ASPECTS OF MICROPARTICLE UTILIZATION FOR POTENTIATION OF NOVEL VACCINES: PROMISES AND RISKS

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Abstract. Many recombinant vaccines against novel (HIV, HCV) or ever-changing (influenza) infectious agents require the presence of adjuvants/delivery vehicles to induce strong immune responses. The necessity of their improvement led to the major effort towards development of vaccine delivery systems that are generally particulate (e.g., nano- and microparticles) and have comparable dimensions to the pathogens (viruses or bacteria). The mode of action of these adjuvants is not fully understood but implies the stimulation of the innate or antigen-specific immune responses, and/or the increase of antigen uptake or processing by antigen-presenting cells (APC). Moreover, enhancement of adjuvant activity through the use of micro- and nanoparticulate delivery systems often resulted from the synergistic effects producing immune responses stronger than those elicited by the adjuvant or delivery system alone. Among particulate adjuvants, biodegradable micro- and nanoparticles of poly(D,L-lactide-*co*-glycoside) (PLGA) or poly(D,L-lactide) (PLA) have been reported to enhance both humoral and cellular immune responses against an encapsulated protein antigen. Cationic and anionic polylactide *co*-glycolide (PLG) microparticles have been successfully used to adsorb a variety of agents, which include plasmid DNA, recombinant proteins and adjuvant active oligonucleotides and are also currently tested in several vaccine applications. Another approach envisions specific targeting of APC, especially peripheral DC and exploitation of particulate systems that are small enough for lymphatic uptake (polystyrene nanobeads). Micro- and nanoparticles offer the possibility of enhancement of their uptake by appropriate cells through manipulation of their surface properties. Still, questions regarding toxicity and molecular interaction between micro- and nano-particles and immune cells, tissues and whole organisms remain to be addressed. These risks and other possible side effects should be assessed in detail especially if mass-production and massive administration of such preparations is to be considered.

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1. Introduction: Vaccination Goes "Nano" – Why?

Prevention of infectious disease through vaccination is one of the most important achievements in the history of medicine. Its effects on the mortality reduction are enormous and may be compared only to those caused by the establishment of safe water supply [1]. Adaptive immunity against infectious disease, i.e. lack of infection in previously exposed individuals has been noted for a long time, most famously by Athenian historian Thucydides in 5th century BC. It is often said that first recorded attempts of vaccination against infectious disease (smallpox) go back to AD 1000. in China, but they cannot be verified till 16th—17th centuries, when the procedure of variolation started to be used on a reasonably large scale. In the end of XVIII pioneering works of Jenner and his less-known predecessor Jesty on vaccination against smallpox with cowpox paved the way to modern vaccination, whose true founder in a scientific sense was Louis Pasteur [1]. Interestingly, first successful vaccines were launched against two viral diseases, smallpox and rabies. Smallpox became first and only infectious disease to be eradicated in 1967–1977 [2]. It is commonly said that Jenner's work constitutes the first deliberate use of live virus vector as a vaccine, but we should also note that it was first recorded use of nanotechnology (virion size of a poxvirus is around 200 nm in diameter and 300 nm in length). We should also mention that the problems of unknown side effects, possibility of human infection with new or incompletely inactivated virus and as well as initial difficulties of social acceptance of vaccination that were faced by both Jenner and Pasteur are not dissimilar from the risks that nanotechnology of vaccination encounters today. In fact, these problems have persisted for more than 200 years and yet many other major diseases have been put under control with the help of vaccination. These include, to name just a few, diphtheria, tetanus, yellow fever, pertussis, poliomyelitis (currently targeted for eradication by WHO), measles, mumps and rubella. And this list is far from complete.

At the same time, it is important to remember that a number of vaccine-related disasters and incidents were caused over this period of time, all of them due to failures in vaccine manufacturing techniques. Specifically, during the 1st century of scientific vaccination (post-Pasteur) three types of vaccines were generally used: live-attenuated (weakened), killed viruses/bacteria and subunit or extract (chemically or biochemically processed and thus detoxified, the technique that mostly important in anti-toxin vaccination, i.e. against diphtheria). It is thus of no surprise that there were instances of incomplete attenuation, sterilization or detoxification of vaccine products (see [2] for details). While historical role of early-generation vaccines cannot be underestimated, current awareness of health and safety issues resulted in much higher standards of safety and it is true that many past vaccines would not even meet the minimum standards of today. New techniques of vaccine development that may enable a generation of safe, effective and economically feasible vaccines are being actively explored. Additionally, it is now clear that successful vaccination against several newly-discovered infectious agents as human immunodeficiency virus (HIV), hepatitis C virus (HCV) or severe acute respiratory syndrome (SARS) will require the development of radically new vaccine concepts and products.

