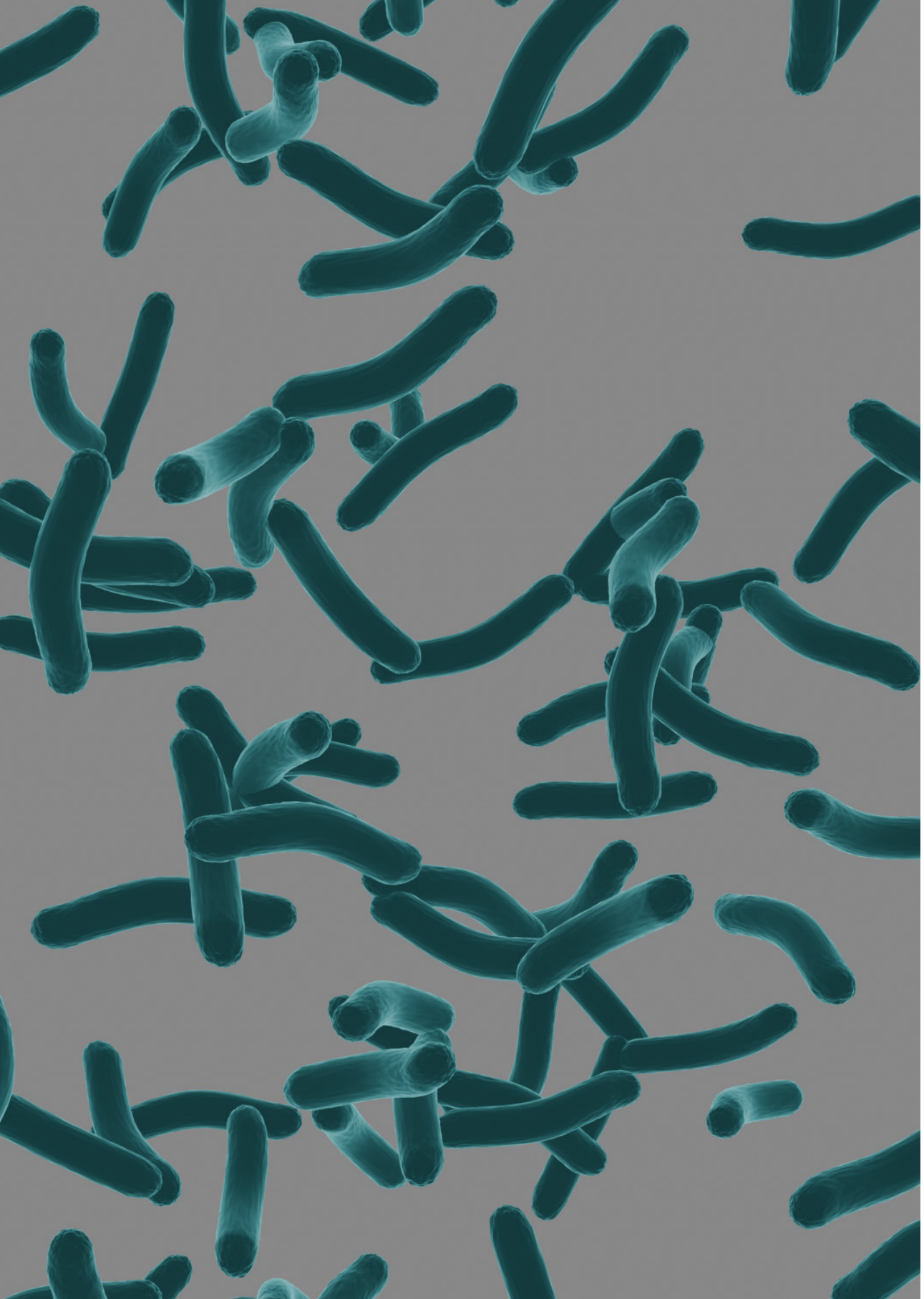
Nanoparticles and microfluidics for future tuberculosis vaccines

Neustrup, M.A.



CHAPTER 1

GENERAL INTRODUCTION
DISSERTATION AIM AND OUTLINE

GENERAL INTRODUCTION

"One can think of the middle of the twentieth century as the end of one of the most important social revolutions in history, the virtual elimination of infectious diseases as a significant factor in social life [1]." Wrote Sir Frank MacFarlane Burnet, the 1960 co-winner of the Nobel Prize in Medicine, in 1962.

This optimistic statement was presumably based on the improved living conditions in the Western World caused by better nutrition, sanitation, housing, and the development of vaccines and antibiotics that had decreased the mortality and morbidity of infectious diseases [2]. However, the statement was shortly after disproven, as a series of outbreaks and epidemics of new, re-emerging, and drug-resistant pathogens, e.g., the AIDS outbreak (1981: first official reporting [3]) and Ebola (1976: first recognition of the disease [4]) refuted the notion that infectious diseases were no longer a threat to human health [2]. We have still not "virtually eliminated" infectious diseases. One of humans' oldest enemies, tuberculosis (TB), which has been with us for at least 10,500 years [5], is still going strong. TB is the deadliest infectious disease in recent times, only recently surpassed (on a death/year basis) by COVID-19 [6, 7].

Tuberculosis

TB is caused by the *Mycobacterium tuberculosis* complex, a group of closely related mycobacteria, including the *Mycobacterium tuberculosis* (Mtb), which is the main cause of TB in humans [8, 9]. Mtb is transmitted through airborne droplets, called liquid aerosols, which expire when individuals with pulmonary TB cough, sneeze, talk, and even breathe [10]. TB mainly affects the lungs, causing symptoms such as fever, chest pain, and cough with sputum and sometimes blood [11]. When Mtb is transmitted to a new host, it can either be eradicated by the immune system [12], lead to the symptomatic (active) disease, or be (mainly) contained in granulomas by the body as an asymptomatic infection that possibly can lead to the active disease later in life [13–15]. If active TB is left untreated, it may cause death by septic shock, respiratory failure, or suffocation [16].

