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Sustained delivery approaches to improving adaptive immune responses

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ABSTRACT

The immune system is one of the most important, complex biological networks regulating and protecting human health. Its precise modulation can prevent deadly infections and fight cancer. Accordingly, prophylactic vaccines and cancer immunotherapies are some of the most powerful technologies to protect against potential dangers through training of the immune system. Upon immunization, activation and maturation of B and T cells of the adaptive immune system are necessary for development of proper humoral and cellular protection. Yet, the exquisite organization of the immune system requires spatiotemporal control over the exposure of immunomodulatory signals. For example, while the human immune system has evolved to develop immunity to natural pathogenic infections that often last for weeks, current prophylactic vaccination technologies only expose the immune system to immunomodulatory signals for hours to days. It has become clear that leveraging sustained release technologies to prolong immunogen and adjuvant exposure can increase the potency, durability, and quality of adaptive immune responses. Over the past several years, tremendous breakthroughs have been made in the design of novel biomaterials such as nanoparticles, microparticles, hydrogels, and microneedles that can precisely control the presentation of immunomodulatory signals to the immune system. In this review, we discuss relevant sustained release strategies and their corresponding benefits to cellular and humoral responses.

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1. Introduction

The immune system serves as the first line of defense that recognizes and responds to pathogens such as parasites, bacteria, and viruses. As such, its dysfunction can result in a plethora of diseases including infections, cancer, autoimmune diseases, and AIDS. One of the first attempts to modulate the immune system was the invention of smallpox vaccines by Edward Jenner in 1796 [1]. Since then, vaccination has become one of the most effective public health interventions and has eradicated or prevented numerous deadly diseases including smallpox, poliomyelitis, measles, rubella, and tetanus. Nonetheless, the development of potent vaccines is still challenging and requires intense investigation for challenging diseases such as HIV, malaria, and tuberculosis as well as ever-changing viral variants like influenza. Robust vaccine design strategies are even more important in the case of emerging pandemics like the one caused by the SARS-CoV-2 virus for which strong protection and rapid worldwide distribution were urgently required. Additionally, new technologies such as cancer immunotherapies and cancer vaccines leverage modulation of the adaptive immune system to better eradicate cancerous cells. To properly mount humoral and cellular responses with lasting memory effector cells would require delicate and precise spatial and temporal control of the immunization processes, from innate cell activation to targeted and prolonged antigen processing and trafficking. Designing delivery carriers able to precisely control the release of vaccine components is therefore a key component in modulating and improving immune responses. Over the years, great advances in biomaterials engineering have resulted in relevant platforms which allow improved stability and loading of multiple drugs as well as controlled and customized delivery.

In this review, we focus on the role of sustained delivery of immunogens through different biomaterials in improving the adaptive immune response. We first describe how the adaptive immune response, comprising both humoral and cellular responses, is activated upon immunization and the ideal immune outcomes for protection against infectious diseases and cancer. We highlight relevant and recent findings that shed light on the importance of sustainably releasing immunomodulatory therapeutics in improving the potency, durability, and quality of the adaptive immune responses. We then discuss relevant macroscale, microscale, and nanoscale biomaterial strategies able to sustain the delivery of immunomodulatory therapeutics to achieve desired protection (Table 1). We finally describe synthetic and naturally derived materials with the ability to provide benefits such as depot formation, directed antigen trafficking, and time-controlled co-delivery of multiple drug payloads. Coordination of these delivery parameters allows heightened adaptive immune responses with the potential to eradicate deadly diseases.

While other strategies on improving the adaptive immune response upon immunization (e.g., enhancing innate cell activations, controlling the spatial/location of the vaccines by targeted delivery of antigens to the lymph nodes) have been reported, this review focuses only on the modulation of the adaptive immune system through the extended delivery of immunomodulatory therapeutics [2]. Therapies such as checkpoint inhibitors that could also benefit from sustained delivery to keep relevant adaptive immune cells active do not activate nor mature adaptive immune cells and have been described elsewhere [3–5]. Therefore, this review focuses on sustained delivery of immunomodulatory therapeutics that are first processed by antigen-presenting cells before activating and maturing adaptive immune cells to harness those effector cells for protection against diseases.

2. The role of time on immune system activation and adaptive immune responses

Upon immunization, proper humoral and cellular immune responses, also known as adaptive immune response, require intricate coordination between cells across the body. Vaccine immunogens, such as antigens for prophylactics or neo-antigens for cancer, are first recognized by innate immune cells in the peripheral tissues. The immunogens are then either passively drained to the lymph nodes through the lymph or first processed by migratory antigen-presenting cells that travel to the lymph nodes (Fig. 1**a/b**). In the lymph nodes (LNs), B cells and T cells are activated and matured into effector cells. Antibodies produced by the effector B cells as well as matured cytotoxic T cells then travel throughout the body providing lasting protection.

The adaptive immune cells' activation in the lymph nodes is an elaborate process. For T cell activation, vaccine immunogens and migratory antigen-presenting cells reach the lymph nodes within hours. These peripheral antigen-presenting cells, such as migratory dendric cells (DCs), can present antigens directly to naïve T cells in the LNs or can transfer it, along with passively drained antigens, to the lymph node resident DCs. The activation and maturation of $CD8^+$ T cells occur upon their interaction with the peptide-Major Histocompatibility Complex (Peptide-MHC) type I complexes on DCs as wells as with co-stimulatory signals and cytokines. Once activated, their interaction with $CD4^+$ T cells directs their fate into short-lived effector cells (cytotoxic T cells) or memory cells. On the other hand, $CD4^+$ T cells have to interact with Peptide-MHC type II complexes on the APCs, including B cells in the LNs (Fig. 1**c**).

B cell activation and maturation occur in B cell follicles. B cells can directly recognize soluble antigens traveling from the peripheral tissues; alternatively, follicular DCs (fDCs) can present antigens to naïve B cells by capturing and retaining antigens that are opsonized. Along with these antigens, B cells require T follicular helper cells (Tfhs), a subset of $CD4^+$ T cells, to provide co-stimulatory signals for activation. Activated B cells then enter germinal centers (GCs) to undergo clonal expansion and somatic hypermutation to expand and diversify their antibodies genes. The positive selection undergone by these mutated B cells is necessary, as they compete for fDCs and Tfhs co-stimulatory signals for survival. Finally, matured B cells exit GCs as effector plasma cells for antibody production or as memory cells (Fig. 1**d**).

A successful prophylactic vaccine affording long-term protection would induce both humoral and cell-mediated immunities. High titers of neutralizing antibodies produced by plasma cells prevent pathogens from infecting host cells while cytotoxic T cells can kill infected cells. For life-long protection, it is necessary to generate robust memory B cells and memory T cells (Fig. 1**e**). Similarly, in the area of cancer immunotherapy, vaccination against cancer neo-antigens is emerging as a powerful therapeutic approach [28]. The vaccination of a patient against antigens

Table 1
Recent advances of biomaterials for sustained delivery of immunotherapeutic.

	Characteristics	Composition	Hydrophobic Cargo	Hydrophilic Cargo	In vivo time scale	Recent improvements	References
Particulates	- Mimic natural pathogens in size and shape - Improve uptake/targeting by APCs	Polymeric materials such as poly(lactic-co-glycolic acid) (PLGA)	++	+	- Months *in vitro* - In vivo not quantified	- Encapsulating protein antigens without organic solvent improves hydrophilic cargo encapsulation - Precise control of the particles' size, shape, and surface chemistry	[51–53,55–59]
	- Improve endosomal escape - Lymph node targeting	Polymeric micelles	++	–	Weeks	- Core-crosslinking micelles to improve stability - Adding specific non-covalent interactions at the end of the hydrophobic blocks to improve cargo loading	[86–88,90,91]
		Polymersomes	++	++	- Months *in vitro* - At least a week *in vivo*	- Improve drug loading by conjugating a hydrophilic polymer to a hydrophobic drug	[32,95–98]
		Lipid based particles	++	++	Weeks	- PEGylation to prevent rapid clearance - Functionalized surface to improve cell-targeting - Cubosomes and other geometries to improve drug loading and retained release	[103–108,114,116,119]
Hydrogels	- Biocompatibility - Depot formation	Synthetic-based polymeric materials such as poly(ethylene glycol) (PEG), poly(D, L-lactide) (PLA), and PLGA	++	++	Months	- Injectable hydrogels - Tunable hydrogels with co-delivery of physical- and chemical- distinct cargos	[148,150,155–157,165]
	- Allow immune cell infiltration and interaction	Naturally derived materials such as hyaluronic acid, chitosan, alginate, DNA, and peptide	++	++	Months	- Initial burst release followed by sustained release mimic a prime-boost vaccination - DNA or peptide hydrogels are naturally adjuvanting	[151,170,175,176]
Microneedles	- Do not require injection - Activate Langerhans and dermal dendric cells	Polymeric materials such as PLGA, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), poly(lactic acid) (PLA), poly (acrylic acid) (PAA), and other composite materials such as silk, chitosan, and sucrose	++	++	Weeks to Months	- Composite materials allow for burst and multistage release - Combining microparticle and microneedle technology - Allow burst release at multiple timepoints with only one administration, eliminating multiple injections	[199–202,205,206]