There are several types of novel vaccines or vaccine components the utilization of which is not mutually exclusive. In fact, their complementary use may be even required in some cases. Firstly, there are recombinant molecules: proteins or nucleic acids (NA; DNA vaccines have been tested extensively over the last decade, RNA vaccines are now actively pursued in several experimental systems, but relevant information is scarce at the moment save for RNA alphaviral replicons). Protein antigens induce immune response directly, while NA vectors encode necessary antigens and produce them upon their introduction into organism. Secondly, these molecules may be carried or delivered by biological or chemically produced vectors, separately or in combination. These vectors are known to tremendously augment the immune response induced by proteins or nucleic acids used alone. Such augmentation is also called adjuvant effect. The size of these vectors is generally within 10–1000 nm and it is a specific mechanism by which our immune system recognizes such particles that underlies their adjuvant potencies (in addition, many carriers protect proteins/NA from rapid degradation *in vivo* and release them into the organism during prolonged periods of time, which also results in higher immunogenicity). Needless to say, many details of this mechanism remain to be elucidated, although current vaccine development fashion is definitely of "going small", i.e. toward micro- and nanoscale [3]. In this paper, I will briefly discuss the current state of the vaccine nanotechnology, promises of the new approaches as well as potential risks that these approaches may contain.

Both recombinant NA and protein vaccines have been designed to be safer than traditional vaccines based on whole viruses and bacteria, but their limited immunogenicity has so far hindered their development for clinical use. This led to the sustained research effort aimed at development of specific adjuvants that may make such vaccines more potent without compromising on their safety. Currently, only one adjuvant, alum (aluminum hydroxide or aluminum phosphate) is used worldwide in diphtheria, tetanus and hepatitis B vaccines. One of the proposed mechanisms for alum action is generation of small salt particles in which vaccinating agent is trapped and then preferentially taken up by cells of immune system, macrophages and dendritic cells (DC), which, in turn, leads to elevated immune response. Multiple attempts to create antigen-bearing particles that will be capable to imitate this suggested avenue of immunogenicity augmentation have been undertaken over the last decade and many of them have been useful. What may be the mechanism of higher immunogenicity exhibited by particulate antigens compared to soluble ones and does the size of these particles matter?

Adjuvanting effects of micro- and nanoscale particulates are now known to be a consequence of them being of suitable size for uptake by antigen-presenting cells (APC), such as DC and macrophages through phagocytosis or pinocytosis. APC then stimulate T-lymphocytes constituting cellular arm of immune response, which among others is responsible for specific antiviral immunity. This APC-mediated activation of T-cells initiates adaptive immune response. Antigens delivered in particulate form are preferentially processed by APC and via so-called MHC class I and II pathways and then presented to CD8+ (cytotoxic lymphocytes) and CD4+ (T-helpers) T-cells, which first serve as effector cells of immune response and then are instrumental in generation memory T-cells, those that are responsible for the defense of the organism in case of

repeated exposure to the infectious agent bearing antigen in question. Those particulate antigens that are not directly taken up by DC, but by other cells of the organism (e.g., by muscle cells after intramuscular injection) may also stimulate immune response by two possible pathways. Either they eventually either find their way to DC after cell death followed by phagocytosis (if they are stable enough) or they may be processed to the cell surface and then recognized by already activated T-cells thus providing further stimulus for their activation and multiplication. Obviously, that particle-driven stability of delivered antigens is a very important part of such an equation.

Moreover, the surface of micro- or nanoparticle may be further modified to increase their targeting specificity, which can be also attained via simple size variation. It is now clear that different subtypes of DC, those residing in skin and in lymph nodes (LN) are differentially affected by nanoparticles of various size with smaller nanoparticles (10–100 nm) being capable of passive migration to lymph nodes and rapid activation of immature LN-resident DC, while particles of the larger sizes (100–1000 nm) are preferentially uptaked by DC at a vaccine injection site. Those skin or mucosal APC residing at those parts of the body where the contact with infectious agents is most likely constitute "the first line of defense" of the organism. It is more than probable that activation of several DC populations done sequentially or simultaneously is a process imperative for generation of potent immune response. Moreover, activation of DC does also trigger non-specific immune responses, known as innate (e.g., induction of interferons and other cytokines), which in turn may further potentiate generation of prolonged and strong specific immune response against vaccinating agent. All of these developments made by fundamental research are now actively transposed into the science of vaccine manufacturing.

