Innovative Approaches in the Characterization and Design of Synthetic Vaccines

Sophia Adams[1], Michael Brooks[1], Nathan Young[2], Charlotte Bennett[3], and Mia Ward*[3]

[1] Department of Chemical Engineering, University of Oxford, UK

[2] Department of Physics, University of Toronto, Canada

[3] School of Electrical Engineering, University of Tokyo, Japan

1. Abstract

Traditional vaccines such as live-attenuated or whole-killed vaccines have been widely used over the past hundred years. However, the safety and effectiveness of traditional vaccines have become a concern as an increasing number of people receive vaccination. In recent decades, a new type of vaccine has emerged called a subunit vaccine which uses only the proteins or peptide epitopes necessary to induce an immune response. Recent studies have demonstrated that subunit vaccines can be synthetically modified to possess different properties such as size, shape, charge, and material chemistry. These synthetic vaccines can be tailored to differentially interact with the immune system to tailor the output response. In order to harness their potential and demonstrate their value, powerful physical and biological characterization tools are needed. This review paper will discuss important methods that can be used to characterize synthetic vaccines. In addition, some important synthetic vaccine design rules determined so far will be discussed and summarized. These two elements can constructively inform the vaccine development process providing future researchers a pathway for evaluating and designing the most effective synthetic vaccines possible.

2. Introduction

Subunit vaccines are a new generation of vaccines which are considered much safer than traditional whole-pathogen vaccines since they only deliver the pathogen component necessary to induce an immune response and lack unnecessary off-target components. However, subunit vaccines alone are typically weak immunogens requiring multiple doses and/or adjuvant co-delivery to induce protection[1, 2]. Over the past few decades, subunit vaccines have been processed and/or chemical modified to yield synthetic vaccines which possess improved immunogenicity[3-7]. Specifically, synthetic vaccine systems such as polymeric nanoparticles[6, 8-10], inorganic nanoparticles[11-13], liposomes[14-16], and micelles[17-19] have all shown significant impact in improving vaccine efficacy. This initial success has led researchers to want to design synthetic vaccines capable of directly modulating corresponding immune responses. In order to achieve this next generation of synthetic vaccines, it is important to understand how the physical properties of synthetic vaccines can be readily modified.

Synthetic vaccine morphology is one of the most heavily studied topic today. Researchers in this field realize that physical characteristics, such as size and shape[6, 8, 20], can play an important role in vaccine immunogenicity. For example, vaccine size impacts its internalization by Antigen Presenting Cells (APCs)[8] and trafficking through the lymphatic system. Vaccine shape can facilitate which cell populations uptake them triggering different types of immune responses[6]. Synthetic vaccine morphology can be readily characterized by electron microscopy and atomic force microscopy.

The secondary structure of synthetic vaccines is also important, especially an antibody-mediated immune response due to the fact that some B cell receptors only recognize peptide epitopes with certain secondary structures[17]. Peptide or protein secondary structures are commonly evaluated by circular dichroism.

Synthetic vaccine surface charge also plays a role in vaccine efficiency and toxicity. Extensive research in this area indicate that highly negatively charged vaccines electrostatically repulse the lipid bi-layer of cells which results in reduced cell internalization[21]. In contrast, highly positive charged materials can easily pass through the cell membrane which can enhance the immune response though can also lead to cell death as a consequence[22-25]. Zeta potential is a powerful surface characterization technique that allows for assessing vaccine charge.

Tracking where synthetic vaccines traffic within cells is a highly important tool in studying the processing mechanism of synthetic vaccines. Specifically, fluorophore-labelled vaccines and fluorescent microscopy allow researchers to be able to track the synthetic vaccine movements within tissues or even a single cell itself.

Circulating antibodies play an important role in clearing out invading pathogens. With the presence of antibodies, individuals may be able to be protected from infection even before the onset of symptoms. Antibody quantity and quality are the two most important characteristics for antibody characterization which can be ascertained by enzyme linked immunosorbent assays.

While many current commercial vaccines are focused on antibody mediated responses, cell mediated responses can be just as important especially prophylaxis against intracellular infections and treatments of certain diseases like cancer. Cell mediated responses are often related to cell proliferation, cell surface marker expression, and cytokine production which can be characterized by flow cytometry.

3. Characterization Methods

3.1. Transmission Electron Microscopy (TEM)

TEM is an important microscopy technique widely used in both the physical and biological sciences including for cancer research, pathological studies, and materials science. Unlike optical microscopes which use light to focus on the sample, the principal of TEM is to use electrons to pass through and interact with the sample. Due to the much shorter wavelength of electrons compared to light, TEM is capable of imaging at considerably higher resolutions than traditional optical microscopy[26].

Important components (**Fig. 1**) of TEM include the electron gun, the specimen, the imaging system, the condenser lens, and the projective lens. The electron gun provides the all-important electron source for TEM. The electrons are pumped through the voltage area and are then shot at the specimen (which includes the sample grid). The imaging system usually consists of a phosphor screen, which reflects the image produced by electrons interacting with the sample, and a digital camera that records the image. The condenser lens form the primary electron beam while the contact lens is responsible for focusing the electrons passed through the specimen.

TEM has been widely used in synthetic vaccine studies as a powerful method to determine vaccine structure. Synthetic vaccines normally include peptide or protein compounds, thus negative staining is the most commonly utilized method in TEM experiments. The atom density of the stains is much higher than synthetic vaccines, therefore, when the electron source hits the stain, the electrons are deflected and filtered by the objective aperture while the electrons hitting the sample will be allowed to pass through the specimen. The stain is shown as a darker area on the screen which provides necessary contrast for acquiring a clear image. A hydrophilic surface that is a part of the grid is required to facilitate stain attachment which can be achieved by glow discharging. In this method, grids are placed inside a partly evacuated chamber where high voltage is applied between the cathode and the anode at each end of the chamber ionizing the gas within the chamber. Those negatively charged ions, called plasma, are deposited on the carbon grid which provides the carbon film with an overall hydrophilic surface. Then the vaccine sample solution is usually incubated on the carbon grid before the solution is blotted with filter paper or other chemical substances[27]. This is followed by the staining process, for which typical stains include uranyl acetate[27-29], ammonium molybdate[30, 31], uranyl formate[32], phosphotungstic acid[33], and many others. If the sample presents a strong negative charge and is found to be difficult to stick on the carbon film, magnesium acetate may be applied to modify the charge on the carbon film before staining.

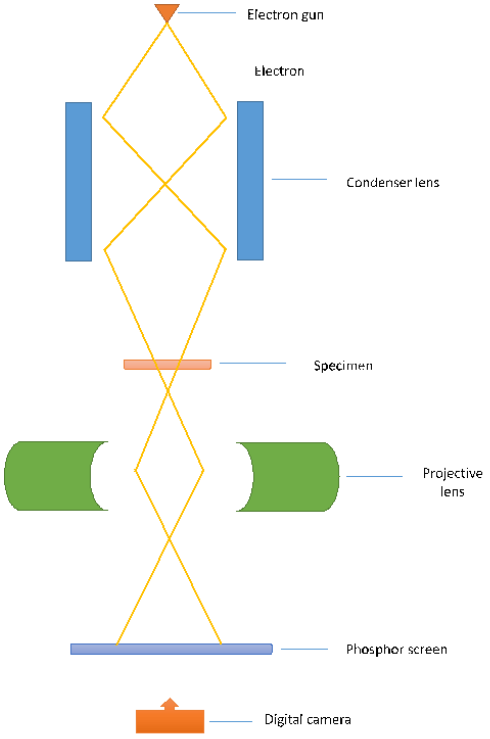

Fig. 1. TEM Schematic. Electrons are shot from the electron gun, cross through the specimen, and eventually hit the phosphor screen or are picked up by the digital camera.

