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CD8+ T-cells in Atherosclerosis: mechanistic studies revealing a protective role in the plaque microenvironment

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Orchestrating immune responses: how size, shape and rigidity affect the immunogenicity of particulate vaccines

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ABSTRACT

Particulate carrier systems are promising drug delivery vehicles for subunit vaccination as they can enhance and direct the type of T-cell response. In order to develop vaccines with optimal immunogenicity, a thorough understanding of parameters that could affect the strength and quality of immune responses is required. Pathogens have different dimensions and stimulate the immune system in a specific way. It is therefore not surprising that physicochemical characteristics of particulate vaccines, such as particle size, shape, and rigidity, affect multiple processes that impact their immunogenicity. Among these processes are the uptake of the particles from the site of administration, passage through lymphoid tissue, and the uptake, antigen processing and activation of antigen-presenting cells. Herein, we systematically review the role of the size, shape, and rigidity of particulate vaccines in enhancing and skewing T-cell responses and attempted to provide a "roadmap" for rational vaccine design.

1. Introduction

The implementation of vaccines has proven to be an affordable and effective strategy to prevent disease. Concerted vaccine efforts in the 20th century have resulted in the reduced occurrence or even elimination of infectious diseases [1, 2]. The first generation of successful vaccines was composed of weakened (attenuated) or inactivated pathogens. Despite their enormous success, some problems arise when using these types of vaccines. Firstly, there is a risk of genetic exchanges with other viruses, which may restore the virulence of live attenuated vaccines [3]. Secondly, due to their complex nature, these vaccines can induce adverse effects such as fever [4–6]. To circumvent these issues, subunit vaccines, containing only the antigen(s) against which the immune response must be targeted, have become more commonly used. These types of vaccines lead to superior safety profiles at the expense of decreased immunogenicity, due to the lack of pathogen-associated molecular patterns (PAMPs). Therefore, these vaccines require the addition of adjuvants [7] and/or the use of a particulate delivery system. The advantages of particulate delivery systems entail the protection of the integrity of antigens until they are delivered to antigen presenting cells (APCs) [8] and co-localisation of adjuvant and antigen to the same APCs, which limits systemic exposure to the adjuvant and thereby minimises adverse effects [9]. Furthermore, uptake of particulate matter by APCs induces an inflammatory response, contributing to the adjuvanticity [10].

Several classes of particulate vaccines have been developed which have been reviewed in great detail [11]. Interestingly, not only the composition of the particle affects its immunogenicity, but a growing number of reports has covered the effect of particle size on vaccine efficiency. More recently, several publications reported the effect of particulate vaccine shape or rigidity on immunogenicity. This is probably due to the fact that techniques to alter and characterise particle shape and rigidity were developed later than those for particle size. There are several ways to alter size, shape, and rigidity. The size of particles is controlled by manufacturing conditions such as extrusion for vesicles [12], centrifugation for vesicles or solid particles [13, 14], and emulsification conditions for polymeric particles [15]. The shape of particles can be altered by mechanical stretching [16] or by producing particles in a mould [17]. Rigidity, a measure of the particle's ability to retain its shape under mechanical stress can be manipulated for instance by varying the density of cross-linking in polymer hydrogel particles, by incorporating cholesterol in liposomes, or by increasing shell layer thickness in capsules [18–20].

In this review, we discuss how particle size, shape, and rigidity affect biodistribution, cellular uptake, antigen presentation and the resulting immune response in murine models (unless stated otherwise), where appropriate as a function of the route of administration. We acknowledge that more parameters, such as surface charge, particle composition, biodegradability or the inclusion of adjuvants are important characteristics that affect immunogenicity. However, the effect of these parameters has been extensively described elsewhere [21–30]. In addition, vaccines equipped with targeting ligands and adjuvants may induce immunological effects solely based on their physicochemical parameters [31].

2. Particle size

2.1. Particle distribution

Vaccination aims to mimic a pathogenic infection and induce immunological memory for possible future encounters. For a vaccine to elicit an immune response, effective delivery of antigens from the site of injection to secondary lymphoid tissue, where APCs, B- and T-cells reside, is the first requirement. Antigens can directly drain to lymphoid organs (such as spleen or lymph nodes, LNs) through the interstitial fluid and the collecting lymphoid vessels. Alternatively, particulate vaccines can be taken up by APCs at the site of injection and subsequently travel through the lymphatic system to interact with T and B cells that reside in the LNs [32, 33].

The size of particulate vaccines plays a crucial role in their transport to the LNs. Upon intradermal injection, interstitial flow (drainage of fluids from the interstitial space) transports small, non-liposomal nanoparticles (< 50 nm) more efficiently into lymphatic capillaries and draining LNs than particles larger than 100 nm. Smaller particles are hypothesised to be convected much easier through the interstitial flow, whereas larger particles require active transport by tissue-resident dendritic cells (DCs) to shuttle them to the LN. These smaller particles show increased retention in the LNs, due to efficient uptake by LN-resident DCs [34–36]. Of note, for larger-sized particles (> 50 nm), the efficiency of DC migration towards the draining LNs creates an extra parameter that might affect the quantity of antigen that is able to reach the LNs [37].

The effect of size on antigen distribution is also evident for liposomal formulations. Oussoren and colleagues reported a negative correlation between lymphatic uptake and liposome size in rats upon s.c. injection [38]. Interestingly, small 40 nm sized liposomes were poorly retained by the LNs compared to larger (> 400 nm) liposomes. This was due to more efficient phagocytosis of larger liposomes, as macrophage-depleted LNs showed reduced LN localisation of large liposomes [39]. Small liposomes are possibly less affected, since they can be taken up by multiple cell types in the LNs via endocytic pathways other than phagocytosis, such as pinocytosis. This, of course, will also affect the immunogenicity of these particles as a lower percentage will reach APCs.

Different routes of administration impose different barriers for the antigen to reach secondary lymphoid tissue. Thereby, different tissues contain different subsets of DCs, such as Langerhans cells in the skin, $CD103^+$ DCs in connective tissue and mucosal DCs in the gut. The type of DC to which the antigen is delivered may influence the skewing of the immune response, but this is outside the scope of this review [40]. A study using orally dosed biodegradable polylactic acid (PLA) microparticles ranging from 1 – 26 µm in diameter showed that the uptake of these particles into intestinal lymphoid structures referred to as Peyer's patches, increased with increasing particle size up to 11 µm, and decreased again hereafter [41]. Microspheres smaller than 5 µm were subsequently translocated via the lymphatic system from the Peyer's patches to the spleen, whereas larger particles remained in the Peyer's patches in the jejunum. The authors suggested that uptake by phagocytes of particles larger than 10 µm was less likely to occur, explaining the decrease in splenic localisation when microparticle size exceeds this limit.

Extending the size into the nanometre range, it was shown that oral administration of nanometre-sized particles results in higher uptake in the rat intestine than microparticles [42, 43]. Nanoparticles are taken up more efficiently by the intestinal epithelial cells and are able to penetrate deeper into the Peyer's patches, which make them more efficient than microparticles for oral delivery [14]. Thus, it appears that for optimal gut barrier passage, particulate vaccines should be designed to have a size in the nanometre range.

Concerning nasal delivery, it has been reported that migration of non-liposomal particles across the nasal mucosa of rats increases with decreasing particle size, resulting in stronger immunoglobulin G (IgG) and IgA responses, which are markers for general and mucosal immune responses, respectively [44]. Possibly, nanoparticles can permeate the epithelial lining more efficiently than microparticles, resulting in enhanced immunity, as shown in rats and mice [13, 45, 46].

Overall, it can be concluded that smaller-sized particles (< 50 nm) can directly drain and penetrate deeper into the LNs. However, larger-sized particles are retained more efficiently in the LNs, which emphasises the need for studies that find the optimal particle size to ensure efficient lymphatic drainage as well as retention. Furthermore, the route of administration can affect the distribution and should be considered in vaccine design as well.

2.2. Cellular uptake

An important step towards inducing a potent immune response is the uptake of antigen-containing particles by APCs. APCs are continuously probing their environment for the presence of pathogens or danger-related signals, which enables them to internalise pathogens or other antigens and process them into peptides. Extracellular fluid, which may contain small antigens, is continuously taken up by APCs through macropinocytosis. Larger particles are generally internalised via phagocytosis due to binding to receptors on the plasma membrane of APCs, which triggers actin assembly and drives particle engulfment. All resulting vesicles travel to endosomes within the APC where their content is processed [47, 48].

Conceivably, due to their exceptional capacity for macropinocytosis, DCs appear to preferentially take up nanoparticles. Studies in DC lines and DCs derived from human mononuclear cells have shown an inverse correlation between particle size and internalisation for particles of different compositions ranging from 20 µm to 150 nm [49–51]. Shima *et al.* have shown that 40, 100 and 200 nm sized poly(γ-glutamic acid)particles also show excellent uptake by DCs in vivo in the LNs upon s.c. administration. Interestingly, they report that the number of DCs that have taken up the 40 nm particles is twice as high as the number of DCs that have taken up the 200 nm particles, while the relative amount of antigen taken up was three times as high for 200 nm particles compared to the 40 nm particles. This suggests that smaller-sized nanoparticles are taken up more efficiently, but larger-sized nanoparticles can deliver a greater amount of antigen to APCs [52]. In a study comparing uptake of polystyrene particles ranging from

20 nm – 1 µm in lung-draining LN upon intranasal administration, it was reported that smaller (< 50 nm) particles were preferentially taken up by LN-resident DCs [53].

