Bio Core
Exploring Scientific Community

International Journal of Pharma Sciences and Scientific Research

ISSN 2471-6782

Review Article Open Access

Nanotechnology For Epicutaneous Delivery Of Vaccine

P. Srinivasa babu[1], Oruganti Sai Koushik[1], Ramadoss Karthikeyan[*1]
[*1]Vignan Pharmacy College, vadlamudi-522213. A.P. India.

*Corresponding author: Ramadoss Karthikeyan, Vignan Pharmacy College, vadlamudi-522213. A.P. India. Email: rkcognosy@gmail.com, Mobile – 9966847127.

Citation: Ramadoss Karthikeyan et al, (2016) Nanotechnology for Epicutaneous Delivery of Vaccine. Int J Pharm Sci & Scient Res.2:5, 197-208

Received November 4, 2016; **Accepted** November 12 , 2016; **Published** November 25, 2016.

Abstract

In vaccine development Nanotechnology plays a significant role increasingly. The formulations that boost antigen effectiveness are increasingly needed, whereas vaccine development orientates toward less immunogenic minimalist compositions. In vaccine formulations the use of nanoparticles permits not simply slow release and targeted delivery, but also immunogenicity and improved antigen stability. However, tasks remain due to a lack of fundamental understanding regarding the in vivo behaviour of nanoparticles, which can operate as either a delivery system to enhance antigen processing and enhance immunity. A broad overview of recent advances in prophylactic nanovaccinology is discussed in this review. Types of nanoparticles and their interaction with immune cells and the biological system are discussed. Due to concerns about the weak intrinsic instability in vivo, toxicity, immunogenicity of these vaccines, and the need for multiple administrations, improvements of conventional vaccines advancements are undoubtedly required. To overcome such problems, vaccine development has been incorporated with nano-technology platforms. The study of nanotechnology for epicutaneous delivery of pharmaceuticals and vaccines is escalating. The possibility of targeted therapy and Combined with novel pharmacokinetics, nanotechnology-based vaccines might have the potential to open therapeutic avenues for treating infectious disease and malignancy than the present vaccines.

Key words: Vaccine, Nanoparticle, Adjuvant, Vaccine delivery

Introduction

Immunization is a way of rendering protective shield to the body, i.e. immunization or vaccination is a prophylactic approach by which the body is shielded or made strong enough to fight against any incoming pathogenic encroachment. Thus immunization generates a force that fights with various microorganisms and their products, which enter by different routes in the body. The immunology is a multidisciplinary science that involves different approaches of vaccination. Immunization is a two-century-old science of prophylaxis. It came into being in 1796 when Jenner studied that inoculation of cowpox virus prevents small pox in human. Later this discovery the cow pox vaccination came up into clinical practice worldwide in 19th century. The science of immunization peaked to new heights in late 19th century to early 20th century and during World War II. After postulation of germ theory by Louis Pasteur, vaccinology never reviewed. The advent of tissue culture techniques revolutionized the immunization accesses. A number of new vaccines with different approaches like live/attenuated bacterial or viral vaccines, belted down bacterial suspension, toxins acquired by bacterial toxoids, rickettesial suspension have been developed By 1929 a number of vaccines had been developed, which lists as cholera, typhoid, plague and diphtheria. Apart from development of vaccine the mechanism behind the generation of the protective shield after immunization such as humoral as well as cellular immunity were also explicated. The development entails for quantification of antibodies by Ehrlich leads to the practical reality of concept of passive immunization by Von Behring. Immunization approaches are basically divided broadly into:

Active immunisation

Passive immunisation

The period after 1930 up to 1950 is called transition period during which various vaccines were in their developmental stage. In this era Good Pasteur showed that virus can grow in embryonated hen's egg. This leads to the development of vaccines for yellow fever

used widely in tropical countries. The period after 1950 is believed a modern era of immunization science. This era has contributed many new and innovative concepts of immunization. During this era bacterial and viral capsular polysaccharide vaccines were developed. This approach is applied for preparation of vaccines against pneumococcus, menin-gococcus and Haemophilus influenza. The poliomyelitis vaccine was developed and the live vaccine for poliomyelitis became the paradigm for the prevention of poliomyelitis worldwide. Several innovative concepts were employed to develop a number of polysaccharide, recombinant and subunit type of vaccines. [1-3]

NOVEL VACCINATION STRATEGIES

Vaccination or immunization against viral diseases is very complicated and largely depends on subunit vaccines. The subunit vaccines are imagined to be the extension of recombinant vaccines. The newer and future vaccines rely on the specific antigens (or) epitopes as well as on in vivo production of antigen using the concepts of genetic immunization (DNA immunization). The current vaccination approaches are based on 'WHAT' to present and 'HOW' to present. Currently emphasis is given to make vaccines more acceptable by understating burden of pain, distress and other common adverse reactions. The topical and oral routes are now considered as preferred routes for delivery of immunogenic substances. The topical route is having an edge over other routes because:

i. It prevents unnecessary invasion to body.

ii. It prevents or bypasses the problems related to degradation of peptidal vaccines as in case of oral route.

iii. It drains the antigens or carrier associated antigens to the lymphatic system and hence to Lymph nodes.

iv. It prevents unnecessary toxicity encountered in case of immunization by other routes.

For topical immunization skin is the target site.

Various routes within the skin are used for the delivery/targeting of antigen to the specialized cells. These include follicular pathway, normal pores present in the skin, lamellar lipid bodies and through corneocytes. Skin can normally allow the molecules not greater than 500Da to penetrate and at fairly low rate, when applied epicutaneously. Therefore, for large molecules, some specialized carrier systems are needed to transport them across the skin in immunologically active form. To develop a carrier for the topical immunization by any immunogen or DNA one must take into account the flux across the skin. The skin like other organs is histologically well-organized into different layers that perform different functions. The surface epithelial layer is epidermis and below this lies connective tissue layer referred to as dermis. The two layers are together called the 'cutis'. The cutis is placed over adipose tissue layer called hypodermis. The epidermis consists of around 90% highly keratinized cells, keratinocytes (corneocytes). These cells differentiate into non-viable tissues and constitute the barrier layer, i.e. stratum corneum on the skin surface. The skin is exploited as a route for immunization, i.e. topical immunization because it shows specific (immunity) as well as non-specific (inflammation) responses for foreign substances. These responses are a result of presence of immunocompetent cells within the skin, which include Langerhan's cells (LC), dendritic epidermal T-cells and epidermotropic lymphocytes. The mast cells also represent the immunocompetent cells of dermis. Other cells present in the skin are resident antigen presenting cells and transient inflammatory lymphoid cells (e.g., poly morphonucleocytes, monocytes and lymphocytes). Skin consists of SALT (skin associated lymphoid tissue) responsible for the specific and non-specific responses. The SALT constitute of the epidermal antigen presenting cells (APC) and migratory T-lymphocytes in circulation, which have avidity for the epidermal tissues. The existence of SALT in the skin is supported by the cytokinins, which have capacity to regulate the immune responses. The antigens that come in contact with the epidermis and hence in contact with the antigen presenting cells are taken to the lymph nodes by means of the lymphatics, because migratory T-cells are attracted towards the peripheral lymph nodes. After binding to high endo the lialvenules (HEV) they enter into the lymph nodes. The accumulation of T-lymphocytes gives rise to immunological response. Cells responsible for immunogenicity of skin Langerhans cells constitute about 3-5% of epidermal cells and have receptor for Fc portion of immunoglobulins and complement protein C3b, and have high ATPase activity. They also have genes for MHC II molecules. The Birbeck granules are marker of Langerhan's cells.

