade (and therefore produce acidic degradation products), but also the acidity of these degradation products and the speed at which they are cleared into the surrounding environment. Bulk degrading materials such as PLGA generate acidic degradation products throughout their volume, which induces a pH gradient between the highly acidic core and well-buffered body. Smaller particles present a shorter diffusion distance that acidic degradation products must travel allowing them to be cleared before producing a major pH gradient. Fu and colleagues used pH sensitive dyes to explore the internal environment of PLGA microspheres and found that the pH at the center of these particles can drop to as low as 1.5 [170]. Therefore, generating small particles containing insoluble buffers may be the best approach to mitigate the deleterious effects of pH on both release kinetics and antigen stability. Alternately, surface eroding polymers such as polyanhydrides are unlikely to have any pH issues since acidic degradation products are only generated near the particle surface where they can quickly diffuse away.

While most single-injection vaccine studies include some in vitro release data, this may not be representative of in vivo release kinetics. In fact, microspheres degradation is commonly reported to be 1.7–2.6 times more rapid in vivo than in vitro [189] due to the differences that include acid-induced hydrolysis [34] and plasticization by lipids and other biological molecules [190]. This should not be particularly surprising since the environment in which in vitro studies are typically conducted (PBS with replacement every few days) is a poor mimic for the in vivo milieu due to different levels of hydration, buffering, and convection. Unfortunately, in vivo vaccine kinetics are difficult to determine due to the minimal antigen load and long duration of release. Traditional blood sampling is inadequate because antigen may not reach circulation and would have a short half-life relative to sampling frequency. Therefore, there is a clear need for a technique to assess in vivo release of antigens for single-injection vaccines. One approach for studying in vivo release kinetics is to use non-invasive imaging with fluorescently-labeled antigens to assess vaccine depletion from controlled release depots over time. This technique has been used for one study, but has yet to be validated [191].

4.8. Perspectives on future work

Despite the many challenges associated with single-administration vaccination, the paradigm-shifting potential of this approach is more than sufficient to warrant further development. In this review, we have purposely excluded a majority of work on model antigens such as OVA and BSA in favor of potentially therapeutic antigens. Although these classic “model” antigens have been widely used in controlled vaccine delivery systems as inexpensive alternatives for studying release kinetics and antibody responses [98,141,192–195], they fail to recapitulate the most significant challenges currently facing single-injection vaccines. Therefore, whenever possible, we would encourage the use of therapeutic antigens as both stability and release kinetics can be highly antigen-dependent. In addition, because the goal of this work is to translate these vaccines to the clinic, the use of currently approved antigens will greatly reduce the barrier to adoption, though it may be substantially more difficult to obtain these reagents.

Further, while in vitro studies are valuable, the true gold standard remains the in vivo immune response [196]. In vitro release studies are very useful for comparing antigen stability and relative release kinetics between a large number of experimental formulations. These studies may be used in combination with chromatographic, spectroscopic, calorimetric, and electrophoretic techniques to determine the mechanisms underlying antigen denaturation and therefore the class of excipient needed to lessen their effect [45]. However, even then the mechanisms that play a major role in vitro may not be the same as in vivo. Measuring native antigen recovery in vitro fails to demonstrate the full benefits of controlled release systems since they do not appreciate the effects of extended release and immune cell targeting, which may be able to overcome imperfect release kinetics or antigen stability [79]. As a result, in vivo studies that provide a direct measurement of vaccine effectiveness should always be used to verify in vitro results.

In general, in vivo studies should allow for the long-term (6+ month) evaluation of antibody titers, ideally ending with a late-stage challenge to view the speed and magnitude of the immune response. Including a dose-sparing group in these studies would also be interesting to evaluate potential adjuvant effects that could offset the cost of the delivery system. This would be especially interesting for vaccines containing expensive antigens such as the HIV bacterial influenza and pneumococcal conjugate vaccines. Lastly, although performing an ELISA for antibodies in serum is a standard way of evaluating titers, a better measurement of functional immunity is to perform a neutralizing antibody experiment. Depending on pathogen availability, these experiments may be performed in-house as a fee-for-service by the CDC or other centralized health organization. In either case, these studies can be performed during the last stages of single-injection vaccine development to verify the results observed in earlier experiments while avoiding a major cost burden.

Moving forward, controlled release systems for single-administration vaccines should build upon existing knowledge in the field. Specifically, every process and component should be thoughtfully selected to achieve the desired effect. The two most important factors in single-injection vaccine design are antigen stability and release kinetics, which together influence efficacy. Formulation techniques that are scalable and reduce antigen interactions with solvent and heat should be chosen to improve commercialization potential and antigen recovery.

Synthetic materials also appear to be quite useful due to their tunable degradation (and thus release) profiles [48]. Combined administration of antigen-containing devices and traditional alum-adsorbed antigens has also been shown to significantly improve titers in numerous studies and therefore merits additional exploration [32,82,84]. Further complicating development is the fact that each alteration affects the delivery system on multiple levels. For example, adding Mg(OH)2 will likely increase antigen stability by neutralizing pH, slow release kinetics by preventing acid-catalyzed hydrolysis, and increase the size of the initial antigen burst by creating pores on the particle surface. Similarly, small particles increase vaccine efficacy by targeting the lymph nodes and minimize particle acidification to improve native antigen recovery, but expose more antigen to solvent during W/O/W formulation, exhibit a larger initial burst, and may also degrade more quickly than larger particles thus minimizing the benefit of extended release [197,198]. Therefore, a systematic, antigen-specific evaluation of formulations in vivo may be the only way to fully determine the optimal formulation parameters. Though these studies are inherently expensive and time-consuming due to their long duration, if successful, development costs will likely be far outweighed by the benefits of single-injection vaccines in terms of both global health and economic impact.

References