3.2. Cryo-Electron Microscopy (cryo-EM)

Cryo-EM is a highly specific type of TEM commonly used to visualize finer features than traditional TEM. Samples are studied at extremely low temperatures while normal TEM samples are studied at room temperature. Cryo-EM is capable of providing images with even higher resolution and contrast than a negative staining TEM, because the

resolution and contrast of TEM is limited by the size of the stain[34, 35]. But limitations for cryo-EM are much less since ice is used instead of stains to provide contrast; the thickness of ice crystals are much smaller than normal stains thus ultra-high resolution images are achievable. The highest resolution that cryo-EM can achieve is 2.2 Å[36] while TEM commonly can only achieve resolution around 10 Å[37]. Cryo-EM is also commonly used to reconstruct three-dimensional images allowing for it to make significant contributions to the field of structure biology (**Fig, 2**). However, cryo-EM images are often noisier than traditional TEM because of the thinner sample grids. This problem could be fixed by averaging images to improve the signal-to-noise ratio in order to retrieve high-resolution information[38].

Cryo-EM can also provide higher resolution and better contrast for synthetic vaccine analysis compared to traditional TEM. The most important part of sample preparation is to keep the sample grids sufficiently frozen throughout the process. To maintain this condition, sample films are usually plunged into liquid nitrogen-cooled liquid ethane which quickly plunges the temperature to -180 degrees Celsius or lower[39]. Vapor contamination can drastically alter sample quality and is often contributed by atmospheric humidity and improper operating conditions so that cryo-EM should be processed in a tightly controlled environment.

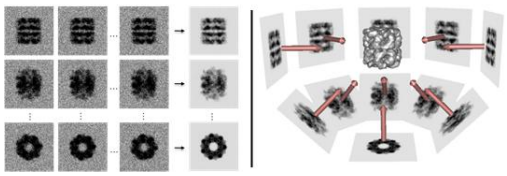

Fig. 2. 3D Reconstruction Scheme. Left panel: For each angle of a single particle, we can take multiple images and average the image to get higher signal-to-noise ratio. Right panel: to obtain a three-dimensional view of a single particle, a sample is tilted in order to be exposed to different angles by the camera, followed by software reconstruction, which yields the three-dimensional image of a single particle. (Reprinted with permission from Greg Pintilie)[40].

3.3. Scanning Electron Microscopy (SEM)

SEM is another important electron microscopy technique which mainly detects secondary electrons, back scattered electrons, and characteristic X-rays (**Fig. 3**). Secondary electrons are the reflected primary electrons shot by an electron gun. This technique can reveal the morphology and surface topography of the sample by analysing the secondary electrons. Back scattered electrons come from the deeper areas of the sample where the atoms are excited by primary electrons. Backscattered electrons allow contrasts to be detected between the background and the elements. X-rays are excited and emitted from an even deeper part of the sample by primary electrons. By detecting and measuring the energy of the X-ray, one can get an accurate elemental distribution of the sample. For traditional SEM, samples are usually required to be solid and conductive. Also, the SEM environment chamber must be maintained under vacuum. However, Environmental scanning electron microscopy (ESEM) eliminates those limitations so that more samples such as those that are in solution or non-conductive can also be used. The absolute finest resolution of SEM is about 10 nm according to FEI (Field Electron and Ion Co.) and with most being even higher than this, SEM cannot measure feature sizes at the same scale as TEM.

Although SEM has lower resolution than TEM or AFM, it can still play an important role in size and shape analysis of synthetic vaccines[9, 41, 42]. Traditional SEM commonly uses dry powder vaccines since these meet the sample requirement of SEM: solid and stable under vacuum[43]. However, for other synthetic vaccines that must be maintained in an aqueous environment, only ESEM can be used to visual them[44]. Due to the limited contrast length, samples typically need to be at least a hundred microns to get a clearly contrasted image. While limiting for many synthetic vaccines, SEM can provide some three-dimensional information through the shadow presented in the image due to the depth of field phenomenon.[45, 46]

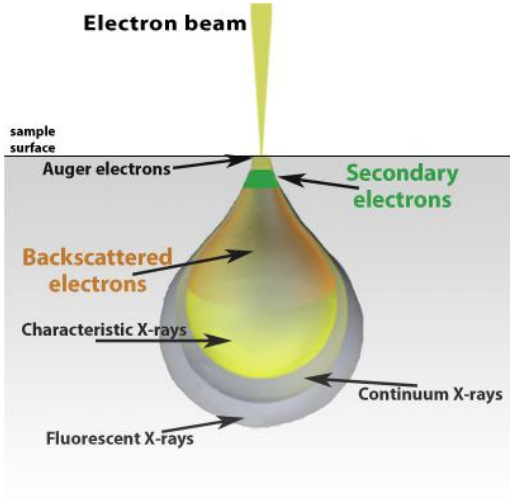

Fig. 3. Working principle of SEM. Sample characteristics from different sample depth can be detected by collecting different sources of information, such as secondary electrons, backscattered electrons, and characteristic X-rays. (Reprinted with permission from Nano Science Instrument)[47].

3.4. Atomic Force Microscopy (AFM)

AFM is a high-resolution surface characterization technique capable of measuring the shape, height, and mechanical properties of samples. The working principle of AFM is based on the deflection of a cantilever caused by the physical forces between the cantilever-bound tip and the sample surface. The cantilever also reflects a laser, emitted from a

laser diode to the photodiode detector, which can be processed and transferred into surface imaging information (**Fig. 4**). Commonly used modes for AFM include contact mode where the AFM tip is dragged across the surface, and non-contact mode where the tip is separated from the sample surface and the deflection of the cantilever is due to the attractive or repulsive forces between the tip and the surface. A key advancement of AFM has been the creation of typing mode which is designed to overcome the difficulties associated with the tip-surface friction, adhesion/repulsion, and electrostatic forces. Each mode can provide different imaging quality and causes different levels of damage to the sample. The tapping mode is the most widely used mode for synthetic vaccine analysis[18, 44].

AFM is a powerful tool when evaluating surface information since it can often provide better 3-D information than electron microscopy by utilizing sample height and the stacking principle. For synthetic vaccine studies, AFM is usually used alongside TEM as a tool to confirm particle morphology. Samples can be dissolved in water or ethanol for which a few drops can be placed on a mica substrate and dried before imaging.

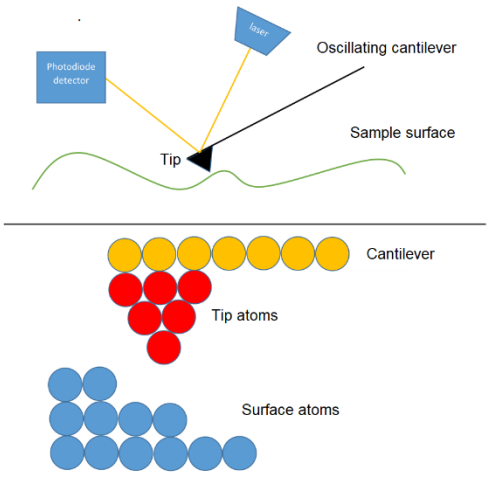

Fig. 4. Working principle of atomic force microscopy. Top panel: the sample surface can be transformed into an image by software processing the signal detected via laser reflected by the AFM tip. Bottom panel: a tip is attached to the cantilever and the cantilever must be soft enough to achieve a certain deflection as shown by the atomic scale image.

3.5. Circular Dichroism (CD)

CD is a technique that can be used to detect various information including secondary structure, geometry, and electronic structure. The theory of CD is based on the different absorption levels of left-handed light and right-handed light from molecules such as proteins that possess different secondary structures[48-51]. For proteins, CD can be used to determine if the molecule is folded by comparing the structures of a protein obtained from different sources or comparing structures for different mutants of the same protein. This information gained through CD can also demonstrate the stability of the protein when exposed to elevated temperatures, pH changes, and denaturing additives. It can also be used to determine whether protein-protein or protein-ligand interactions alter the conformation of protein [48].