Examining the behaviour of particles in the extremely small size ranges, le Guével *et al.* produced gold nanoparticles of 12 nm and nanoclusters (clusters of gold atoms) of 2 nm in size [54]. Comparing the number of particles per human-derived DC, the nanoclusters showed a higher uptake compared to the nanoparticles. However, only the nanoparticles induced DC maturation and subsequent Th1-mediated immunity. Of interest, the nanoclusters have a higher diffusion capacity than the nanoparticles. This suggests the nanoparticles are taken up by receptor-mediated endocytosis, which is less efficient than diffusion, resulting in lower uptake, but the particles taken up via this process are able to induce immunity.

Of note, the mechanism of antigen delivery has been reported to differ between nano- and microparticles. Here we discuss the consequences of particle size on uptake, however, it must be noted that attachment of microparticles to the APCs, without endocytosis of the delivery system, appears to be sufficient to deliver the antigen to the APCs [55, 56].

2.3. Antigen presentation and APC activation

Following antigen uptake by APCs, these cells need to become activated via the recognition of PAMPs by pattern recognition receptors (PRRs) [57]. Effective processing leading to robust antigen presentation is required to induce potent immune responses. After uptake, antigen loaded particles are deposited in the endosome, where the particle and antigen are broken down by enzymatic degradation upon acidification of the endosome, resulting in short peptide sequences. These small protein fragments are loaded upon major histocompatibility complex (MHC) class II molecules, leading to $CD4^+$ T-cell activation. Alternatively, particles can be modified to facilitate endosomal escape, after which the antigenic peptide can reach the cytosol and, after proteasomal degradation, can be loaded upon MHC class I molecules, which can activate $CD8^+$ T-cells. This process, referred to as cross-presentation, can occur via two pathways: the presently described 'phagosome to cytosol pathway' and via the 'vacuolar pathway' in which antigens are loaded onto MHC class I molecules within the phagosome, which is not necessarily TAP-dependent [58–60].

Nanoparticles appeared to be efficient at inducing class I antigen presentation *in vitro*, whereas microparticles induced almost no MHC class I antigen presentation [61]. Particles larger than 500 nm were delivered into phagosomes, which subsequently fused with early endosomes, whereas smaller (< 200 nm) particles localised rapidly into late endosomes which fused with lysosomes. MHC class II complexes were recruited to both compartments, but delivery to the prelysosomal (early) compartment was shown to be more efficient in processing and presenting an encapsulated antigen. Consequently, the larger particles (> 500 nm) produced enhanced $CD4^+$ T-cell activation compared to smaller particles [12]. It has been suggested that the accumulation of nanoparticles within the lysosomes may have caused lysosomal overload, which

resulted in defective lysosomal degradation, which may explain the reduced MHC class II antigen-presenting capacity [62].

Considering cross-presentation, it was suggested that particles in the nanometre size range induced MHC class I presentation via the phagosome-to-cytosol pathway, whereas the larger micrometre-sized particles were processed via the vacuolar pathway, which yielded relatively fewer MHC class I complexes [60]. Together, these studies provide strong evidence for a size-dependent effect of both liposomal and non-liposomal particles on endosomal antigen processing and subsequent presentation.

2.4. Skewing immune responses

Pathogens can infect host cells via various routes, occupy different (intracellular or extracellular) compartments and cause acute or chronic infections. Therefore, clearance of pathogens requires a specific approach. $CD8^+$ T-cells play a seminal role in detecting and clearing intracellular pathogens as they recognise infected cells through specific epitopes presented upon MHC class I molecules, upon which they exert inflammatory and cytotoxic functions [63, 64]. $CD4^+$ T-cells recognize MHC class II via their T-cell receptor (TCR) and can be subdivided into different classes, the principal of which are T helper 1 (Th1), Th2 and T-regulatory (Treg) cells, characterised by the expression of T-bet, GATA-3, and FoxP3, respectively. Th1 cells produce inflammatory cytokines and are major producers of interferon-γ (IFN-γ) and TNF-α, which are pivotal for cell-mediated immunity (e.g. macrophage activation, $CD8^+$ T-cell help). The Th2 subset is characterised by a different cytokine profile, including cytokines such as interleukin 4 (IL-4), IL-5, IL-10 and IL-13, and is associated with the induction of humoral (antibody-mediated) immunity. Finally, Treg cells are a tolerogenic subset that suppresses inflammatory responses through secretion of anti-inflammatory cytokines (e.g. IL-10, TGF-β) [65].

As clearing a pathogen requires a specific type of immune response, skewing of the response after immunisation is an important aspect that particles can influence. As the size of a particulate vaccine affects the extent of MHC class I or MHC class II presentation, this directly influences effective $CD8^+$ and $CD4^+$ T-cell priming. However, the size of particles also appears to influence the type $CD4^+$ T-cell that is induced. Nanoparticles (ranging in size from $100-600$ nm) induce the most prominent activation of DCs (as measured by CD80 expression) compared to micro-sized particles. As a result, nanoparticles generated the highest antigen-specific $CD8^+$ T-cell response and a higher proportion of IgG2a antibodies relative to IgG1 antibodies, which indicates skewing towards a Th1 phenotype. Other studies showed that particles of $40-50$ nm in size are most potent in inducing IFN-γ mediated Th1 immunity compared to particles in the 100 nm range, which induced stronger IL-4 responses. It has been suggested that smaller (<100 nm) particles may enter APCs through one of the mechanisms used by viruses, such as clathrin-coated pit-mediated uptake, which may induce a stronger Th1 immune response [66, 67]. This suggests there is an optimal particle size of around 50 nm that triggers Th1 responses.

Microparticles (2 – 8 µm) were not taken up but instead attached to the surface of the APC, releasing their antigen into the cell in both mouse and rat models. This favoured IL-4 secretion and showed a higher IgG1/IgG2a ratio and higher antibody titres. Thereby, microparticles upregulated MHC class II expression, whereas nanoparticles induced more MHC class I. This suggests that nanoparticles induce a Th1 type immune response while microparticles induce a Th2 type response [49, 55, 68]. There appears to be an upper size limit for effective Th2 response; 5 µm poly(lactic-co-glycolic) acid (PLGA) microspheres containing hepatitis B surface antigen in pulmonary immunisation in rats induced higher antibody titres compared to larger (12 µm) PLGA particles, which could be due to less efficient uptake or adherence of particles that are larger in size than DCs [56]. Thus, the optimal size for inducing Th2 responses is approximately 1 – 5 µm.

Thus far we have seen a trend that smaller solid (polymeric or gold) particles induce stronger Th1 and CD8-mediated responses, whereas larger particles seem to skew towards a Th2 and B-cell mediated response (Fig. 1). Lipid vesicles, however, have been reported to show an opposite trend; small (< 200 nm) liposomes appear to induce Th2 mediated immunity, whereas larger liposomes skew towards Th1 responses [12, 69, 70].

The reason for the apparently contradictory effects observed for liposomal and non-liposomal particles could be explained by the differences in lysosomal degradation rates. Tran and colleagues studied the intracellular trafficking of 50 nm, 500 nm or 3 µm particles and showed that OVA conjugated to 50 nm polystyrene beads was rapidly exposed to an acidic environment in the lysosome [71]. This led to fast degradation of the antigen in the lysosome and, therefore, inefficient presentation. Furthermore, antigens bound to 500 nm and 3 µm particles remained in a less acidic environment within the phagosomes for a longer period of time, resulting in more efficient MHC class I presentation.

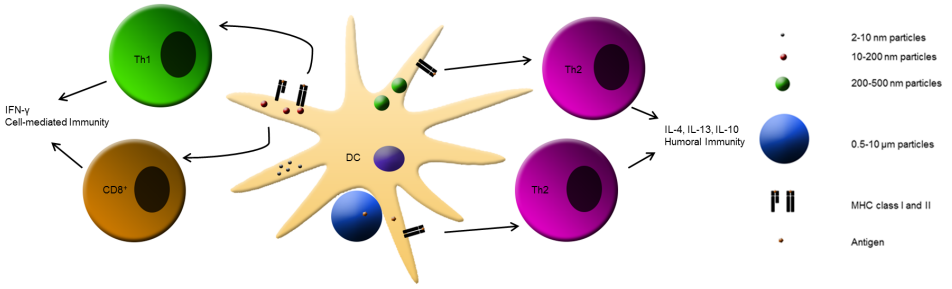

Figure 1: Schematic overview of the effect of non-liposomal particle size on inducing T-cell immunity. Ultra-small particles (2 – 10 nm) are taken up very efficiently by APCs but are poorly immunogenic. 10 – 200 nm nanoparticles are most efficient at inducing Th1 and CD8-mediated immunity, whereas larger 200 – 500 nm nanoparticles tend towards Th2 mediated responses. Microparticles adhere to the cell membrane and release antigen into the cell, which is presented upon MHC class II molecules and skews the immune response towards Th2 mediated responses.