The mast cells of dermis take part in contact hypersensitivity responses and produce various vasoactive compounds such as serotonin and histamine. These granules when brought out cause enhanced permeability of the blood vessels resulting in edema and erythema. The mast cells on stimulation initiate inflammatory response and IgE mediated immediate type hypersensitivity. The cells bearing avidity for skin are epidermotropic; include lymphocytes, monocytes and polymer pho nuclear cells. The urticaria seen at the immune and inflammatory region is a result of accumulation of epidermotropic T-cells. The epidermotropic T-cells having power to present antigen in the form of CD3complex have receptors for skin endothelium. Skin endothelium expresses cell adhesion molecules, which facilitate their extravasation. Dendritic epidermal T-cells act as antigen presenting cells (APCs) and possess activity similar to natural killer cells (NKC) but their accurate function is yet unidentified. Keratinocytes (corneocytes) represent another cell type, which under normal conditions do not convey any immuno-competency but when induced by certain skin diseases they express HLD-DR antigens. The stimulus by g-interferon may also lead to the induction of cell surface class II molecule (MHC II). Even though they are having ability to express MHC II molecules, they have very little capacity for antigen presentation. This is because they are not active under normal conditions. These immuno-competent cells are targeted by means of topical delivery of immunogens in order to give immunity. But afterwards the skin, especially the stratum corneum acts as a barrier for the transfer of immunogen, a delivery system or any other physical method to carry over is essential.

TOPICAL DELIVERY

Topical and transdermal delivery is a field of concern for researchers to hand over the drugs, bioactive molecules (enzymes, DNA, RNA) and immunogens. The topical route has been employed by the pharmaceutical industry for delivery of various low molecular weight drugs. Some of the approaches used for smaller compounds may also have potential for delivery of either protein or polynucleotide vaccines. However, there is a greater challenge in delivering large molecular weight molecules through the skin due to size, charge and other physicochemical properties. The topical application of antigen and adjuvant directly onto intact skin, can safely and effectively elicit systemic immune responses in mice and humans against a variety of antigens. But since stratum corneum is a barrier for channelize of drugs to the dermal and epidermal sites, number of approaches has been researched for the efficient delivery of bioactive molecules. These approaches include:

Non-invasive approaches

Invasive approaches

TOPICAL IMMUNIZATION

One of the major impediments in ensuring vaccine efficacy and compliance is its safe and effective delivery. Now, most of the vaccines are given by intramuscular injection. However, they suffer from certain drawbacks, such as requirement of periodic boosters, need of qualified personnel, patient discommode and possibility of transmission among patients of certain diseases, like AIDS, due to needle pricking. On the other extreme, topical immunization puts up some potential advantages, i.e. it is a needle free method and ensues increased patient compliance and obviates complexities related to physical skin penetration. TI provides access to skin immune system, which is prevailed by potent Antigen Presenting Cells (APCs), Langerhans cells that can be manipulated by adjuvants to orchestrate specific, robust immune responses ensuing in high IgA/IgG ratio in serum. TI has potential for human vaccination and no serious vaccine associated with adverse reaction has been reported. Furthermore, simple method of administration in TI excludes the necessity of any trained personnel for vaccination. The topical and oral routes are now regarded as preferred routes for immunogen delivery. The topical route is having an edge over other routes because:

1. It forbids unneeded invasion to body.

2. It prevents the problems related to degradation of peptidal vaccines as in the case of oral route.

3. It drains the antigens or carrier related antigens to the lymphatic system and therefore to lymph nodes.

4. It prevents unnecessary toxicity encountered in case of immunization by other routes.

Skin is the target site for topical immunization. TI alters efficient delivery of vaccine antigen to epidermis harbouring immunocompetent Langerhans cells. Skin can normally allow the molecules not greater than 200 -350 Da to penetrate when applied epicutaneously. Therefore, for large molecule, some specialized carrier systems are needed to transport them across the skin in immunologically active form.

DELIVERY CONSIDERATIONS

In order for vaccine to be effective, it is significant that it should be delivered to epidermis, where the APCs are localized. The stratum corneum is the main obstacle against effective transdermal delivery. Each animal species possesses unique skin barriers that must be overcome to evoke a potent immune response by TI. In addition to antigen characteristics that may influence their delivery via the skin, several parameters important for optimizing TI for each species include differences in skin structure, the total surface area of the skin and the appropriate location for topical application.

SKIN COMPOSITION

For successful immunization by TI, it is crucial to understand the skin characteristics of each species of interest. Substantial variation occurs between species both in structural characterization and in lipid composition of skin. To help the development of targeted and topically employed vaccine formulation, a thorough understanding of the composition of the stratum corneum is required. The stratum corneum has a complex structure composed of closely associated cellular and lipid components. In the lipid composition of human stratum corneum is presented. Each species has a unique composition of lipid in the stratum corneum, which needs to be taken into consideration when designing TI formulations. For TI to provide maximum efficacy, the vaccine constituent (adjuvant and a co-administered antigen) must penetrate the surface lipid, the sebum and the stratum corneum, containing both cells and lipids, for adequate uptake and processing by immune cells, most likely LCs.