For synthetic vaccines, CD is an important technique for evaluating the peptide or protein secondary structure. It can be used to detect the relative percentages of α-helix, β-sheet, random coils, and other secondary structures of synthetic vaccines, which helps characterize the impact synthetic vaccine formulation has on biomolecule functionality. In specific, different secondary structures possess unique light absorbance behavior. For example, α-helical peptides and proteins absorb polarized light most strongly at around 208 nm and 222 nm, β-sheet peptides and proteins possess maximum absorption at around 218 nm, and random coil peptides and proteins absorb polarized light most strongly near 195nm. The resulting curve detected from wavelength sweeps can be compared to known secondary structure spectra to determine a sample's relative secondary structure.

3.6. Dynamic Light Scattering (DLS)

DLS is a technique used in evaluating size distribution of small particles in suspension or polymers in solution[49]. DLS works by directing monochromatic light from a source to the sample so that they are diffracted and their "speckle pattern" can be collected on a screen. The dark areas in the speckle pattern show regions where diffracted light interferes with and cancels itself out which is known as destructive interference. In contrast, the light areas in the speckle pattern demonstrate constructive interference, regions where the diffracted light interferes with and enhances each other. The particles are suspended and can randomly move due to the collision with solvent molecules which obey the rule of Brownian Motion meaning that Stokes-Einstein equation can be applied to relate the velocity of a particle in solution to its hydrodynamic radius[52]. DLS can be utilized to measure different nanomaterials including proteins, polymers, and others.

DLS solutions are comprised of dissolved or suspended samples and usually include some ions which will surround the particles to help the screening process. However, some ions such as chloride are too aggressive and could potentially alter the particle surface so care must be taken when choosing ions. Synthetic vaccines are usually made of modified peptides or proteins, so sonication is not always necessary for creating homogeneous solutions, but can be used for particles that tend to aggregate. The sample

solution should not be too concentrated otherwise viscosity effects come into play skewing the results though in some studies the concentration effects have been shown to have limited to no effect on DLS measurements[53].

3.7. Zeta-Potential

Zeta-Potential, also known as the electro-kinetic potential of colloidal dispersions, is a value that represents the surface charge of particles. One of the most effective methods to measure Zeta-potential is by electrophoresis. Electrophoresis can be used to measure the velocity of particles moving in an electric field which directly relates to particle surface charge. Zeta-potential also reflects the stability of the particles in which particles with greater zeta-potential values provide more repulsion force and prevent aggregation ,hence are generally more stable.[54, 55] Samples should be prepared in low ion concentration solution to prevent false readings. The zeta-potential value is dependent on solution pH, so pH should be measured both before and after calculating the zeta-potential[56].

3.8. Critical Micelle Concentration (CMC)

CMC is the lowest concentration at which amphiphilic molecules, such as polymeric micelles or peptide amphiphile micelles, start to undergo self-assembly (**Fig. 5**). Since the discovery of self-assembled structures, CMC has become a widespread method for micelle formation characterization for which two common techniques are used. One way to measure CMC is through fluorophore sequestration polarization. The principle of this method is the chosen fluorophore strongly fluoresces when confined in a hydrophobic structure but not when free in solution. Therefore, the fluorophore entrapped within a self-assembled structure can emit a significant amount of light compared to those that are not. A plot comparing concentration and fluorescence intensity is then used to calculate the CMC value[57, 58]. The other approach to measuring CMC is through analyzing solution surface tension. Since the solution surface tension will not change after amphiphilic molecules reach the CMC value, CMC can be measured using surface tensionmeter measurements of serially diluted samples.

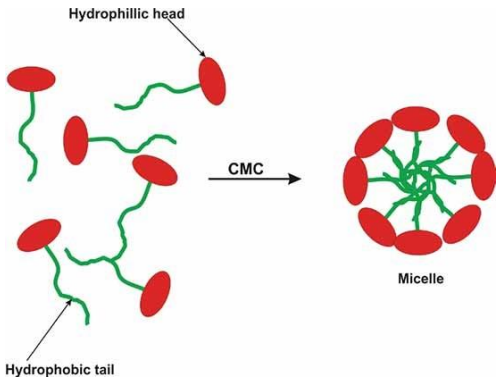

Fig. 5. Scheme of hydrophobically-drive self-assembly. Self-assembled monomers are usually amphiphilic consisting of a hydrophilic region and a hydrophobic region which form hydrophobic-core micelles once reaching a certain concentration, the CMC. (Reprinted with permission from BioTek)[59].

3.9. Widefield Fluorescent Microscopy and Confocal Fluorescent Microscopy

Widefield fluorescent microscopy is an optical microscopy method that utilizes fluorescence and phosphorescence instead of optical reflection or absorption to yield an image of a sample. A mercury arc lamp acts as the light source and an excitation filter allows monochromatic light to pass through. A dichroic mirror reflects the excitation light and allows the emission light reflected from the sample to pass through. A sample holder contains the fluorophore-labelled sample and an emission filter allows emitted light to pass through after which it can be focused by an ocular lens and reach the detector (**Fig. 6**). Widefield fluorescent microscopy is commonly used in materials science and biomedical research for its capability to create a fluorophore-enabled two-dimensional image.

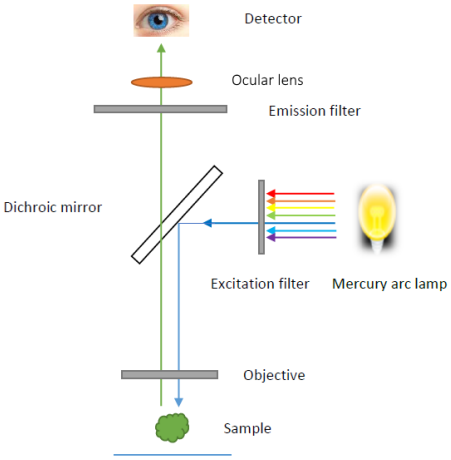

Fig. 6. Widefield fluorescence microscopy scheme. The sample is excited by absorbing higher energy light (lower wavelength) after which it relaxes and emits lower energy light (higher wavelength). The long wavelength light (emission light) can pass through the dichroic mirror, while the short wavelength light (excitation light) is reflected by the dichroic mirror.

Confocal fluorescent microscopy is a specific kind of fluorescent microscopy which allows for higher resolution and depth-selective capability than widefield fluorescent microscopy. This specifically allow for the technique to provide a three-dimensional image. The major differences between confocal fluorescent microscopy and widefield fluorescent microscopy are the depth selectivity function and a confocal pinhole that blocks the reflected light from outside the confocal plane only allowing emitted light from the confocal plane to pass through the pinhole (**Fig. 7**). Confocal

fluorescent microscopy has been quite valuable in giving special resolution to where materials are located around and within one another.

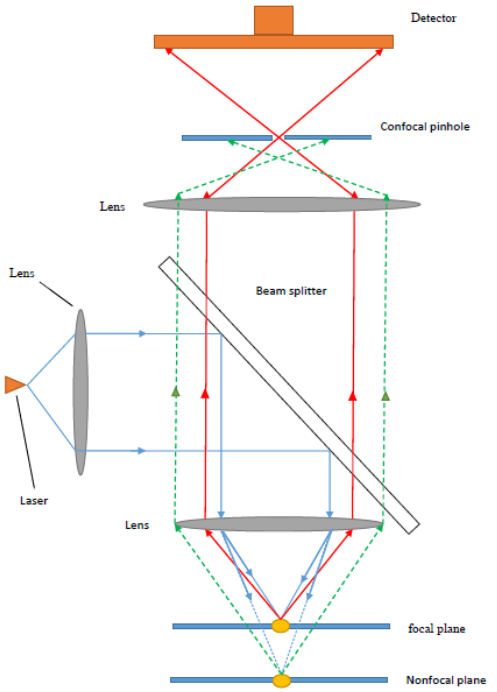

Fig. 7. Confocal fluorescent microscopy scheme. The confocal pinhole allows the emission light (red line) from the confocal plane to pass through the confocal pinhole up to the detector. The beam splitter reflects the excitation light while passing through the emission light. Confocal fluorescent microscopy can provide images with higher resolution than widefield fluorescent microscopy as well as render a three-dimensional image of the sample.