3. Particle shape

3.1. Particle distribution

Besides particle size, shape is an important parameter influencing the immune response. A common way of characterising particle shape is by using the ratio between the height and width of the particle, denoted as the aspect ratio (AR). Huang *et al.* reported that the shape of mesoporous silica nanoparticles affects the biodistribution of these particles after intravenous (i.v.) administration in mice [72]. Both short-rod-shaped particles (185 nm, low AR) and long-rod particles (720 nm, high AR) were trapped in the spleen and liver. However, compared to the short-rod particles, the long-rod particles were more prominent in the spleen. Furthermore, short-rod particles were cleared faster from the body by urine and faeces than long-rod particles. Likely, the shape of the particles affects the ability for uptake by tissue-residenT-cells, which in turns affects the biodistribution and retention ability in the tissues. However, a size effect cannot be excluded in this experiment [72]. Injected filomicelles, micelles with a tubular shape (high AR), remained in circulation in rats and mice for up to one week [73]. Short tubular micelles were cleared from the circulation within two days. Filomicelles longer than 3 µm could not be taken up by human macrophages, whereas shorter filomicelles could be taken up via phagocytosis. The authors suggest that longer circulation time of the long filomicelles can be explained by the theory that they are stretched out by the blood flow, thereby minimising interactions with phagocytes and the blood vessel wall. Shorter cylinders will be less affected by the blood flow and interact more with phagocytes, resulting in more efficient uptake and thus faster clearance from the circulation. However, it must be noted that these particles were injected i.v. and therefore, this study focused on the uptake from the circulation, instead of lymphatic trafficking [73]. Upon oral administration of mesoporous silica nanoparticles, different effects were observed; decreasing ARs (5, 1.75 and 1) of the particles resulted in increased absorption by the small intestine, whereas urinary secretion was decreased [74]. Indeed, particles with the smallest AR showed the highest content in the spleen compared to the other particles, which were mainly deposited in the liver, lungs and kidneys. These results suggest that upon oral administration, spherical particles will exhibit a more favourable biodistribution profile than non-spherical ones, emphasising that the route of administration is an important parameter influencing the effect of particle properties on immunogenicity.

3.2. Cellular uptake

Similar to particle size, particle shape also plays a major role in the uptake of particulate vaccines by APCs. Non-spherical long-rod polystyrene particles stretched from 3 µm spheres were shown to exhibit negligible phagocytosis in a macrophage cell line, as observed by time-lapse imaging [75]. Moreover, spherical particles of similar size were internalised efficiently by macrophages. Spheres and rods of 1 µm in size showed the same differential phagocytosis as 3 µm spheres and rods. The authors suggested that macrophages cannot take up the rod-like particles, as the shape is mostly flat and

only contains curvatures on extreme ends, which hinders phagocytosis. Niikura *et al.* tested macrophage uptake of gold particles of different shapes; spheres of 20 nm and 40 nm in diameter, 40 nm × 10 nm rods (AR = 4) and 40 × 40 × 40 nm cubes [76]. Interestingly, rod-shaped particles appeared to be taken up more efficiently by macrophages than spherical or cubic particles (with cubic particles being the least effective), but in fact, the spherical particles had more efficient uptake per weight. Sharma *et al.* produced initially spherical polystyrene particles that were stretched to either prolate ellipsoids (high AR) or oblate ellipsoids (lower AR) [77]. The phagocytosis efficiency was in the order of oblate ellipsoids>>spheres>prolate ellipsoids. Even though oblate ellipsoids did not have the highest cell attachment, almost 90% of the attached particles were internalised, compared to 50% of the prolate ellipsoids and 70% of spheres. The combination of relatively high attachment and internalisation gives oblate ellipsoids a clear advantage for phagocytosis. Champion *et al.* reported that the particle shape at the point of cell contact dictated whether or not phagocytosis was initiated [78] (Fig. 2). Polystyrene particles were fabricated in the shapes of spheres, oblate ellipsoids, prolate ellipsoids, elliptical discs, rectangular discs or flying saucer shapes. The orientation of the particle towards the phagocyte was of great importance in Fc receptor-mediated phagocytosis by macrophages. Actin polymerisation in the shape of a cup occurs beneath the particle, which then forms an actin ring that forces the membrane along the particle surface until it is engulfed. When this initial actin attachment forms on the flat side of a particle, the formation of the actin ring is not supported. The contact angle between the membrane normal and the particle, therefore, is an important determinant of the internalisation efficiency. Particles for which this angle is small are phagocytosed more efficiently, as only gradual expansion of the actin ring is required, which is a metabolically intensive process. If the contact angle is too large, the cell will spread across the surface of the particle but cannot internalise it. Therefore, the uptake of (near) spherical particles is always favourable, whereas that of rod-shaped particles depends on the likelihood of the particle approaching at a favourable contact angle, thereby negatively influencing the uptake of such particles (Fig. 2). Indeed, Huang *et al.* manufactured mesoporous silica nanoparticles of different lengths and ARs; 100 nm spherical (AR = 1), 240 nm short rod (AR = 2) and 450 nm long rod (AR = 4) [72]. Incubation with human melanoma cells showed the formation of well-organised F-actin bundles for the particles with ARs 1 and 2. However, F-actin was disorganised for cells incubated with the particles with an AR of 4. This may explain why near-spherical particles are taken up more effectively as described in the aforementioned papers.

Yi and Gao created a theoretical model for membrane wrapping of particles of different shapes [79]. Keeping rigidity constant, they found that longer and thinner rods require more energy for cellular wrapping than more spherical particles. Furthermore, non-spherical particles undergo an orientation change during wrapping, which also contributes to increased energy expenditure. Both *in vitro* and *in silico* models suggest that spherical and slightly ellipsoidal nanoparticles are most efficiently taken up due to favourable energy expenditure during actin membrane wrapping. It can also be noted that small spheres inherently require less polymerisation of the actin cytoskeleton, compared to larger spheres; hence, less energy is expended in this process. This might explain the preferential uptake of smaller compared to larger spheres.

3.3. Antigen presentation and APC activation

The effect of particle shape on antigen presentation is currently poorly described. In one study, rod-shaped gold particles (40 nm long, AR = 4) coated with West Nile virus induced production of IL-1β and IL-18 in bone marrow-derived DCs; these cytokines are secreted upon inflammasome activation. It is known that lysosomal rupture can induce inflammasome activation and indeed, rod-shaped particles were able to escape from the lysosome into the cytosol, suggesting lysosomal rupture could have occurred. In contrast, spherical and cubical particles induced production of tumour necrosis factor-α (TNF-α), IL-6 and IL-12, which are not associated with inflammasome activation [76]. Mathaes *et al.* reported that both nano- (150 nm) and micro-sized (1.5 µm) spherical PLGA particles induced stronger activation of DCs as measured by upregulation of CD83 and CD86 than similar sized, non-spherical, stretched particles [50]. As these molecules provide important co-stimulatory signals during antigen presentation, this finding may suggest that spherical particles result in more efficient antigen presentation. However, more research is required to study this relationship.

3.4. Skewing immune responses

Recent observations suggest particle shape directly influences the type of immune response. In the aforementioned study by Niikura *et al.*, spherical 40 nm gold particles coated with antigen derived from West Nile virus, induced superior levels of West Nile-specific IgG as compared to cubical and rod-shaped particles of similar size [76]. Kumar *et al.* performed an elegant study in which they used spherical polystyrene ovalbumin conjugated particles of 190 and 520 nm in diameter, which they stretched into rod-shaped particles of 380 and 1530 nm in length [16]. They found that the 190 nm spheres were most potent at inducing IgG2a antibody responses, whereas the 1530 nm rods induced the highest IgG1 antibody responses. Moreover, they showed that the small spheres were most potent at inducing IFN-γ responses, whereas IL-4 was consistently produced in all groups. From this, it can be concluded that the smaller-sized nanoparticles are more effective than larger particles are inducing Th1 and $CD8^+$ T-cells, and this effect is most pronounced when these small particles are spherical. In contrast, the larger-sized nanoparticles are more potent at inducing Th2 responses, which are most effective when using rod-shaped particles.

4. Particle rigidity

4.1. Particle distribution

Apart from size and shape, rigidity can also affect the biodistribution and elimination rate of particles. Merkel *et al.* produced red blood cell mimics (RBCM); hydrogels containing particles of a similar shape, size (6 µm) and rigidity as compared to RBCs [80]. By altering the rigidity, they observed that circulation time was inversely correlated with rigidity, with the most rigid RBCMs being eliminated much faster than the least rigid

ones. This was likely due to the less rigid particles being able to reach areas with constricted blood flow, increasing circulation times. Similarly, "soft" polyethylene glycol (PEG) based hydrogel nanoparticles were formulated which had a longer distribution half-life (rate of particle distribution from plasma into tissues) and elimination half-life (rate of particle clearance from plasma) than rigid particles [81]. Analysis of tissues after 30 min and 12 hours showed that soft particles were found at a higher concentration in almost all tissues (spleen, kidney, heart, lungs, brain, and blood) except for the liver. The differences in biodistribution were attributed to the longer circulation time of the soft nanoparticles, which led to increased retention in organs with high blood flow. Possibly, the soft nanoparticles were degraded in the liver, explaining their reduced retention in this tissue. Moreover, it was found that more rigid particles accumulated in the capillaries in the lungs while the less rigid particles avoided lung filtration and instead were found mostly in the spleen, suggesting that more rigid particles become trapped in the first tissue with microvasculature they encounter [80].