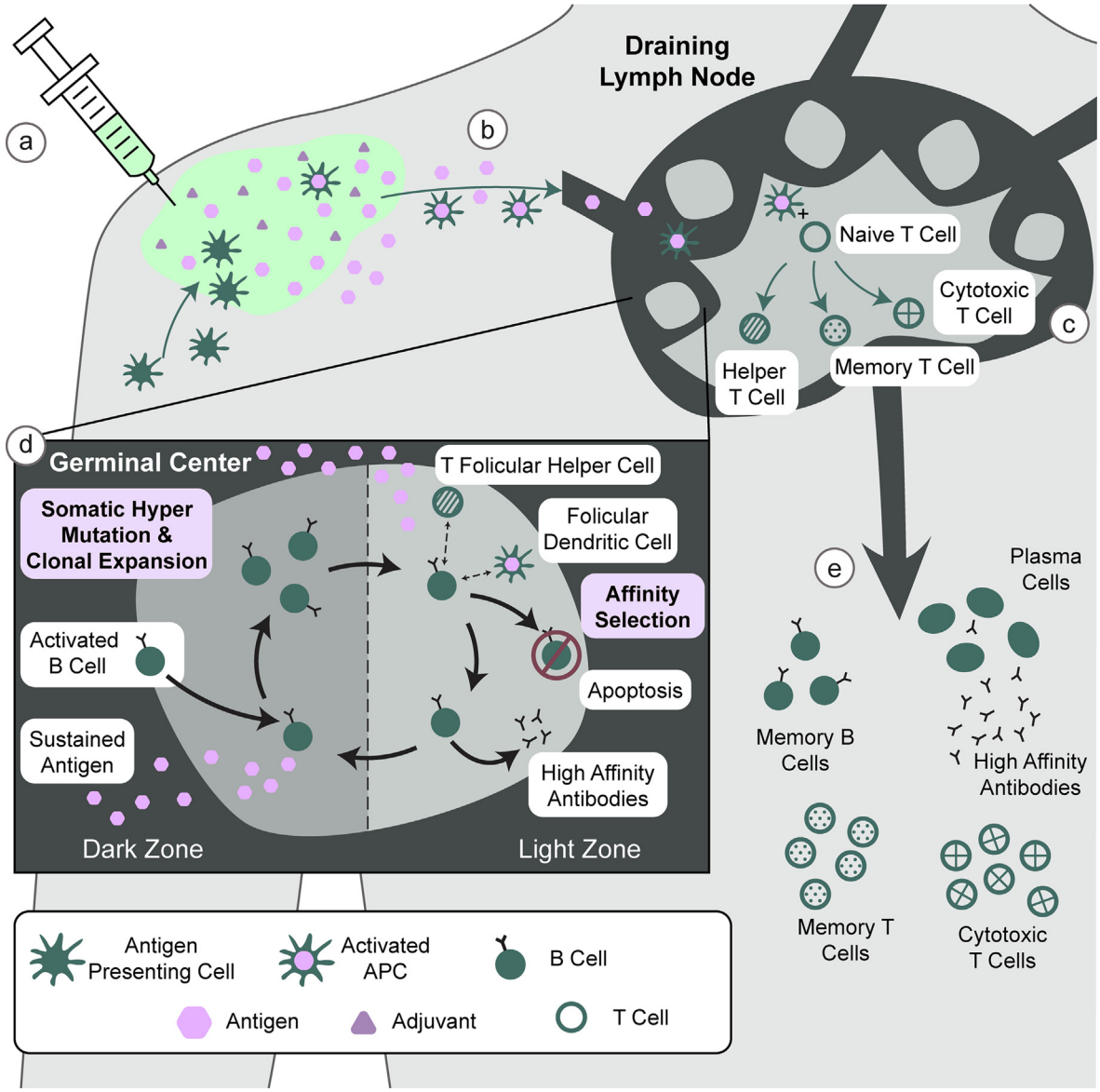

Fig. 1. Major components of the adaptive immune response to immunotherapeutic drug delivery. (a) Delivery of antigen and adjuvant attracts antigen-presenting cell (APC) migration to the initial site resulting in antigen uptake. (b) Activated APCs and soluble antigens traffic to the draining lymph node though the lymphatic system. (c) Within the draining lymph node, antigen-presenting cells activate naïve T cells to differentiate into helper, memory, and cytotoxic T cells specific to the antigen. Alongside T follicular helper cells, soluble antigens and APCs enter the B cell follicles to activate B cells. (d) B cells enter germinal centers and undergo an iterative process of positive selection for affinity maturation. In the dark zone, B cells undergo somatic hypermutation and clonal expansion. In the light zone, T follicular helper cells and follicular dendritic cells facilitate affinity selection, inducing apoptosis of low affinity B cells and promoting high affinity B cell production. Sustained antigenic information allows for numerous cycles of somatic hypermutation, clonal expansion, and affinity selection resulting in B cells that produce higher affinity antibodies. (e) Adaptive immune cells exit the lymph node in the form of memory B and T cells, cytotoxic T cells, and plasma cells that continue to produce high affinity antibodies.

occurring on cancerous cells could provide long term immunity against cancer recurrence. Potent cancer vaccines should mount a large population of effective cytotoxic T cells that could recognize and kill cancerous cells.

The duration of an administered immunomodulatory therapeutic can have great effect on the immune response to these inputs [3]. While natural infections can result in persisting viral materials in the LNs for up to weeks to even months, antigens and other therapeutics from the initial administration of traditional vaccines are often cleared within days [6–9]. Mimicking natural infections by prolonging the presentation of immunomodulatory therapeutics to immune cells is therefore a key element to achieve potent and robust immune responses. A long-used supplement to a vaccine is the adjuvant, a moiety that can draw migratory immune cells to the site of injection [10]. One of the first used and most prevalent adjuvants, alum (aluminum hydroxide or aluminum phosphate), was initially chosen to stimulate immune response and was thought to act as a depot that could adsorb and slowly release antigens [11]. However, numerous studies confirmed that alum's success in initiating immune response is not tied to its adsorption and retention of antigens. Early work showed that antigens adsorbed on alum stayed for less than 3 days at the injection site [12]. More recently, Brewer and co-workers confirmed that an alum depot site was not required by showing similar immune responses when the alum-antigen depot was surgically removed 2 h after injection in mice compared to no resection [13]. Though not all antigens bind securely with alum, phosphorylated proteins interact tightly to alum via electrostatic interactions [14]. This strategy was used to adsorb HIV envelope (Env) immunogens to alum, allowing their presence at the injection site for 3 weeks,

compared to the 3-day duration of free antigen [15]. Moreover, greatly increased germinal center B cell responses as well as neutralizing antibody responses have been observed. Though alum is not always a depot forming vehicle, this work demonstrates the potential for depot antigen retention and slow release to improve vaccine response.

A number of groups have developed relevant strategies to provide extended exposure of immunomodulatory therapeutics [9,16–18]. A novel approach by the Irvine group using osmotic pumps to deliver vaccine continuously over periods of days to weeks elegantly demonstrated the immunomodulatory effects of sustained delivery. Exponentially increasing doses of HIV antigens over a period of 1–2 weeks elicited over 10-fold increase in antibody production as compared to bolus injection. Moreover, the prolonged presence of antigens in LNs resulted in the production of higher affinity antibodies as well as increased amount of Tfhs and germinal center B cells in murine [9] and rhesus monkey models by fine needle aspiration [16]. Slow delivery has a drastic effect on B and T cell development in GCs of the draining LNs, starting with inducing higher frequencies of total and antigen specific GC Tfhs. The larger population and duration of Tfhs in GCs combined with a longer duration of antigen presence by fDCs enable greater and more diverse antigen specific B cell production. Extended B cell production and somatic hypermutation exponentially lead to more opportunities for B cells to develop specificity for less immune dominant antigen epitopes that are usually outcompeted by easily accessible but non neutralizing epitopes. As a result, antibodies produced by the extended timeline vaccination in rhesus monkeys targeted both neutralizing and non-neutralizing epitopes, whereas antibodies from the bolus group heavily favored non-neutralizing epitopes. This finding demonstrates that sustained delivery of antigens is essential for developing neutralizing responses to historically difficult pathogens. In context, B cells only undergo around 10–20 rounds of somatic hypermutation from traditional bolus vaccines, while at least 40–100 rounds of mutations are necessary to generate broadly neutralizing antibodies against HIV, demonstrating the importance of slow delivery and extending GCs activity [19–22]. Other studies have also pointed out the importance of sustained release on cellular immunity, showing that slow delivery of antigens significantly increased the number of cytotoxic and memory T cells [23–27]. This led to improved anti-tumor activities as well as enhanced recall activities.

However, the delivery of vaccines by osmotic pump requires the patient to either undergo surgical implantation or wear an external device for the entire duration of delivery, being therefore cumbersome and challenging with regards to patient compliance. Alternative materials carriers that can be administered without the necessity of a wearable device have been developed to fill this need and involve common delivery methods such as injection or application of a small patch. This review will discuss the use of micro-nanoparticles, injectable hydrogels, microneedles, and other novel materials to provide sustained delivery of immunomodulatory therapeutics to elicit adaptive and protective immune responses.

3. Particulate delivery methods to achieve sustained delivery

Nanoparticles (NPs) and microparticles (MPs) have been extensively explored as drug delivery platforms in immunomodulatory biomaterials research for the release of immunomodulatory therapeutics in vaccines and cancer therapy over the past 30 years [28–40]. The control of their size, shape, and surface composition as well as their ease of manufacturing, biocompatibility, and high surface area make these particles ideal candidates for targeted antigen-presenting cell (APC) uptake and lymphatic drainage while maintaining lower systemic concentrations (Fig. 2) [33,41]. More importantly, the encapsulation or adsorption of vaccine materials and cancer therapeutics can provide sustained kinetics compared to traditional bolus administration eliciting more potent immune responses. This section describes the latest achievements of sustained and targeted delivery using particles-based carriers.

3.1. Polymeric Nano/Microparticles

Poly(lactic-*co*-glycolic acid) (PLGA) is one of the longest and most extensively studied synthetic polymers due to its biocompatibility as demonstrated by US FDA approval and its clinical human use in resorbable sutures as well as grafts and prosthetic devices [42]. To date, PLGA is considered one of the most successful biodegradable polymers for the controlled delivery of a wide range of molecules including drugs, proteins, and vaccines [43,44]. The controlled release of materials is predominantly dictated by the erosion of the co-polymer by ester hydrolysis leading to biocompatible and metabolizable monomers (lactic acid and glycolic acid). Moreover, the degradation of the co-polymer and thereby the rate of release can be precisely tuned by altering the composition, the molecular weight, and the chain-end functionality of the co-polymer as well as the crystallinity of the particles [45–50]. The benefits of PLGA nano- and microparticles in altering the time-frame of release from days to months and in enhancing cellular uptake of cargos have been widely reported in literature. However, while the encapsulation of small hydrophobic drugs can be easily performed in organic solvents, vaccine antigen entrapment would require mixing aqueous antigens with dissolved polymers in organic media, therefore limiting the compatible antigen choices. A number of groups have circumvented this issue by using organic solvent-free methods and demonstrated improved protein stability in PLGA particles [45]. Schwendeman and co-workers have designed a method of microencapsulating model vaccine antigens ovalbumin and tetanus toxoid in aqueous conditions [51,52]. Biomacromolecules diffusing through open pores of PLGA particles can be trapped by spontaneous pore closing due to the passive healing of PLGA above the glass transition temperature. The resulting self-healing PLGA microparticles improved antigen structural stability and bioactivity with sustained release over 28 days and longer. Moon and co-workers successfully applied this technique to the sustained release of ovalbumin antigen (OVA) over more than 40 days [53]. A single dose of microspheres elicited a comparable cellular and humoral responses in mice to that measured from prime-boost vaccinations with an equivalent total dose of antigen. Notably, compared to both prime-boost PBS control and calcium phosphate gel clinical control, the single immunization microparticle group with extended release elicited greater number of antigen specific $CD8^+$ T cells as well as comparable total IgG titers.