2. Approach 1: Imitation or Modification of Nature - Viruses and, Rarely, Bacteria

There are two types of vectors of viral origin that have been extensively explored lately, first being viral vectors per se (replication-competent or -deficient) and second, virus-like particles (VLP). Viral vectors consist of replicating or non-replicating virus that contains genetic material (in form of DNA or RNA) encoding the desired antigen. These vectors maintain the ability to penetrate cells and disseminate in the organism and sometimes even replicate, but are rendered harmless by specific changes or mutations. Advantages of virally-vectored vaccines include their ease of production, a good safety profile (at least in some cases), ability to potentiate strong immune responses, potential for nasal or epicutaneous delivery and mucosal immunization [3]. They are also more potent than pure DNA vaccines (since DNA in this case is protected from degradation by viral capsid and directly delivered to virus-susceptible cells), but are especially good if used sometime after initial DNA immunization (in so-called prime-boost regimen).

Adenoviruses are probably among the best-studied viruses and they were heavily exploited for vaccine development over last two decades [4]. There are many promising experimental results using either replication-competent or -incompetent (the latter, by definition, are very safe) adenoviral vectors. However, consistent presence of adenoviruses

(the agents of common cold) in human population resulting in pre-exisitng immunity (the ability of human immune system to recognize adenoviral vector and expeditiously remove it from the organism) has somewhat damped the enthusiasm for their clinical use. Still, experimental data, especially in the immunization against HIV and influenza were sufficiently promising to enable large-scale clinical trial of adenoviral-based HIV vaccine manufactured by Merck (USA). This, as we know, has recently ended in a complete failure. The trial was aborted [5, 6]. Importantly, not only this vaccine was not protective, it has apparently also increased risk of HIV infection in vaccinated subjects that were previously exposed to adenovirus (those with pre-existing immunity). Notably, we do not currently know the fundamental reason for the latter (and it was not foreseen by anyone based on current immunological knowledge). This example shows that vaccines of novel generation, however promising, may cause side effects of completely unpredictable nature.

Another type of viral vectors is based on various poxviruses, closely related to vaccinia virus that has been used for many years for vaccination against smallpox. It is known that immunization with vaccinia viruses induces potent immune response against recombinant antigen [7] and many experimental vaccines containing proteins from HIV, influenza, malaria, etc. have been produced and tested. It is important to stress that unmodified live vaccinia virus has never been proposed as delivery vehicle for vaccines aimed at general use (application of such vaccines as anti-cancer therapeutics is outside of this review) since the risks of its utilization would have been enormous. In particular, live vaccinia virus can not be used to vaccinate people with immune deficiencies, suffering from eczema, etc. However, its replication-deficient strain, called modified vaccinia virus Ankara (MVA) is much safer and still very immunogenic. Another approach envisions the utilization of avian poxviruses, e.g. fowlpox or canarypox viruses, which are also incapable of replication in humans, but still immunogenic. Similar to adenoviral vectors, the issue of pre-existing immunity should be addressed regarding poxviral-based vaccines since nearly all humans over 40 years old have been vaccinated against smallpox at least once and may therefore respond poorly to new poxviral vaccination. The possibility of adverse effects similar to the one observed in Merck anti-HIV vaccine trial can not be discounted as well, although what is true for one group of viruses may not be automatically applied to another. Many MVA-vectored vaccines have been already developed, but only one of them, against malaria, is now under active clinical investigation, which at the moment appears to be highly successful. In this case prime vaccination with DNA molecule is followed by boost immunization with recombinant poxvirus. Currently we do not know why poxviral-based vaccine is especially potent against malaria and if these results could be translated into other infectious systems.

Other replicating virus vectors are based on measles and vesicular stomatitis virus and they suffer from similar drawbacks: prior immunity and safety concerns. The same could be said about two more novel non-replicating viral vectors based on alphaviruses and herpesviruses [4]. It appears that viral vaccine vectors will require considerable breakthrough in order to enable their extensive clinical application.