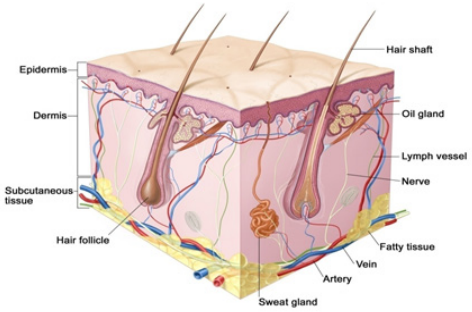

Figure 1 Shows the composition of the skin with possible delivery route

Transportation Route through Skin

Normal skin is impervious to most substances. Stratum corneum contributes over 80% to this transport resistance. The stratum corneum (10-20µm thick) consists of terminally differentiated keratinocytes called corneocytes. Corneocytes are 0.3µm thick coordinated in parallel, partly overlapping multicellular stacks perpendicular to the skin surface. Group of 3 to 10 corneocytes stacks forms a cluster that is separated from the other cluster by clefts or gorges. Each gorges has a uniform width (4-6µm) and depth (3-5µm). Lipid material between the corneocytes is not only ample but also highly organized and this behaves as extra intercellular "glue" sealing the spaces between the cells in the skin. The multicellular lipids in the horny layer mainly encompass the relatively non-polar substances, such as free fatty acid, cholesterol and cholesteryl esters, in addition to more than a dozen ceramides. Owing to the fairly long aliphatic chains of the latter and due to the low overall lipid polarity in the skin, the inter corneocyte lipids are tightly packed and at least locally seen as the lipid multi lamellae. All these contribute to the tightness and impermeability of the intact skin; hence, it is very difficult to bring molecule with a molecular mass greater than 200-350 Da size efficiently across the intact skin. Accomplishing the similar task for the molecule greater than 750 Da size is practically impossible, even when these molecules have an ideal solubility in the skin. Only after elimination of the stratum corneum from the skin, e.g. by tape stripping or after lipid extraction from the horny layer (e.g. with ethanol or acetone), it does allow material transport across the skin with a dramatic increase. The possible macro routes comprise the trans-epidermal pathway (across the horny layer either intracellularly or inter cellularly) or via hair follicles and sweat glands (the appendageal way). The specific pathway that a vaccine antigen will follow depends on the physico-chemical characteristics of antigen as well as the method of delivery used. There are three possible pathways for penetration of compounds through the skin the intercellular pathway, the transcorneocyte pathway and the transappendageal pathway.

INTER CELLULAR PATHWAY

In this pathway, the molecules travel through extracellular surrounding the corneocytes. Compounds delivered in liposomes generally use intercellular lipids, which form continuous pathways pathway for entry. Penetration of liposomes through extracellular lipids has been shown by liposome-skin interaction studies using a fluorescent lipid bilayer marker and confocal laser scanning microscopy.

TRANSCORNEOCYTE PATHWAY

In this pathway, the delivery occurs through the corneocytes. Physical method of delivery such as gene gun and electroporation use the transcorneocyte pathway.

TRANSAPPENDAGEAL PATHWAY

In this route, the compound travels through hair follicles and sweat glands. This pathway allows the stratum corneum to be evaded by allowing the biomolecules to travel around it to the cells surrounding the hair follicles. Epithelial cells surrounding the hair follicles constitute a much less resistant barrier than the stratum corneum. Diameter of hair follicles ranges from 50 to 100µm and hair follicles range in density from 10% on areas such as scalp to 0.1% in area with low follicle density. The transappendageal route is thought to be the major route by which large molecules such as oligonucleotide or liposome-complexed DNA enter the skin.

TRANSPORTATION INTO AND ACROSS THE SKIN

Material administration on the skin creates a concentration gradient between the application site and the skin interior. This drives the applied molecule through the skin in proportion to the skin permeability and the involved skin area Any epicutaneously administered solute is thus pushed across the skin by its inward directed concentration gradient. Simultaneously, any such entity attracts water towards the skin surface while being itself attracted into the water rich (subcutaneous) compartment by intrinsic hydration pressure difference. In general, the efficacy of the permeant transport across the skin, hence, depends on the relative magnitude of the trans-barrier water and permeant flow (or permeability). The maximum useful permeant mass on the skin is limited by permeant solubility. This also limits the maximum achievable material flow across the intact skin, when the permeant flux is driven merely by the permeant concentration gradient. This problem can be overcome mainly by three approaches:

1. By enlarging the application area.

2. By enhancing the skin permeability using permeation enhancer.

3. By activating concentration-independent transport driving forces. This approach has been realized by applying an electric (in ionotophoresis) or mechanical force on the skin (in various jet devices). Non-occlusively applied drug carrier on the skin is the most recent and an elegant variation of this principle.

For a lipid vesicle to be driven through an opening smaller than vesicle diameter, the activation energy rises with the cost of bilayer deformation. The latter is deduced from the standard model of membrane elasto-mechanics to increase with some power of the effective membrane elasticity. Lipid vesicle penetration through the skin is a function of membrane deformability and thus inversely proportional to the energy of elastic membrane deformation, which in turn is composition dependent.

Topical and transdermal delivery is a field of interest for delivery of bioactives and immunogens. Since stratum corneum is a barrier for transfer of drugs to the dermal and epidermal sites, number of approaches has been explored for efficient delivery of bioactives. These approaches include:

1. Physical–Ionotophoresis, Gene gun, Laser pulse, Ultra sound waves.

2. Chemical (Permeation enhancer)–Azones, Dimethyl formamide (DMF), Dimethyl suphoxide (DMSO).

3. Vesicular–Liposomes, Niosomes, Transfersomes.

4. Topical Genetic Immuno stimulation.

The physical approaches such as electroporation can be applied for the transfer of bioactive molecules across the stratum corneum. Electroporation is used for the delivery of genes to the keratinocytes for immunization as well as for gene therapy. The majority of protocols to raise the permeability of the epidermis include utilization of chemicals, such as surfactants, alcohols and polyols. They increase the permeability of the stratum corneum by any of the following mechanisms or combination of them:

a) Enhancing the fluidity of skin lipids,

b) Hydrating the polar pathways,

c) Opening heterogeneous multi laminate pathways, and keratolytic action.

These chemicals do not increase the permeability of the bio actives to the coveted extent. Only up to five times, the permeability can be enhanced by this method. However, permeability is still less in case of high molecular weight molecules. The chronic use of these chemicals for permeability enhancement may have dangerous side effects.

An ideal vaccine

1. It should not be toxic or pathogenic, i.e. it should be safe.

2. It should have very low levels of side effects in normal individuals.

3. It should not cause problems in individuals with impaired immune system.

4. It should not spread either with in the vaccinated individual or to other individuals live vaccine.

5. Should produce long lasting humoral and cellular immunities.

6. The technique of vaccination should be simple.

7. The vaccine should be cheap so that it is generally

8. It should not contaminate the environment.

9. It should be effective in affordable.

10. So far, such an ideal vaccine has not been developed.

NEEDFORNEWVACCINES

There are certain infectious diseases for which no vaccines are available e.g. HIV, tuberculosis, malaria, Neisseria meningitides Type B, etc. Using traditional approaches to vaccine development, these kinds of diseases are found to be extremely difficult to control. Thus, there is a clear need for the development of new vaccines against these kinds of diseases. New vaccines are requisite to protect against the influenza virus and antimicrobial resistant organisms. Threat of bioterrorism is also an important reason for the development of new vaccines. There are various emerging infectious diseases, including West Nile, SARS, and Ebola & (anta, which, if not controlled, would lead to a mass destruction of human kind. New vaccines have to be developed for the prevention of these diseases. There are various microorganisms which cause chronic diseases (Hepatitis B&C viruses cause (hepatocellular carcinoma, (human papilloma virus causes cervical, anal & vulvar cancer & Epstein-Barr virus causes Burkitt lymphoma. These chronic diseases can be prevented only by novel vaccines. Vaccines are the potential therapeutic agents which can be used to treat established infections. Thus, novel vaccine delivery technologies will be required to enable the development of these new vaccines. Traditional vaccines, although highly effective and relatively easy to produce at low cost, suffer from the following limitations:

• Inman cases, live vaccines have to be used since killed pathogen vaccines are in effective.