Additional novel approaches such as PRINT technology (particle replication in nonwetting templates) have also been developed to improve antigen stability and to precisely modulate PLGA particles' physicochemical characteristics in terms of size, shape and surface chemistry [54]. This platform has been commercialized by Liquidia Technology and several vaccine formulations for dengue virus and influenza both in animal and human models showed promising results such as robust neutralizing titers [55–57]. Similarly, Jacklenec and co-workers have designed original PLGA cubic microparticles using SEAL process (StampEd Assembly of polymer Layers) [58,59]. In contrast to single-layer geometries such as PRINT, each cube displays an internal enclosed cavity which allowed a sustained and pulsatile release of STING agonist cGAMP at anticipated time points mimicking multiple injections. A single injection of these microparticles showed an antitumor efficacy comparable with multiple injections of STING agonist solutions such as increase in tumor infiltrating lymphocytes (TILs). Similar number

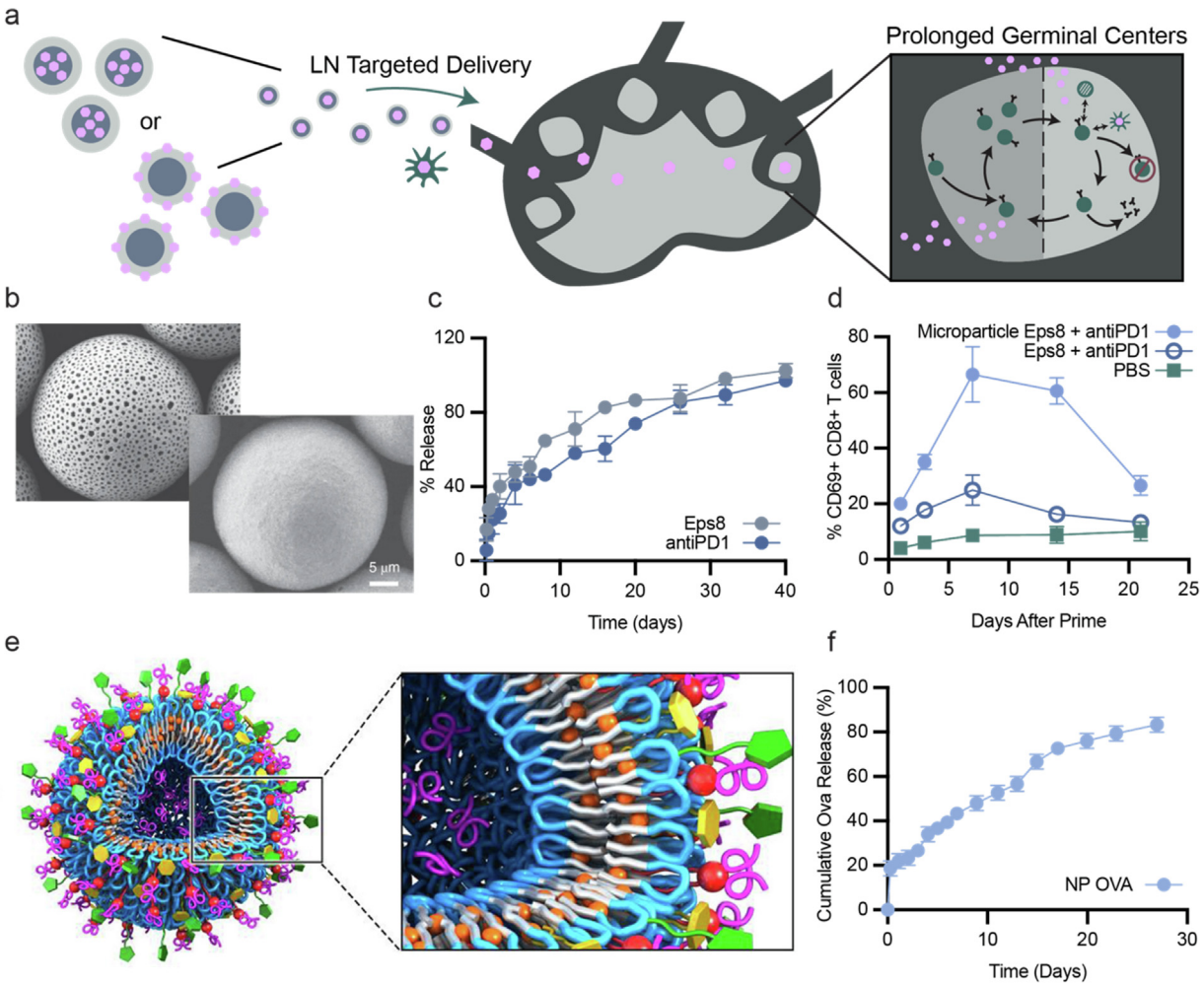

Fig. 2. Particulate Delivery Methods to Achieve Sustained Delivery. (a) Particles can encapsulate or tether vaccine and drug cargo to prolong exposure by reducing clearance as well as improve delivery to lymph nodes. (b) Self-healing poly(lactic acid) (PLA) microspheres can be used for controlled encapsulation and prolonged delivery. Upon infrared irradiation to increase local temperature to 38 °C, above the glass transition temperature of the PLA, rearrangement and healing of the pores on the surface of the microspheres leads to the formation of microcapsules for efficient encapsulation of molecular cargo [73]. (c) Sustained co-delivery of leukemia-associated epitope peptide (Eps8) and checkpoint inhibitor (anti-PD-1) was achieved with these microspheres. (d) Delivering microspheres co-encapsulating Eps8 and anti-PD-1 significantly increased activity of $CD8^+$ T cells (indicated by $CD69^+$ population) compared to soluble group, demonstrating superior cytotoxic effects. (e) Illustration of mannose-functionalized lipid-hybrid polymersomes for co-delivery of antigens and dual agonist molecules [95]. (f) Sustained release of vaccine antigen ovalbumin (OVA) was achieved with these polymersomes, where 80% of the antigen was released over the course of roughly one month. Only when the polymer matrixes were dissolved or degraded to form pores can the encapsulated OVA diffuse through the barriers and be released.

of $CD4^+$ and $CD8^+$ T cell populations were found within the TILs between the microparticle and the control 3-times injection groups (with equivalent total cCAMP dosage) after inoculating mice with B16F10 tumor cells. Increased number of memory T cells and circulating IFN-γ^+ $CD8^+$ T cells were also observed, inducing systematic antitumor immunity and preventing metastasis. This system can be applied to any hydrophilic or hydrophobic drugs and can deliver different drugs for combination cancer therapies, opening a new exciting and promising field in cancer treatments.

Particulate delivery vehicles also allow precise sustained co-delivery of antigens with adjuvants for subunit vaccines to improve immunogenicity [32,60–62]. For example, Hu and co-workers designed a viral capsid-like hollow PLGA nanoparticle for synchronized delivery of vaccine antigen and adjuvant [63]. The particles are comprised of cyclic diguanylate monophosphate (cdGMP), a canonical STING agonist adjuvant encapsulated in the aqueous core, and the MERS-CoV RBD antigen grafted onto the surface for the development of a MERS vaccine. Compared to soluble RBD antigens formulated with free STING agonists or MF59, a potent adjuvant used in influenza vaccine, these nanoparticles enhanced antigen presentation and cellular uptake by APCs and induced a balanced Th1/Th2 immune responses while reducing systemic reactogenicity. A protection against lethal MERS-CoV challenges in highly MERS-CoV-permissive transgenic mice has also been demonstrated. Other examples of utilizing particles to sustainably co-deliver therapeutics to improve humoral or cellular responses include Allahverdiyev and co-workers who reported a strong humoral responses against visceral leishmaniasis when co-delivering lipophosphoglycan and leishmania antigens over more than 30 days compared to both antigens released separately [64]. Similarly, Cruz and co-worked showed the synergistic antitumor effect of co-delivering TLR agonists R848 and poly(I:C) with CCL20 chemokine MIP3α with PLGA nanoparticles alongside TC1-vaccine upon inoculating mice with TC1 tumor [65]. Sustainably delivering these TLR agonists increased the number of circulating antigen-specific $CD8^+$ T cells leading to better mice survival compared to vaccination alone.

Over the years, numerous efforts have been made to functionalize PLGA particles, mostly for targeting purposes to achieve more specific and efficient responses. One of the most common

modifications the is surface PEGylation to avoid rapid clearance in the blood stream [66,67]. The release of small hydrophobic molecules can be controlled by tuning the surface texture of PLGA- *b*-PEG microparticles [68]. As example, PLGA-*b*-PEG blended with a small amount of PLGA increased microparticles roughness and thereby the rate of drug release. In addition to its stabilization effect, PEGylation can be used to tune and optimize the release timeframe. Other targeted approaches involve the use of mannose for improved antigen-presenting cell uptake, hyaluronic acid for CD44 targeting, chitosan for enhanced cell adhesion and uptake, and antibodies for specific cell receptors targeting [69]. Zheng and co-worked demonstrated the benefit of using surface mannose-modified PLGA nanoparticles to improve humoral and cellular responses compared to unmodified nanoparticles when slowly delivering hepatitis B surface antigen (HBsAg) [69]. Panyam and co-workers reported the sustained co-release of a TLR7/8 adjuvant derivative encapsulated into PLGA nanoparticles mixed with ovalbumin antigen for cancer immunotherapy [70]. The nanoparticles induced a better antigen presentation due to the aggregation of the positively charged antigen on the surface of the nanoparticles. Moreover, a more potent T cell response with increased dendritic cell migration and activation in draining lymph nodes as compared to the free molecules has been observed leading to metastasis reduction in different tumor models. Similarly, Appel and co-workers demonstrated an anticancer efficacy by tethering the TLR 7/8 agonist on the surface of nanoparticles [71].

Besides PLGA, other polymers have been developed as carriers for the delivery of immunomodulatory therapeutics. Its main derivative, poly(lactic acid) (PLA), has also been extensively used for years to control the release of biomolecules in a wide range of biomedical applications [72]. Li and co-workers designed gigaporous PLA microspheres encapsulating leukemia-associated epitope peptide (epidermal growth factor receptor pathway substrate 9 gene (Eps8)) as neo-antigen and PD-1 antibody checkpoint inhibitor for new therapeutic leukemia vaccines (Fig. 2**b-d**) [73]. Microspheres allowed for the sustained release of both biomolecules over 5 weeks compared to the rapid clearance observed after a bolus injection. They also demonstrated higher effect on chemokines upregulation and increased number of APCs recruitment. According to the authors, this phenomenon could be due to the inflammatory response caused by the exogenous macroscale formation, implying thereby the retention of microcapsules at the injection site. With favorable APC responses and five-fold increase of therapeutics found in the lymph nodes released sustainably, $CD8^+$ T cells population was massively increased with improved activity ($CD69^+$ $CD8^+$ T cells) for the microcapsules treated mice compared to control groups. A single subcutaneous injection of microcapsules in mice inoculated with human leukemia cells (Nalm6/$Eps8^+$ cells) suppressed leukemic blast populations in peripheral blood, bone marrow and spleen, thereby increasing survival rate compared to bolus administration. Similar results were obtained with different types of leukemia cell lines, xenograft models, antigens or even mixtures of neo-antigens, highlighting the potential of this platform as immunotherapy approach for the treatment of leukemia. Polyanhydride-based particles have also been explored due to their biocompatibility and have been used for bovine brucellosis, pneumonic plague, as well as influenza vaccines delivery [74–76]. While the hydrophobic monomers resist bulk erosion due to water penetration, anhydride bonds hydrolyze causing the particles to undergo surface degradation [77]. This slow degradation can result in month long release of the encapsulated vaccine cargos [78]. Notably, Narasimhan and co-workers developed polyanhydride nanoparticles for internasal delivery of F1-V antigen against respiratory disease caused by Yesinia pestis [79]. F1-V release kinetics were found to be sustained for up to 70 days and led to improved antibody titers and long-term protection against lethal challenge. Poly(propylene) sulfide is another well studied polymer due to its sensitivity to a variety of chemicals as well as oxidoreduction conditions, making it a good candidate for precise and controlled drug delivery [80]. Thomas and co-workers developed a programmable two stage drug delivery using poly(propylene) sulfide nanoparticles and thiol-reactive oxanorbornadiene linkers carrying small molecular cargos [81]. These sensitive linkers can undergo fragmentation by retro Diels-Alder reaction at different and controlled rates depending on the substitution pattern on the oxanorbornadiene. Following the uptake of nanoparticles and their transport to lymph nodes, the cargos could be sustainably released in a programmable manner within the lymph nodes, facilitating lymphatic delivery and access of molecules to lymphocyte subpopulations. Even though poly (propylene) sulfoxide has been already used for several years [50,82,83], applying a double-stage controlled delivery could further modulate the adaptive immune response.