It was found that liposomes containing phosphatidylcholine (PC) with a high transition temperature (i.e., high rigidity) injected i.v. remained in the blood for a longer period of time than similar liposomes containing PC with a low transition temperature. This was combined with a decreased uptake in the liver and spleen [82, 83]. Similar results were observed by Senior *et al.* who hypothesised that longer circulation was due to less interaction between the liposomes and high-density lipoprotein in the blood [84]. There is evidence that ApoA-I and ApoA-II on high-density lipoprotein react with PC and cholesterol-containing liposomes, which results in faster clearance [85]. After i.m. injection, rigid cationic liposomes remained at the site of injection longer than less rigid ones. This corresponded with higher amounts of non-rigid liposomes found in the draining LNs [86]. In contrast, Kaur *et al.* observed no effects of cholesterol content (which also affects rigidity) in cationic liposomes on drainage from the site of injection or transport to LNs after i.m. injection [87].

It appears that similar to particles of increasing size, lymphatic trafficking of particles with increasing rigidity will be hindered by a decreased ability to navigate through narrow lymphatic vessels.

4.2. Cellular uptake

To understand the importance of particle rigidity on cellular uptake, one must first examine the interplay between the cell membrane and the particle. The first theoretical model of adhesive wrapping of a vesicle by the cell membrane was created by Yi *et al.* [88]. They theorised that the degree of wrapping was dependent on adhesion energy between the vesicle and the cell surface, vesicle size, the surface tension of the cell membrane upon contact with the vesicle, and the difference in rigidity of the cell membrane and the vesicle. They concluded that rigid particles are in general more easily wrapped by the cell membrane due to cell membrane deformation by the particles and that flexible particles spread out more across the cell membrane. This was supported by molecular dynamics simulations by Sun *et al.* [89]. Experimentally, Beningo and Wang reported that macrophages preferentially phagocytosed $1-6\mu$m-sized rigid

particles over softer particles due to rigid particles stimulating actin filament assembly in macrophages [90]. The previously described RBCM hydrogels also showed minimal (< 10%) uptake by human umbilical vein endothelial cells, probably due to a combination of low rigidity and large (6 µm) particle size [80]. Similarly, Anselmo *et al.* found that PEG-based rigid particles had significantly higher uptake than flexible particles (both spheres of 200 nm) in an endothelial brain cell line (bEnd.3), an epithelial tumour cell line (4T1) and macrophages (J774) [81]. As previously stated, phagocytosis by macrophages is important for retention of particles in the LNs, which improves the overall immunogenicity of the particles [39]. Similarly, cationic gel-state liposomes with higher cholesterol contents (i.e., lower rigidity) showed reduced uptake by THP-1 macrophages [87] and gel-state liposomes consisting of high transition temperature lipids had increased APC uptake compared to fluid-state liposomes made up of low transition temperature lipids [86].

Generally, it can be stated that rigid particles are most efficiently taken up, whereas more flexible particles are deformed by the membrane, resulting in increased energy expenditure and consequently reduced uptake (Fig. 2).

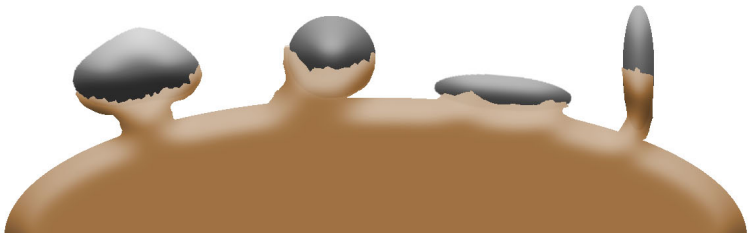

Figure 2: Cellular uptake of a flexible sphere, rigid sphere and rigid rod approaching the cellular membrane at a perpendicular or tangential angle. The rigid sphere is taken up more efficiently than the flexible sphere; while the membrane envelopes both particles, the flexible particle deforms leading to slower uptake. For rod-shaped particles, the angle at which the particle approaches the cell is important; when the particle arrives at a tangential angle, it would require too much energy to form an actin cup around the particle leading to no uptake. Therefore, an orientation change is needed, which also requires high energy expenditure. The rigid rod approaching the cell at a perpendicular angle requires much less energy to be taken up.

4.3. Antigen presentation and APC activation

Once a particle has been taken up by a cell, intracellular processing can also be affected by rigidity. Hartmann *et al.* produced microcapsules of about 4 µm with varying shell thickness that altered their rigidity [91]. By observing uptake and acidification of the microcapsules in HeLa cells, they found that more rigid capsules had longer endosomal processing times and reached the lysosome later than more flexible capsules. Unfortunately, it was not reported how this affects the efficiency of antigen processing. Cui *et al.* prepared capsules of around 1 µm composed of polyglycolic acid (PGA) cross-linked to the adjuvant CpG [92]. They altered rigidity by increasing the cross-linker concentration. Incubation with plasmacytoid DC (pDCs) showed increased particle association to pDCs with increasing rigidity. The authors also showed a rigidity-dependent increase in pDC activation as measured by CD86 and CD40 levels. Thus, the effect of rigidity on

uptake by macrophages and DCs may have important implications for immunity. In the case of liposomes, Christensen *et al.* showed that more rigid cationic liposomes injected i.m. resulted in increased activation of DCs in draining lymph nodes, as measured by CD40 and CD86 upregulation [86].

To our knowledge, no reports have been published that specifically examine antigen presentation as a function of particle rigidity. However, since antigen presentation is largely dependent on particle uptake by APCs, and it was shown above that rigid particles are more likely to be taken up, we suggest rigid particles shall have more efficient antigen presentation. Thereby, it can be speculated that the shorter endosomal processing time of rigid particles shall enhance antigen stability and lead to more efficient MHC presentation compared to flexible particles. This will mainly affect MHC class II epitopes as they require endosomal processing, whereas MHC class I epitopes are derived from the cytosol.

4.4. Skewing immune responses

Several studies have shown that particle rigidity can affect the skewing of the immune response. In two studies by the same group, the immune response was measured in mice after immunisation with liposomes composed of phospholipids with different transition temperatures. They found that liposomes containing high transition temperature lipids elicited higher antibody responses [93, 94]. A similar rigidity effect on antibody [95, 96] and T-cell responses [97, 98] has been found by other groups. There is some evidence that reducing liposome rigidity by the addition of cholesterol or by selecting lipids with lower phase transition temperatures leads to reduced Th1 responses after i.m. immunisation. In contrast to the studies mentioned above, the authors state that there is no measurable effect of particle rigidity on Th2 or antibody responses. However, this was hypothesised to be due to reduced APC uptake of non-rigid liposomes from the site of injection [86, 87].

Arnal and colleagues reported that the presence of virulence factors in Bordetella pertussis increased rigidity; a non-infectious mutant deficient of filamentous haemagglutinin (FHA) had lower rigidity [99]. The authors postulate that FHA increases the rigidity of the cell, specifically by creating rigid nanodomains that could enhance the adhesion of B. pertussis to cells. Studying different strains of *Lactobacillus* and *Bifidobacterium*, Mokrozub *et al.* found that *Lactobacillus* strains with elastic cell walls were more effectively digested by macrophages *in vitro* and enhanced their ability to produce nitric oxide and accumulate reactive oxygen species. However, the more rigid strains had higher IL-12 and IFN-γ production (indicative of a Th1 immune response). In the case of *Bifidobacterium* strains, uptake of the more rigid strains increased macrophage effector functions while also enhancing IFN-γ production [100]. The authors hypothesise that strains with more rigid cell walls remain viable within macrophages longer, prolonging cytokine production. For viruses, it was shown in two separate papers by Kol *et al.* that rigidity differs between the immature (non-infectious, viral budding) and mature (infectious, entry into cells) stage, for both murine leukaemia virus (MLV) and human immunodeficiency virus (HIV) (about 100 nm in size). In the case of MLV, the mature form

of the virus is more rigid. Conversely, immature HIV is much more rigid than mature HIV, suggesting that a more flexible viral structure is beneficial for cell entry [101, 102].

It can be concluded that, for lipid vesicles, immunisation with more rigid particles results in higher antibody and T-cell responses. Studies that examine the effect of particle rigidity on the immune response would be extremely valuable to further understanding and the role of this parameter in vaccine design.

5. Summary and conclusions

Here we reviewed how the immunogenicity of particulate vaccines is directed by their shape, size, and rigidity. Clearly, the choice of the optimal physicochemical parameters depends on multiple factors, such as the route of administration, which immune cells are targeted and what type of immune response is preferred. Importantly, in most of the studies discussed here, not only the shape, size or rigidity of the particles differ, but other parameters are also (indirectly) altered. Particle shape and rigidity are especially closely related, since highly deformable particles can alter their shape during circulation or cellular uptake. Additionally, differences in rigidity measurements and calculations can result in different definitions of "soft" or "rigid" particles. We strongly plead for systematic investigations where only one particle parameter is changed and all others are kept constant. This will help to accurately define the relationship between particle size or shape and the immunogenicity of particulate vaccines.

This review suggests it is important to take the physicochemical characteristics of particulate vaccines into account in order to induce maximal antigen responses. For instance, what may be a favourable characteristic for, e.g., transport towards the LN may not be ideal for inducing the desired skewing of the immune response. Therefore, the choice of particle size, shape and rigidity must involve a careful consideration of the effects of these on all of the events influencing the immunogenicity. Figure 3 could function as a "roadmap" and when the desired immunologic outcome is known, it might provide a model for rational vaccine development.