• Live vaccines are generally based on cultured animal cells; hence expensive tissue culture setup is essential.

• Live vaccines are heat labile.

• Traditional vaccines carry a variable risk of disease development due to the occasional presence of active virus particles or reversion to virulence after replication in the vaccinated individuals.

• In many cases, they are difficult to produce, e.g., hepatitis virus does not grow in highlighter in cultured cells.

• These limitations have prompted the development of new vaccines, which are rather costly, at least for the present.

NEEDLE¬FREEVACCINEDELIVERY

Importance and need

• The World health Organization (WHO) estimates that 12 billion injections are given annually, and that 5% of these are immunizations.

• The development of needle-free delivery systems for vaccines has been named one of the Gr and Challenges in Global health.

• Needles are associated with an increased risk of infection, especially in developing countries, where there are problems with needles being reused and issues with waste management and disposal. Needle free delivery systems would make vaccines easier to deliver.

• Compliance would be significantly improved, with people more likely to avoid an injected vaccine because of fear and pain associated with the needle, especially children.

• Needle-free vaccines can reduce cost of immunization as they can be delivered without medical intervention.

• Topical vaccines are cheaper and easier to transport and store, than injectable vaccines, which generally require refrigeration.

• Needle stick injuries area significant problem in both developed and developing countries. It is estimated that 5 in every 100 injections worldwide result in a needle stick accident. The introduction of needle-less vaccines would significantly reduce the risk and incidence to health care workers.

• Some progress has been made with oral polio, cholera and rotavirus vaccines. But diphtheria, tetanus, pertussis, varicella, measles, mumps, rubella, tuberculosis and yellow fever are all still

injectable vaccines.

• The current market for vaccines is worth approximately $9 billion globally with a 1012% annual growth rate.

TYPES OF NANO PARTICLES

Polymeric nano particles

A great assortment of synthetic polymers are used to prepare nanoparticles, such as poly (d,l-lactide-co-glycol ide) (PLG), poly (d,l-lactic-co-glycolic acid) (PLGA), poly (g-glutamic acid) (g-PGA), poly (ethylene glycol) (PEG), and polystyrene. PLG and PLGA nano particles have been the most widely investigated due to their excellent biocompatibility and biodegradability. These polymeric nano particles entrap antigen for delivery to certain cells or genre lease by virtue of theirs low biodegradation rate. PLGA has been used to carry anti gen derived from various pathogens including Plasmodium vivax with mono-phosphoryl lipid A as adjuvant hepatitis virus (HBV), Bacillus anthracis, and

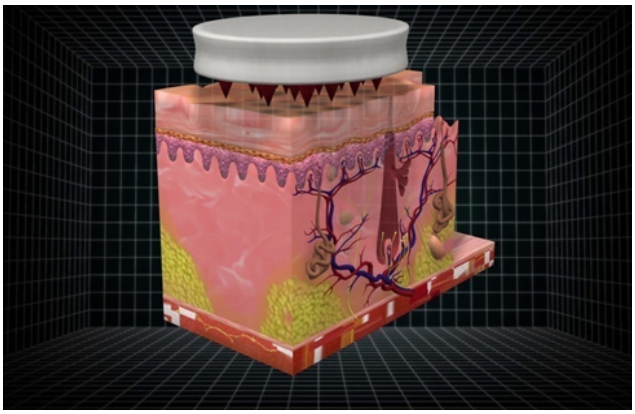

Figure 3: Shows the clear mechanism of needle free vaccine

model anti gens such as ovalbumin and tetanus toxoid. g-PG A nano particles are comprised of amphiphilic poly (amino acid)s, which self-assemble into nano-micelles with a hydrophilic outer shell and a hydro phobic inner core. g-PG A nano particles are generally used to encapsulate hydrophobic antigen. Polystyrene nanoparticles can conjugate to a variety of antigens as they can be surface-modified with various functional groups. Natural polymers based on polysaccharide have also been used to prepare nano particle adjuvants, such as pullulan, alginate, inulin, andchitosan. In particular, chitosan-based nano particles have been widely studied due to their bio compatibility, biodegrade ability, nontoxic nature and their ability to be easily modified into desired shapes and sizes. These nano particles have been used in the preparation of various vaccines including HBV, Newcastle disease vaccines, and DNA vaccines. Inulin, a well-known activator of complement via the alternative pathway, is also a potent adjuvant. Nano particle adjuvants derived from inulin, such as AdvaxTM, have shown enhancement of immune response in vaccines against various viruses including influenza and hepatitis B. Polymers, such as Poly(L-lactic acid) (PLA), PLGA, PEG, and natural polymers such as polysaccharides, have also been used to synthesize hydro gel nanoparticles, which area type of nano-sized hydrophilic three-dimensional polymer network. Nano gels have favourable properties including flexible mesh size, large surface area for multivalent conjugation, high water content and high loading capacity for antigens. Chitosan nano gels have been widely used in antigen delivery, such as Clostridium botulinum type-A neuro toxinubun it antigen Hc for an adjuvant-free intranasal vaccine, and recombinant Nc PDI antigen for Neospora caninum vaccination.

IN ORGANIC NANO PARTICLES

Many inorganic nano particles have been studied for their use in vaccines. Although these nanoparticles are mostly non-biodegradable, the advantage of them lies in their rigid structure and controllable synthesis. Gold nanoparticles (Au NPs) are used in vaccine delivery, as they can be easily fabricated into different shapes (spherical, rod, cubic, etc.)With a size range of 2–150nm, and can be surface-modified with carbohydrates. Gold nano rods have been used as a carrier for an anti gen derived from respiratory syncytial virus by conjugating the antigen to the surface. Other types of gold nanoparticles have been used as carriers for anti gens derived from other viruses such as influenza and foot-and-mouth disease, or as a DNA vaccine adjuvant for human immunodeficiency virus (HIV). Carbon nanoparticles are another commonly-studied composition for drug and vaccine delivery. They are known for their good biocompatibility and can be synthesized into a variety of nanotubes and mesoporous spheres. The diameter of carbon nanotubes (CNTs) used as carriers is generally 0.8–2nm with a length of 100–1000nm, while the size of mesoporous carbon spheres is around 500 nm. Multiple copies of protein and peptide antigens can be conjugated on to CNTs for delivery and have enhanced the level of IgG response. Mesoporous carbon nano particles have been studied for application as an oral vaccine adjuvant. One of the most promising inorganic materials for nano vaccinology and delivery system design is silica. Silica-based nano particles (SiNPs) are bio compatible and have excellent properties as nano carriers for various applications, such as selective tumor targeting, real-time multi modal imaging, and vaccine delivery.