It is estimated that one-quarter of the world's population is infected (mostly latently) with TB and that it killed around 1.3 million people in 2022 [7]. This is despite TB being manageable/curable with a strict regimen of antibiotics [7]. According to the World Health Organization (WHO), TB is especially a problem in the regions of Southeast Asia (46%), Africa (23%), and the Western Pacific (18%), regions which mainly consist of low-income countries [7]. TB is a severe health problem in these countries due to, among others, low TB case detection [17] and low adherence to and availability of medication [18–20].

Vaccination can potentially reduce the TB burden, as it has lowered the incidence rates of other diseases, e.g. measles and polio, and even eradicated smallpox [21]. However, the only vaccine registered against TB, the live-attenuated vaccine *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) (already administered since 1921 [22]), is inadequate, as it has a high variable efficiency (the protection varies between 0 and 80%), fails to prevent active pulmonary TB in adults, and can cause severe side effects in immunocompromised individuals [23, 24]. The variable efficacy of the BCG vaccine against TB has several controversial and diverse reasons. Such as, but not limited to, i) TB not only being caused by one, but several mycobacteria in the Mtb complex, ii) previous exposure to mycobacteria may interfere with the BCG response, iii) lack of essential antigens in the vaccine, and iv) vaccine manufacturing differences [25]. In the past century, these differences in manufacturing have been caused by the usage of different strains of the BCG vaccine, e.g., BCG Russia, Copenhagen, and Japan, and batch-to-batch variations [25, 26]. Therefore, a new, more effective vaccine and/or vaccination strategy is needed to increase the effectiveness and protection rate of TB vaccines. The studies described in this dissertation will explore different vaccine formulations, administration routes, and methodologies, as a first step toward a new vaccine against TB.

A promising antigen for a new TB vaccine is Ag85B-ESAT6-Rv2034 (AER), which we have focussed on in this dissertation. AER is a fusion protein consisting of Antigen 85B (Ag85B), one of the proteins that is most abundant in Mtb and BCG culture supernatants [27, 28], the 6 kDa early secretory antigenic target (ESAT-6); a protein secreted by Mtb and other mycobacteria, but not BCG [29–31], and Rv2034; a protein that is expressed during inflammatory pulmonary infections in mice [32] and strongly recognised by human T cells [33]. By choosing a protein, the possibility of strain differences is complety avoided. In a preclinical study, AER adjuvanted (from Latin: adjuvare; to help) with CAF01 exhibited promising results in HLA-DR3 mice, which are transgenic mice that have the human leucocyte antigen variant DR3 (a major allele present in 20% of the human population) instead of murine major histocompatibility complex (MHC) class II [33]. It reduced the colony-forming units of Mtb in lungs and spleens in guinea pigs to the same level as BCG 30 days post-infection, which is better than described in other studies performed with Ag85B-ESAT6 [33]. However, the protective efficacy is still only at the same level as BCG, the suboptimal vaccine. Therefore, the vaccine could be further optimised.

Mounting of an immune response against TB

To formulate a vaccine against TB, it is important to know how Mtb infects the body and which response is necessary to eradicate the bacteria. Mtb is (usually) transmitted after inhalation of Mtb-containing aerosols into the respiratory tract, which comprises the upper respiratory tract (the oral and nasal cavity down to the larynx above the vocal cords [34]) and the lower respiratory tract (the larynx below the vocal cords down to the alveoli [35, 36]). The respiratory tract is mostly lined with a mucous membrane, the first line of defence against Mtb [35]. The mucous membrane consists of the lamina propria (a connective tissue layer) and tightly bound epithelial cells covered in mucus [37].

Little is known about the early Mtb infection [38]. However, it is believed that if the mucosal barrier functions do not clear the Mtb bacteria, they can travel to the mucosa-free alveolar sacs and infect alveolar macrophages, which are both the primary immune cell type in the alveoli and the cell type that Mtb mainly infects [38, 39]. Alveolar macrophages recognise pathogen-associated molecular patterns located (PAMPs) on Mtb with their pattern-recognition receptors (PRRs) that, among others, initiate phagocytosis [40]. Phagocytosis is a process by which the pathogen is taken up through an extension of the cell membrane, which fuses and creates a vesicle inside the macrophage called the phagosome. The phagosome fuses with other vesicles inside the macrophage called lysosomes, which contain enzymes and other substances, which the macrophage uses to kill the pathogen [41]. However, Mtb avoids destruction in various ways, e.g., by preventing phagosome-lysosome fusion [41]. Therefore, Mtb is able to reproduce slowly (an approximate generation time of 20 h [38]) inside the phagosomal compartments [41].

Mtb-infected alveolar macrophages can traverse the airway epithelium and gain access to the lung interstitium [39]. Mtb replicates for approximately one week inside the macrophages, whereafter the bacteria escape and are phagocytosed by other cells, e.g., neutrophils, migratory inflammatory monocytes, and the professional antigen-presenting cell dendritic cells (DCs) [42]. The inflammatory monocytes and the DCs transport Mtb to the draining lymph node in the lung and transfer the antigen to uninfected lymphoid-tissue resident immature DCs [42]. The immature DCs differentiate into mature DCs when their PRRs interact with PAMPs on the Mtb [43, 44].

Antigens that the DCs have sampled are presented on their MHC molecules, which can interact with T cells through their α:β T cell receptor (TCR) [45]. Classical MHC molecules are polymorphic and can be divided into MHC class I molecules and MHC class II molecules [46]. For antigen presentation to the cluster of differentiation (CD)4$^+$ T cell, the antigen has to be presented on a MHC class II molecule, and the T cell's co-receptor CD4 has to interact with the MHC molecule [45]. For the T cell type CD8$^+$ T cell, the antigen has to be presented on a MHC class I molecule, with the co-receptor CD8 interacting with the MHC molecule [45]. Additional costimulatory signals are transmitted by the interaction of surface molecules and the secretion of cytokines, see Fig. 1. The best characterised costimulatory signals for the CD4$^+$ T cells are provided by CD80 and CD86 molecules, which are present in high levels on activated antigen-presenting cells (APCs) [47]. These molecules bind to the CD28 molecule on the CD4$^+$ T cell, and additional costimulatory signals in the form of cytokines, produced by the DC or other cells, bind to the CD4$^+$ T cell's cytokine receptors and direct the differentiation into various subsets of effector CD4$^+$ T cells [47].