For instance, the development of a $CD8^+$ T-cell activating vaccine (e.g. cancer vaccines) may be most efficient when a small (< 200 nm), rigid, elliptical non-liposomal nanoparticle is used. The elliptical shape, as well as rigidity, will ensure efficient uptake by APCs and a size smaller than 200 nm will skew the immune response towards Th1 and $CD8^+$ T-cell immunity. Alternatively, the development of a Th2-directed vaccine (e.g. hepatitis B vaccines), could benefit most from a small (< 200 nm), spherical, slightly more flexible liposomal particulate formulation. Since liposomes show opposite trends compared to non-liposomal formulations in skewing immune responses, this will ensure a Th2-directed response. Thereby, the uptake of this particle by APCs will still be efficient due to the small size. As rigid bacterial strains and liposomes are known to skew the immune response towards type 1 immunity, it could be desirable to use a more flexible particle. However, more energy is needed for uptake of soft particles, which may have a negative influence on the antigen presentation. Thus, depending on the application, one could also choose to use a larger, non-liposomal particle. Micro-sized particles are

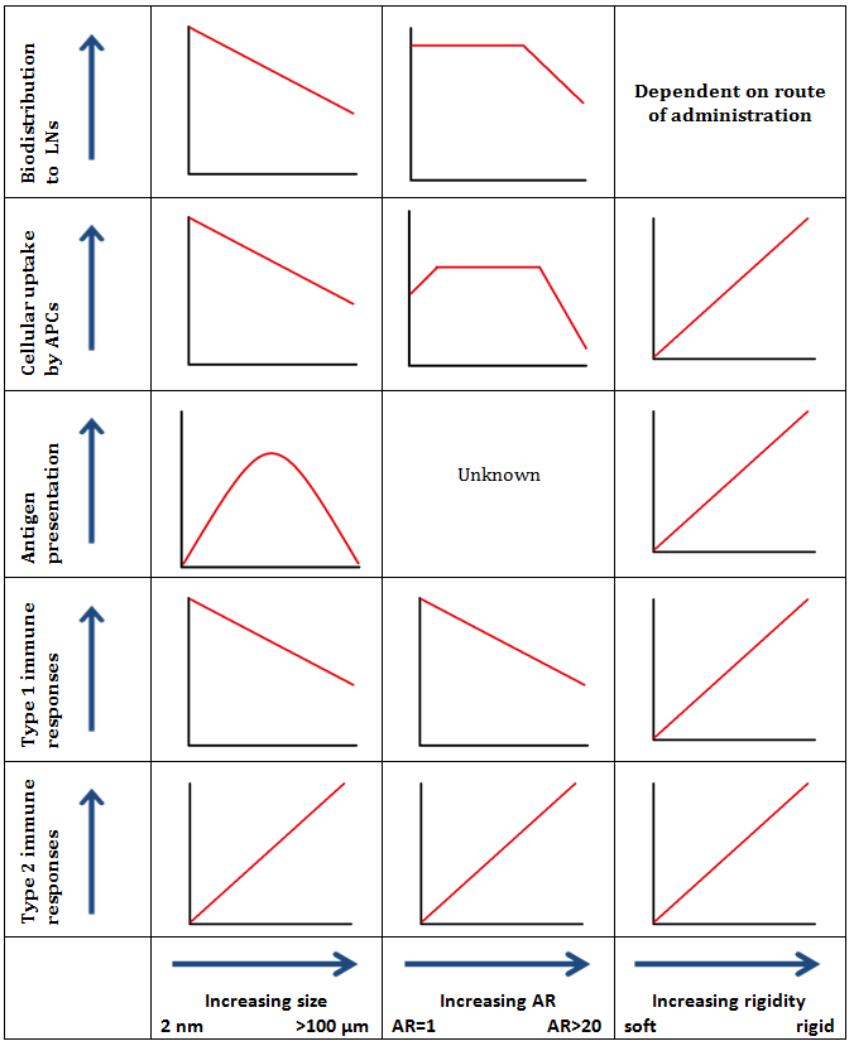

Figure 3: Summary of the general trends of the effects of shape, size and rigidity on different parameters affecting the immunogenicity of particulate vaccines. The height of the red line represents the efficiency per parameter. Size ranges from left to right from ultra-small (2 nm) to large (> 100µm) microparticles. Shape ranges from left to right from spherical (AR = 1) to long filomicelles (AR >> 20). Rigidity ranges from left to right from very elastic particles to non-deformable particles

known to tether to the membrane of APCs and deliver their antigens without being internalised. This also results in skewing towards a Th2-mediated immunity.

Overall, it appears that small, rigid, near spherical nanoparticles are the most favourable particles to reach APCs in the LNs and induce strong immune responses. The exact size can be tailored based on the type of particle used, to skew towards either Th1 or Th2 mediated immunity. Due to the scarcity of research, particularly

in the field of shape and rigidity, more studies will inevitably contribute to a more thorough understanding of how these parameters influence the immune response. This knowledge, in turn, will be of great importance for the rational design of more efficient vaccines, especially for diseases for which there is currently no vaccine, such as HIV, cancer or tuberculosis.