The SiNPs can be prepared with tunable structural parameters. By controlling the sol–gel chemistry, the particle size and shape of SiNPs can be adjusted to selectively alter their interaction with cells. The abundant surfaces silanol groups are beneficial for further modification to introduce additional functionality, such as cell recognition, absorption of specific biomolecules, improvement of interaction with cells, and enhancement of cellular uptake. In addition, porous SiNPs such as mesoporous silica nanoparticles (MSNs) and hollow SiNPs can be prepared by templating methods, which can be applied as a multi-functional platform to simultaneously deliver cargo molecules with various molecular weights. MSNs with sizes in the range of 50–200nm have been studied as both nano-carriers and adjuvants for delivery of effective antigens, such as those derived from porcine circovirus and HIV. MSNs can be used to control their lease of antigens by controlling the shape, pore size and surface functionalization. Compared to solid SiNPs, MSNs have higher loading capacity for their larger specific surface area, and better performance in delivery and controlled release due to the tunable hollow and mesoporous structure. In addition, MSNs can be degraded which can then be excreted in the urine. With these properties, MSNs show potential to become high-efficiency, controlled-release nano-carriers in future vaccine formulations. Calcium phosphate nano particles can be created by mixing calcium chloride, di basic sodium phosphate and sodium citrate under specific conditions. They are non-toxic and can be formed into a size of 50–100nm. These nanoparticles are useful adjuvants for DNA vaccines and mucosal immunity, and show excellent bio compatibility.

LIPOSOMES

Liposomes are formed by bio de grad able and nontoxic phosphor lipids. Liposomes can encapsulate antigen with in the core for delivery and incorporate viral envelope glycoproteins to form virosomes including for influenza. Combination of 1, 2-dioleoyl-3-trimethyl ammonium propane (DOTAP) modified cationic liposome and a cationic polymer (usually protamine) condensed DNA are called liposome poly cation DNA nanoparticles (LPD), a commonly used adjuvant delivery system in DNA vaccine studies. The components of LPD spontaneously re arrange into a nano-structure around 150n min size with condensed DNA located inside the liposome. Liposomes modified with maleimide can be synthesized into inter bi layer-cross linked multi- lamellar vesicles (ICMVs) by cation driven fusion and crosslinking enabling slowed release of entrapped antigen. A number of liposome systems have been established and approved for human use, such as Inflexal V and Epaxal, which have been discussed in other reviews.

IMMUNO STIMULATING COMPLEX (ISCOM)

ISCOMsarecagelikeparticlesabout40nmlargeinsize, made of the saponin adjuvant Quil A, cholesterol, phospholipids and protein antigen. These spherical particles can trap the antigen by a polar interaction. ISCOMATRIX comprises ISCOMs without antigen. ISCOMATRIX can be mixed with antigen, enabling more flexible application than is possible for ISCOMs, by removing the limitation of hydrophobic antigens. Various antigens have been used to form ISCOMs, including antigens derived from influenza, herpes simplex virus, HIV, and Newcastle disease.

VIRUS-LIKE PARTICLES

Virus-like particles (VLP) are self-assembling nano particles, lacking infectious nucleic acid, formed by self-assembly of biocompatible capsid proteins. VLPs are the ideal nano vaccine system as they harness the power of evolved viral structure, which is naturally optimized for interaction with the immune system, but avoid the infectious components. VLPs take the good aspects of viruses and avoid the bad. The naturally-optimized nano particle size and repetitive structural order means that VLPs induce potent immune responses, even in the absence of adjuvant. VLP based vaccines are the first nanoparticle class to reach market the first VLP vaccine for hepatitis B virus was commercialized in 1986 and have become widely administered in healthy populations. In nano vaccinology, VLP nanoparticles have the strongest evidence base for safe use in healthy humans. Newer VLP vaccines for human papilloma virus and hepatitis E have been approved for use in humans in 2006 and 2011, respectively. VLPs can be derived from a variety of viruses, with sizes ranging from 20nm to 800nm, and can be manufactured with a variety of process technologies. The historical approach to VLP manufacture involves an in-vivo route, where the assembly of capsid proteins into VLPs occurs inside the expression host. The assembled particle is then purified away from adherent and encapsulated contaminants. In some cases it becomes necessary to disassemble and then re-assemble the VLP to improve quality; recently approved VLP vaccines typically include some aspect of extra cellular assembly with in the processing regime. An emerging approach for VLP assembly is through cell free in-vitro processing. This approach inverts the traditional assemble then purify paradigm; large scale purification of the VLP building blocks from contaminants occurs first, then these are assembled in-vitro, avoiding the need tod is assemble VLP structures after assembly in a cell. Further review of VLP manufacturing approaches is available elsewhere. VLPs commercialized to date are based on self-assembly of proteins derived from the target virus. However, VLPs can also act as a delivery plat form where a target antigen from a virus unrelated to the VLP used is modularized on the surface of a VLP. These modular VLPs exploit known benefits of VLPs (optimized particle size and molecular structure) to target disease in an engineered fashion. With many VLP vaccines currently in clinical or pre-clinical trials, an increase in the number of approved VLP-based vaccines can be expected.

SELF-ASSEMBLED PROTEINS

Recognizing the power of the VLP approach, self-assembling systems that attempt to drive higher levels of protein quaternary structuring have emerged for the preparation of nano particle-based vaccines. Ferritin is a protein that can self-assemble into nearly spherical 10nm structure. By genetically fusing influenza virus haem-agglutin (HA) to ferritin, the recombined protein spontaneously assembled into an octahedrally symmetric particle and reformed 8 trimeric HA spikes to give a higher immune response than trivalent inactivated influenza vaccine, which typically is processed to destroy rather than build viral structure. This example highlights the importance of driving higher order

molecular structure in modern vaccines. The major vault protein (MVP) is another kind of self-assembling protein. Ninety six units of MVP can self-assemble into a barrel-shaped vault nano particle, with a size of approximately 40nm wide and 70nm long. Antigens that are genetically fused with a minimal inter action domain can be packaged inside vault nano particles by self-assembling process when mixed with MVPs. Vault nano particles have been used to encapsulate the major outer membrane protein of Chlamydia muridarum for studies of mucosal immunity.

EMULSIONS

Another type of nano particles used as adjuvants in vaccines delivery is nano sized emulsions. These nano particles can exist as oil-in-water or water-in-oil forms, where the droplet size can vary from 50nm to 600nm. Emulsions can carry antigens inside their core for efficient vaccine delivery or can also be simply mixed with the antigen. One commonly used emulsion is MF59, an oil-in-water emulsion which has been licensed as a safe and potent vaccine adjuvant in over 20 countries. It has been widely studied for use in influenza vaccines. Another is Montanide, a large family of both oil-in-water and water-in-oil emulsions, including ISA 50V, 51, 201, 206 and 720. Montanide ISA 51 and 720 have been used in Malaria vaccines, Montanide ISA 201 and 206 have been used in foot-and-mouth disease vaccines. Recently, a tailorable nano sized emulsion (TNE) platform technology has been developed using non-covalent click self-assembly for anti gen and drug delivery. An oil-in-water nano emulsion is formed using designed bio surfactant peptides and proteins. Using a self-assembling peptide-protein system, immune-evading PEG and a receptor-specific antibody can be arrayed in a selectively proportioned fashion on the aqueous interface of a nano-sized oil-in-water emulsion. Targeted delivery of protein antigen to dendritic cells was achieved. This work demonstrates a new and simple way to make biocompatible designer nano emulsions using non-covalent click self-assembly by sequential top-down reagent addition.