To date, polymeric nanoparticles are considered as one of the most successful nanocarriers for drug delivery thanks to their biocompatibility, tunability, long term safety, size control as well as surface functionalization for targeting purposes. This is especially useful to maximize targeting to APCs for improved adaptive immune cells' modulation. Depending on their nature, they can encapsulate and deliver a wide range of biomolecules, in a sustained and even co-sustained manner. The choice of polymers is an important key factor that should be precisely chosen and tuned to achieve relevant encapsulation efficiency, stability of nanoconstructs and release profiles [84].

3.2. Polymeric micelles and polymersomes

Self-assembled polymeric systems composed of amphiphilic block co-polymers have been widely studied for drug delivery. Among them, polymeric micelles are core shell nanostructures comprising a hydrophilic shell and a hydrophobic core that can encapsulate a variety of hydrophobic molecules [85]. They have been widely used as nanocarriers mostly for the delivery of cancer therapeutics and some of them have entered in preclinical trials [86,87]. Chen and co-workers designed polymeric micelles from an amphiphilic deblock co-polymer of poly(2-ethyl-2-oxazoline)-PLA combined with carboxylterminated-Pluronic F127 for the co-delivery of OVA and TLR7 agonist CL264 [88]. A sustained delivery of the adjuvant encapsulated into the hydrophobic core for more than 2 days as well as the maintenance of the antigen conjugated onto the surface over 3 days have been observed. Interestingly, micelles displaying a carboxylated surface were more effectively internalized by dendritic cells than bolus or hydroxylated-micelles due to scavenger receptors known to recognize negatively charged substances. Moreover, the pH-sensitive property of poly (oxazoline) facilitated endosome escape and cytosolic release of antigens, improving thereby MHC I antigen presentation. Carboxylated micelles led to higher Th1 responses due to increasing antigen cross-presentation to $CD8^+$ T cells by elevated numbers of $CD4^+$ T cells. Immunization with these micelles in E.G7-OVA-bearing mice demonstrated an inhibition of tumor growth and better survival. However, the *in vivo* instability of micelles in large dilution volumes and their dissociation by proteins leading to their rapid clearance once intravenously injected can limit their use [89]. Core-crosslinked micelles have demonstrated an improved stability for the sustained release of drugs [90]. The loading ability of small hydrophobic molecular cargos can also be improved by adding specific non-covalent interactions at the end of hydrophobic blocks such as π-π stacking [91].

In contrast to polymeric micelles which can only encapsulate hydrophobic drugs, polymersomes can be used to simultaneously encapsulate and deliver both hydrophilic and hydrophobic drugs

thanks to their double hydrophobic layer and aqueous core [92]. Often compared to liposomes, they have shown to have a better colloidal stability as well as a better cargo retention efficiency [93]. While a multitude of studies have reported the use of stimulus-responsive polymersomes for the rapid release of immunomodulatory therapeutics within a specific location [94], some groups have highlighted their use for sustained delivery. A sustained co-delivery of antigens and TLR agonist adjuvants from mannose-functionalized lipid-hybrid polymersomes has been reported by Zhang and co-worker (Fig. 2**e-f**) [95]. The nanoconstructs comprise a combination of two TLR agonists activating different pathways encapsulated within the hydrophobic bilayer (TLR 7/8 agonist imiquimod, R837) and the lipid layer (TLR4 agonist monophosphoryl lipid A, MPLA). OVA was encapsulated in the aqueous core and adsorbed on the outer surface via electrostatic interaction with cationic lipids. This strategy allowed a double stage of release of the antigen with a rapid initial release (20%) for antigen exposure to prime immune responses followed by an extended release over more than 20 days for long-term memory antibody and cellular responses. The polymersome-formulated vaccines activated dendritic cells by enhancing antigen uptake and lysosome escape compared to the free molecules. Moreover, the use of mannose enhanced the cellular uptake and the migration to the draining lymph nodes for better dendritic cells activation compared to non-targeted nanoparticles. Improved APC uptake and activation as well as prolonged and targeted antigen delivery by these mannose-targeted polymersomes resulted in elevated splenocyte and lymphocyte proliferation and activation, boosting antigen specific T cell responses. A significant reduction and suppression of tumor growth as well as the improvement of survival time even after re-challenging have been demonstrated, offering new promising vaccine formulation for cancer immunotherapy. Interestingly, compared to solid-core poly(propylene sulfide) nanoparticles, Hubbel and co-workers have demonstrated enhanced frequencies of antigen-specific $CD4^+$ T cells when using OVA-encapsulated polymersomes [96]. In contrast to the nanoparticles, polymersomes failed to elicit robust $CD8^+$ T cell responses. The observed differences in T cell activation by these two types of particles were speculated to be due to the activation of different subsets of antigen-presenting cells. Interestingly, a synergistic effect combining strong $CD4^+$ and $CD8^+$ T cell responses when co-administrating these two systems has been demonstrated, giving new insights on the direct role of the nanoconstruct's effect on the immune response. Similarly, antigen encapsulated in polymersomes showed better $CD4^+$ T cell activation and induced a higher frequency of $CD4^+$ T follicular helper cells compared to antigens grafted onto the surface of nanoparticles [97]. Recently, a polymer-prodrug conjugate of poly(ethylene glycol) and TLR7/8 agonist self-assembling into a polymersome-like vesicle showed potent and long-lasting immune stimulation in terms of interferon expression at the injection site and in draining lymphoid tissue when slowly delivering the adjuvant over several days [98].

However, while polymersomes show great promises especially thanks to their ability to escape from endosomes, they can also suffer from low drug loading, implying successive injections are necessary to reach a therapeutic effect. Some strategies to improve drug loading have been reported such as the conjugation of a hydrophilic polymer to a hydrophobic drug which self-assembles into polymersomes [32].

3.3. Lipid-Based Nano/Microparticles

Lipid-based particles such as liposomes, lipid nanoparticles and nanoemulsions have been extensively exploited for the delivery of immunomodulatory therapeutics [99–102]. Their inherent biocompatibility combined with their adjuvanting properties make them successful as adjuvant systems as demonstrated by the FDA approved lipid-based adjuvants such as MF59 (squalene-based emulsion) and AS01 (saponin and TLR4a MPLA based-liposome) or the well-known Freund's adjuvants or ISCOMatrix [60]. In addition to their potency, the surface of lipid-based particles are often functionalized with carbohydrates such as mannose for cell-targeting approaches [103]. Liposomes hold great promises due to their ability to mimic the morphology of cellular membranes and to deliver both hydrophobic and hydrophilic bioactive molecules [104]. Several liposome formulations are under clinical trials for cancer treatment or clinically used as vaccines delivery platforms for influenza, human papilloma virus, hepatitis A and SARS-CoV-2 virus [105,106]. Interestingly, cationic lipid-based liposomes have been shown to form a depot at the injection site once injected, avoiding the rapid clearance of antigens and thereby improving their exposure to immune cells [104,107]. The cationic nature of lipids allows the electrostatic adsorption of the antigen and the aggregation of vesicles at the injection site. Similarly, pegylation is the gold standard to prevent the rapid clearance of nanoparticles *in vivo* resulting in improved antibody response [108]. However, Perrie and co-workers observed that highly pegylated functionalized liposomes resulted in a faster drainage and a lower immune response compared to the non-pegylated ones [109]. The steric stabilization induced by pegylation reduced the aggregation of vesicles and thereby the depot effect. Therefore, an adequate balance of pegylation should therefore be used to take advantage of both the depot effect and enhanced antibody titers. Recently, Dietrich and co-workers developed a two-stage lipid-based delivery system for enteric diseases [110]. After a parenteral immunization, a fast release of retinoic acid from neutral liposomes and its drainage to lymph nodes allowed for their preconditioning followed by the sustained release of OVA from the depot formed by the cationic liposomal with CAF01 adjuvant. This two-stage delivery platform induced an antigen-specific intestinal IgA response, making it a relevant strategy for the development of vaccines against enteric diseases.

Despite their structural similarities with liposomes, lipid nanoparticles are considered a separate class of lipid-based particles in which ionizable lipids are organized into inverted micelles to encapsulate nucleic acids. Lipid nanoparticles are the most extensively used lipid-based system for gene regulation and the delivery of nucleic acids in cancer therapy and vaccines [111–114]. However, even if the use of lipid-based particles for the sustained release of drugs has been widely reported, only a few studies investigated its quantification [115–118]. Irvine and co-workers developed interbilayer-crosslinked multilamellar vesicles for pulmonary vaccination [115]. The lipid nanocapsules were formed from an equivalent mixture of DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and MPB (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide]) in which OVA and either TLR3 agonist poly(I:C), TL4 agonist MPLA or the combination of the two adjuvants were encapsulated. One week after intratracheal immunization, nanocapsule-delivered antigen was still detectable in the draining mediastinal lymph nodes, allowing sustained exposure of vaccine components and prolonged antigen presentation. Pulmonary immunization increased antigen transport to draining lymph nodes compared to subcutaneous vaccination. Compared to bolus administration, nanocapsules enhanced the frequency of OVA-specific $CD8^+$ T cells in the reproductive tract and gut. Hook and co-workers originally designed cubosomes composed of phosphatidylcholine in which TLRs agonists imiquimod (TLR7a) and MPLA (TLR4a) were entrapped [116]. When cubosomes were mixed with OVA, a significantly extended release over more than 10 days could be observed compared to a liposome formulation. Combined with a retained

release, a higher encapsulation efficiency as well as OVA-specific cellular responses and equivalent humoral responses were reported compared to liposomes, making cubosomes an interesting candidate for the sustained delivery of vaccines [119].

As shown in the previous examples, the ability of cationic or ionizable lipids to interact with nucleic acids via electrostatic interactions has consequently been widely explored for the delivery of mRNA vaccines [120,121]. Liposomes and lipid nanoparticles show great promises as delivery carriers for the effective encapsulation and protection of mRNA. Naked mRNA can be degraded by enzymes leading to its rapid clearance *in vivo* and suffers from low translation efficiency into the cytoplasm [121]. When delivered in lipid nanoparticles in mice, mRNA displayed a protein production half-life of 30 h and high protein protection for up to 10 days [122]. Moreover, the sustainably delivery of mRNA into the cytosol for its translation into proteins can be easily achieved via endosomal escape by these particles. These advantages have led to the design of a multitude of lipid-based systems that are in clinical trials or clinically used as mRNA vaccines carriers against Influenza, Rabies, or Zika viruses [102,123]. Notably, this technology has been adopted and is currently broadly distributed by Moderna and Pfizer-BioNTech who rapidly received an emergency use authorization for their lipid nanoparticles carriers for the prevention of COVID-19. Its rapid development, clinical translation and distribution have proven to be crucial during a pandemic. Similarly, to mRNA prophylactic vaccines, lipid-based nanocarriers have been widely reported for mRNA cancer vaccines [114], highlighting these nanoparticles as highly valuable platforms in immunotherapy.