Another approach envisions the utilization of VLPs. VLPs are non-infective virus particles (capsids) consisting of self-assembled viral proteins without NA, which mimic the structure of native virions [8]. These particles fall in the general size range of viruses (22–150 nm) [9], albeit it is fair to say that full viral size range is within 15–400 nm [10]. They also maintain a specific virus-like morphology and are effectively consumed by cells similarly to infective viral particles. They may be formed by a single viral protein or by co-expression of many, in the latter case creating complex virus-like structures. It appears that particulate nature of VLPs drives their efficient presentation to the cells of immune system. Moreover, those VLPs that closely resemble infectious viruses may trigger the same array of antiviral immune responses (e.g., via viral receptor) without causing immune suppression (as living viruses do).

VLPs can be produced in many expression systems, those of mammalian cells, yeast, bacteria, baculovirus (viruses of insects) and plants. They have been manufactured for many viruses including, but not limited to HIV, hepatitis B virus (HBV), HCV, Ebola, influenza, rotavirus, human papillomavirus (HPV) and Norwalk virus [8]. Several VLP-based vaccines have been licensed for general use, many of them against HBV, which are composed of HBV surface antigen (HBsAg), which is a main component of currently used protein-based, alum adjuvant-potentiated vaccine. Upon its production *in vitro* (e.g., in yeast) HBsAg protein aggregates in 22 nm particles (single-component VLP), which are both safe and highly immunogenic [11]. In fact, HBsAg is now actively pursued as molecular adjuvant on its own ("VLP as platform" approach). Fusion of several less immunogenic proteins to HBsAg, most notably, influenza M2 protein capable of cross-protective immunity, resulted in significant immunogenicity augmentation [12]. Similar data has been reported for HBV VLP containing proteins from the circumsporozoite stage of the malaria *Plasmodium* [13]. Improvements of single-protein HBV VLPs by inclusion of large and middle viral envelope proteins are also actively sought [9].

The most recently approved VLP vaccine is Gardasil that is protective against several types of HPV, which are known to be associated with cervical cancer and genital warts. This vaccine is composed of self-assembled particles of HPV major capsid protein [14–16] and it also contains an aluminum salt adjuvant. It has been shown to reduce infection of HPV by 90% and is almost 100% effective HPV types 6, 11, 16 and 18. This vaccine if administered early in life is expected to drastically reduce the occurrence of this life threatening disease in women and has generated significant excitement. Therefore, it is currently recommended that girls at 9–14 years group age are vaccinated and many U.S. states will likely make this vaccination mandatory. Anti-HPV vaccine is clearly the most dramatic example of recent success of recombinant vaccine developers. At the same time, there was a significant fight-back against general utilization of this vaccine stemming from different social groups that we will describe in the final part of this review.

Several other VLP vaccines are now close to clinical use, those against Norwalk virus and rotavirus (both causing acute gastrointestinal infections). Rotavirus is the leading cause of diarrhea-related deaths (400–600,000) in children worldwide and clinical trials of VLP-based vaccine against rotavirus has long been advocated [8]. VLP-based vaccine

against Norwalk virus is now assessed in phase I clinical trials. A variety of other VLP vaccines have been evaluated in preclinical studies include HIV, influenza, HCV, Ebola and SARS coronavirus [9]. It should be added that non-vaccine nanoparticle VLP applications also exist such as using them as scaffolds to allow assembly of biopolymers (large plant virus-derived VLPs are utilized, notably from tobacco and cowpea mosaic viruses), including their utilization as templates to polymerize nanowires as building blocks for semiconductors or manufacture nanowires for the construction of smaller lithium ion batteries [10].

Liposomes (phospholipids-based vesicles) are roughly of the similar size as VLP (usually 50–200 nm) and they were used extensively as drug delivery vehicle for nearly four decades. Liposomes transport their contents across cellular membranes and release it following fusion with internal cellular compartments called endosomes. Thus, they are capable of delivering of encapsulated or adsorbed antigen for vaccine use and were investigated as adjuvants for vaccines against influenza, HIV and malaria, among others. However, it seems that no unmodified liposome-based vaccine possesses an adjuvant effect strong enough to substantiate its clinical development. Conversely, modified liposomal vaccines based on viral membrane proteins (virosomes) are sufficiently potent and are approved for use in Europe as vaccines against hepatitis A virus (HAV) and influenza [17]. Immunopotentiating influenza virosomes are liposomes containing main influenza surface protein hemagglutinin intercalated into their membrane. Their approximate mean diameter is 120–170 nm. The glycoproteins of influenza virus stabilize the liposomal particles and maintain their receptor-binding actrivities when reconstituted into such protein lipid vesicles and are therefore actively taken up by cells *in vivo* and actively conveyed to immunocompetent cells [18]. They have an excellent safety profile. Other virosomal vaccines produced include not only those against HAV, but also combined HAV-HBV vaccine in which inactivated HAV virions and HBV HBsAg cores were covalently coupled to the surface of virosome [18].