In synthetic vaccine studies, both techniques are incredibly important for the labelling and tracking of biomaterials. For cell imaging, different components of a cell such as its membrane or nucleus can be labelled by specific dyes[11, 60] and products can be labelled with other fluorophores. For identifying protein localization within a cell, fluorophore conjugated antibody can be used to bind those proteins which can be detected by direct fluorophore conjugation or secondary fluorescently-tagged antibodies[61, 62]. In order to track a sample to be measured, fluorescent dyes can be attached to a biomaterial sample such as a peptide, a protein, or a polymer before it is delivered to the cells[11]. For imaging biological samples, Near Infrared Region (NIR) light is preferred because it is capable of deep penetration into a sample providing better imaging than visible light[63].

3.10. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an important characterization technique which detects the presence of proteins, such as antigens or antibodies. The principle behind ELISA is to bind enzyme-linked antibodies to the proteins being measured followed by exposure to a substrate which can be processed yielding a notable colorimetric change **(Fig. 8)**. Sometimes the antibody can be detected directly by a secondary antibody which is linked to an enzyme through bioconjugation. ELISAs are commonly used for vaccine studies to assess antibody or cytokine content.

There are five kinds of common ELISA methods, including direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA, and ELISPOT. The direct ELISA is achieved by attaching primary fluorophore-labelled antibodies to the antigen to detect antigen concentration. An indirect ELISA utilizes pre-labelled secondary antibodies binding the primary antibody instead of direct labelling the primary antibody. A sandwich ELISA consists of multiple antibodies for which one traps the protein of interest and other detects it. Finally, competitive ELISAs are ones where an antigen and a purified immobilized antigen compete to bind to the capture antibody. By comparing the signal of competitive ELISAs versus the signal with purified antigens, one can demonstrate the antigen's presence and binding efficiency. ELISPOT is similar to the sandwich ELISA and is -most commonly used for cytokine detection.

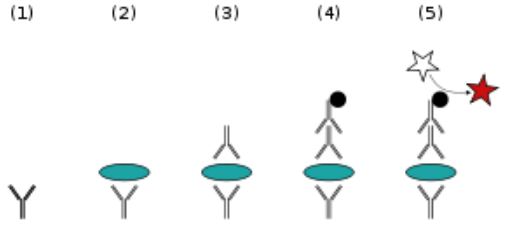

Fig. 8. Sandwich ELISA scheme. (1) Capture antibodies are coated on the plate; (2) the sample to be assayed is added; (3) the primary detection antibodies are included; (4) the enzyme-linked secondary antibodies are bound to primary detection antibodies; (5) substrate is provided and converted by the enzyme to a detectable form. (Reprinted with permission from wikipedia).[64]

3.11. Flow Cytometry

Flow cytometry is one of the most commonly used single-cell characterization techniques that can provide a great deal of cellular information. Flow cytometry is widely used by researchers to study cell mediated responses such as cell proliferation, cell surface marker expression, and cytokine production. The theory of flow cytometry is to allow only one cell to pass through the analyser at a time so that single-cell analysis can be achieved. Every cell is exposed to a pulsed laser for which two detectors receive light scattered and/or emitted from the cells. Forward scattered light (FSC) differentiates healthy cells from dead cells based on size differences while side scattered light (SSC) detects granularity of the cells and emitted fluorescent signals. Flow cytometry can also be

used as a cell sorting or cell purification technique. This is achieved by the aid of an electromagnet field which can move the cell to a desired collection vessel. Different cell types are recognized and bound with different types of fluorophore-labelled antibodies. The antibodies are electrostatically-charged so they can be readily displaced by the electromagnet field (**Fig. 9**). Overall, flow cytometry is an important method used for cell counting, sorting, analysis, and biomarker analysis.

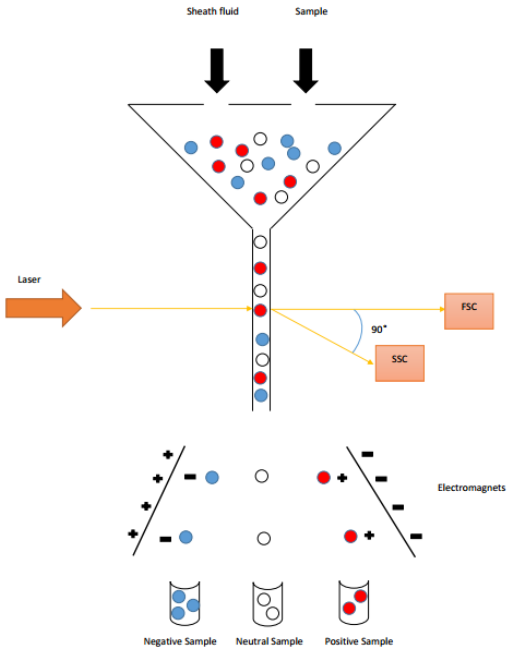

Fig. 9. Working principle of flow cytometry. Only a single cell at a time can pass through the laser scanning tunnel. Cells can also be separated through the use of a magnetic field.

4. Synthetic Vaccines Design Rules

4.1. Vaccine Size

Numerous research has been carried out exploring the optimal size of particle vaccines to dictate their uptake by APCs specifically dendritic cells (DCs) and macrophages. A broad conclusion that has been drawn relatively independent of materials chemistry is that nanoparticles tend to possess better internalization capacity than macro- or microparticles with optimal internalization found for 25 – 30nm diameter nanoparticles[65]. Following uptake by APCs, particles will reach the endosomal-lysosomal compartment where they are processed allowing for their antigen payload to be processed into small peptide fragments. These peptides are then loaded onto MHC-II molecules where they can be transported to the cell surface for the stimulation of helper T cells. The size of the synthetic vaccine can also determine its ability to travel to secondary immune organs (*i.e.* lymph nodes) via the lymphatic system. Initial lymph vessels are 10 - 60 μm in diameter whereas large lymphatic vessels can be up to 2 mm in diameter. The size of lymph vessels is therefore large enough to transport particles and cells up to several microns. However, initial lymphatic vessels are blind-ended structures that are lined with overlapping endothelial cells so that only quite small particles are able to pass the initial lymphatic endothelial cell junctions while larger particles are not able to directly enter[66, 67]. The optimal size of particles for passing lymphatic endothelial cell junction is around 40 nm in diameter[68, 69]. Therefore, research in this area have been focused on creating subunit vaccines within this diameter ranges in order to be effective.

4.2. Vaccine Shape

Generally, spherical nanoparticles were first generated due to their micro structural isotropy. However, natural biological interactions, especially cell-cell interactions, do not always occur between two spherical components. Inspiration from natural systems led to the extensive study of a promising research area, bioinspired materials, where researchers believe that materials that mimic natural structures would have enhanced bioactivity. Cellular cytoskeletal geometry and spatial organization are critical components that then can determine processing fate[70, 71]. This effect is seen within the immune system in a variety of ways. Dendritic cells for example undergo dramatic morphological changes upon activation. This change increases their overall cell surface area and affects their interactions with naïve T cells. More specifically, researchers have evaluated polymer based “artificial APCs” of different shapes and the results show that this variable can directly influence T cell fate in turn modulating the resulting immune response[6]. In addition to mimicking the natural biological system, the shape of the drugs can also affect ligand interactions which affect material cell uptake[72]. Gold nanoparticles are another important tool widely used in drug delivery for which different shapes have been extensively studied. Interestingly, gold nanoparticles with different shapes can direct APCs to produce different types of cytokines due to the fact that their shapes influences their internalization and processing by APCs differently[73, 74]. This, in turn, may result in different types of T helper cell responses[6, 75, 76]. Thus, finding the appropriate vaccine shape can help drive immune responses towards the desired outcome.