3.4. Other Particulate delivery vehicles

Other nanoparticle systems have been developed for the sustained release of immunomodulatory therapeutics. Dendrimers such as poly(amidoamine), poly(ethylenimine) and poly(L-lysine) have been explored due to their ability to form complex with nucleic acids and proteins [124–127]. These hyperbranched symmetric polymers arranged in a tree-like fashion around a core can escape from endosomes and be sensitive to low pH environments through sensitive linkers or protonation, facilitating the release of drugs. Notably, cationic dendrimers have been reported as nanocarriers for the delivery of mRNA vaccines [128]. A few studies have reported a sustained and extended release over several days [129–133]. Recently, Steinmetz and co-workers enhanced the antitumor efficacy of the plant viral nanoparticle cowpea mosaic virus (CPMV) when slowly delivered from polyamidoamine generation 4 dendrimer [133]. The sensitivity of aggregates formed by electrostatic interactions between negatively charged CPMV and positively charged polyamidoamine towards salts allowed for the disassembly of microparticles once injected *in vivo* leading to the release of the virus. Following an intraperitoneal injection in a mouse model of ovarian cancer, CPMV-based dendrimer particles remained in the intraperitoneal space over more than 14 days and have been proved to be as effective at reducing disease burden as compared to weekly administration of soluble virus. While the interaction of CPMV-based dendrimers with the immune system has not been reported yet, CPMV has been shown to improve effector and memory $CD4^+$ and $CD8^+$ T cell responses and to promote systemic tumor-specific cytotoxic $CD8^+$ T cell activity with the dendrimer providing continuous antigen presence in the intraperitoneal (IP) space leading to prolonged immune stimulation [134,135].

Similarly to dendrimers, nanogels have also been designed for the delivery of biomolecules. Nanogels are tri-dimensional networks of chemically or physically crosslinked polymers entrapping a large volume of water. Even if the majority of hydrogels are macroscopic in size, they can be designed as nano sized particles combining therefore the advantages of hydrogels and nanoparticles [136]. While a wide variety of nanogels have been developed to effectively and rapidly deliver drugs within specific areas under a stimulus [137–141], only a few studies have reported and quantified a sustained delivery of antigens and its impact on modulating the immune response [142]. Nanogels have the ability to by-pass the blood brain barrier to deliver drugs when administrated intranasally, making them a promising alternative to deliver vaccines in a non-invasive manner [143,144].

3.5. Summary of particle technologies

In summary, on top of sustained and prolonged delivery of antigens for weeks to months, particulate delivery vehicles allow for co-delivery of vaccine antigens and adjuvants, enhanced APC cell targeting and activation, endosomal escape for better cellular responses, and precise size control for improved drainage to the lymph nodes. Nano- and micro-particles as sustained delivery carriers have been successfully used for years, some of them being currently used in clinic. However, many reported sustained delivery time scales were determined via *in vitro* studies. Consequently, the timescale for sustained delivery may be significantly shorter due to innate immune cells phagocytosing the particles. Nonetheless, some reports of tracking of polymersomes *in vivo* in mice demonstrated prolonged and persistence depot at the injection site as well increased accumulation of vaccine components in the draining LN, albeit cleared quicker than what was found *in vitro* [95]. Moreover, the PLA microparticle based Lupron Depot, a clinical used hormone therapeutics, could maintain its therapeutics for up to 6 months, further demonstrating the ability for nano- and micro-particles to sustained delivery.

Another challenge is the influence of a specific nano-construct in terms of morphologies and the improvement of the adaptive immunity remains unclear considering the large number of studies reporting most of the time different polymer compositions, adjuvants, or injection routes. Additionally, it remains unclear to what extent does sustainably delivering immune therapeutics via these particles improves the adaptive immune response given their other positive attributes in improving targeting and drainage. A better understanding on the temporal control of antigen availability through the use of these particles should therefore be investigated.

4. Hydrogels and depot forming delivery methods to achieve sustained delivery

Hydrogels provide a highly tunable platform for the spatiotemporal control of drug delivery. Consisting of a polymer network swollen in a large amount of water, they lend itself to biocompatibility with many tissue systems [145]. Their biggest strength relies on their injectability (shear-thinning and self-healing properties) and the formation of instantaneous depots within the body for a sustained release of drugs and even immune cell infiltration and interaction with the network [146–149]. For prophylactic vaccine delivery, depot formations better mimic natural viral infection by creating a local high antigen concentration for a long duration, thereby eliciting a stronger immune response. They allow for sustained exposure of the viral protein antigen to the immune system, imitating the weeks-to-months persistence time of a virus in the body and allowing for prolonged affinity maturation [150]. The polymeric materials for hydrogel, whether synthetic, natural or bio-derived, are selected not only for their biocompatibility but also for their mechanical properties, ability to contain and release drugs on relevant timescales, and potential for immune cell infiltration [3,150]. Tuning these parameters allows the temporal con-

trolled delivery of a wide variety of drugs, from viral subunit vaccines to cancer immunotherapeutics, for modulation of the adaptive immune system (Fig. 3, Fig. 4) [145,148,150,151].

4.1. Synthetic-based macroscopic hydrogels

Synthetic-based hydrogels have been extensively developed as carriers thanks to their reliability, stability, ease of manufacturing and high tunability [41,152,153]. Among the multitude of existing polymers, poly(ethylene glycol) (PEG) as well as poly(lactic-*co*-glycolic acid) (PLGA) and their derivative are the most commonly used due to their FDA approval or their extensive use as drug delivery carriers [154]. Qian and co-workers designed a biodegradable poly(D, L-lactide)-poly(ethylene glycol)-poly(D, L-lactide) hydrogel vaccine for cancer immunotherapy [155]. B16F10 tumor cell lysates antigen, CpG-ODN TLR9 agonist, and granulocyte–macrophage colony-stimulating factor (GM-CSF) mixed in the hydrogel could be slowly released over the course of 5 to 7 days. Compared to a bolus administration, hydrogel vaccines improved activation and maturation of dendric cells as well as prolonged inflammatory cytokines secretion, all of which are important factors in improve cellular immune responses. Improved tumor inhibition and increased survival rate in C57BL/6 or BALB/c mice bearing B16F10 melanoma or C26 colorectal tumor models were observed. Similarly, Yang and co-workers delivered OVA along with an aluminum based adjuvant in Vitamin E-poly(ethylene glycol)-Vitamin E 'ABA' triblock hydrogel to C57BL/6 mice [156]. Gel-vaccinated mice showed higher concentration of anti-OVA antibodies than mice given bolus vaccine injections, demonstrating the ability of a depot forming hydrogel to prolong antigen release. Recently, Wang and co-workers developed an original poly (ethylenimine)-graphene oxide hydrogel for the sustained release of OVA encoding mRNA and TLR7/8 agonist resiquimod (R848) for durable cancer therapy [157]. Both antigens and adjuvants were effectively encapsulated in the network thanks to strong non-covalent electrostatic and π- stacking interactions. Interestingly, the supramolecular hydrogel is not stable at the interface when embedded in liquid solution and transformed into nanoparticles slowly releasing from the network over 30 days. This technology combined with a sustained co-release of vaccine components over 14 days improved the level of OVA-specific antibodies in B16-OVA melanoma C57BL/6 mice, suggesting that hydrogels could inhibit tumor and prevent tumor recurrence or metastasis formation.

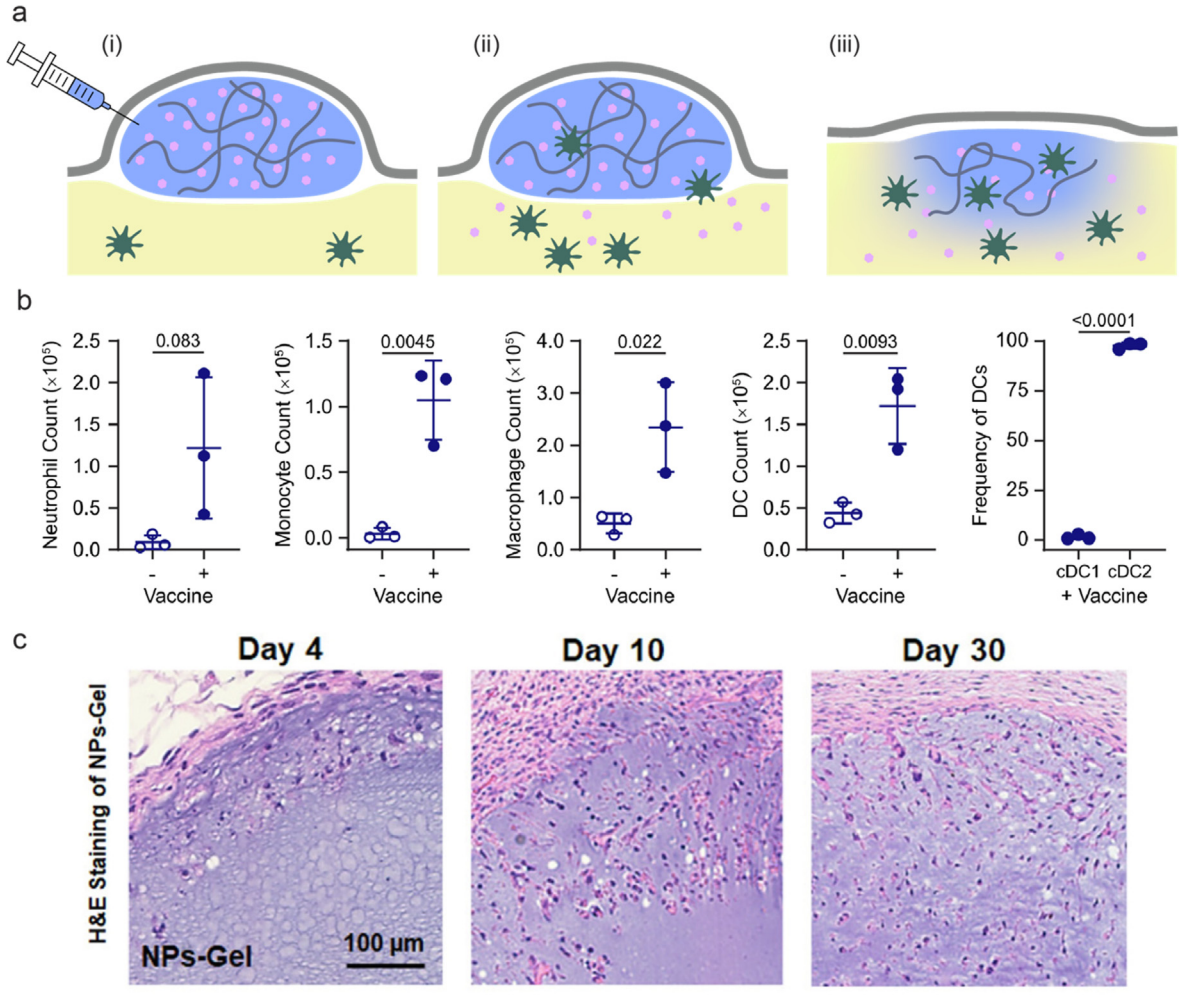

Fig. 3. Injectable hydrogels can simultaneously prolong the release of drug cargo and attract immune cells to create an immunological niche. (a) Diagram of (i) drug-loaded hydrogel injection into subcutaneous space, (ii) slow release of drug cargo into the body accompanied by immune cell recruitment and gel infiltration, and (iii) eventual dissolution and clearance of the hydrogel niche. (b) Appel and co-workers characterized the local inflammatory niche resulting from *in vivo* administration of subunit vaccines in injectable polymer-nanoparticle (PNP) hydrogels [148]. Numerous immune cell types, including a very large number of APCs, were recruited and found to infiltrate into vaccine-loaded hydrogels than empty gels. The choice of adjuvant in this PNP hydrogel system resulted in recruitment of a specific population of DCs called cDC2s, which are known to be important for initiating Tfh cell responses and enhancing humoral immunity. (c) Sung and co-workers observed immune cell infiltration of nanocomposite hydrogels containing TMC nanoparticles. Histological samples taken over a month-long period showed increasing cell count in the materials over time [151].