Adjuvant properties of virosomes can be further increased by integration of co-stimulatory molecules (immune response mediators and activators) or their specific targeting to particular DC subtype. Moreover, they can additionally carry DNA or RNA and thus provide for multifaceted immune stimulation via different pathways. Therefore, virosomes are becoming more and more popular in modern vaccine concepts since they combine the safety and flexibility of subunit vaccines with the biological and immunogenic properties of VLPs [10].

Another mode of imitating nature is the creation of sometime misleadingly called Proteosomes (note the capital "P"), nanoparticles composed of the outer membrane proteins (OMPs) of *Neisseria meningitides* and other *Neisseria*. Proteosome particles are considered to serve as potent vaccine delivery vehicles based on their nanoparticulate (20–800 nm size), vesicle-like nature [19]. In addition, hydrophobicity of Proteosomes may inhibit antigen degradation facilitate its uptake and processing by immunocompetent cells. OMPs have been used successfully in a marketed meningococcal vaccine since 1981 and are considered non-toxic and well-tolerated [3]. Meningococcal OMPs have been safely given parenterally to hundreds of million of children in a *Haemophilis influenza* Type b conjugate vaccine [19]. In several cases, a novel adjuvant known as

Protollin consisting of Proteosomes non-covalently complexed with immune stimulant lipopolysaccharide (LPS) has been used [20]. Proteosome-based influenza vaccines for nasal application have been also generated using this technology and successfully tested and many others (against *Shigella*, HIV, staphylococcal and *Brucella* toxins are under active development). There are other bacteria-based adjuvants, but they may not be called particles in a true sense of words, rather being biomolecules (modified proteins, peptides, components of bacterial wall). It is for their efficient delivery that some of synthetic micro- and nanoparticles can be successfully used.

3. Approach 2: Imitation or Modification of Nature via Physical Chemistry, Biochemistry or Natural Products Chemistry

The only nano-sized vaccine adjuvant that is approved for human use (in Europe, but not in the U.S.) as a vaccine component is MF59. It was the first new adjuvant to be licensed for human use (in 1997) 70 years after introduction of alum [21]. MF59 is an oil-in-water emulsion composed of <250 nm uniform and stable microparticles made by a drop of oil surrounded by a coat of water droplets held onto oil by surface detergents. Specifically, these droplets are formed when squalene (4.3% v/v) and two surfactants, polysorbate 80 (0.5% v/v, Tween 80) and sorbitan trioleate (0.5% v/v, Span 85) are emulsified in citrate buffer [3]. This oil is obtained from shark liver and is also found in humans as natural metabolite [21]. The strong immunogenicity enhancement of MF59 has been repeatedly demonstrated with some researchers showing an effect even stronger than that of alum. The mechanism of adjuvanticity of MF59 is believed to be through cytokine production, although no precise information is available as of yet. In general, it is thought that adjuvant emulsions potentiate immune responses via formation of antigen depots and stimulation of antibody-producin plasms cells. As noted above, an MF59-adjuvanted influenza vaccine, Fluad, is licensed in Europe and experi-mental vaccines for avian influenza have been produced. Their strong immunogenicity and protectivity has been demonstrated experimentally. Other vaccines strongly potentiated by MF59 include those against HBV, HCV, HIV, herpes simplex virus (HSV), *Haemophilis influenzae* type b and *N. meningitides*.

Montanide is a different, water-in-oil emulsion (one modification is a water-in-oil-in-water emulsion). Emulsions of Montanide51 and 720 are composed of a metabolizable squalene-based oil with a mannide monooleate emulsifier, which augments immunogenicity via formation of antigen depot at the site of injection [3]. While similar to Incomplete Freund's adjuvant (IFA), a well-known but extremely reactogenic adjuvant that contains a mineral oil and an emulsifying agent, in its physical character, Montanide is biodegradable. This eliminates many of the cytotoxic properties inherent for IFA. ISA 51 and 720 emulsions have been in phase I and/or II clinical trials for vaccines against malaria and HIV and various cancers and in most cases they were found to be safe and fairly well-tolerated.