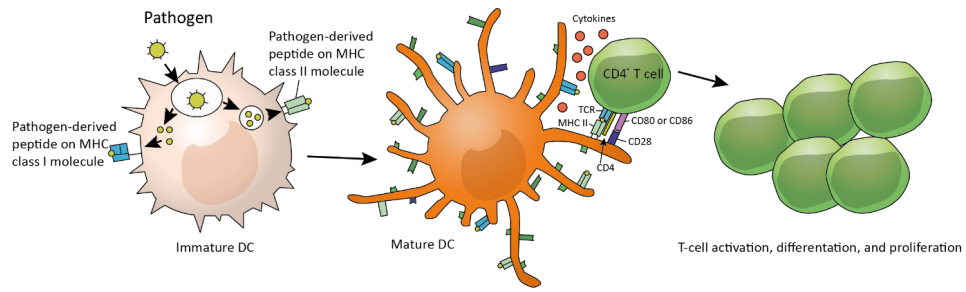


Figure 1. Simplified illustration of (pathogen-derived) antigen presentation on an MHC class I molecule and an MHC class II molecule on a DC. The DC is activated via PRRs from the pathogen and can hereafter activate T cells by presenting the antigen on MHC molecules to the TCR while supplying costimulatory signals (here shown for CD4$^+$ T cells), which leads to activation, differentiation, and proliferation of the T cell. CD: cluster of differentiation, DC: dendritic cell, MHC: major histocompatibility complex.

Based on the costimulatory signals given during the activation of CD4$^+$ T cells, they develop into different effector cells. The major subsets are T helper (Th)1, Th2, Th17, regulatory T (T_{reg}) cells, and T follicular helper (T_{FH}) cells. The subsets are characterised by their cytokine profiles associated with their effector functions [47]. Th1 cells help macrophages clear intravesical infections; Th2 cells help to control infections by extracellular organisms, e.g., parasites [47, 48]; Th17 cells help to protect against extracellular organisms and have been associated with autoimmunity and stimulation of neutrophils [49, 50]; T_{FH} cells provide help to the B cells [51], and; T_{reg} cells have a role in dampening immune responses and maintaining immunological self-tolerance by inhibiting conventional T-cell responses [52]. The activated CD8$^+$ T cell differentiates into the effector cell, the cytotoxic T cell [53]. Once pathogens have entered host cells, they are inaccessible to antibodies and phagocytic cells. By recognising pathogen-derived antigens on MHC class I molecules of the infected cell, the cytotoxic T cell reorganises its cytoskeleton and exocytose granules, which fuse with the cell membrane and release its content in the synapse between the cytotoxic T cell and the infected cell [54]. The content in the granules consists of preformed effector molecules such as granzymes and perforins, which penetrate the cell membrane and induce the apoptosis, programmed cell death, of the host cell [54].

The inflammatory milieu of the Mtb infection promotes Mtb-specific T_{reg} cells that restrict priming and proliferation of effector T cells, therefore delaying their arrival in the lung (T-cell responses against Mtb are on average detected 45 days after Mtb exposure, whereas T-cell response typically peak at 7-14 days for most infections), and when these effector T cells finally reach the site of infection, there is a sizeable bacterial burden, regulatory cell types and immunosuppressive factors [42]. Therefore, Mtb gets encapsulated in large granulomas consisting of various cell types, where it can be contained for decades [55]. Sometimes, the structural organisation in the granuloma is altered, which can lead to necrosis of the granuloma and, thereby, the release of bacteria [55]. As the normal immune response against TB often is insufficient, it is important to identify the essential factors in containing/

eradicating the TB infection. However, which kind of immune responses correlate with protection against TB is not entirely determined.

It has been shown that B cell-deficient mice (B cells can develop into plasma cells that produce antibodies) demonstrate increased susceptibility to TB and more severe lesion formation [56]. However, other groups have shown little to no role of B cells [56]. Therefore, the role of B cells and antibodies in protection against TB is yet to be determined. $CD4^+$ T cells are, on the other hand, crucial as HIV-infected individuals depleted of $CD4^+$ T cells are restricted in their ability to contain TB [42]. Mice with a deletion of the interferon (IFN)-γ gene have shown extreme susceptibility to TB; however, IFN-γ-deficient BCG-vaccinated mice have also shown to exhibit significant protection against Mtb, which indicates that an IFN-γ independent mechanism to limit Mtb does exist and that IFN-γ might not be a reliable correlate of protection against TB [57].

As the immunological response to BCG seems to depend on the animal model, dose, BCG strain, and administration route, among others, it is hard to determine what makes BCG inadequate to protect against TB [57]. BCG induces both humoral and cellular responses, but it might be that the response is not adequate in regards to the central-memory T-cell response, Th17 response, and/or $CD8^+$ T-cell response [57]; however, it is not determined. Therefore, it is hard to conclude exactly which kind of response a vaccine should elicit to confer protection. However, it is believed that an effective vaccine against TB should comprise both a $CD4^+$ and a $CD8^+$ T-cell response [24].

Different vaccine types and adjuvants

Early vaccines were based on live-attenuated or inactivated-whole pathogens. Live-attenuated (weakened) vaccines are based on living viruses or bacteria that have been modified, usually by repeated culturing [58]. They cause a mild infection and induce an immune response similar to that of the wild-type strain infections; however, they can cause disease in immunosuppressed people, as seen with disseminated BCGosis in HIV-infected infants [26], and have the potential for reversion to the virulent form, as it has been documented with the oral polio vaccine [59]. Vaccines based on inactivated whole pathogens, produced by inactivating/killing the pathogens with heat, chemicals, or radiation [60], are safer than live-attenuated vaccines as they cannot revert to the virulent form while still containing pathogen components, which act as intrinsic adjuvants [61]. However, inactivated-whole pathogen vaccines are unsuitable when the natural infection does not convey long-standing immunity [61] and can sometimes not be produced in large quantities, as seen with hepatitis B [62].