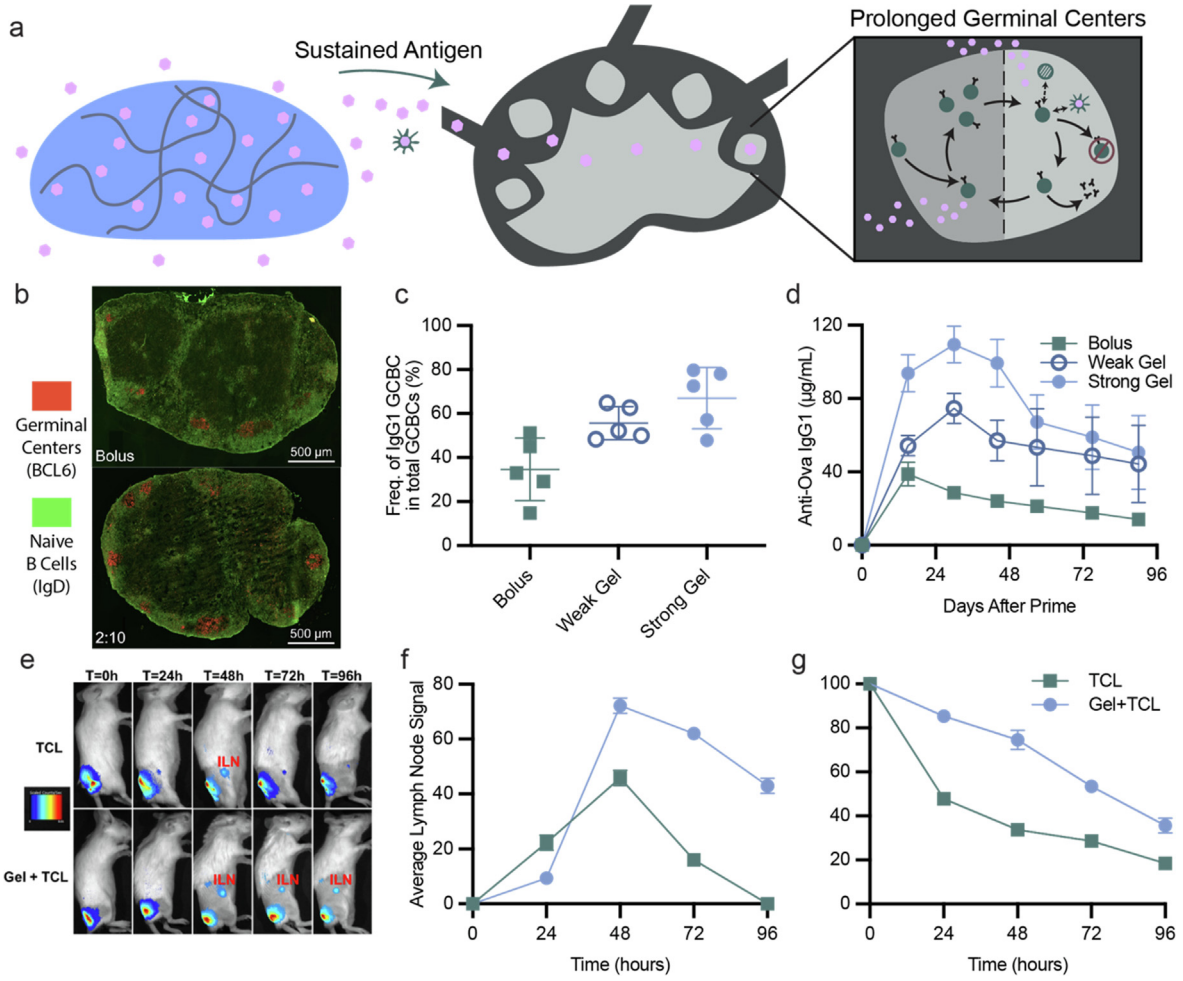

Fig. 4. Hydrogels can release antigen for extended periods of time, resulting in increased immune activation. (a) Antigens released over prolonged timeframes from hydrogels are taken by APCs to the lymph nodes. The sustained antigen presence here induces prolonged germinal center responses where more cycles of somatic hypermutation and affinity selection result in higher affinity antibodies. (b-d) Appel and co-workers imaged germinal center responses to prolonged, hydrogel-based vaccination [148]. (b) Immunohistochemistry images of explanted inguinal lymph nodes 15 days after vaccine administration either in a saline bolus or in one of two hydrogel formulations (a "weak" gel releasing over the course of roughly 2 weeks, and a "strong" gel releasing over the course of roughly 4 weeks) shows germinal centers (red) and naïve B cells (green). (c) Hydrogel groups show increased presence of IgG1 + class switched germinal center B cells, indicating gel administration leads to more protective humoral immune responses. (d) Vaccine administration in stronger gels with prolonged exposure led to the highest number of antibodies against administered antigen as compared to weaker gels and a simple saline bolus. (e-g) Wang and co-workers delivered tumor cell lysate (TCL) antigen and adjuvant in an injectable polypeptide hydrogel [176]. (e) Fluorescently labeled TCL antigen images show that the hydrogel (f) enhances antigen accumulation in lymph nodes, and (g) extends antigen presence at the injection site and in the lymph nodes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Besides, some hydrogel networks can be formed through non-covalent interactions between nanoparticles and polymers [158–161]. They leverage the benefit of hydrogels as depots for sustained and controlled drug delivery as well as nanoparticles for the potential improved presentation to dendritic cells and drainage into the lymph nodes. Appel and co-workers designed an injectable supramolecular polymer-nanoparticle (PNP) hydrogel composed of dodecyl-modified hydroxypropylmethylcellulose and poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-PLA) nanoparticles for the sustained co-delivery of subunit vaccine components (Fig. 3**b**, Fig. 4**b-d)** [148]. The dynamic nature of the network allowed for rapid depot formation once injected and allowed for extended co-delivery of physicochemically distinct vaccine cargo by physically constraining their diffusion within the network's polymer mesh. The sustained delivery of co-formulated model antigen OVA and poly(I:C) adjuvant in C57BL/6 mice resulted in infiltration of immune cells into the hydrogel, enhanced magnitude and duration of germinal center response as well as antibody affinity maturation leading to increased antibody titers compared to bolus injection. Hydrogels displaying the highest stiffness demonstrated the persistence of OVA at the injection site depot for weeks rather than days, with a retention half-life of 7.7 days. Moreover, the co-delivery of the clinically relevant hemagglutinin influenza antigen and a TLR7/8 agonist derivative adjuvant enhanced the potency and breadth of humoral immune response compared to clinical controls [150]. As a small molecule, TLR7/8 agonist was tethered on the nanoparticles surface to achieve matched diffusion kinetics and co-delivery of adjuvant with antigen. This modular platform allows the cargo diffusivity and thereby the immune response be tuned by changing the hydrogel mechanical properties [162]. This PNP hydrogel has the potential as a sustained delivery platform for a diverse array of vaccines and immunomodulatory cargo as Appel and co-workers have also demonstrated improved SARS-CoV-2 vaccine immune responses [163,164]. Other examples include Wei and co-workers' utilization of hydrogels composed of poly(D, L-lactide)-poly(ethylene glycol)-poly(D, L-lactide) (PLA-PEG-PLA) triblock copolymers with the patient's own tumor tissues neoantigens and light-responsive adjuvant-encapsulated nanoparticles containing TLR7/8 agonist resiquimod (R848) and TLR9 agonist CpG-ODN for cancer therapeutics [165]. Following its injection into

the tumor resection cavity in BALB/c mice bearing 4 T1 tumor, the hydrogel formed a depot and retained nanoparticles at the injection site. Upon exposure to near-infrared light, the light-sensitive unit led to ablation of tumor tissue to produce antigenic materials and to the sustained release of adjuvants over more than 6 days. This cancer vaccine produced strong DC and $CD8^+$ T-cell response and inhibited lung metastasis in a breast cancer mouse model.

4.2. Naturally-derived microscopic hydrogels

In contrast to synthetic-based materials, natural systems such as hyaluronic acid, chitosan or alginate have become a great interest in immunotherapy thanks to their inherent biocompatibility and bioactivity [166–171]. Sung and co-workers developed an injectable hyaluronic acid-catechol-based hydrogel to provide sustained delivery of the model antigen OVA encased in *N*-trimethyl chitosan nanoparticles (Fig. 3**c**) [151]. The authors hypothesized that initial burst release of OVA nanoparticles followed by gradual uptake of the remaining antigen by gel-infiltrating immune cells would mimic a prime-boost vaccination schedule in which two doses of vaccine are administered 4 weeks apart. Hydrogels increased dendritic cell maturation and cytokine release and generated higher antibody titers than both single dose OVA and prime-boost OVA in C57BL/6 mice as a result of extended delivery. Incorporation of OVA nanoparticles into the hydrogel greatly extended its *in vitro* release from 70% in one day to 40% in 6 days. Moreover, remaining OVA nanoparticles trapped in the gel could recruit dendritic cells and macrophages to infiltrate the injection site for 30 days. The hypothesized burst release likely occurred because of non-instantaneous gelation after injection in liquid like-form, leading to an unformed network minutes post injection. Mooney and co-workers developed injectable alginate-based porous and tough cryogels for cancer vaccines [170]. Tough materials were obtained through covalent crosslinking of methacrylated-alginate and ionic interactions with a high concentration of calcium ions. The gels displaying both crosslinking approaches showed to be injectable compared to covalently-crosslinked ones thanks to the dynamic nature of ionic interactions. After an initial burst release, a sustained delivery of TLR9 agonist CpG-ODN and granulocyte macrophage colony-stimulating factors over at least 2 weeks has been demonstrated. When co-encapsulating OVA as a model antigen, cryogels induced stronger antigen-specific cytotoxic T-lymphocyte, higher anti-OVA IgG and anti-HER2/neu antibody titers, and significantly higher survival rate compared to blank cryogels in C57BL/6 mice inoculated with HER2/neu-overexpressing DD breast cancer cells. Interestingly, the distance from the vaccine scaffold to the draining lymph nodes was found to affect the kinetics of the antibody response. While 80% of the vaccinated mice remained tumor free for more than 150 days after tumor challenge when cryogels were injected near the lymph nodes, only 40% survived when implanted farther. The injection site is then a key aspect for effective immune responses.