4.3. Vaccine Charge

The surface charge of vaccines plays a critical role in determining their immunogenicity, cytotoxicity, and payload carrying capacity especially for nucleic acids and proteins[77-79]. Liposomal vaccine surface charge has been widely studied and has been shown to influence their efficiency in many respects including their retention time[80], cell uptake ability[81], and DC activation and maturation capability[82].

Although in rare cases, anionic liposomes have shown a capability to induce an immune response[83], most research has found cationic liposomes preferentially promote desirable immune responses. Positively charged nanoparticles have been widely used as nucleic acid carriers to facilitate electrostatic interactions to associate the components with one another. This same electrostatic interaction strategy has also been successfully utilized in vaccines including for polyethyleneimine (PEI) nanoparticles[84, 85], cationic lipid nanoparticles[86-88], and surface charge modified inorganic particles[12, 89]. Despite the excellent cell-penetrating ability of cationic materials, one major issue associated with using cationic particles is their toxicity[24, 90, 91]. Fortunately, after years of studying the issue, researchers have found successful techniques for modifying cationic materials to limit their toxicity[92-95]. Overall, neutral or moderately positively charged vaccines have shown a better ability to induce a robust immune response in many cases.

4.4. Vaccine Secondary Structure

Although T cell responses rely on the recognition of antigen fragments processed and presented by APCs, antibody mediated responses can be closely related to the recognition of intact antigen through the B cell receptor. These receptors often best recognize a peptide fragment only with a certain secondary structure, so this factor can play an important role in vaccine effectiveness. Soluble antigens, especially short peptides, have too many degrees of freedom limiting their ability to form desirable secondary structures[5, 17]. Modulating antigen secondary structure has become possible with the successful development of novel engineering methods. Most of these techniques including micellization involve the use of nanostructures which cluster antigens in ways that create an artificial tertiary structure facilitating desirable secondary structure formation. A secondary benefit of this approach is that the condensing of so many antigens together enhances local concentration mimicking the multivalent repetitive epitope structure found on the surface of pathogens[96]. This may favor the high avidity interactions found between antigens and B cell receptors helping to activate the B cells. Thus, designing subunit vaccines that preserve native antigen secondary structure will be beneficial for increasing vaccine efficacy.

4.5. Vaccine Adjuvants

Vaccine adjuvants have been utilized in commercial vaccine formulas to enhance the body's immune response to the antigen of interest. Currently, physical adjuvants such as alum and Freund's adjuvant have received widespread clinical adoption. It is believed that physical adjuvants create an antigen depot at the injection site facilitating controlled antigen release[97] with some formulations leading to injection site inflammation with can also enhance the corresponding immune response[51, 98]. However, physical adjuvants commonly possess minimal capacity to facilitate T_H1 and cytotoxic T cell responses[98-102] limiting their ability to be used for vaccines against intracellular pathogens (*i.e.* viruses) and cancer. This major disadvantage can be overcome through the use of molecular adjuvants which function by mimicking microbial components (pathogen associated molecular patterns - PAMPs) such as bacterial cell wall components, flagella, or nucleic acid fragments that stimulate Toll Like Receptors (TLRs) on APCs to release danger signals[103-105]. Delivery strategies for molecular adjuvants have been extensively studied. One of the most important discoveries from the past decade is that the co-delivery of antigen and molecular adjuvants tends to produce stronger immune responses than delivering them separately[106, 107]. This work has led to extensive research into the development of polymer particles[8, 108], liposomes[109], and inorganic particles[11, 12] that are able to carry both antigen and molecular adjuvants to the same site of interest.

The adjuvant delivery strategy also greatly influences its efficiency. This is likely due to the fact that different classes of TLRs are located in different compartments within APCs. For example, bacterial cell wall based adjuvants like $Pam_2C\text{-}SK_4$, and Monophosphoryl Lipid A (MPLA) stimulate TLR-2 and TLR-4, respectively, which can be found on the APC surface. By contrast, nucleic acid based adjuvants such as CpG oligodeoxynucleotide and polyinosinic:polycytidylic acid (poly I:C) stimulate endogenous TLRs (TLR-3 and TLR-9 respectively) located with the APC endosome. Most recently, researchers discovered that a potentially more effective way to deliver adjuvants is by mimicking the bacterial natural structure. In specific, adjuvants that simulate bacterial surface structures should be located on the exterior of the carrier while adjuvants that simulate bacterial genetic structures are best to be delivered within the carrier[110]. Choosing the right type of adjuvant and delivering it with an appropriate platform can aid in enhanced adjuvanticity in turn improving overall vaccine efficacy.

5. Conclusion

Current advancements in material characterization techniques allows research scientists and engineers to create synthetic vaccines with optimal physicochemical properties to manipulate the immune system. Recent research has focused on biological inspiration which has been shown to greatly improve corresponding host immune responses. The field of synthetic vaccines has become increasingly promising yielding more and more design rules which can be utilized to expedite

research progress. Synthetic vaccines will hopefully become the next generation of vaccines and help human beings overcome emerging diseases.

6. Acknowledgements

The authors greatly acknowledge supports from University of Missouri Start-Up Funds, the University of Missouri Research Board, and the PhRMA Foundation. We thank Professor Heather Hunt and Mr. David Budde for their helpful comments and discussion regarding the manuscript.

7. References and Notes

1 M. Black, A. Trent, M. Tirrell, and C. Olive, Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. *Expert review of vaccines*. 9, 157-173 (**2010**)

2 R. Zhang, J. S. Kramer, J. D. Smith, B. N. Allen, C. N. Leeper, X. Li, L. D. Morton, F. Gallazzi, and B. D. Ulery, Vaccine Adjuvant Incorporation Strategy Dictates Peptide Amphiphile Micelle Immunostimulatory Capacity. *The AAPS journal*. 20, 73 (**2018**)

3 J. J. Moon, B. Huang, and D. J. Irvine, Engineering nano‐and microparticles to tune immunity. *Advanced materials*. 24, 3724-3746 (**2012**)

4 R. Kuai, L. J. Ochyl, K. S. Bahjat, A. Schwendeman, and J. J. Moon, Designer vaccine nanodiscs for personalized cancer immunotherapy. *Nature Materials*. (**2016**)

5 J. C. Barrett, B. D. Ulery, A. Trent, S. Liang, N. A. David, and M. V. Tirrell, Modular Peptide Amphiphile Micelles Improving an Antibody-Mediated Immune Response to Group A Streptococcus. *ACS Biomaterials Science & Engineering*. (**2016**)

6 J. C. Sunshine, K. Perica, J. P. Schneck, and J. J. Green, Particle shape dependence of CD8+ T cell activation by artificial antigen presenting cells. *Biomaterials*. 35, 269-277 (**2014**)

7 Y. Ge, and L. Liu, Cancer-testis antibodies mediated superparamagnetic nanoprobes as tumor targeting contrast agents for in vitro magnetic resonance imaging. *Journal of Bionanoscience*. 7, 606-611 (**2013**)

8 V. B. Joshi, S. M. Geary, and A. K. Salem, Biodegradable particles as vaccine delivery systems: size matters. *The AAPS journal*. 15, 85-94 (**2013**)

9 B. D. Ulery, D. Kumar, A. E. Ramer-Tait, D. W. Metzger, M. J. Wannemuehler, and B. Narasimhan, Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases. *PloS one*. 6, e17642 (**2011**)

10 A. Verma, R. Pandey, A. Chanchal, I. Siddiqui, and P. Sharma, Encapsulation of Antigenic Secretory Proteins of Mycobacterium tuberculosis in Biopolymeric Nanoparticles for Possible Aerosol Delivery System. *Journal of Bionanoscience*. 5, 88-95 (**2011**)

11 P. Zhang, Y.-C. Chiu, L. H. Tostanoski, and C. M. Jewell, Polyelectrolyte multilayers assembled entirely from immune signals on gold nanoparticle templates promote antigen-specific T cell response. *Acs Nano*. 9, 6465-6477 (**2015**)

12 Y.-C. Chiu, J. M. Gammon, J. I. Andorko, L. H. Tostanoski, and C. M. Jewell, Modular vaccine design using carrier-free capsules assembled from polyionic immune signals. *ACS biomaterials science & engineering*. 1, 1200-1205 (**2015**)