Another particulate vaccine vehicle is immunostimulating complex (ISCOM). ISCOMs are based on triterpenoid saponins (Q saponins). They form particles of approximately 40 nm. These are essentially cage-like structures containing protein antigen, cholesterol,

phospholipid and the saponin adjuvant Quil A or its purified component Quil21, which is derived from the aqueous extract from the bark of the South American tree *Quillaia saponaria Molina*. These components are held together by hydrophobic interactions. Saponins have been tested and used in both human and veterinary medicine for decades and they are known to efficiently induce T-helper responses. They have not been extensively used because of their reactogenicity (Quil A composed of more than 23 different saponins is too toxic for human use), although recently semi-synthetic saponins were created that are apparently less toxic [22]. ISCOM matrix traps the protein antigens (typically hydrophobic membrane proteins) through apolar interactions. A similar vaccine delivery vehicle and adjuvant has also been developed that uses the same material minus the antigen (ISCOMATRIX) and still possesses preferential targeting to APC [23]. A clinical study that compared a classical trivalent subunit influenza vaccine with an ISCOM adjuvanted version revealed a stronger immune response with the ISCOM vaccine eliciting rapid antibody responses as well as T helper (CD4+ T-cell) and some CTL (CD8+ T-cell) responses [24]. Additional ISCOM/ISCOMATRIX vaccines have been tested in the clinic for HIV, HSV, HPV and HCV [3]. In all cases, these studies have shown a good safety and tolerability profile in humans as well as effective induction of both humoral and cellular immune responses. Still, the actual use of ISCOMs in human vaccines has been deterred by concerns regarding safety since some saponins are toxic at elevated levels [3, 22].

Another completely new mode of biochemical modification of antigen is its attachment to so-called protein aggregating domains that form intracellular inclusions of several hundred nm. Such inclusions are then digested via the process called autophagy, which is now known to be linked to effective antigen presentation via MHC class II pathway. Initial data provided evidence of dramatic increase of immunogenicity when a test soluble antigen was attached to such aggregating domain [25], although this very promising approach is still in the very first stages of possible development.

There are several types of synthetic micro- and nanoparticles that are effective in vaccine delivery and potentiation. Calcium phosphate microparticles are relatively less-known. They can be generated by combining (while stirring) of calcium chloride, sodium phosphate and sodium citrate [3]. Since calcium phosphate is naturally occurring in the body, these materials are thought not to present a danger of significant side-effects. Calcium phosphate microparticles are less than ~1.2 μm in diameter (<1,000 nm according to other authors, [26]). Phase I study showed that these microparticles are safe and non-toxic when administered subcutaneously. Vaccines utilizing CaP, which are currently in preclinical studies include anthrax, HBV, influenza (H5N1 avian and seasonal) and HSV-2 [3].

Much better known are polymeric biodegradable microparticles. Those used most frequently are poly(D,L-lactide-*co*-glycolide) (PLG/PLGA) and polylactide (PLA) and their copolymers as well as polyorthoesters, polyanhidrids and polycarbonates [27]. Microparticles may be loaded by many types of recombinant vaccines, i.e., DNA (which is thus protected from degradation), proteins and also other adjuvants of smaller molecular size (of protein, peptide or oligonucleotide nature). These biodegradable, biocompatible polymers have been approved for use in humans (e.g., as sutures, bone

implants, screws and implants for sustained drug delivery) and have been extensively studied for use in the formulation of vaccine antigens. PLG-based microparticles are the primary candidates for the development of microparticles as vaccine adjuvants since they have been safely used in humans for many years. The direct uptake of biodegradable microparticles into DC has been demonstrated both *in vitro* and *in vivo* and it appears that their appropriate size is within 1–3 μm range and that cationic microparticles are particularly effective in this regard [28]. In these formulations, antigen can be either entrapped or adsorbed to the surface of the particles. Furthermore, these particles can be tailored to degrade over a range of rates, which is especially important since several of those systems degrade rather slowly. Thus, additional triggering of their dissolution is necessary for efficient antigen delivery. On a positive side, they can therefore act as a depot from which the encapsulated antigen is gradually released [28]. Additionally, polymeric particles may offer high degree of protection to encapsulated antigens delivered orally and nasally and thus subjected to many degrading enzymes residing at these sites of the body. It appears that DCs respond to biomaterials via an innate immune response, which then stimulates an adaptive response to an antigen delivered by polymeric particles.