NANOPARTICLE INTERACTIONS WITH ANTIGEN

Vaccine formulations comprising nanoparticles and antigens can be classified by nanoparticle action into those based on delivery system or immune potentiator approaches. As a delivery system, nanoparticles can deliver antigen to the cells of the immune system, i.e. the antigen and nanoparticle are co-ingested by the immune cell, or act as a transient delivery system, i.e. protect the antigen and then release it at the target location. For nano particles to function as a delivery system, association of antigen and nano particle is typically necessary. For immune potentiator approaches, nanoparticles activate certain immune pathways which might then enhance antigen processing and improve immunogenicity. Hard material nanoparticles, such as those based on silica, gold, and calcium phosphate, have predominantly been examined for use

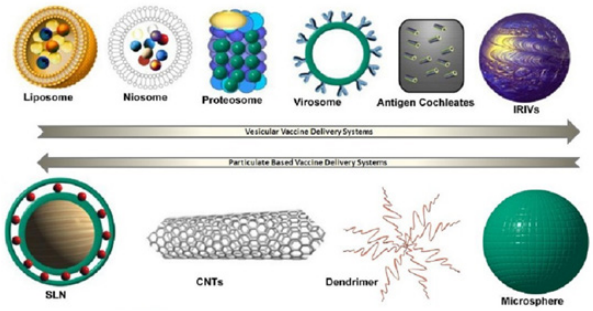

Figure 2: Shows the currently available Nano carriers for epicutaneous delivery

as a delivery system and have thus been engineered to promote antigen attachment. Attachment of antigen has been achieved through simple physical adsorption or more complex methods, such as chemical conjugation or encapsulation. Adsorption of antigen onto a nanoparticle is generally based simply on charge or hydrophobic interaction. Therefore, the interaction between nano particle and antigen is relatively weak, which may lead to rapid is association of antigen and nanoparticle invivo. Encapsulation and chemical conjugation provide for stronger interaction between nanoparticle and antigen. In encapsulation, antigens are mixed with nano particle precursors during synthesis, resulting in encapsulation of antigen when the precursors particulate into a nanoparticle. Antigen is released only when the nanoparticle has been decomposed invivo or inside the cell. On the other hand, for chemical conjugation, antigen is chemically cross-linked to the surface of a nanoparticle. Anti gen is taken up by the cell together with the nano particle and is then released inside the cell. In soft matter nanoparticle delivery system, such as those based on VLPs, ISCOM, IS COMATRIX, or liposomes, attachment of antigen is achieved through chemical conjugation, adsorption, encapsulation, or fusion at DNA level. For nanoparticles to act as an immune potentiator, attachment or interaction between the nanoparticle and antigen is not necessary, and maybe undesirable in cases where modification of antigenic structure occurs at the nanoparticle interface. Soft-matter nano particles, such as emulsion-based adjuvants MF 59 and AS03, have been shown to adjuvant a target antigen even when they are injected independently of, and before, the antigen. Building on this idea, formulation of immune potentiator nanoparticles with a target antigen could be possible through simple mixing of nano particle and adjuvant, shortly prior

to injection, with minimal association between nano particle and antigen needed. This approach has only recently been investigated for hard-material nanoparticle adjuvants, with results suggesting that nanoparticles may act as a size-dependent immune potentiator adjuvant even when not conjugated to the antigen. This new finding is consistent with a number of other studies that have demonstrated induction inflammatory immune responses after injection of hard material nano particles alone and without antigen. Further studies into the use of nano particles as immune potentiating adjuvants are clearly needed. As the interaction of nano particles with the immune system becomes more fully understood, we expect their impact to be broadened.

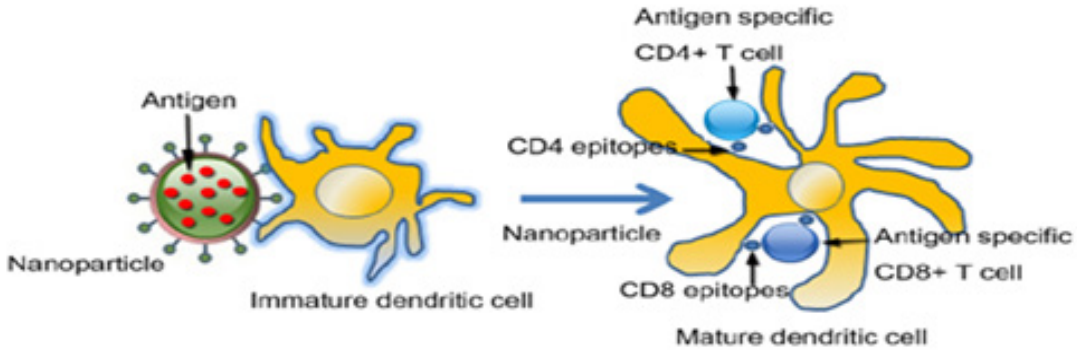

Figure 4. Shows the Nano particle interactions with antigen

NANO PARTICL EINTERACTIONS WITH ANTIGEN PRESENTING CELLS

Incorporatingantigeniccomponentsintonanoparticleshasattractedextensiveinterestwithafocusonhowtodeliverantigenmoreefficientlytoantigenpresentingcells(APCs)andsubsequentlyinducetheirmaturationandcrosspresentationofantigenforactivationofapotentimmuneresponse.AsspecializedAPCswhichefficientlyuptakeandprocessantigen,dendriticcells(DCs)andmacrophagesareoftentargetedinvaccinedesign.GoodunderstandingofDCandmacrophageuptakemechanismsandinteractionsofNPswiththesecellsisthereforeveryimportantfordevelopingefficaciousnanoparticlevaccines. Studieshavereportedthatsize,chargeandshapeofnanoparticlesplaysignificantrolesinantigen uptake. Generally, nanoparticles having a comparable size to pathogens can be easily recognized and are consequently taken up efficiently by APCs for induction of immune response. DCs preferentially uptake virus-sized particles (20–200nm) while macrophages preferentially up take larger particles (0.5–5m). In an Invitro study using polystyrene particles ranging from 0.04m to 15m, the optimum size for DC uptake was found to be smaller than 500nm. Similarly, 300nm sized PLGA particles also showed higher internalization and activation of DC sin comparison to 17, 7 and 1 m particles. Higher uptake of smaller PLA particles (200–600nm) in comparison to larger ones (2–8m) has also been reported for uptake by macrophages. Different studies however, show discrepancies in optimum nano particle vaccine size. Amphiphilic poly (amino acid) (PAA) nanoparticles of 30nm were shown to have a lower DC uptake than that of 200nmnano particles. Polyacrylamide hydro gel particles of 35nm and 3. 5min size showed no difference in macrophages uptake. These discrepancies may be related to the intrinsic differences in the material properties, with each material having an optimum size for induction of potent immune response. In addition to particle size, surface charge also plays a significant role in the activation of immune response. Cationic nanoparticles have been shown to induce higher APC uptake due to electrostatic inter actions with anionic cell membranes. Invitro studies suggested that a cationic surface could significantly enhance the uptake of polystyrene particles of micron size (~1m) by macrophages and DCs in comparison with a neutral or negative surface, but not for the smaller nanoparticles (100nm). However, other invivo studies revealed that either positively or negatively charged liposomes could act as efficient adjuvants to induce cell-mediated immune response. Furthermore, due to their electro static inter action with an ionic cell membranes, cationic particles are more likely to induce haemolysis and platelet aggregation than neutral or an ionic particles.