13 V. L. Praba, M. Kathirvel, K. Vallayyachari, K. Surendar, M. Muthuraj, P. J. Jesuraj, S. Govindarajan, and K. Raman, Bactericidal effect of silver nanoparticles against Mycobacterium tuberculosis. *Journal of Bionanoscience*. 7, 282-287 (**2013**)

14 H. H. Tam, M. B. Melo, M. Kang, J. M. Pelet, V. M. Ruda, M. H. Foley, J. K.

Hu, S. Kumari, J. Crampton, and A. D. Baldeon, Sustained antigen availability during germinal center initiation enhances antibody responses to vaccination. *Proceedings of the National Academy of Sciences*. 201606050 (**2016**)
15 K. Nerome, Y. Yoshioka, M. Ishida, K. Okuma, T. Oka, T. Kataoka, A. Inoue, and A. Oya, Development of a new type of influenza subunit vaccine made by muramyldipeptide-liposome: enhancement of humoral and cellular immune responses. *Vaccine*. 8, 503-509 (**1990**)
16 J. Dietrich, R. Billeskov, T. M. Doherty, and P. Andersen, Synergistic effect of bacillus calmette guerin and a tuberculosis subunit vaccine in cationic liposomes: increased immunogenicity and protection. *The Journal of Immunology*. 178, 3721-3730 (**2007**)
17 A. Trent, B. D. Ulery, M. J. Black, J. C. Barrett, S. Liang, Y. Kostenko, N. A. David, and M. V. Tirrell, Peptide amphiphile micelles self-adjuvant group A streptococcal vaccination. *The AAPS journal*. 17, 380-388 (**2015**)
18 M. Black, A. Trent, Y. Kostenko, J. S. Lee, C. Olive, and M. Tirrell, Self‐Assembled Peptide Amphiphile Micelles Containing a Cytotoxic T‐Cell Epitope Promote a Protective Immune Response In Vivo. *Advanced Materials*. 24, 3845-3849 (**2012**)
19 P. Balamohan, C. Anjali, and A. Ravindran, Nanoemulsion: Synthesis, characterization and its applications. *Journal of Bionanoscience*. 7, 323-333 (**2013**)
20 L. Tang, X. Yang, Q. Yin, K. Cai, H. Wang, I. Chaudhury, C. Yao, Q. Zhou, M. Kwon, and J. A. Hartman, Investigating the optimal size of anticancer nanomedicine. *Proceedings of the National Academy of Sciences*. 111, 15344-15349 (**2014**)
21 Y. Wen, A. Waltman, H. Han, and J. H. Collier, Switching the Immunogenicity of Peptide Assemblies Using Surface Properties. *ACS nano*. 10, 9274-9286 (**2016**)
22 F. Madani, S. Lindberg, Ü. Langel, S. Futaki, and A. Gräslund, Mechanisms of cellular uptake of cell-penetrating peptides. *Journal of Biophysics*. 2011, (**2011**)
23 S. Console, C. Marty, C. García-Echeverría, R. Schwendener, and K. Ballmer-Hofer, Antennapedia and HIV transactivator of transcription (TAT)“protein transduction domains” promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *Journal of Biological Chemistry*. 278, 35109-35114 (**2003**)
24 D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, and T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials*. 24, 1121-1131 (**2003**)
25 T. Thomas*, and T. Thomas, Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cellular and Molecular Life Sciences*. 58, 244-258 (**2001**)
26 C. B. Carter, and D. B. Williams, *Transmission electron microscopy,* Springer-Verlag US, (**2009**)
27 G. A. Hudalla, T. Sun, J. Z. Gasiorowski, H. Han, Y. F. Tian, A. S. Chong, and J. H. Collier, Gradated assembly of multiple proteins into supramolecular nanomaterials. *Nature materials*. 13, 829-836 (**2014**)
28 N. Norlin, M. Hellberg, A. Filippov, A. A. Sousa, G. Gröbner, R. D. Leapman, N. Almqvist, and O. N. Antzutkin, Aggregation and fibril morphology of the Arctic mutation of Alzheimer’s Aβ peptide by CD, TEM, STEM and in situ AFM. *Journal of structural biology*. 180, 174-189 (**2012**)

29 R. R. Pompano, J. Chen, E. A. Verbus, H. Han, A. Fridman, T. McNeely, J. H. Collier, and A. S. Chong, Titrating T‐Cell Epitopes within Self‐Assembled Vaccines Optimizes CD4+ Helper T Cell and Antibody Outputs. *Advanced healthcare materials*. 3, 1898-1908 (**2014**)
30 J. Mašek, E. Bartheldyová, Z. Korvasová, M. Škrabalová, Š. Koudelka, P. Kulich, I. Kratochvílová, A. D. Miller, M. Ledvina, and M. Raška, Immobilization of histidine-tagged proteins on monodisperse metallochelation liposomes: Preparation and study of their structure. *Analytical biochemistry*. 408, 95-104 (**2011**)
31 M. Kanekiyo, C.-J. Wei, H. M. Yassine, P. M. McTamney, J. C. Boyington, J. R. Whittle, S. S. Rao, W.-P. Kong, L. Wang, and G. J. Nabel, Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature*. 499, 102-106 (**2013**)
32 X. Ye, Z. Ku, Q. Liu, X. Wang, J. Shi, Y. Zhang, L. Kong, Y. Cong, and Z. Huang, Chimeric virus-like particle vaccines displaying conserved enterovirus 71 epitopes elicit protective neutralizing antibodies in mice through divergent mechanisms. *Journal of virology*. 88, 72-81 (**2014**)
33 J. F. Correia-Pinto, N. Csaba, J. T. Schiller, and M. J. Alonso, Chitosan-poly (I: C)-PADRE based nanoparticles as delivery vehicles for synthetic peptide vaccines. *Vaccines*. 3, 730-750 (**2015**)
34 S. De Carlo, and J. R. Harris, Negative staining and cryo-negative staining of macromolecules and viruses for TEM. *Micron*. 42, 117-131 (**2011**)
35 S. Brenner, and R. Horne, A negative staining method for high resolution electron microscopy of viruses. *Biochimica et biophysica acta*. 34, 103-110 (**1959**)
36 E. Callaway, The revolution will not be crystallized. *Nature*. 525, 172 (**2015**)
37 M. Rames, Y. Yu, and G. Ren, Optimized negative staining: a high-throughput protocol for examining small and asymmetric protein structure by electron microscopy. *JoVE (Journal of Visualized Experiments)*. e51087-e51087 (**2014**)
38 J. Frank, *Three-dimensional electron microscopy of macromolecular assemblies: visualization of biological molecules in their native state,* Oxford University Press, (**2006**)
39 J. Davidsen, I. Rosenkrands, D. Christensen, A. Vangala, D. Kirby, Y. Perrie, E. M. Agger, and P. Andersen, Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6, 6′-dibehenate)—a novel adjuvant inducing both strong CMI and antibody responses. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1718, 22-31 (**2005**)
40 G. Pintilie,
41 M. J. Kipper, J. H. Wilson, M. J. Wannemuehler, and B. Narasimhan, Single dose vaccine based on biodegradable polyanhydride microspheres can modulate immune response mechanism. *Journal of Biomedical Materials Research Part A*. 76, 798-810 (**2006**)
42 K. A. Ross, H. Loyd, W. Wu, L. Huntimer, M. J. Wannemuehler, S. Carpenter, and B. Narasimhan, Structural and antigenic stability of H5N1 hemagglutinin trimer upon release from polyanhydride nanoparticles. *Journal of Biomedical Materials Research Part A*. 102, 4161-4168 (**2014**)
43 P. T. Ingvarsson, S. T. Schmidt, D. Christensen, N. B. Larsen, W. L. J. Hinrichs, P. Andersen, J. Rantanen, H. M.