Biodegradable and biocompatible micro- and nanoparticles of PLGA or PLA have been reported to enhance both humoral and cellular immune responses against an encapsulated protein antigen. PLG nanoparticles can induce systemic antibody titers comparable to those of aluminum salts. It was demonstrated that PLG nanoparticles loaded with MPL (an immune stimulant composed of detoxified lipopolysaccharide (LPS) from *Salmonella minnesota*) were efficiently taken up by DC. Recently, it was shown that the adsorption of antigens at the surface of PLA particles also leads to elevated immune response. Cationic and anionic PLG microparticles have been successfully used to adsorb a variety of agents, including DNA, recombinant proteins and adjuvant active oligonucleotides and are also currently tested in several vaccine applications.

Another approach envisions specific targeting of APC, especially peripheral DC and exploitation of particulate systems that are small enough for lymphatic uptake (polystyrene nanobeads). These are in contrast non-degradable nanoparticles (represented also by gold, latex and silica beads). It appears that solid synthetic, particulate vaccines can induce strong immune responses imitating classical "danger signals" generated by infectious agents and microparticles. In this application 40–50 nm particles preferentially taken by some types of DC are often used and are known to generate potent and broad immune response [23]. Moreover, it was demonstrated that for polysterene beads even miniscule difference in size can influence the breadth and type of induced immune response [29].

Recently, ultra-small (25 nm) nanoparticle systems were shown to be capable of interstitial-to-lymphatic flow to deliver antigen and adjuvant to LN-resident DC via lymphatic capillaries, whereas 100 nm nanoparticles were only 10% as efficient [30]. Also, these ultra-small, ovalbumin-conjugated, polyhydroxylated nanoparticles based on Pluronic (a block copolymer of polyethylene glycol and polypropylene glycol) were shown to induce antigen-specific cellular immunity since their surface chemistry has

activated complement system, a pathway of innate immunity that serves as a biochemical defense system that clears pathogens nonspecifically, but can also play a role in promoting antigen-specific responses.

In general, nanoparticles as vaccine vehicles might have three different advantages over microparticles. Most importantly, they have increased surface area for adsorption allowing for a higher antigen/polymer ratio and are also easier to prepare and process. As for their higher immunogenicity, the jury is still out since different groups of investigators are presenting conflicting evidence [31]. It should be also noted that sometimes the utilization of preposition "nano" to describe nearly any type of vaccine component is a bit overdone [3].

Since non-degradable nanoparticles may remain in the tissues for extended periods of time, it is thought that it will therefore enhance the time of immobilized antigen presentation and thus augment immunogenicity. Gold particles have been frequently described for vaccine delivery both with and without the aid of electroporation, a method which is unlikely to be employed in humans. An alternative approach to delivering DNA vaccines employing non-degradable nanoparticles is through particle bombardment also referred to as "gene gun" approach. This is essentially firing the DNA-coated gold nanoparticles into the epidermis. While the delivery efficiency of this technique is quite low, only small amounts of DNA are required to achieve a significant immune response. This method has been tested for vaccines against HBV, influenza and malaria.

Furthermore, micro- and nanoparticles offer the possibility of enhancement of their uptake by appropriate cells through manipulation of their surface properties. As delineated above, upon their inoculation, particulate compounds, microspheres or nanoparticles, must reach the secondary lymphoid organs, which are the sites of the immune response. This led to design of novel methods of dermal or transcutaneous vaccination, including the use of micro- and nano-particles to target the skin APC that will then deliver consumed antigens to the LN.

Finally, pulmonary delivery (which may be especially potent and useful against influenza and other respiratory viruses) requires dry forms of vaccines that are low cost, temperature-tolerant, efficiently aerosolized, and APC-directed. Therefore, nanoparticles can play a critical role in the formulation, development and delivery of needle-less pulmonary vaccines and these are now actively pursued as well. Additionally, nanoparticles containing vaccines for the oral delivery are also investigated. These particles are 100–200 nm in diameter and are likely needed to be targeted towards a special subtype of APC called M cells that reside within gut-associated lymphoid tissue.

4. Micro-/Nanoparticle-Based Vaccines – Is There Any Risk and Can We Foresee It?

It is apparent that novel micro- and nanoparticle-based delivery vehicles are being actively evaluated in many vaccine systems. Promising results have been reported from many directions. Still, questions regarding toxicity and molecular interaction between micro- and nanoparticles and immune cells, tissues and whole organisms remain to be

addressed. There are many regulatory hurdles for new adjuvants and they are there for a reason. One of the greatest hurdles is the sheer size of population that needs to be tested to prove safety of a new adjuvant or vaccine. These numbers have dramatically increased in recent years since it became apparent that some approved drugs have rare serious and even fatal side effects that were not identified because of inadequate sample sizes during their clinical development [23]. During that time the association of adverse reactions with two vaccines resulted in their withdrawal from the market. These are nasal inactivated influenza vaccine associated with increase of cases of Bell's Palsy and also rotavirus vaccine, the administration of which lead to higher intussusception [23]. The case of adenoviral-based HIV vaccine has been mentioned above.