Particle shape plays an equally important role in the interaction between nano particles and APCs. For big particles (>1m), particles plays a dominant role in phagocytosis by macrophages as the uptake of particles is strongly dependent on locals hap eat the interface between particles and APCs. Worm like particles with high a spect ratios (>20) exhibited negligible phagocytosis compared to spherical particles. On the other hand, spherical gold nano particles (Au NPs) (40nm) were more effective in inducing antibody response than other shapes (cube androd) or the 20nm sized Au NPs, even though the rods (40nm×10nm) were more efficient in APC uptake than the spherical and cubic Au NPs. A number of studies also reported the effect of hydrophobicity, showing high-

er immune response for hydrophobic particles than hydro philic ones. A number of other factors such as surface modification (pegylation, targeting-ligands) and vaccine cargo have been shown to affect the interaction between nanoparticles and APCs as well.

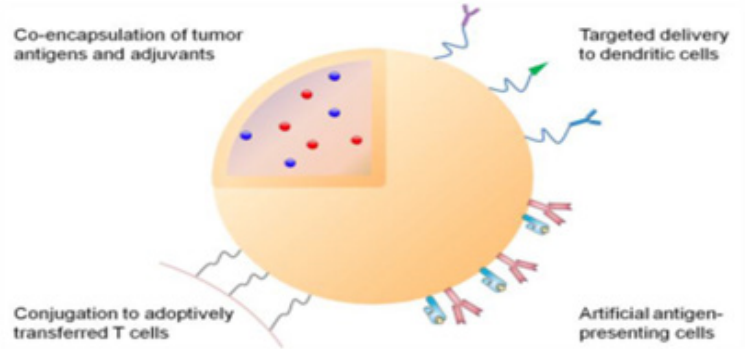

Figure 5: shows the Nanoparticle interactions with antigen presenting cells

NANOPARTICLE-BIOSYSTEM INTERACTIONS

Designing safe and efficacious nanoparticle vaccines requires a thorough understanding of the interaction of nanoparticleswithbiologicalsystemswhichthendeterminesthefateofnanoparticles invivo. Physicochemical properties of nanoparticles including size, shape, surface charge and hydrophobicity influence the interaction of nano particles with plasma proteins and immune cells. These interactions as well as morphology of vascularendotheliumplayanimportantroleindistributionofnanoparticlesinvariousorgansandtissuesofthebody.Thelymphnode (LN) is a target organ for vaccine delivery since cells of the immune system, in particular B and T cells, reside there. Ensuring delivery of antigen to LNs, by direct drainage or by migration of well-armed peripheral APCs, for optimum induction of immune response is therefore an important aspect of nanoparticle vaccine design. Distribution of nanoparticles to the LN is mainly affected by size. Nanoparticles with a size range of 10–100nm can penetrate the extra cellular matrix easily and travel to the LNs where they are taken up by resident DCs for activation of immune response. Particles of larger size (>100nm) linger at the administration point and are subsequently scavenged by local APCs, while smaller particles (<10nm) drain to the blood capillaries. The route of administration and biological environment to which nanoparticles are exposed could also affect the draining of nano particles to the LN. It was reported that small PEG coated liposomes (80–90nm) were significantly present in larger amounts in LNs after subcutaneous administration as compared to intravenous and intraperitoneal administration. In addition to targeting lymphatic organ for efficient activation of immune response, design of nanoparticle vaccines also needs to consider nanoparticle clearance from the body. Adverse effects may occur when nano particles are not degraded or excreted from the body and hence, accumulate in different organs and tissues. Clearance of nanoparticles could be achieved through degradation by the immune system or by renal or biliary clearance. Renal clearance through kidneys can excrete nano particles smaller than 8nm. Surface charge also plays an important role in determining renal clearance of nanoparticles. Few reports have suggested that for appropriate identically sized articles, based on surface charge, ease frenal clearance follows the order of positively charged<neutral<negatively charged. This may be attributed to the presence of negatively charged membrane of glomerular capillary. On the other hand, biliary clearance through liver allows excretion of nanoparticles larger than 200nm. Surface charge also plays role in biliary clearance with increase in surface charges showing increased distribution of nano particles in the liver. Furthermore, a study reported shape dependent distribution of nanoparticles where short rod nanoparticles were predominantly found in liver, while long rods were found in spleen. Short rod nanoparticles were excrete data faster rate than longer ones. In order to aid understanding of interaction of nanoparticles with immune cells and the Biosystems, many different invivo molecular imaging techniques including magnetic resonance imaging (MRI), positron emission tomography (PET), fluorescence imaging, single phot one mission computed tomography (SPECT), X-ray computed tomography (CT) and ultra sound imaging could be employed. Owing to its excellent soft tissue contrast and non-invasive nature, MRI imaging is extensively used for obtaining three-dimensional images invivo. Super para magnetic iron oxide nanoparticles (SPION) have been extensively used as contrast agents form or pho logical imaging. PET usually employs an imaging device (PET scanner) and a radio tracer that is usually intravenously injected into the blood stream. Due to high sensitivity of this technique, it is used to study the bio-distribution of particles of interest. The only d is advantage of this technique is relatively low spatial resolution as compared to their techniques. PET imaging of64Cu radio shell-crosslinked nanoparticles has been demonstrated. Fluorescence imaging facilitates imaging of nanoparticles using fluorescent tags. Dye-doped silica nanoparticles as contrast imaging agents for invivo fluorescence imaging in small animals have been reported .Now a days, more attention is being paid to synergize two or more imaging techniques that complement each other and provide an opportunity to overcome shortcomings of individual techniques in terms of resolution or sensitivity. For instance, simultaneous PET-MRI imaging is a new emerging hybrid imaging system that combines them or pho logical imaging component of MRI with the functional imaging component of PET. Multi functionality of nano particle scan be utilized for such hyphenated imaging.