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; neutral, poloxamer 407-stabilised poly(propylene sulphide)	BALB/c and C57BL6 mouse	i.d.	Size	SAINNEHNI	[35]
Spherical; neutral; PEG-poly(propylene sulphide)	BALB/c mouse	s.c.	Size	Distribution	[34]
Spherical; anionic; polystyrene	C57BL/6 mouse	i.c.	Size	Distribution	[36]
Spherical; anionic; egg PC:egg phosphatidylglycerol(PG):cholesterol (10:1:4 molar ratio) liposomes	Wistar rat	s.c.	Size	Distribution	[38]
Spherical; neutral; egg PC:cholesterol (6:1 molar ratio)	Wistar rat	s.c.	Size	Distribution	[39]
Spherical; anionic; polystyrene	Sprague-Dawley rat	oral	Size	Distribution	[42]
Spherical; anionic; PLGA (50:50, MW 100000)	Sprague-Dawley rat	*In situ* intestinal tissue loop	Size	Distribution	[43]
Spherical; anionic; PLA (MW 7000)	BALB/c mouse	oral	Size	Distribution	[41]
Spherical; anionic; PLGA (75:25, MW 98000)	CD1 mouse	oral	Size	Distribution	[14]
Spherical; neutral; PLA-PEG (31:69, MW 28000)	Sprague-Dawley rat	intranasal	Size	Distribution	[13]
Spherical; cationic; N-trimethyl chitosan:tripolyphosphate (10:1.7 weight ratio)	BALB/c mouse rat	intranasal	Size	Distribution	[46]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; γ-PGA-g-phenylalanine (MW 380 000, 50:50)	C57BL/6j mouse	s.c.	Size	Distribution, cell uptake, APC activation	[52]
Spherical; anionic; carboxyl-modified polystyrene	C57BL/6j mouse	s.c.	Size	Distribution, cell uptake, APC activation	[53]
Spherical; anionic; poly(sulfobutyl-vinyl alcohol)-g-PLGA (50:50 MW 221 000)	BALB/c mouse	oral, intranasal, i.p.	Size, administration, route	Distribution, immune response	[45]
Spherical; anionic; PLGA (50:50, MW 24 000)	C57BL/6 mouse	i.p.	Size	Cell uptake, APC activation, immune response	[49]
Spherical and elliptical; anionic; polystyrene	JAWSII DC cell line	-	Size, shape	Cell uptake, APC activation	[50]
Spherical and elliptical; anionic; polystyrene	JAWSII DC cell line	-	Size, shape	Cell uptake, APC activation	[50]
Spherical; anionic and cationic; polystyrene	Human monocyte derived DCs	-	Size	Cell uptake	[51]
Spherical; anionic; PLA (MW 45 000)	Wistar rat	i.m.	Size	Cell uptake, antigen presentation, immune response	[55]
Spherical; neutral; glutathione-conjugated gold)	Human monocyte derived DCs	-	Size	Cell uptake, APC activation, immune response	[54]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; PLGA (50:50, MW 43 500 and 4500)	Sprague-Dawley rat	pulmonary	Size, weight	Cell uptake, immune response	[56]
Spherical; anionic; PLGA (50:50 MW 5000 and 75:25 MW 4000)	C57BL/6 mouse	s.c.	Size	Cell uptake, immune response	[61]
Spherical; anionic; monopalmitoyl glycerol:cholesterol:dicetyl phosphate (DCP) (5:4:1 molar ratio) liposomes	BALB/c or CBA/ca mouse derived macrophages	-	Size	Antigen presentation, cell uptake	[12]
Spherical; anionic; carboxyl-modified polystyrene	BALB/c and C57BL/6 mouse	i.d.	Size	Cell uptake, immune response	[66]
Spherical; anionic; carboxyl-modified polystyrene	C57BL/6 mouse	i.d.	Size	Distribution, immune response	[67]
Spherical; anionic; soy lecithin:glycerol monostearate (7:1 weight ratio)	C57BL/6 mouse	s.c.	Size	Distribution, antigen presentation, cell uptake, immune response	[68]
Spherical; anionic; monopalmitoyl glycerol:cholesterol:DCP (5:4:1 molar ratio) liposomes	BALB/c mouse, ferret	oral, i.m.	Size, administration route	Immune response	[69]
Spherical; cationic; dimethyldioctadecylammonium (DDA): trehalose dibehenate (TDB) (5:1 weight ratio) liposomes	BALB/c mouse	i.m.	Size	Distribution, cell uptake, immune response	[70]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Elliptical (AR 1.5 and 5); cationic; tetraethyl orthosilicate mesoporous silica	ICR mouse	i.v.	Shape	Distribution	[72]
Filamental; neutral; PEG-polyethylethylene or PEG-polycaprolactone	Sprague-Dawley rat, C57BL/6 mouse	i.v.	Shape, size	Distribution, cell uptake	[73]
Elongated (AR > 20); anionic; polystyrene	Rat alveolar macrophage	-	Shape, size	Cell uptake	[75]
Spherical, cubic and rod-like (AR 4); anionic; gold	C3H/HeN Jc1 mouse	i.p.	Shape, size	Cell uptake, APC activation, immune response	[76]
Spherical, oblate elliptical, prolate elliptical; anionic; polystyrene	RAW264.7 macrophage	-	Shape, size	Cell uptake	[77]
Spherical, oblate elliptical, prolate elliptical, elliptical disk, rectangular disk, UFO-shaped; anionic; polystyrene	Rat alveolar macrophage	-	Shape, size	Cell uptake	[78]
Spherical, short rod (AR 2), long rod (AR 4); cationic; tetraethyl orthosilicate mesoporous silica	A375 human melanoma	-	Shape	Cell uptake	[72]
Spherical, rod-shaped (AR 3 and 7); anionic; carboxyl-modified polystyrene	BALB/c mouse	s.c.	Shape, size	Cell uptake, immune response	[16]
Discoid; anionic; 2-hydroxyethyl acrylate and 2-carboxyethyl acrylate cross-linked with PEG-diacrylate hydrogel	BALB/c mouse	i.v.	Rigidity	Distribution, cell uptake	[80]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; PEG-diacrylate and 2-carboxyethyl acrylate hydrogel	BALB/c mouse	i.v.	Rigidity	Distribution, cell uptake	[81]
Spherical; anionic; polyacrylamide	Murine BMDC	-	Rigidity	Cell uptake	[90]
Spherical; cationic; poly(sodium 4-styrenesulphonate):poly(allylamine hydrochloride) (MW 70000 and 56000) and dextran sulphate:poly-L-arginine (MW 40000 and 15-70.000) capsules	A549 cells, HeLa cells, SH-SY5Y cells, HUVECs and human monocyte-derived macrophages and DCs	-	Rigidity	Cell uptake	[91]
Spherical; anionic; PGA (MW 3-15000 and 45000) capsules	PBMCs	-	Rigidity	Cell uptake, APC activation	[92]
Spherical; neutral and anionic; several liposomes containing egg PC, sphingomyelin (SM), DSPC, phosphatidylinositol, phosphatidic acid, DPPG, PS and monosialoganglioside	ICR mouse	i.v., i.p., s.c.	Rigidity	Distribution	[83]
Spherical: neutral; liposomes containing egg PC, egg SM, DSPC and cholesterol	T.O. mouse	i.v. and i.p.	Rigidity	Distribution	[84]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; cationic; DDA:TDB (1.98:0.25 molar ratio) and dimethyldioleoylammonium:TDB liposomes	C67BL/6 and BALB/c mouse	i.m.	Rigidity	Distribution, cell uptake, APC activation, immune response	[86]
Spherical; cationic; DDA:TDB:cholesterol (8:1:0, 8:1:2 and 8:1:4 molar ratio) liposomes	C67BL/6 and BALB/c mouse	i.m.	Rigidity	Distribution, cell uptake, immune response	[87]
Spherical; anionic; various PC:cholesterol:DCP:dinitrophenyl-aminocaproyl phosphatidylethanolamine (PE)(2:1.5:0.2:0.1 molar ratio)	AKR mouse	i.p.	Rigidity	Immune response	[93]
Spherical; neutral, anionic or cationic; various PC and PE, DCP and stearylamine liposomes	AKR mouse	i.p.	Rigidity	Immune response	[94]
Spherical; anionic; PC:cholesterol:PG, DPPC:cholesterol:DPPG, DSPC:cholesterol:DSPG liposomes of various molar ratios	Cpb:SE mouse	s.c.	Rigidity	Immune response	[95]
Spherical; anionic; various PC/PE, DPPG/DCP and cholesterol (7:2:1 molar ratio) liposomes	W/Fu rat	s.c.	Rigidity	Immune response	[96]
Spherical; anionic; cholesterol:DCP:DLPC or cholesterol:DCP:DSPC (1:2:7 molar ratio) liposomes	BALB/c mouse	s.c.	Rigidity	Immune response	[97]
Spherical; neutral; various PC:cholesterol (7:2 molar ratio) liposomes	Hamster	i.p.	Rigidity	Immune response	[98]

References

[1] K. A. Smith, *Smallpox: can we still learn from the journey to eradication?* Indian J Med Res **137**, 895 (2013).

[2] J. B. Milstien and J. J. Gibson, *Quality control of bcg vaccine by who: a review of factors that may influence vaccine effectiveness and safety,* Bull World Health Organ **68**, 93 (1990).

[3] S. Guillot, V. Caro, N. Cuervo, E. Korotkova, M. Combiescu, A. Persu, A. Aubert-Combiescu, F. Delpeyroux, and R. Crainic, *Natural genetic exchanges between vaccine and wild poliovirus strains in humans,* J Virol **74**, 8434 (2000).

[4] S. Donnelly, C. E. Loscher, M. A. Lynch, and K. H. Mills, *Whole-cell but not acellular pertussis vaccines induce convulsive activity in mice: evidence of a role for toxin-induced interleukin-1beta in a new murine model for analysis of neuronal side effects of vaccination,* Infect Immun **69**, 4217 (2001).

[5] T. V. Murphy, P. M. Gargiullo, M. S. Massoudi, D. B. Nelson, A. O. Jumaan, C. A. Okoro, L. R. Zanardi, S. Setia, E. Fair, C. W. LeBaron, B. Schwartz, M. Wharton, and J. R. Livingood, *Intussusception among infants given an oral rotavirus vaccine,* New England Journal of Medicine **344**, 564 (2001).

[6] E. A. Cherkasova, E. A. Korotkova, M. L. Yakovenko, O. E. Ivanova, T. P. Eremeeva, K. M. Chumakov, and V. I. Agol, *Long-term circulation of vaccine-derived poliovirus that causes paralytic disease,* J Virol **76**, 6791 (2002).

[7] P. M. Moyle and I. Toth, *Modern subunit vaccines: development, components, and research opportunities,* ChemMedChem **8**, 360 (2013).

[8] B. Slutter, P. C. Soema, Z. Ding, R. Verheul, W. Hennink, and W. Jiskoot, *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen,* J Control Release **143**, 207 (2010).

[9] D. T. O'Hagan and M. Singh, *Microparticles as vaccine adjuvants and delivery systems,* Expert Rev Vaccines **2**, 269 (2003).

[10] F. A. Sharp, D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, M. Singh, D. T. O'Hagan, V. Petrilli, J. Tschopp, L. A. O'Neill, and E. C. Lavelle, *Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome,* Proc Natl Acad Sci U S A **106**, 870 (2009).

[11] M.-L. De Temmerman, J. Rejman, J. Demeester, D. J. Irvine, B. Gander, and S. C. De Smedt, *Particulate vaccines: on the quest for optimal delivery and immune response,* Drug Discovery Today **16**, 569 (2011).

[12] J. M. Brewer, K. G. Pollock, L. Tetley, and D. G. Russell, *Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles,* J Immunol **173**, 6143 (2004).

[13] A. Vila, A. Sanchez, C. Evora, I. Soriano, O. McCallion, and M. J. Alonso, *PLA-PEG particles as nasal protein carriers: the influence of the particle size,* Int J Pharm **292**, 43 (2005).

[14] M. Shakweh, M. Besnard, V. Nicolas, and E. Fattal, *Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by peyer's patches in mice,* Eur J Pharm Biopharm **61**, 1 (2005).

[15] J. A. Cohen, T. T. Beaudette, W. W. Tseng, E. M. Bachelder, I. Mende, E. G. Engleman, and J. M. Frechet, *T-cell activation by antigen-loaded ph-sensitive hydrogel particles in vivo: the effect of particle size,* Bioconjug Chem **20**, 111 (2009).

[16] S. Kumar, A. C. Anselmo, A. Banerjee, M. Zakrewsky, and S. Mitragotri, *Shape and size-dependent immune response to antigen-carrying nanoparticles,* J Control Release **220**, 141 (2015).

[17] S. E. Gratton, P. D. Pohlhaus, J. Lee, J. Guo, M. J. Cho, and J. M. Desimone, *Nanofabricated particles for engineered drug therapies: a preliminary biodistribution study of print nanoparticles,* J Control Release **121**, 10 (2007).

[18] K. Nakano, Y. Tozuka, H. Yamamoto, Y. Kawashima, and H. Takeuchi, *A novel method for measuring rigidity of submicron-size liposomes with atomic force microscopy,* Int J Pharm **355**, 203 (2008).