Since the early days of vaccine production, other types of vaccines have emerged, such as subunit vaccines [63]. Subunit vaccines are based on purified antigens, usually proteins [62], and often have fewer adverse effects than whole inactivated pathogens, e.g., as seen for the pertussis vaccine [63]. Subunit vaccines often lack adjuvant effects and are, therefore, usually poorly immunogenic [61]. It is thus necessary to add adjuvants to these vaccines, as adjuvants can help improve the potency and redirect the immune system toward an effective response [61]. Adjuvants can be divided into two groups: particulate and molecular [64, 65]. In 1926, the immune-enhancing effects of the particulate adjuvant type aluminium salts, also called alum, were reported [62]. This led to the first licensed adjuvanted vaccine, which remained the only licensed adjuvant for more than half a century [61]. Aluminium adjuvants are associated with enhanced antibody responses and are skewed towards a Th2 response [62, 66]. Since then, other classes of adjuvants have entered the market. For example, emulsions, e.g., the oil-in-water emulsion adjuvant MF59® based on squalene first licensed in 1997 [67, 68], as well as liposomes, e.g., AS01, which also contains monophosphoryl lipid A and Quillaja Saponaria-21, used against shingles [62]. This dissertation will focus mainly on the three particulate adjuvant types: liposomes, poly(lactic-*co*-glycolic acid) (PLGA) particles, and lipid-PLGA hybrids.

Adjuvants: liposomes, PLGA (nano)particles, and lipid-PLGA hybrids

Particulate adjuvants consist of small particles. Different types of particulate adjuvants exist, but some common denominators have been identified. Particulate adjuvants can act as carrier systems for the antigens and molecular adjuvants, thereby ensuring co-delivery, which has been shown to enhance the immune response [69]. Factors such as size, charge, and rigidity are important determinants for generating a particular immune response [70].

There are discrepancies among experimental results regarding nanoparticle size in different studies, probably due to differences in the experimental setup [71]. However, when it comes to uptake in immune cells such as macrophages and Langerhans cells, phagocytosis is most efficient for particles with a size of around 3 μm, and clathrin-mediated endocytosis is optimal for particles of about 100 nm [71]. Nanoparticles also seem to be better at cross-presenting their antigenic cargo in DCs and hereby being better at activating $CD8^+$ T cells, as particles around 800 nm were processed by the proteasome in DCs, indicating that they escaped from the lumen of the endosome into the cytosol, whereas 3 μm sized particles were processed by endolysosomal proteases [72]. Furthermore, nanoparticles (around 50 nm and also 300-600 nm) are more likely to induce Th1 responses than micrometre-sized particles (2-8 μm) that are more prone to trigger Th2 responses [71, 73]. Therefore, nanoparticles seem to be preferred for a vaccine that should induce Th1 and $CD8^+$ T-cell responses.

Particles can be neutral, positively or negatively charged. Positively charged particles typically form a depot at the injection site and are efficiently taken up by APCs [70]. It has been shown that positively charged particles are taken up more efficiently than neutral and negatively charged particles and induce higher $CD4^+$ and $CD8^+$ T-cell responses [74]. Regarding rigidity, studies have shown that rigid particles are more readily taken up by macrophages, endothelial cells, and DCs than less rigid particles [70, 75]. Only a few studies have been published on the relationship between rigidity and immunogenicity;

however, more rigid particles tend to induce Th1 responses [70, 71]. The particulate adjuvant types this dissertation will focus on (cationic liposomes, PLGA particles, and lipid-PLGA hybrids) have different physicochemical properties and have been shown to affect the immune system in various ways.

Cationic liposomes can induce maturation of DCs that subsequently can trigger $CD4^+$ (skewed towards a Th1-response) and $CD8^+$ T-cell responses [74, 76, 77]. A subgroup of cationic liposomes is the pH-sensitive cationic liposomes. They are stable at physiological pH; however, when they are internalised by APCs and exposed to the decreasing pH in the endosomes, where the liposomal bilayer becomes unstable, fuses with the endosomal membrane and the content leaks into the cytosol [78, 79]. This can promote $CD8^+$ T-cell responses [80].

PLGA nanoparticles have an excellent safety profile, being both biodegradable and biocompatible [81], and their properties (hydrophilicity/hydrophobicity, drug loading, drug release rate, etc.) are tuneable, which allows for the customisation of their properties to fit specific applications [82]. PLGA nanoparticles, without any added molecular adjuvants, do not elicit much of an immune response [76]. The small response can be slightly Th2-biased [73]. However, with a molecular adjuvant included, PLGA nanoparticles can induce Th1-response in mice [76].

Lipid-PLGA hybrids combine liposomes and PLGA nanoparticles by being nanoparticles with a PLGA core covered by lipids [73]. Lipid-PLGA hybrids have successfully been used in drug and vaccine delivery preclinical research, where they induced equal $IFN\gamma^+CD4^+CD44^{high}$ (Th1)-cell responses to liposomes with the same lipid composition in vivo [73].

Microfluidics and PLGA particle production

Currently, PLGA particles are mainly produced by two methods: emulsion-based methods, where a water-immiscible or partly water-immiscible organic solvent containing dissolved PLGA is emulsified in an aqueous solution with a surfactant, and nanoprecipitation methods, where a water-miscible organic solvent containing dissolved PLGA is mixed with an aqueous solution [83, 84]. While these techniques allow for manipulation of the nanoparticle diameter by varying factors, such as the PLGA concentration and the surfactant concentration [84], the batch-to-batch reproducibility is low [85], and the nanoparticles are rarely below 100 nm [84].

Microfluidics, a technique that enables the manipulation of fluid streams through microscale fluidic channels, has emerged to overcome these problems, offering precise control of the nanoparticle diameter, greater batch-to-batch reproducibility, and a narrower particle size distribution [86]. Therefore, microfluidic methods seem to be the future of PLGA particle production.