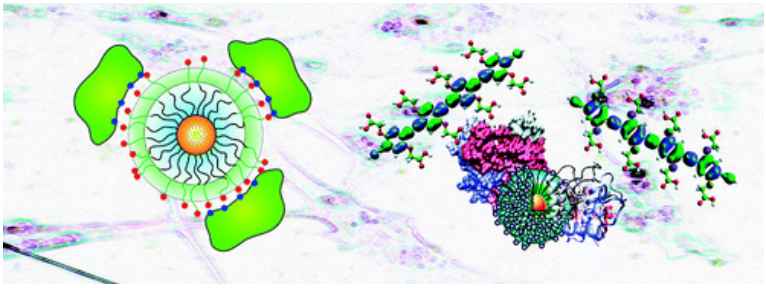

Figure 6: shows the nanoparticle bio-system interactions

FUTURE DEVELOPMENTS

Numerous studies have stated enhanced immunogenicity of nano carrier-based vaccines upon co-delivery with an immune-modulator. Single immunization of OVA and MPL co-formulated with the PLGA nano carrier induced much higher system Ic and mucosal immune responses after oral delivery than OVA alone. Micelle formulations that include a PEG-modified cationic polypeptide can co-deliver OVA and TLR3 agonists while increasing vaccine induced anti body production by more than a factor of 70. The subcutaneous vaccination of pH-responsive micelle nano particles containing amphiphilic di block copolymers conjugated to OVA and CpG oligonucleotides displayed remarkably higher CD8þT cell responses equated with the free form or a physical mixture. In addition the co-delivery of antigens and immuno-modulators, modifying the surfaces of nano carriers can contribute to the delivery of antigens specifically to relevant immune cells. Imiquimod (TLR7agonist)-entrapped PLGA nano carriers we recoated with a chitosan derivative (N, N, N-tri methylated chitosan) to improve the protective response generated by mucosal immunization. Another chitosan derivative, glycol-chitosan, was decorated on to the surfaces of PLGA nanoparticles for use in a nasal vaccination. Glycol-chitosan coated PLGA nanoparticles showed a lower clear an cerate and a higher local up take in the nasal cavity compared with chitosan-coated PLGA nano carriers. Multi-functional nano vaccines can significantly increase the immune response generated by the target-specific, effective and stable delivery of an antigen. Surface-modified nano particulate vaccines require complicated synthesis procedures that can require complex purification processes and high expenses. Scale-up processes tend to be time consuming for pharmaceutical applications. Self-assembled nano vaccine technologies would be beneficial for reducing obstacles to the development of industrial-scale manufacturing protocols.

CONCLUSION

Delivery to the skin, a specialized organ long targeted for delivery of vaccines, is blossoming into a new frontier for expanded application for vaccine delivery and for delivery of bio-therapeutics. An increased understanding of the potential advantages associated with trans-epicutaneous delivery and a growing number of components and devices with demonstrated utility around meeting the delivery challenges for these large classes of drugs have fueled interest in broader commercial application of trans-epicutaneous delivery techniques. The trans-epicutaneous devices discussed here have the potential to provide the comfort and convenience that meet the emotional needs of patients along with the delivery performance that address more fundamental therapeutic needs for many bio-therapeutics.

REFERENCES

1. Oberg AL, Kennedy RB, Li P, Ovsyannikova IG, Poland GA, Systems biology approaches to new vaccine development, Current Opinion in Immunology, 2011,vol 23, issue1, 436–43.

2. Rappuoli R, Mandl CW, Black S, De Gregorio E, Vaccines for the twenty-first century society, Nature Reviews Immunology, 2011, vol 1, issue1, 865–72.

3. Mamo T, Poland GA, Nano vaccinology: the next generation of vaccines meets 21st century materials science and engineering, Vaccine 2012, vol 30, issue2, 6609–11.

4. Couvreur P, Vauthier C, Nanotechnology: intelligent design to treat complex disease. Pharmaceutical Research, 2006, vol 23, issue1, 1417–50.

5. Moghimi SM, Hunter AC, Murray JC, Nano medicine: current status and future prospects, The FASEB Journal, 2005, vol 19, issue 1, 311–30.

6. Treuel L, Jiang X, Nienhaus GU, New views on cellular uptake and traffic-king of manufactured nanoparticles, Journal of the Royal Society Interface, 2013, vol 10, issue 1, doi 20120939.

7. Wagner V, Dullaart A, Bock A-K, Zweck A, The emerging nanomedicine landscape, Nature Biotechnology, 2006, vol 24 issue1, 1211–7.

8. Pankhurst QA, Connolly J, Jones SK, Dobson J, Applications of magnetic nanoparticles in biomedicine, Journal of Physics D: Applied Physics, 2003, vol 36 issue1, 167–81.

9. Dobrovolskaia MA, McNeil SE, Immunological properties of engineered nano-materials, Nature Nanotechnology, 2007, vol 2, issue1, 469–78.

10. Roldao A, Mellado MCM, Castilho LR, Carrondo MJ, Alves PM, Virus-like particles in vaccine development, Expert Review of Vaccines, 2010, vol 9, issue1, 1149–76.

11. Bolhassani A, Safaiyan S, Rafati S, Improvement of different vaccine delivery systems for cancer therapy, Molecular Cancer, 2011, vol 10 issue1, 3–22.

12. Krishnamachari Y, Geary S, Lemke C, Salem A, Nanoparticle delivery systems in cancer vaccines, Pharmaceutical Research, 2011, vol 28, issue1, 215–36.

13. Correia-Pinto JF, Csaba N, Alonso MJ, Vaccine delivery carriers: insights and future perspectives, International Journal of Pharmaceutics, 2013, vol 440, issue1, 27–38.

14. Plummer EM, Manchester M, Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design, Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2011, vol 3, issue1, 174–96.

15. Kalkanidis M, Pietersz GA, Xiang SD, Mottram PL, Crimeen-Irwin B, Ardipradja K, et al, Methods for nano-particle based vaccine formulation and evaluation of their immunogenicity, Methods, 2006, vol 40, issue1, 20–9.

16. Minigo G, Scholzen A, Tang CK, Hanley JC, Kalkanidis M, Pietersz GA, et al, Poly-l-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy, Vaccine, 2007, vol 25 issue1, 1316–27.

17. Peek LJ, Middaugh CR, Berkland C, Nanotechnology in vaccine delivery, Advanced Drug Delivery Reviews, 2008, vol 60, issue1, 915–28.

18. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V., PLGA-based nanoparticles: an overview of biomedical applications, Journal of Controlled Release, 2012, vol 161, issue1, 505–22.

19. Cevc G, Schatzlein A, Blume G, Transdermal drug carrier: Basic properties, optimization and transfer efficiency in case of epicutaneously applied peptides, J Control Rel, 1995, vol 36, iisue1, 3-16.

20. Gregoriadis G, Liposomes as immunological adjuvants, In: Stewart DES's The theory and application of adjuvants. Chichester, John Wiley, 1994, vol 1, iisue1, 145-69.

21. Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin, Nature, 1998, vol 391, issue1, 851.

22. www.medicalnewstoday.com

23. Moingeon P, Cancer vaccines, Vaccine, 2001, vol 19, issue1, 1305–26.

24. Wohlleben G, Erb KJ, Barnes P, Atopic disorders: a vaccine around the corner,TrendsImmunol, 2001, vol 22, issue 11, 618–26.