[19] J. P. Best, J. Cui, M. Müllner, and F. Caruso, *Tuning the mechanical properties of nanoporous hydrogel particles via polymer cross-linking,* Langmuir **29**, 9824 (2013).

[20] H. Sun, E. H. H. Wong, Y. Yan, J. Cui, Q. Dai, J. Guo, G. G. Qiao, and F. Caruso, *The role of capsule stiffness on cellular processing,* Chem Sci **6**, 3505 (2015).

[21] A. Albanese, P. S. Tang, and W. C. Chan, *The effect of nanoparticle size, shape, and surface chemistry on biological systems,* Annu Rev Biomed Eng **14**, 1 (2012).

[22] D. S. Watson, A. N. Endsley, and L. Huang, *Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens,* Vaccine **30**, 2256 (2012).

[23] J. Wang, J. D. Byrne, M. E. Napier, and J. M. DeSimone, *More effective nanomedicines through particle design,* Small **7**, 1919 (2011).

[24] T. B. H. Geijtenbeek and S. I. Gringhuis, *Signalling through c-type lectin receptors: shaping immune responses,* Nat Rev Immunol **9**, 465 (2009).

[25] T. Kawai and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on toll-like receptors,* Nat Immunol **11**, 373 (2010).

[26] C. R. Alving, *Liposomes as carriers of antigens and adjuvants,* Journal of Immunological Methods **140**, 1 (1991).

[27] K. Zaks, M. Jordan, A. Guth, K. Sellins, R. Kedl, A. Izzo, C. Bosio, and S. Dow, *Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes,* J Immunol **176**, 7335 (2006).

[28] D. M. Smith, J. K. Simon, and J. Baker, J. R., *Applications of nanotechnology for immunology,* Nat Rev Immunol **13**, 592 (2013).

[29] J. J. Moon, B. Huang, and D. J. Irvine, *Engineering nano- and microparticles to tune immunity,* Adv Mater **24**, 3724 (2012).

[30] N. Kamaly, Z. Xiao, P. M. Valencia, A. F. Radovic-Moreno, and O. C. Farokhzad, *Targeted polymeric therapeutic nanoparticles: design, development and clinical translation,* Chem Soc Rev **41**, 2971 (2012).

[31] T. J. Moyer, A. C. Zmolek, and D. J. Irvine, *Beyond antigens and adjuvants: formulating future vaccines,* The Journal of Clinical Investigation **126**, 799 (2016).

[32] J. Banchereau and R. M. Steinman, *Dendritic cells and the control of immunity,* Nature **392**, 245 (1998).

[33] U. H. von Andrian and T. R. Mempel, *Homing and cellular traffic in lymph nodes,* Nat Rev Immunol **3**, 867 (2003).

[34] S. T. Reddy, A. Rehor, H. G. Schmoekel, J. A. Hubbell, and M. A. Swartz, *In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles,* J Control Release **112**, 26 (2006).

[35] S. T. Reddy, A. J. van der Vlies, E. Simeoni, V. Angeli, G. J. Randolph, C. P. O'Neil, L. K. Lee, M. A. Swartz, and J. A. Hubbell, *Exploiting lymphatic transport and complement activation in nanoparticle vaccines,* Nat Biotech **25**, 1159 (2007).

[36] V. Manolova, A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M. F. Bachmann, *Nanoparticles target distinct dendritic cell populations according to their size,* Eur J Immunol **38**, 1404 (2008).

[37] A. Martín-Fontecha, S. Sebastiani, U. E. Höpken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto, *Regulation of dendritic cell migration to the draining lymph node: Impact on T lymphocyte traffic and priming,* The Journal of Experimental Medicine **198**, 615 (2003).

[38] C. Oussoren, J. Zuidema, D. J. Crommelin, and G. Storm, *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. influence of liposomal size, lipid compostion and lipid dose,* Biochim Biophys Acta **1328**, 261 (1997).

[39] C. Oussoren, M. Velinova, G. Scherphof, J. J. van der Want, N. van Rooijen, and G. Storm, *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection . iv. fate of liposomes in regional lymph nodes,* Biochim Biophys Acta **1370**, 259 (1998).

[40] M. Merad, P. Sathe, J. Helft, J. Miller, and A. Mortha, *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting,* Annu Rev Immunol **31**, 563 (2013).

[41] Y. Tabata, Y. Inoue, and Y. Ikada, *Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres,* Vaccine **14**, 1677 (1996).

[42] P. Jani, G. W. Halbert, J. Langridge, and A. T. Florence, *The uptake and translocation of latex nanospheres and microspheres after oral administration to rats,* J Pharm Pharmacol **41**, 809 (1989).

[43] M. P. Desai, V. Labhasetwar, G. L. Amidon, and R. J. Levy, *Gastrointestinal uptake of biodegradable microparticles: effect of particle size,* Pharm Res **13**, 1838 (1996).

[44] H. W. Schroeder and L. Cavacini, *Structure and function of immunoglobulins,* The Journal of allergy and clinical immunology **125**, S41 (2010).

[45] T. Jung, W. Kamm, A. Breitenbach, K. D. Hungerer, E. Hundt, and T. Kissel, *Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice,* Pharm Res **18**, 352 (2001).

[46] B. Slutter, S. M. Bal, I. Que, E. Kaijzel, C. Lowik, J. Bouwstra, and W. Jiskoot, *Antigen-adjuvant nanoconjugates for nasal vaccination: an improvement over the use of nanoparticles?* Mol Pharm **7**, 2207 (2010).

[47] I. Mellman and R. M. Steinman, *Dendritic cells: Specialized and regulated antigen processing machines,* Cell **106**, 255 (2001).

[48] E. S. Trombetta and I. Mellman, *Cell biology of antigen processing in vitro and in vivo,* Annu Rev Immunol **23**, 975 (2005).

[49] V. B. Joshi, S. M. Geary, and A. K. Salem, *Biodegradable particles as vaccine delivery systems: size matters,* AAPS J **15**, 85 (2013).

[50] R. Mathaes, G. Winter, T. J. Siahaan, A. Besheer, and J. Engert, *Influence of particle size, an elongated particle geometry, and adjuvants on dendritic cell activation,* Eur J Pharm Biopharm **94**, 542 (2015).

[51] C. Foged, B. Brodin, S. Frokjaer, and A. Sundblad, *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model,* Int J Pharm **298**, 315 (2005).

[52] F. Shima, T. Uto, T. Akagi, M. Baba, and M. Akashi, *Size effect of amphiphilic poly(γ-glutamic acid) nanoparticles on cellular uptake and maturation of dendritic cells in vivo,* Acta Biomaterialia **9**, 8894 (2013).

[53] F. Blank, P. A. Stumbles, E. Seydoux, P. G. Holt, A. Fink, B. Rothen-Rutishauser, D. H. Strickland, and C. von Garnier, *Size-dependent uptake of particles by pulmonary antigen-presenting cell populations and trafficking to regional lymph nodes,* American Journal of Respiratory Cell and Molecular Biology **49**, 67 (2013).

[54] X. le Guével, F. Palomares, M. J. Torres, M. Blanca, T. D. Fernandez, and C. Mayorga, *Nanoparticle size influences the proliferative responses of lymphocyte subpopulations,* RSC Advances **5**, 85305 (2015).

[55] V. Kanchan and A. K. Panda, *Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response,* Biomaterials **28**, 5344 (2007).

[56] C. Thomas, V. Gupta, and F. Ahsan, *Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles,* Pharm Res **27**, 905 (2010).

[57] J. Janeway, C. A. and R. Medzhitov, *Innate immune recognition,* Annu Rev Immunol **20**, 197 (2002).

[58] J. A. Villadangos and P. Schnorrer, *Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo,* Nat Rev Immunol **7**, 543 (2007).

[59] A. K. Varkouhi, M. Scholte, G. Storm, and H. J. Haisma, *Endosomal escape pathways for delivery of biologicals,* Journal of Controlled Release **151**, 220 (2011).

[60] A. Mant, F. Chinnery, T. Elliott, and A. P. Williams, *The pathway of cross-presentation is influenced by the particle size of phagocytosed antigen,* Immunology **136**, 163 (2012).

[61] A. L. Silva, R. A. Rosalia, E. Varypataki, S. Sibuea, F. Ossendorp, and W. Jiskoot, *Poly-(lactic-co-glycolic-acid)-based particulate vaccines: Particle uptake by dendritic cells is a key parameter for immune activation,* Vaccine **33**, 847 (2015).

[62] E. Seydoux, B. Rothen-Rutishauser, I. M. Nita, S. Balog, A. Gazdhar, P. A. Stumbles, A. Petri-Fink, F. Blank, and C. von Garnier, *Size-dependent accumulation of particles in lysosomes modulates dendritic cell function through impaired antigen degradation,* Int J Nanomedicine **9**, 3885 (2014).

[63] J. C. Nolz, *Molecular mechanisms of $CD8^+$ T cell trafficking and localization,* Cellular and Molecular Life Sciences **72**, 2461 (2015).

[64] D. C. Tscharke, N. P. Croft, P. C. Doherty, and N. L. La Gruta, *Sizing up the key determinants of the $CD8^+$ T cell response,* Nat Rev Immunol **15**, 705 (2015).