In addition, hydrogels derived from DNA or peptide are of great interest thanks to their bioactivity and adjuvanting properties [172–174]. Nishikawa and co-workers designed cholesterol-modified DNA hydrogels for the sustained release of antigens to increase antigen-specific cancer immunity [175]. Modified urea-denatured OVA antigen was used to better interact with cholesterol-functionalized DNA through hydrophobic interaction. The chemical modification of OVA and DNA hydrogels greatly delayed the release of antigens from almost 100% of release in 5 h to 50% in 25 h and increased the remaining antigen quantity at the injection site. While hydrogels induced little acute local inflammation, they inhibited the growth of EG7-OVA tumor in C57BL/6 mice compared to saline solutions of OVA or modified-OVA as well modified-OVA encapsulated in unmodified DNA hydrogels. According to the authors, the higher antitumor effect of modified DNA-OVA system could be due to the enhancement of resident time of antigens at the injection site and its sustained release from the immunostimulatory DNA hydrogel. Wang and co-workers delivered tumor cell lysate with TLR3 agonist and poly(I:C) adjuvants in a polypeptide hydrogel to prolong the duration of antigen persistence at the injection site and recruit immune cells to the depot (Fig. 4**e-g**) [176]. The delivery system was able to recruit, activate, and mature dendritic cells and promote strong $CD8^+$ T-cell response, as well as suppress tumor growth in C57BL/6 mice bearing B16 melanoma tumor model.

4.3. Microgels

Not only have depot-forming macroscopic hydrogels been extensively used for sustained vaccine delivery, but microgels as drugs carriers have also been reported [177,178]. These materials consist of granular discrete hydrogels on the scale of 10′s to 100′s of microns and can encapsulate various drug cargo similarly to macroscopic hydrogels [179]. Compared to conventional hydrogels, they benefit from a larger surface area which can be functionalized, and they do not undergo liquid-to-gel transition during injection through needles thanks to their small size, potentially reducing the incidence of burst release. Several groups have designed microgels for the controlled delivery of biomolecules and more particularly of proteins thanks to their hydrophilic nature [180–185]. Even if microgels have not been clearly defined as depots, they can be used as reservoirs for the sustained release of vaccine components and form depots after injection if individual microgels interact and aggregate. Ma and co-workers developed pH-responsive chitosan-based hydrogel microparticles to release H5N1 split antigen [186]. Microgels were able to slowly deliver antigens over 48 h at neutral pH and allowed immune cell infiltration due to their discontinuous nature. The implanted materials formed an antigen depot which could recruit inflammatory cells at the injection site, resulting in increased bone marrow derived dendritic cell activation as well as higher IgG antibodies and hemagglutination inhibition titers compared to standard formulations comprising alum or liposaccharide adjuvants. While this class of materials is growing in interest and demonstrating utility for vaccine delivery, they require precise synthesis approaches as particles that are too large can be challenging to administer by injection.

4.4. Summary of hydrogel technologies

In summary, Hydrogels are promising platforms for the delivery of immunomodulatory therapeutics. The variability and countless structures available as well as their high tunability in terms of mechanical properties enable adaptation to a wide range of different cargos. The formation of depots *in vivo* allows for a sustained release of antigens and cells infiltration, being the key factors for promoting better immune responses. Though natural polymers offer great biocompatibility, they can suffer from batch-to-batch dependency and low mechanical properties, limiting therefore their use as delivery cargos. Synthetic and naturally derived hydrogels have been extensively reported thanks to their tunability and robustness. Some constructs derived from peptides, DNA or carbohydrates can be made bioactive or immunostimulatory by the presence of specific moieties such as RGD peptides, CpG-ODN, or mannose, enhancing immune cell activation and response. Often injectable, hydrogels can be easily administered through the ubiquitous route of needles, allowing for easy adoption by medical professionals. Recent findings suggest that the geometry of the injected hydrogel depot could affect release kinetics, with spherical depot demonstrating the most sustained release time scale,

highlighting that care must be taken in the design of the mechanics of the materials to enable robust depot formation and precise depot persistence to ensure consistency of administration to achieve desired immunological outcomes [187].

5. Microneedles to achieve sustained delivery

Microneedles consist of patches of micron scale needles that can be placed on the surface of the skin for transcutaneous delivery of drug cargos [188–199]. These devices consist of needle-like structures with diameters microns wide and up to 900 μm in length [191]. Microneedles pierce the uppermost layer of the skin (epidermis) to allow the delivery of drug cargos that would not travel across the skin by passive diffusion alone. They provide an attractive alternative to standard hypodermic needle vaccine delivery route which comes with the risk of accidental needle sticks, production of hazardous sharps, and prevalent fear of needles across patients. Microneedles allow for near painless administration due to their microscale size and can be self-administered. They can serve as an extended delivery vehicle by slowly dissolving to release cargos embedded in the needle structure or carrying cargos coated on the surface of non-dissolving needles. Unlike hydrogels, they do not allow for immune cell infiltration, but still form a depot that dissolves slowly to release drugs over many days. Additionally, microneedles provide direct access to the populations of antigen-presenting immune cells that reside in the skin including Langerhans cells and dermal dendritic cells [189]. Furthermore, extensive network of lymphatic vessels in the dermis help promote immune activation [189]. Numerous microneedles have been adapted and developed for the temporal delivery of vaccines and showed promising results to enhance the immune response (Fig. 5) [200–209].

Prausnitz and co-workers have measured the immune response following the application of dissolving sodium carboxymethyl cellulose/sucrose microneedles as well as the release of antigens compared to bolus injections. After 15 min, microneedles dissolved off of the patch backing and were sufficiently hydrated and soft to remain inside the skin. The complete dissolution of microneedles and antigen release occurred over a longer unspecified period. This study provided a unique way of investigating extended delivery: microneedles, intradermal injections, and intramuscular injections were given for multiple days over an extended period of time and compared to bolus single administration of the same vaccines. A low dose of Fluvirin subunit influenza vaccine administered to BALB/c mice every other day for 28 days via dissolving microneedles resulted in higher total influenza-specific IgG titers than bolus prime, bolus prime-boost, and shorter microneedle deliveries. This work laid the groundwork for using microneedles as extended vaccine delivery vehicles but did not examine the kinetics of single administration microneedles. Chen and co-workers provided more insights on the dissolution and delivery kinetics of microneedles through the design of poly(lactic acid) supporting array to support model antigen OVA-loaded chitosan microneedles, with a fast-dissolving polyvinylpyrrolidone (PVP) layer in between (Fig. 5**c-e**). Upon insertion into Sprague Dawley rats dorsal skin, the interfacial PVP coating dissolved, leaving the chitosan microneedles embedded in the skin. Histological analysis showed antigen presence in the surrounding skin for up to 14 days. A single microneedle dose induced long-lasting and significantly higher OVA-specific antibody titers than intramuscular vaccination. Similarly, Hammond and co-workers used a combination of burst and sustained release to induce OVA-specific $CD8^+$ T-cell response and anti-OVA antibody production (Fig. 5**f-h**) [202]. Composite microneedles consisting of poly(acrylic acid) (PAA) and silk, each mixed with OVA were fabricated. Following their implantation into C57BL/6 mice, the PAA block dissolved and its OVA loading was cleared from the application site in 24 h while OVA embedded in the silk remained present for over two weeks. This combination of burst and extended releases resulted in higher levels of both $CD8^+$ T-cell response and anti-OVA antibody production than bolus intradermal injection.

Vaccination against influenza has the potential to greatly benefit from injection free delivery methods such as microneedles [200,205–208]. Annual flu shots create copious amounts of sharps waste and cause needle-related distress in children and adults alike. Early studies showed that delivering inactivated influenza through dissolving microneedles in mice induced superior protective immune responses compared to those obtained with intramuscular injection at the same dose [205]. Birchall and co-workers delved further into the immune response in the skin itself and analyzed gene expression in human skin samples immunized with influenza virus-like-particles coated on microneedles [206]. The authors observed that genes responsible for cell recruitment, migration, activation, and T cell stimulation were upregulated in surrounding cells. This evidence supports the use of microneedles as an effective influenza vaccination system and improves modulation of the immune system. A phase I clinical trial reported the successful administration of inactivated influenza vaccine via dissolvable polymer microneedle patch, resulting in the same efficacy of seroprotection as achieved by intramuscular injection [208]. Furthermore, patients reported less pain and a preference for the microneedle patch over the shot. These findings indicate that microneedle patches are a viable alternative to administer the influenza vaccine.

Not only have microneedles been extensively developed as vaccines carriers, but Gu and co-workers have also investigated their use for cancer immunotherapy [199]. Hyaluronic acid-based patch encapsulating tumor lysates as neo-antigen, granulocyte–macrophage colony-stimulating factor (GM-CSF) as adjuvants, and melanin were fabricated. Upon remotely near-infrared (NIR) light emission, melanin allowed for local release of heat between 38 °C and 42 °C, generating a hyperthermic-mimicking environment that recruited and activated immune cells such as dendritic cells at the injection site. *In vitro* studies showed that while adjuvants were rapidly released from the microneedles within 48 h, antigens were slowly delivered over 5 days, suggesting the formation of antigens depot. In a prophylactic mouse model, patches loaded with B16F10 whole tumor lysate were transdermally implanted on the caudal-dorsal area of C57BL/6 mice and submitted to localized NIR irradiation for 10 mins every day for 5 days. Patches remained in the skin for at least 5 days. 10 days after vaccination, mice were inoculated with B16F10 melanoma cells. Mice receiving the combo patches containing tumor lysates, adjuvants, and NIR irradiation showed long-term survival and enhanced tumor rejection compared to blank patches or incomplete vaccination. Moreover, vaccination delayed distant tumor growth and improved survival rate in other tumor models such as $BRAF^{V600E}$-mutated BP melanoma and triple-negative breast cancer 4 T1 carcinoma tumor highlighting the versatility of this approach in inducing effective immune responses toward different tumors.