Nielsen, M. Yang, and C. Foged, Designing CAF-adjuvanted dry powder vaccines: spray drying preserves the adjuvant activity of CAF01. *Journal of Controlled Release*. 167, 256-264 (**2013**)
44 A. Vangala, D. Kirby, I. Rosenkrands, E. M. Agger, P. Andersen, and Y. Perrie, A comparative study of cationic liposome and niosome ‐ based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. *Journal of pharmacy and pharmacology*. 58, 787-799 (**2006**)
45 J. T. Y. Lee, and K. L. Chow, SEM sample preparation for cells on 3D scaffolds by freeze ‐ drying and HMDS. *Scanning*. 34, 12-25 (**2012**)
46 C. Sheppard, and T. Wilson, Depth of field in the scanning microscope. *Optics Letters*. 3, 115-117 (**1978**)
47
48 P. W. Atkins, and J. De Paula, *The elements of physical chemistry,* Oxford University Press New York, NY, USA:, (**2005**)
49 E. I. Solomon, and A. Lever, *Inorganic Electronic Structure and Spectroscopy, Volume II: Applications and Case Studies,* Wiley, New York, (**1999**)
50 R. Zhang, J. D. Smith, B. N. Allen, J. S. Kramer, M. Schauflinger, and B. D. Ulery, Peptide Amphiphile Micelle Vaccine Size and Charge Influence the Host Antibody Response. *ACS Biomaterials Science & Engineering*. (**2018**)
51 R. Zhang, L. D. Morton, J. D. Smith, F. Gallazzi, T. A. White, and B. D. Ulery, Instructive Design of Triblock Peptide Amphiphiles for Structurally Complex Micelle Fabrication. *ACS Biomaterials Science & Engineering*. (**2018**)
52 R. Pecora, *Dynamic light scattering: applications of photon correlation spectroscopy,* Springer Science & Business Media, (**2013**)
53 S. M. Alam, S. M. Dennison, B. Aussedat, Y. Vohra, P. K. Park, A. Fernández-Tejada, S. Stewart, F. H. Jaeger, K. Anasti, and J. H. Blinn, Recognition of synthetic glycopeptides by HIV-1 broadly neutralizing antibodies and their unmutated ancestors. *Proceedings of the National Academy of Sciences*. 110, 18214-18219 (**2013**)
54 G. Sonavane, K. Tomoda, and K. Makino, Biodistribution of colloidal gold nanoparticles after intravenous administration: effect of particle size. *Colloids and Surfaces B: Biointerfaces*. 66, 274-280 (**2008**)
55 J. D. Smith, L. N. Cardwell, D. Porciani, J. A. Nguyen, R. Zhang, F. Gallazzi, R. R. Tata, D. H. Burke, M. A. Daniels, and B. D. Ulery, Aptamer-displaying peptide amphiphile micelles as a cell-targeted delivery vehicle of peptide cargoes. *Physical biology*. 15, 065006 (**2018**)
56 J. D. Clogston, and A. K. Patri, Zeta potential measurement. *Characterization of nanoparticles intended for drug delivery*. 63-70 (**2011**)
57 X. Zhang, J. K. Jackson, and H. M. Burt, Determination of surfactant critical micelle concentration by a novel fluorescence depolarization technique. *Journal of biochemical and biophysical methods*. 31, 145-150 (**1996**)
58 A. Chattopadhyay, and E. London, Fluorimetric determination of critical micelle concentration avoiding interference from detergent charge. *Analytical biochemistry*. 139, 408-412 (**1984**)
59 P. Held, (**2014**)
60 B. G. De Geest, M. A. Willart, B. N. Lambrecht, C. Pollard, C. Vervaet, J. P. Remon, J. Grooten, and S. De Koker,

Surface‐Engineered Polyelectrolyte Multilayer Capsules: Synthetic Vaccines Mimicking Microbial Structure and Function. *Angewandte Chemie International Edition*. 51, 3862-3866 (**2012**)
61 R. A. Rosalia, E. D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J. W. Drijfhout, A. L. Silva, W. Jiskoot, T. Hall, and P. A. Veelen, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T‐cell activation. *European journal of immunology*. 43, 2554-2565 (**2013**)
62 S. Fallarini, T. Paoletti, C. O. Battaglini, P. Ronchi, L. Lay, R. Bonomi, S. Jha, F. Mancin, P. Scrimin, and G. Lombardi, Factors affecting T cell responses induced by fully synthetic glyco-gold-nanoparticles. *Nanoscale*. 5, 390-400 (**2013**)
63 S. Rahimian, J. W. Kleinovink, M. F. Fransen, L. Mezzanotte, H. Gold, P. Wisse, H. Overkleeft, M. Amidi, W. Jiskoot, and C. W. Löwik, Near-infrared labeled, ovalbumin loaded polymeric nanoparticles based on a hydrophilic polyester as model vaccine: In vivo tracking and evaluation of antigen-specific CD8+ T cell immune response. *Biomaterials*. 37, 469-477 (**2015**)
64
65 S. Zhang, J. Li, G. Lykotrafitis, G. Bao, and S. Suresh, Size‐dependent endocytosis of nanoparticles. *Advanced materials*. 21, 419-424 (**2009**)
66 M. A. Swartz, D. A. Berk, and R. K. Jain, Transport in lymphatic capillaries. I. Macroscopic measurements using residence time distribution theory. *American Journal of Physiology-Heart and Circulatory Physiology*. 270, H324-H329 (**1996**)
67 V. Manolova, A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M. F. Bachmann, Nanoparticles target distinct dendritic cell populations according to their size. *European journal of immunology*. 38, 1404-1413 (**2008**)
68 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. *Nature biotechnology*. 25, 1159-1164 (**2007**)
69 C. Oussoren, J. Zuidema, D. Crommelin, and G. Storm, Lymphatic uptake and biodistribution of liposomes after subcutaneous injection.: II. Influence of liposomal size, lipid composition and lipid dose. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1328, 261-272 (**1997**)
70 C. A. Parent, Making all the right moves: chemotaxis in neutrophils and Dictyostelium. *Current opinion in cell biology*. 16, 4-13 (**2004**)
71 P. Naumanen, P. Lappalainen, and P. Hotulainen, Mechanisms of actin stress fibre assembly. *Journal of microscopy*. 231, 446-454 (**2008**)
72 S. Barua, J.-W. Yoo, P. Kolhar, A. Wakankar, Y. R. Gokarn, and S. Mitragotri, Particle shape enhances specificity of antibody-displaying nanoparticles. *Proceedings of the National Academy of Sciences*. 110, 3270-3275 (**2013**)
73 K. Niikura, T. Matsunaga, T. Suzuki, S. Kobayashi, H. Yamaguchi, Y. Orba, A. Kawaguchi, H. Hasegawa, K. Kajino, and T. Ninomiya, Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo. *ACS nano*. 7, 3926-3938 (**2013**)
74 W. Jiang, B. Y. Kim, J. T. Rutka, and W. C. Chan, Nanoparticle-mediated cellular response is size-dependent. *Nature nanotechnology*. 3, 145-150 (**2008**)