[65] J. Geginat, M. Paroni, F. Facciotti, P. Gruarin, I. Kastirr, F. Caprioli, M. Pagani, and S. Abrignani, *The CD4-centered universe of human T cell subsets,* Seminars in Immunology **25**, 252 (2013).

[66] P. L. Mottram, D. Leong, B. Crimeen-Irwin, S. Gloster, S. D. Xiang, J. Meanger, R. Ghildyal, N. Vardaxis, and M. Plebanski, *Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus,* Mol Pharm **4**, 73 (2007).

[67] T. Fifis, A. Gamvrellis, B. Crimeen-Irwin, G. A. Pietersz, J. Li, P. L. Mottram, I. F. McKenzie, and M. Plebanski, *Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors,* J Immunol **173**, 3148 (2004).

[68] X. Li, B. R. Sloat, N. Yanasarn, and Z. Cui, *Relationship between the size of nanoparticles and their adjuvant activity: data from a study with an improved experimental design,* Eur J Pharm Biopharm **78**, 107 (2011).

[69] J. F. Mann, E. Shakir, K. C. Carter, A. B. Mullen, J. Alexander, and V. A. Ferro, *Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection,* Vaccine **27**, 3643 (2009).

[70] M. Henriksen-Lacey, A. Devitt, and Y. Perrie, *The vesicle size of dda:tdb liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production,* J Control Release **154**, 131 (2011).

[71] K. K. Tran and H. Shen, *The role of phagosomal ph on the size-dependent efficiency of cross-presentation by dendritic cells,* Biomaterials **30**, 1356 (2009).

[72] X. Huang, L. Li, T. Liu, N. Hao, H. Liu, D. Chen, and F. Tang, *The shape effect of mesoporous silica nanoparticles on biodistribution, clearance, and biocompatibility in vivo,* ACS Nano **5**, 5390 (2011).

[73] Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko, and D. E. Discher, *Shape effects of filaments versus spherical particles in flow and drug delivery,* Nat Nanotechnol **2**, 249 (2007).

[74] L. Li, T. Liu, C. Fu, L. Tan, X. Meng, and H. Liu, *Biodistribution, excretion, and toxicity of mesoporous silica nanoparticles after oral administration depend on their shape,* Nanomedicine: Nanotechnology, Biology and Medicine **11**, 1915 (2015).

[75] J. A. Champion and S. Mitragotri, *Shape induced inhibition of phagocytosis of polymer particles,* Pharm Res **26**, 244 (2009).

[76] K. Niikura, T. Matsunaga, T. Suzuki, S. Kobayashi, H. Yamaguchi, Y. Orba, A. Kawaguchi, H. Hasegawa, K. Kajino, T. Ninomiya, K. Ijiro, and H. Sawa, *Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo,* ACS Nano **7**, 3926 (2013).

[77] G. Sharma, D. T. Valenta, Y. Altman, S. Harvey, H. Xie, S. Mitragotri, and J. W. Smith, *Polymer particle shape independently influences binding and internalization by macrophages,* J Control Release **147**, 408 (2010).

[78] J. A. Champion and S. Mitragotri, *Role of target geometry in phagocytosis,* Proceedings of the National Academy of Sciences of the United States of America **103**, 4930 (2006).

[79] X. Yi and H. Gao, *Phase diagrams and morphological evolution in wrapping of rod-shaped elastic nanoparticles by cell membrane: a two-dimensional study,* Phys Rev E Stat Nonlin Soft Matter Phys **89**, 062712 (2014).

[80] T. J. Merkel, S. W. Jones, K. P. Herlihy, F. R. Kersey, A. R. Shields, M. Napier, J. C. Luft, H. Wu, W. C. Zamboni, A. Z. Wang, J. E. Bear, and J. M. DeSimone, *Using mechanobiological mimicry of red blood cells to extend circulation times of hydrogel microparticles,* Proc Natl Acad Sci U S A **108**, 586 (2011).

[81] A. C. Anselmo, M. Zhang, S. Kumar, D. R. Vogus, S. Menegatti, M. E. Helgeson, and S. Mitragotri, *Elasticity of nanoparticles influences their blood circulation, phagocytosis, endocytosis, and targeting,* ACS Nano **9**, 3169 (2015).

[82] A. Gabizon and D. Papahadjopoulos, *Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors,* Proc Natl Acad Sci U S A **85**, 6949 (1988).

[83] T. M. Allen, C. Hansen, and J. Rutledge, *Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues,* Biochim Biophys Acta **981**, 27 (1989).

[84] J. Senior and G. Gregoriadis, *Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components,* Life Sci **30**, 2123 (1982).

[85] M. Wroblewska, M. Czyzewska, A. Wolska, B. Kortas-Stempak, and A. Szutowicz, *Apo a-ii participates in hdl-liposome interaction by the formation of new pre-beta mobility particles and the modification of liposomes,* Biochim Biophys Acta **1801**, 1323 (2010).

[86] D. Christensen, M. Henriksen-Lacey, A. T. Kamath, T. Lindenstrom, K. S. Korsholm, J. P. Christensen, A. F. Rochat, P. H. Lambert, P. Andersen, C. A. Siegrist, Y. Perrie, and E. M. Agger, *A cationic vaccine adjuvant based on a saturated quaternary ammonium lipid have different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity compared to its unsaturated analog,* J Control Release **160**, 468 (2012).

[87] R. Kaur, M. Henriksen-Lacey, J. Wilkhu, A. Devitt, D. Christensen, and Y. Perrie, *Effect of incorporating cholesterol into dda:tdb liposomal adjuvants on bilayer properties, biodistribution, and immune responses,* Molecular Pharmaceutics **11**, 197 (2014).

[88] X. Yi, X. Shi, and H. Gao, *Cellular uptake of elastic nanoparticles,* Phys Rev Lett **107**, 098101 (2011).

[89] J. Sun, L. Zhang, J. Wang, Q. Feng, D. Liu, Q. Yin, D. Xu, Y. Wei, B. Ding, X. Shi, and X. Jiang, *Tunable rigidity of (polymeric core)–(lipid shell) nanoparticles for regulated cellular uptake,* Advanced Materials **27**, 1402 (2015).

[90] K. A. Beningo and Y. L. Wang, *Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target,* J Cell Sci **115**, 849 (2002).

[91] R. Hartmann, M. Weidenbach, M. Neubauer, A. Fery, and W. J. Parak, *Stiffness-dependent in vitro uptake and lysosomal acidification of colloidal particles,* Angewandte Chemie International Edition **54**, 1365 (2015).

[92] J. Cui, R. De Rose, J. P. Best, A. P. R. Johnston, S. Alcantara, K. Liang, G. K. Such, S. J. Kent, and F. Caruso, *Mechanically tunable, self-adjuvanting nanoengineered polypeptide particles,* Advanced Materials **25**, 3468 (2013).

[93] T. Yasuda, G. F. Dancey, and S. C. Kinsky, *Immunogenicity of liposomal model membranes in mice: dependence on phospholipid composition,* Proc Natl Acad Sci U S A **74**, 1234 (1977).

[94] G. F. Dancey, T. Yasuda, and S. C. Kinsky, *Effect of liposomal model membrane composition on immunogenicity,* J Immunol **120**, 1109 (1978).

[95] G. F. Kersten, A. M. van de Put, T. Teerlink, E. C. Beuvery, and D. J. Crommelin, *Immunogenicity of liposomes and iscoms containing the major outer membrane protein of neisseria gonorrhoeae: influence of protein content and liposomal bilayer composition,* Infect Immun **56**, 1661 (1988).

[96] O. Bakouche and D. Gerlier, *Enhancement of immunogenicity of tumour virus antigen by liposomes: the effect of lipid composition,* Immunology **58**, 507 (1986).

[97] F. Garnier, F. Forquet, P. Bertolino, and D. Gerlier, *Enhancement of in vivo and in vitro T cell response against measles virus haemagglutinin after its incorporation into liposomes: effect of the phospholipid composition,* Vaccine **9**, 340 (1991).

[98] T. Mazumdar, K. Anam, and N. Ali, *Influence of phospholipid composition on the adjuvanticity and protective efficacy of liposome-encapsulated leishmania donovani antigens,* J Parasitol **91**, 269 (2005).

[99] L. Arnal, D. O. Serra, N. Cattelan, M. F. Castez, L. Vazquez, R. C. Salvarezza, O. M. Yantorno, and M. E. Vela, *Adhesin contribution to nanomechanical properties of the virulent bordetella pertussis envelope,* Langmuir **28**, 7461 (2012).

[100] C. V. V., L. M. Lazarenko, L. M. Sichel, L. P. Babenko, P. M. Lytvyn, O. M. Demchenko, Y. O. Melnichenko, N. V. Boyko, B. Biavati, D. DiGioia, R. V. Bubnov, and M. Y. Spivak, *The role of beneficial bacteria wall elasticity in regulating innate immune response,* Epma j **6**, 13 (2015).

[101] N. Kol, M. Gladnikoff, D. Barlam, R. Z. Shneck, A. Rein, and I. Rousso, *Mechanical properties of murine leukemia virus particles: effect of maturation,* Biophys J **91**, 767 (2006).

[102] N. Kol, Y. Shi, M. Tsvitov, D. Barlam, R. Z. Shneck, M. S. Kay, and I. Rousso, *A stiffness switch in human immunodeficiency virus,* Biophys J **92**, 1777 (2007).