Microneedles have proven to be a valuable strategy to provide extended delivery of vaccine components and immunotherapies. They can be tuned to provide steady extended release or time-controlled burst release of their cargoes. They are well-tolerated, causing minimal skin irritation or pain, and most of the time preferred compared to injections [210,211]. However, these platforms pose some challenges in terms of manufacturing and safety. Microneedles require careful fabrication to create such sharp structures, implying most of the time several steps of manufacturing. They must also be kept safely packaged to avoid damaging their microstructure. Additionally, they often require formulation of

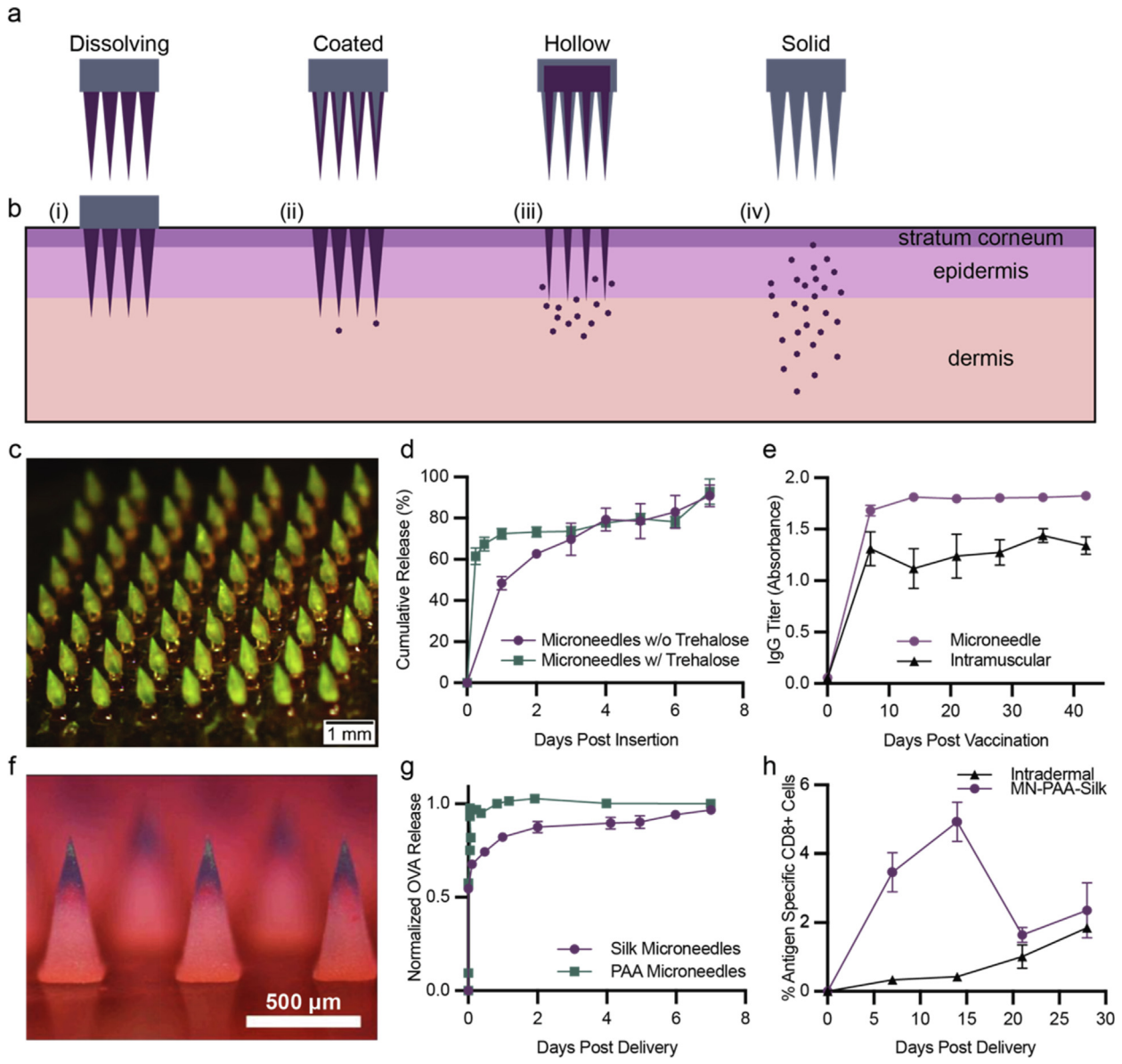

Fig. 5. Microneedles allow for prolonged delivery of drug cargo without injection via hypodermic needle. (a) The main types of microneedles are dissolving (drug formulated in solid form that makes up the needles themselves), coated (needle substrate coated with drug in solid film form), hollow (hollow needle conduit allows liquid drug formulation diffusion or injection), and solid (solid microneedles are inserted and removed to permeabilize skin, then drug is applied topically). (b) Dissolving microneedles containing antigen are (i) applied to the skin and (ii) release needles from the supporting array (typically within minutes). (iii) These microneedles dissolve over time, releasing antigen and (iv) eventually disappear. (c) Chitosan microneedles on top of a PLA base array allow for stable delivery and quick separation from the base into the skin where microneedles can persist for up to 14 days [201]. (d) Microneedles containing OVA formulated with and without a protein stabilizer (trehalose) demonstrated extended release in a week-long *in vitro* experiment, though following different release profiles. (e) Microneedle-administered OVA elicited higher antigen-specific IgG responses than did a standard intramuscular saline bolus. (f) Microneedles consisting of a rapidly dissolving PAA base and longer persisting silk tip were fabricated to provide an initial burst release followed by extended delivery [202]. (g) Pure PAA microneedles were shown to release nearly all ovalbumin cargo in the first hours after injection, acting as a burst, while silk microneedles extended delivery of a portion of the OVA antigen over the course of a week. (h) Administration of antigen-loaded PAA-silk microneedles (MN-PAA-Silk) elicited a significant increase in antigen specific CD8 + cells compared to intradermal injection of a saline bolus.

solid cargos and may require additional steps to support the encapsulation of new cargos. Even though microneedles are well tolerated, the degradation and metabolism of polymer fragments *in vivo* is still unclear and needs more investigation to ensure their complete safety as delivery carriers.

6. Other approaches to achieve sustained delivery

Besides the materials mentioned in the previous sections, original peptide nanofibers displaying adjuvanting properties have been reported by Collier and co-workers [212–216]. These engineered nanosystems can be internalized in antigen-presenting cells and raised strong antibody responses without the need of adjuvants. Besides their use as intranasal vaccines [212], hybrid polymer-peptide nanofibers formulated with sugar and adjuvants as tablet vaccines easily dissolvable under the tongue induced antibody responses against the model epitope pOVA and and the *M. tuberculosis* epitope ESAT6 [215,216]. These self-adjuvanting fibers are promising nanomaterials delivery platforms that can be administrated via several routes without adjuvants. Moreover, inorganic materials have also proven to be interesting candidates for the sustained delivery of immunotherapeutic materials and have been reported elsewhere [41,217,218]. Briefly, mesoporous silica rods as well as gold, iron, silica nanoparticles or quantum

dots are one of the most reported scaffolds. They usually combine the properties of organic-based systems such as hydrogels and some specific and relevant physico-chemical features for drug delivery. The high porosity, injectability and formation of depots after injection provided by mesoporous silica rods allow them to act as a reservoir for the controlled release of immunotherapeutics [13,25–27]. Moreover, their ability to rearrange after injection induces dendritic cell infiltration and eventual draining to lymph nodes, in which they have shown to enhance T cell response [219]. On the other hand, the ease of functionalization, well-defined structures, versatility, and optical, electric or hyperthermic properties of inorganic nanoscale systems create unique opportunities to modulate the immune response [220]. Furthermore, some inorganic materials can display adjuvanting properties, such as alum, which allow them to serve as delivery vehicles and immunostimulatory systems [221]. However, the toxicity and clearance of inorganic scaffolds *in vivo* is still under debate and can limit their translation into advanced clinical trials. Over the years, numerous efforts have been made to design biodegradable or clearable inorganic nanoparticles but challenges in terms of metabolism processes and fragments toxicity still need to be fully elucidated [222].

Hybrid organic–inorganic scaffolds such as metal organic frameworks have also been reported as delivery carriers [223–225]. These highly crystalline structures composed of zinc, cobalt or iron display some relevant characteristics such as easy modification, large surface area, and tunability in terms of pore sizes. Numerous groups have been interested in using metal organic frameworks to protect and improve stability of proteins and to control the delivery immunotherapeutics [226–230]. Recently, Sung and co-workers reported biomimetic aluminum-based metal organic frameworks encapsulated in yeast capsules for the sustained release of OVA through oral vaccination, giving exciting perspectives for new delivery vaccination routes [231]. However, the lack of data regarding the biocompatibility and toxicity of metal organic frameworks can limit their further use in clinical applications.

7. Outlook

Over the years, vaccine technology has benefited from the tremendous efforts of research groups and industries in designing more effective strategies to modulate the immune system. Besides choosing the right combination of antigens and adjuvants, the nature of the delivery platform is a key element in improving the quality, potency, and durability of immune responses. An increased number of vaccine delivery nanocarriers have proven their efficacy and are currently being studied in clinical trials or are already in use. This field is growing even more with the recent FDA approval of mRNA vaccines which marks a new era in vaccinology and offers exciting opportunities to bring new material scaffolds into clinical trials. While vaccines have become the gold standard in the treatment of infectious diseases and are expanding into cancer therapy, certain challenges remain. The modulation of the adaptive immune responses when slowly delivering immunomodulatory therapeutics has been shown to be effective in creating stronger and broader humoral and cellular responses. The formation of a depot for slow cargo release into which immune cells can infiltrate and better interact with the bioactive molecules appears to be a valuable asset for improving immune responses. The different delivery platforms ranging from nanoparticles to microneedles and hydrogels presented in this review constitute the exciting frontiers of sustained drug delivery. While hydrogels directly fall into depot forming vehicles, growing evidence suggests that microneedles as well as nano- and microparticles can remain at the injection site for long period of time, allowing for immune cell interaction even if they don't necessarily involve cell infiltration into a 3D matrix. While nano- and microparticles typically have a much shorter *in vivo* release time scale than other sustained delivery platforms, they nonetheless still present other attractive characteristics such as improved uptake by APCs as well as endosomal escape to improved cross-presentation. Numerous synthetic and engineering strategies for all sustained delivery technologies have been proposed to precisely control drug release in time and space, some of them even mimicking multiple injections. These great achievements must first succeed in clinical trials before becoming available for public use.

To facilitate broad adoption and improve patient compliance, concerns have been turned to the administration method. While injections are still the medical standard, their ubiquitous use suffers from patient fatigue and/or fear and production of biohazardous sharps waste. Traditional hydrogels still mostly require to be injected subcutaneously or implanted surgically. Over the past few years, the formulation of sprayable systems in the mucosal cavity has proven to be a promising alternative to overcome these limitations. Nanoparticles and microneedles benefit from additional administration routes. Microneedles can be self-applied to the skin and have been reported to be preferred and less painful than a traditional injection; nonetheless, their potential in scaling up is limited by their cumbersome manufacturing process. Similarly, while nanoparticles can still be injected intravenously, intramuscularly, or subcutaneously, they can also be sprayed in the nasal cavity, applied topically or mucosally (e.g., buccal), or administrated orally. As seen through the studies referenced in this work, innovation in the drug delivery materials space provides not only greatly improved immune responses through sustained delivery, but also varied routes of administration that can revolutionize yearly vaccines and cancer immunotherapies alike.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Competing Interests

E.A.A is listed as an author on several pending patent applications related to work described in this review filed by Stanford University (PCT/US2019/054070; PCT/US2021/032575; PCT/US2021/055897; Provisional 63/159,416). All other authors declare that they have no competing interests.

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