75 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. *Molecular pharmaceutics*. 4, 73-84 (**2007**)
76 R. A. Meyer, J. C. Sunshine, K. Perica, A. K. Kosmides, K. Aje, J. P. Schneck, and J. J. Green, Biodegradable Nanoellipsoidal Artificial Antigen Presenting Cells for Antigen Specific T‐Cell Activation. *Small*. 11, 1519-1525 (**2015**)
77 S. Pasche, J. Voros, H. J. Griesser, N. D. Spencer, and M. Textor, Effects of ionic strength and surface charge on protein adsorption at PEGylated surfaces. *Journal of Physical Chemistry B*. 109, 17545 (**2005**)
78 C. G. Oster, N. Kim, L. Grode, L. Barbu-Tudoran, A. K. Schaper, S. H. Kaufmann, and T. Kissel, Cationic microparticles consisting of poly (lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. *Journal of Controlled Release*. 104, 359-377 (**2005**)
79 M. R. Garzón, P. Berraondo, J. Crettaz, L. Ochoa, M. Vera, J. J. Lasarte, A. Vales, N. Van Rooijen, J. Ruiz, and J. Prieto, Induction of gp120-specific protective immune responses by genetic vaccination with linear polyethylenimine–plasmid complex. *Vaccine*. 23, 1384-1392 (**2005**)
80 M. Henriksen-Lacey, D. Christensen, V. W. Bramwell, T. Lindenstrøm, E. M. Agger, P. Andersen, and Y. Perrie, Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. *Journal of controlled release*. 145, 102-108 (**2010**)
81 A. J. Schroit, J. Madsen, and R. Nayar, Liposome-cell interactions: in vitro discrimination of uptake mechanism and in vivo targeting strategies to mononuclear phagocytes. *Chemistry and physics of lipids*. 40, 373-393 (**1986**)
82 D. P. Vangasseri, Z. Cui, W. Chen, D. A. Hokey, L. D. Falo Jr, and L. Huang, Immunostimulation of dendritic cells by cationic liposomes. *Molecular membrane biology*. 23, 385-395 (**2006**)
83 A. Kohli, and H. Alpar, Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge. *International journal of pharmaceutics*. 275, 13-17 (**2004**)
84 A. Zintchenko, A. Philipp, A. Dehshahri, and E. Wagner, Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjugate chemistry*. 1448-1455 (**2008**)
85 H. K. de Wolf, C. J. Snel, F. J. Verbaan, R. M. Schiffelers, W. E. Hennink, and G. Storm, Effect of cationic carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous administration. *International journal of pharmaceutics*. 331, 167-175 (**2007**)
86 R. N. Majzoub, K. K. Ewert, and C. R. Safinya, Quantitative Intracellular Localization of Cationic Lipid–Nucleic Acid Nanoparticles with Fluorescence Microscopy. *Non-Viral Gene Delivery Vectors: Methods and Protocols*. 77-108 (**2016**)
87 M. Pizzuto, M. Gangloff, D. Scherman, N. J. Gay, V. Escriou, J.-M. Ruysschaert, and C. Lonez, Toll-like receptor 2 promiscuity is responsible for the immunostimulatory activity of nucleic acid nanocarriers. *Journal of Controlled Release*. 247, 182-193 (**2017**)

88 P. Pierrat, A. Casset, P. Didier, D. Kereselidze, M. Lux, F. Pons, and L. Lebeau, Cationic DOPC–Detergent Conjugates for Safe and Efficient in Vitro and in Vivo Nucleic Acid Delivery. *ChemBioChem*. 17, 1771-1783 (**2016**)
89 V. Sokolova, and M. Epple, Inorganic nanoparticles as carriers of nucleic acids into cells. *Angewandte chemie international edition*. 47, 1382-1395 (**2008**)
90 T. Xia, M. Kovochich, M. Liong, J. I. Zink, and A. E. Nel, Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. *ACS nano*. 2, 85-96 (**2007**)
91 C. M. Goodman, C. D. McCusker, T. Yilmaz, and V. M. Rotello, Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. *Bioconjugate chemistry*. 15, 897-900 (**2004**)
92 J. S. Choi, E. J. Lee, H. S. Jang, and J. S. Park, New cationic liposomes for gene transfer into mammalian cells with high efficiency and low toxicity. *Bioconjugate chemistry*. 12, 108-113 (**2001**)
93 V. Floch, S. Loisel, E. Guenin, A. C. Hervé, J. C. Clément, J. J. Yaouanc, H. des Abbayes, and C. Férec, Cation substitution in cationic phosphonolipids: a new concept to improve transfection activity and decrease cellular toxicity. *Journal of medicinal chemistry*. 43, 4617-4628 (**2000**)
94 A. Hirko, F. Tang, and J. A. Hughes, Cationic lipid vectors for plasmid DNA delivery. *Current medicinal chemistry*. 10, 1185-1193 (**2003**)
95 S. J. Soenen, A. R. Brisson, and M. De Cuyper, Addressing the problem of cationic lipid-mediated toxicity: the magnetoliposome model. *Biomaterials*. 30, 3691-3701 (**2009**)
96 J. J. Moon, H. Suh, A. V. Li, C. F. Ockenhouse, A. Yadava, and D. J. Irvine, Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand Tfh cells and promote germinal center induction. *Proceedings of the National Academy of Sciences*. 109, 1080-1085 (**2012**)
97 S. Awate, L. A. B. Babiuk, and G. Mutwiri, Mechanisms of action of adjuvants. *Frontiers in immunology*. 4, 114 (**2013**)
98 H. HogenEsch, Mechanism of immunopotentiation and safety of aluminum adjuvants. *Frontiers in immunology*. 3, 406 (**2013**)
99 E. Oleszycka, and E. C. Lavelle, Immunomodulatory properties of the vaccine adjuvant alum. *Current opinion in immunology*. 28, 1-5 (**2014**)
100 X. Wang, X. Li, K. Yoshiyuki, Y. Watanabe, Y. Sogo, T. Ohno, N. M. Tsuji, and A. Ito, Comprehensive Mechanism Analysis of Mesoporous‐Silica‐Nanoparticle‐Induced Cancer Immunotherapy. *Advanced healthcare materials*. 5, 1169-1176 (**2016**)
101 S.-h. Moon, E.-C. Shin, Y.-W. Noh, and Y. T. Lim, Evaluation of hyaluronic acid-based combination adjuvant containing monophosphoryl lipid A and aluminum salt for hepatitis B vaccine. *Vaccine*. 33, 4762-4769 (**2015**)
102 R. Zhang, C. N. Leeper, X. Wang, T. A. White, and B. D. Ulery, Immunomodulatory vasoactive intestinal peptide amphiphile micelles. *Biomaterials science*. (**2018**)
103 C. C. Lee, A. M. Avalos, and H. L. Ploegh, Accessory molecules for Toll-like receptors and their function. *Nature Reviews Immunology*. 12, 168-179 (**2012**)
104 S.-W. Lee, P. F. Markham, M. J. Coppo, A. R. Legione, J. F. Markham, A. H. Noormohammadi, G. F. Browning, N. Ficorilli, C. A. Hartley, and J. M. Devlin, Attenuated vaccines can recombine to

form virulent field viruses. *Science*. 337, 188-188 (**2012**)
105 P. M. Moyle, Biotechnology approaches to produce potent, self-adjuvanting antigen-adjuvant fusion protein subunit vaccines. *Biotechnology Advances*. (**2017**)
106 N. O. Fischer, A. Rasley, M. Corzett, M. H. Hwang, P. D. Hoeprich, and C. D. Blanchette, Colocalized delivery of adjuvant and antigen using nanolipoprotein particles enhances the immune response to recombinant antigens. *Journal of the American Chemical Society*. 135, 2044-2047 (**2013**)
107 S. Nierkens, M. H. den Brok, R. P. Sutmuller, O. M. Grauer, E. Bennink, M. E. Morgan, C. G. Figdor, T. J. Ruers, and G. J. Adema, In vivo colocalization of antigen and CpG within dendritic cells is associated with the efficacy of cancer immunotherapy. *Cancer research*. 68, 5390-5396 (**2008**)
108 J. J. Moon, H. Suh, A. Bershteyn, M. T. Stephan, H. Liu, B. Huang, M. Sohail, S. Luo, S. H. Um, and H. Khant, Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. *Nature materials*. 10, 243-251 (**2011**)
109 Y. Fan, P. Sahdev, L. J. Ochyl, J. J. Akerberg, and J. J. Moon, Cationic liposome–hyaluronic acid hybrid nanoparticles for intranasal vaccination with subunit antigens. *Journal of controlled release*. 208, 121-129 (**2015**)
110 A. L. Siefert, M. J. Caplan, and T. M. Fahmy, Artificial bacterial biomimetic nanoparticles synergize pathogen-associated molecular patterns for vaccine efficacy. *Biomaterials*. 97, 85-96 (**2016**)