Intradermal administration

The subcutaneous, intramuscular, and intradermal routes are among the most conventional vaccine administration routes. Among these, the dermis is more densely populated by different subsets of DCs compared to subcutaneous and muscle tissue, which contain fewer, less investigated DCs [87]. Therefore, intradermal delivery of a new TB vaccine could be of interest.

The BCG vaccine is already mainly administered intradermally. While the oral and respiratory administration routes seem to induce better mucosal and systemic responses compared to the subcutaneous and intradermal delivery (for BCG), they do not come without obstacles [56, 57]. The oral route is associated with cervical adenitis [56], and the efficacy of orally delivered BCG is lower in the developing world, where helminth and *Helicobacter Pylori* infections have been shown to decrease the effect [57]. For the respiratory route, intranasal vaccination (with *Escherichia coli* heat-labile toxin adjuvant) has been associated with facial nerve paralysis [56], intratracheal and endobronchial are challenging to deliver [56], and in aerosol delivery, it is hard to control the delivered dose and the uniformity of the distribution [57]. Therefore, the intradermal administration route remains a viable option.

Among the intradermal administration forms are dissolvable microneedle arrays (dMNAs). dMNAs are made of a dissolvable material, such as polymers or sugars. They are favourable, as they can: i) secure the stability of loaded drugs by keeping them in their dry form, ii) are possible to self-administer because of the easy application of the microneedle patch, which has needle lengths that would target the dermis, and iii) create zero needle waste as the needle dissolve leading to no spread of blood-borne pathogens [88].

DISSERTATION AIM AND OUTLINE

This dissertation describes how to design and optimise nanoparticulate vaccine formulations and dMNAs and assess if the formulations can induce $CD4^+$ and $CD8^+$ T-cell responses, which are deemed important in a vaccine against TB.

Chapter 2 reports on how the lipid composition of liposomes affects the immune system. AER-containing liposomal formulations were formulated with different cationic lipids and cholesterol contents, and their physicochemical characteristics and immunological potential in vitro were assessed. The formulations were added to DCs, and the uptake of the formulations and their ability to upregulate DC surface markers were evaluated. The most promising formulations were tested in a T-cell assay to test if the formulation-exposed DCs could upregulate T cells' activation markers and IFN-γ production.

Chapter 3 describes a novel modular microfluidic system to prepare PLGA nanoparticles. The system was set up to establish a low-cost method that circumvents bulk nanoparticle preparation methods, which are typically time-consuming and have high batch-to-batch variability. Parameters in the modular microfluidic system that affected physicochemical characteristics, such as the nanoparticle diameter, were determined, and it was possible to incorporate proteins into the PLGA nanoparticles. Furthermore, this microfluidics system was also used to prepare the lipid-PLGA hybrids used in **Chapter 4** and the PLGA nanoparticles incorporated in the dMNAs described in **Chapter 5**.

Chapter 4 describes the immunological responses of PLGA particles, lipid-composition-optimised liposomes, and lipid-PLGA hybrids. The three particulate adjuvant types formulated with AER were compared in vitro with and without the molecular adjuvants monophosphoryl lipid A and cytosine-phosphate-guanine motifs oligodeoxynucleotides (CpG ODN) 1826 to measure the uptake of the formulations in DCs and their ability to activate them. The three particulate formulations with AER and the molecular adjuvants' ability to induce protection against Mtb in mice are hereafter described.

Chapter 5 reports on the comparison of two administration forms: intradermal injection of an aqueous formulation and intradermal administration with dMNAs. The aqueous formulations and dMNAs contained the antigen ovalbumin and the molecular adjuvant CpG ODN 1826 with or without PLGA particles. These formulations were tested in mice to determine if the formulations could induce $CD4^+$ and $CD8^+$ T-cell responses.

Finally, **Chapter 6** contains the summary, general discussion, prospects, and conclusions learned from the research described in this dissertation.