Reaction of parts of the society to a new vaccine may be also caused by imaginary side-effects. We have already noted that this is a case with Gardasil, a highly efficient anti-HPV vaccine, which is likely to tremendously diminish the incidence of cervical cancer and genital warts. Several conservative groups and think-tanks in the U.S. have questioned if such a vaccination will spark more promiscuity in young women. Statements like "What message are we sending to our elementary students when we inoculate them for a sexually transmitted disease in the third grade?" or "This means even Christian children who are brought up knowing that sexual activity before marriage is a sin would still be forced to be vaccinated against this STD or they could not attend school" [32] are not uncommon. But conservatives are not the only ones, who are in opposition to a mandatory vaccination against deadly disease and throughout the history of vaccination such reactions have been noted many times. It was aptly observed that groups fighting against Gardasil are very diverse and include Christian conservatives, who have long argued that safe sex encourages profligate sex, the growing antivaccine movement, which objects to all school-entry requirements and the parental-rights adherents, incensed by any mandates regarding their children's health [33]. These arguments may seem laughable or medieval to some, but they are very valid to others and it is advisable that the introduction of this great novel product of vaccine technology is done employing all possible precautions and public-relations instruments. Un-questionably, there is a lesson to be drawn. No new medicinal technology can be successful unless public is educated and well-informed of it.

It is apparent that nanotechnology is currently driving the development of novel vaccines and adjuvants. At the same time, some concerns regarding the toxicity of such small particles have appeared. Currently, there is a keen interest in nanotoxicology research since the processing of nanostructures in biological systems could lead to unpredictable and hence unknown toxic effects [34]. One may draw some lesson from the history of polio vaccine when during 1955–1963 more than 98 million Americans received one or more of its doses contaminated with polyomavirus SV40, which was then simply unknown being identified and isolated only in 1960. When it became apparent that under specific set of conditions SV40 may cause cancer in laboratory animals, this led to a serious public scare. Fortunately, subsequent studies of vaccinated humans over many years have shown that there was no causal relationship between receipt of SV40-contaminated vaccine and cancer. Such a completely unforeseen event should not be discounted when we in fact are actively engaged in genetical modification

of humans (immunized individual always contains cells of immune system, which genome is partially rearranged in a way that cells of a non-immunized individual are not).

Other potential problems include high surface area and reactivity of small particles, their involvement in catalytic and oxidative reaction, ability to cross biological membranes as well as slow biodegradability of some materials used for their manufacturing. Possibility of all of these being potential toxicity issues are at least partially supported by data describing the effects of pollutants on human health [3]. Still, it seems reasonable to anticipate that in the case of vaccines, the infrequent and low-level exposure to nanoparticles that an individual will encounter during immunization is not enough to cause adverse health problems such as those potentially attributed to nanotoxicity effects. With that being said, the development of any novel vaccine adjuvant or delivery platform should undergo all the necessary safety tests. They will also need to withstand public and political scrutiny. Recently, regulatory authorities and the general public start to be concerned with products that cross traditional lines, i.e. combine biomaterials with cells, DNA or proteins as in non-viral polymeric carriers for vaccines [35].

Currently, the most probable reason for the micro- and nano-based vaccines not flooding the market is linked to the cost of their safety testing. Necessary clinical trials required for their approval are very long and difficult. Unlike animal studies, human trials often require significant waiting period before protection can be analyzed. Furthermore, since many vaccines are often administered to healthy individuals, and frequently to infants, it is critical that they are proven safe and well-tolerated in non-human primates before entering human trials.

These risks and other possible side effects should be assessed in detail especially if mass-production and widespread administration of novel preparations is to be considered. Currently, there is an uncertainty about nanoparticle processing *in vivo* and also near-complete absence of understanding of mechanisns of their interactions with biological systems (although the latter was never necessary in the history of medicine provided that the safety of this or that useful approach is demonstrated experimentally). Therefore, continued animal research dealing with nanoparticle *in vivo* pharmakinetics and tissue distribution, nanotoxicity investigated in the same vein as drug toxicity currently is, as well as human trials for the safety evaluation of the experimental micro- and nanoparticle-based vaccine regimens will be of immense importance.

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