References

1. Pier GB. On the greatly exaggerated reports of the death of infectious diseases. Clin Infect Dis. 2008;47(8):1113-4. https://doi.org/10.1086/592123.
2. Cohen ML. Changing patterns of infectious disease. Nature. 2000;406:762-7. https://doi.org/10.1038/35021206.
3. HIV.gov. A Timeline of HIV and AIDS [Internet]. https://www.hiv.gov/hiv-basics/overview/history/hiv-and-aids-timeline#year-1981. Accessed Nov 1 2024.
4. CDC. Ebola, Outbreak History [Internet]. https://www.cdc.gov/ebola/outbreaks/index.html. Accessed Nov 1 2024.
5. Baker O, Coqueugniot H, Dutour O, Chamel B, Coqueugniot É, Stordeur D, et al. Prehistory of human tuberculosis: Earliest evidence from the onset of animal husbandry in the Near East. Paléorient. 2017;43(2):35-51. https://doi.org/10.3406/paleo.2017.5765.
6. Coronavirus Death Toll [Internet]. 2022. https://www.worldometers.info/coronavirus/coronavirus-death-toll/. Accessed Mar 24 2022.
7. World Health Organization. Global tuberculosis report 2023: World Health Organization; 2023. https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023.
8. Sinha P, Gupta A, Prakash P, Anupurba S, Tripathi R, Srivastava GN. Differentiation of Mycobacterium tuberculosis complex from non-tubercular mycobacteria by nested multiplex PCR targeting IS6110, MTP40 and 32kD alpha antigen encoding gene fragments. BMC Infect Dis. 2016;16:123. https://doi.org/10.1186/s12879-016-1450-1.
9. Brites D, Gagneux S. Co-evolution of Mycobacterium tuberculosis and Homo sapiens. Immunol Rev. 2015;264(1):6-24. https://doi.org/10.1111/imr.12264.
10. Dinkele R, Gessner S, McKerry A, Leonard B, Leukes J, Seldon R, et al. Aerosolisation of Mycobacterium tuberculosis by Tidal Breathing. Am J Respir Crit Care Med. 2022;206(2):206-16. https://doi.org/10.1164/rccm.202110-2378OC.
11. World Health Organization. Tuberculosis 2021. https://www.who.int/news-room/fact-sheets/detail/tuberculosis. Accessed Mar 23 2022.
12. Lerner TR, Borel S, Gutierrez MG. The innate immune response in human tuberculosis. Cell Microbiol. 2015;17(9):1277-85. https://doi.org/10.1111/cmi.12480.
13. Gengenbacher M, Kaufmann SH. Mycobacterium tuberculosis: success through dormancy. FEMS Microbiol Rev. 2012;36(3):514-32. https://doi.org/10.1111/j.1574-6976.2012.00331.x.
14. Connolly LE, Edelstein PH, Ramakrishnan L. Why is long-term therapy required to cure tuberculosis? PLoS Med. 2007;4(3):e120. https://doi.org/10.1371/journal.pmed.0040120.
15. Andersen P, Scriba TJ. Moving tuberculosis vaccines from theory to practice. Nat Rev Immunol. 2019;19(9):550-62. https://doi.org/10.1038/s41577-019-0174-z.
16. Lin C-H, Lin C-J, Kuo Y-W, Wang J-Y, Hsu C-L, Chen J-M, et al. Tuberculosis mortality: patient characteristics and causes. BMC Infect Dis. 2014;14(1):5. https://doi.org/10.1186/1471-2334-14-5.
17. Abayneh M, HaileMariam S, Asres A. Low Tuberculosis (TB) Case Detection: A Health Facility-Based Study of Possible Obstacles in Kaffa Zone, Southwest District of Ethiopia. Can J Infect Dis Med Microbiol. 2020;2020:7029458. https://doi.org/10.1155/2020/7029458.
18. Sazali MF, Rahim S, Mohammad AH, Kadir F, Payus AO, Avoi R, et al. Improving Tuberculosis Medication Adherence: The Potential of Integrating Digital Technology and Health Belief Model. Tuberc Respir Dis (Seoul). 2023;86(2):82-93. https://doi.org/10.4046/trd.2022.0148.
19. Bagchi S, Ambe G, Sathiakumar N. Determinants of poor adherence to anti-tuberculosis treatment in mumbai, India. Int J Prev Med. 2010;1(4):223-32.
20. Simbwa BN, Katamba A, Katana EB, Laker EAO, Nabatanzi S, Sendaula E, et al. The burden of drug resistant tuberculosis in a predominantly nomadic population in Uganda: a mixed methods study. BMC Infect Dis. 2021;21(1):950. https://doi.org/10.1186/s12879-021-06675-7.
21. Greenwood B. The contribution of vaccination to global health: past, present and future. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130433. https://doi.org/10.1098/rstb.2013.0433.
22. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, et al. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci U S A. 2003;100(21):12420-5. https://doi.org/10.1073/pnas.1635213100.
23. Fine PEM. Variation in protection by BCG:

implications of and for heterologous immunity. Lancet. 1995;346(8986):1339-45. https://doi.org/10.1016/S0140-6736(95)92348-9.
24. Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog. 2012;8(5):e1002607. https://doi.org/10.1371/journal.ppat.1002607.
25. Sierra VG. Is a new tuberculosis vaccine necessary and feasible? A Cuban opinion. Tuberculosis. 2006;86(3):169-78. https://doi.org/10.1016/j.tube.2006.03.003.
26. Dockrell HM, Smith SG. What Have We Learnt about BCG Vaccination in the Last 20 Years? Front Immunol. 2017;8:1134. https://doi.org/10.3389/fimmu.2017.01134.
27. Ernst JD, Cornelius A, Bolz M, Sher A, Ehrt S, Edelstein P. Dynamics of Mycobacterium tuberculosis Ag85B Revealed by a Sensitive Enzyme-Linked Immunosorbent Assay. mBio. 2019;10(2):e00611-19. https://doi.org/10.1128/mBio.00611-19.
28. Wiker HG, Harboe M. The antigen 85 complex: a major secretion product of Mycobacterium tuberculosis. Microbiol Rev. 1992;56(4):648-61. https://doi.org/10.1128/mr.56.4.648-661.1992.
29. Gey van Pittius NC, Warren RM, van Helden PD. ESAT-6 and CFP-10: what is the diagnosis? Infect Immun. 2002;70(11):6509-10; author reply 11. https://doi.org/10.1128/IAI.70.11.6509-6511.2002-a.
30. Li W, Deng G, Li M, Zeng J, Zhao L, Liu X, et al. A recombinant adenovirus expressing CFP10, ESAT6, Ag85A and Ag85B of Mycobacterium tuberculosis elicits strong antigen-specific immune responses in mice. Mol Immunol. 2014;62(1):86-95. https://doi.org/10.1016/j.molimm.2014.06.007.
31. Andersen P, Andersen AB, Sørensen AL, Nagai S. Recall of long-lived immunity to Mycobacterium tuberculosis infection in mice. J Immunol. 1995;154(7):3359-72. https://doi.org/10.4049/jimmunol.154.7.3359.
32. Commandeur S, van Meijgaarden KE, Prins C, Pichugin AV, Dijkman K, van den Eeden SJF, et al. An Unbiased Genome-Wide *Mycobacterium tuberculosis* Gene Expression Approach To Discover Antigens Targeted by Human T Cells Expressed during Pulmonary Infection. J Immunol. 2013;190(4):1659-71. https://doi.org/10.4049/jimmunol.1201593.
33. Commandeur S, van den Eeden SJF, Dijkman K, Clark SO, van Meijgaarden KE, Wilson L, et al. The in vivo expressed Mycobacterium tuberculosis (IVE-TB) antigen Rv2034 induces CD4+ T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. Vaccine. 2014;32(29):3580-8. https://doi.org/10.1016/j.vaccine.2014.05.005.
34. Bustamante-Marin XM, Ostrowski LE. Cilia and Mucociliary Clearance. Cold Spring Harb Perspect Biol. 2017;9(4). https://doi.org/10.1101/cshperspect.a028241.
35. Li W, Deng G, Li M, Liu X, Wang Y. Roles of Mucosal Immunity against Mycobacterium tuberculosis Infection. Tuberc Res Treat. 2012;2012:791728. https://doi.org/10.1155/2012/791728.
36. Mettelman RC, Allen EK, Thomas PG. Mucosal immune responses to infection and vaccination in the respiratory tract. Immunity. 2022;55(5):749-80. https://doi.org/10.1016/j.immuni.2022.04.013.
37. Britannica TeoE. Mucous membrane. Encyclopedia Britannica [Internet]. https://www.britannica.com/science/mucous-membrane. Accessed May 29 2024.
38. Tobin EH TD. Tuberculosis [Internet]. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK441916/. Accessed May 29 2024.
39. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, et al. Alveolar Macrophages Provide an Early Mycobacterium tuberculosis Niche and Initiate Dissemination. Cell Host Microbe. 2018;24(3):439-46.e4. https://doi.org/10.1016/j.chom.2018.08.001.
40. Chai Q, Wang L, Liu CH, Ge B. New insights into the evasion of host innate immunity by Mycobacterium tuberculosis. Cell Mol Immunol. 2020;17(9):901-13. https://doi.org/10.1038/s41423-020-0502-z.
41. Warner DF, Mizrahi V. The survival kit of Mycobacterium tuberculosis. Nat Med. 2007;13(3):282-4. https://doi.org/10.1038/nm0307-282.
42. Urdahl KB. Understanding and overcoming the barriers to T cell-mediated immunity against tuberculosis. Semin Immunol. 2014;26(6):578-87. https://doi.org/10.1016/j.smim.2014.10.003.
43. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialisation through signaling specificity and transcriptional programming. EMBO J. 2014;33(10):1104-16. https://doi.org/10.1002/embj.201488027.
44. Worbs T, Hammerschmidt SI, Forster R. Dendritic cell migration in health and disease. Nat Rev Immunol. 2017;17(1):30-48. https://doi.org/10.1038/nri.2016.116.
45. Grommé M, Neefjes J. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol Immunol. 2002;39(3-4):181-202. https://doi.org/10.1016/s0161-5890(02)00101-3.
46. Adams EJ, Luoma AM. The adaptable major histocompatibility complex (MHC) fold: structure and function of nonclassical and MHC class I-like molecules. Annu Rev Immunol. 2013;31:529-61. https://doi.org/10.1146/annurev-immunol-032712-095912.
47. Corthay A. A three-cell model for activation of naive T helper cells. Scand J Immunol. 2006;64(2):93-6. https://doi.org/10.1111/j.1365-3083.2006.01782.x.
48. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6(11):1123-32. https://doi.org/10.1038/ni1254.
49. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood. 2010;115(2):335-43. https://doi.org/10.1182/blood-2009-04-216085.
50. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517. https://doi.org/10.1146/annurev.immunol.021908.132710.
51. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. J Exp Med. 2012;209(7):1241-53. https://doi.org/10.1084/jem.20120994.
52. Loser K, Beissert S. Regulatory T cells: banned cells for decades. J Invest Dermatol. 2012;132(3 Part 2):864-71. https://doi.org/10.1038/jid.2011.375.
53. 53. Cui W, Kaech SM. Generation of effector CD8+ T cells and their conversion to memory T cells. Immunol Rev. 2010;236:151-66. https://doi.org/10.1111/j.1600-065X.2010.00926.x.
54. Chang HF, Bzeih H, Schirra C, Chitirala P, Halimani M, Cordat E, et al. Endocytosis of Cytotoxic Granules Is Essential for Multiple Killing of Target Cells by T Lymphocytes. J Immunol. 2016;197(6):2473-84. https://doi.org/10.4049/jimmunol.1600828.
55. Silva Miranda M, Breiman A, Allain S, Deknuydt F, Altare F. The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clin Dev Immunol. 2012;2012:139127. https://doi.org/10.1155/2012/139127.
56. Tanner R, Villarreal-Ramos B, Vordermeier HM, McShane H. The Humoral Immune Response to BCG Vaccination. Front Immunol. 2019;10. https://doi.org/10.3389/fimmu.2019.01317.
57. Singh S, Saavedra-Avila NA, Tiwari S, Porcelli SA. A century of BCG vaccination: Immune mechanisms, animal models, non-traditional routes and implications for COVID-19. Front Immunol. 2022;13. https://doi.org/10.3389/fimmu.2022.959656.
58. Kumar A. 7 - Different vaccination approach for vaccine development. In: Kumar A, editor. Visceral Leishmaniasis: Academic Press; 2021. p. 77-81. https://doi.org/10.1016/B978-0-323-91124-5.00006-0.
59. Zepp F. Principles of vaccine design-Lessons from nature. Vaccine. 2010;28(Supplement 3):C14-24. https://doi.org/10.1016/j.vaccine.2010.07.020.
60. Seo HS. Application of radiation technology in vaccines development. Clin Exp Vaccine Res. 2015;4(2):145-58. https://doi.org/10.7774/cevr.2015.4.2.145.
61. Di Pasquale A, Preiss S, Tavares Da Silva F, Garçon N. Vaccine Adjuvants: from 1920 to 2015 and Beyond. Vaccines (Basel). 2015;3(2):320-43. https://doi.org/10.3390/vaccines3020320.
62. Reyes C, Patarroyo MA. Adjuvants approved for human use: What do we know and what do we need to know for designing good adjuvants? Eur J Pharmacol. 2023;945:175632. https://doi.org/10.1016/j.ejphar.2023.175632.
63. Delany I, Rappuoli R, De Gregorio E. Vaccines for the 21st century. EMBO Mol Med. 2014;6(6):708-20. https://doi.org/10.1002/emmm.201403876.
64. Hafner AM, Corthésy B, Merkle HP. Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. Adv Drug Deliv Rev. 2013;65(10):1386-99. https://doi.org/10.1016/j.addr.2013.05.013.
65. Johnston D, Zaidi B, Bystryn JC. TLR7 imidazoquinoline ligand 3M-019 is a potent adjuvant for pure protein prototype vaccines. Cancer Immunol Immunother. 2007;56(8):1133-41. https://doi.org/10.1007/s00262-006-0262-3.
66. Tritto E, Mosca F, De Gregorio E. Mechanism of action of licensed vaccine adjuvants. Vaccine. 2009;27(25-26):3331-4. https://doi.org/10.1016/j.vaccine.2009.01.084.

67. O'Hagan DT, Ott GS, De Gregorio E, Seubert A. The mechanism of action of MF59 - an innately attractive adjuvant formulation. Vaccine. 2012;30(29):4341-8. https://doi.org/10.1016/j.vaccine.2011.09.061.
68. Ko EJ, Kang SM. Immunology and efficacy of MF59-adjuvanted vaccines. Hum Vaccin Immunother. 2018;14(12):3041-5. https://doi.org/10.1080/21645515.2018.1495301.
69. Lim JW, Na W, Kim HO, Yeom M, Kang A, Park G, et al. Co-delivery of antigens and immunostimulants via a polymersome for improvement of antigen-specific immune response. J Mater Chem B. 2020;8(26):5620-6. https://doi.org/10.1039/d0tb00892c.
70. Benne N, van Duijn J, Kuiper J, Jiskoot W, Slütter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release. 2016;234:124-34. https://doi.org/10.1016/j.jconrel.2016.05.033.
71. Baranov MV, Kumar M, Sacanna S, Thutupalli S, van den Bogaart G. Modulation of Immune Responses by Particle Size and Shape. Front Immunol. 2020;11:607945. https://doi.org/10.3389/fimmu.2020.607945.
72. Mant A, Chinnery F, Elliott T, Williams AP. The pathway of cross-presentation is influenced by the particle size of phagocytosed antigen. Immunology. 2012;136(2):163-75. https://doi.org/10.1111/j.1365-2567.2012.03558.x.
73. Rose F, Wern JE, Ingvarsson PT, van de Weert M, Andersen P, Follmann F, et al. Engineering of a novel adjuvant based on lipid-polymer hybrid nanoparticles: A quality-by-design approach. J Control Release. 2015;210:48-57. https://doi.org/10.1016/j.jconrel.2015.05.004.
74. Nakanishi T, Kunisawa J, Hayashi A, Tsutsumi Y, Kubo K, Nakagawa S, et al. Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. J Control Release. 1999;61(1-2):233-40. https://doi.org/10.1016/s0168-3659(99)00097-8.
75. Benne N, Leboux RJT, Glandrup M, van Duijn J, Lozano Vigario F, Neustrup MA, et al. Atomic force microscopy measurements of anionic liposomes reveal the effect of liposomal rigidity on antigen-specific regulatory T cell responses. J Control Release. 2020;318:246-55. https://doi.org/10.1016/j.jconrel.2019.12.003.
76. Du G, Hathout RM, Nasr M, Nejadnik MR, Tu J, Koning RI, et al. Intradermal vaccination with hollow microneedles: A comparative study of various protein antigen and adjuvant encapsulated nanoparticles. J Control Release. 2017;266:109-18. https://doi.org/10.1016/j.jconrel.2017.09.021.
77. Barnier Quer C, Elsharkawy A, Romeijn S, Kros A, Jiskoot W. Cationic liposomes as adjuvants for influenza hemagglutinin: More than charge alone. Eur J Pharm Biopharm. 2012;81(2):294-302. https://doi.org/10.1016/j.ejpb.2012.03.013.
78. Karanth H, Murthy RS. pH-sensitive liposomes--principle and application in cancer therapy. J Pharm Pharmacol. 2007;59(4):469-83. https://doi.org/10.1211/jpp.59.4.0001.
79. Balamuralidhara V, Pramodkumar T, Srujana N, Venkatesh M, Gupta NV, Krishna K, et al. pH sensitive drug delivery systems: a review. Am J Drug Discov Dev. 2011;1(1):24-48.
80. Liu G, Zhu M, Zhao X, Nie G. Nanotechnology-empowered vaccine delivery for enhancing CD8+ T cells-mediated cellular immunity. Adv Drug Deliv Rev. 2021;176:113889. https://doi.org/10.1016/j.addr.2021.113889.
81. Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev. 2012;64:72-82. https://doi.org/10.1016/j.addr.2012.09.004.
82. Prabhu P, Patravale V. Potential of nanocarriers in antigen delivery: the path to successful vaccine delivery. Nanocarriers. 2014;1:10-45. https://doi.org/10.2478/nanca-2014-0001.
83. Operti MC, Bernhardt A, Grimm S, Engel A, Figdor CG, Tagit O. PLGA-based nanomedicines manufacturing: Technologies overview and challenges in industrial scale-up. Int J Pharm. 2021;605:120807. https://doi.org/10.1016/j.ijpharm.2021.120807.
84. Astete CE, Sabliov CM. Synthesis and characterisation of PLGA nanoparticles. J Biomater Sci Polym Ed. 2006;17(3):247-89. https://doi.org/10.1163/156856206775997322.
85. Streck S, Neumann H, Nielsen HM, Rades T, McDowell A. Comparison of bulk and microfluidics methods for the formulation of poly-lactic-co-glycolic acid (PLGA) nanoparticles modified with cell-penetrating peptides of different architectures. Int J Pharm X. 2019;1:100030. https://doi.org/10.1016/j.ijpx.2019.100030.
86. Valencia PM, Farokhzad OC, Karnik R, Langer R. Microfluidic technologies for accelerating the clinical translation of nanoparticles. Nat Nanotechnol. 2012;7(10):623-9. https://doi.org/10.1038/nnano.2012.168.
87. Romani N, Flacher V, Tripp CH, Sparber F, Ebner S, Stoitzner P. Targeting skin dendritic cells to improve intradermal vaccination. Curr Top Microbiol Immunol. 2012;351:113-38. https://doi.org/10.1007/82_2010_118.
88. Kim Y-C, Park J-H, Prausnitz MR. Microneedles for drug and vaccine delivery. Adv Drug Deliv Rev. 2012;64(14):1547-68. https://doi.org/10.1016/j.addr.2012.04.005.