Chapter 1: Introduction

Vaccines are the preparations given to patients to evoke immune responses leading to the production of antibodies (humoral) or cell-mediated responses that will combat infectious agents or noninfectious conditions such as malignancies. Alarming safety profile of live vaccines, weak immunogenicity of sub-unit vaccines and immunization, failure due to poor patient compliance to booster doses which should potentiate prime doses are few strong reasons, which necessitated the development of new generation of prophylactic and therapeutic vaccines to promote effective immunization. Attempts are being made to deliver vaccines through carriers as they control the spatial and temporal presentation of antigens to immune system thus leading to their sustain release and targeting. Hence, lower doses of weak immunogens can be effectively directed to stimulate immune responses and eliminate the need of the administration of prime and booster doses as a part of conventional vaccination regimen. Development of stable and immunogenic formulation is one of the critical goals of formulation for development of any vaccine. Hence, this research primarily focused on the use of Niosomes as potential vaccine delivery systems.

**HPV infection & cervical cancer:** Cervical cancer is the second most common cause of female cancer death worldwide (Parkin et al., 2005). Persistent infection with 'high risk' HPV genotypes is the major etiological factor in cervical cancer and thus effective vaccination against HPV provides an opportunity to reduce the morbidity and mortality associated with HPV. The FDA has approved two preventive vaccines to limit the spread of HPV. However, these are unlikely to impact upon HPV prevalence and cervical cancer rates for many years. Furthermore, preventive vaccines do not exert therapeutic effects on pre-existing HPV infections and HPV-associated lesions. In order to further impact upon the burden of HPV infections worldwide, therapeutic vaccines are being developed. These vaccines aim to generate a cell-mediated immune response to infected cells.

**Limitations of current strategies to prevent cervical cancer and other HPV-related disease:** Most HPV-related precancerous lesions of the cervix are asymptomatic; thus, they can only be detected through screening tests that rely on cytologic or histologic examination of cervical cells or tissue collected by a health professional during an internal pelvic examination.

i) In settings where cervical cancer screening is absent or limited, most women present with symptomatic late-stage disease that is often complex and costly to treat, untreated or fatal.
ii) In many high-income countries, and a few middle-income countries, organized cervical cytology screening programs with high population coverage have substantially reduced cervical cancer incidence and mortality. However, even when screening is available, many women are unaware of it, do not access it or cannot afford it. Cytology screening is more effective in detecting pre-cancers and cancers of squamous cell type than AIS or adenocarcinoma, because the endo-cervical glands from which these lesions arise are more difficult to sample using cytology.

iii) Screening programs are absent or very limited in most low and middle-income countries. They are complex, costly, labor intensive, and hard to sustain with quality. Five percent of women in developing countries have been screened in the last 5 years, compared with 75% in other countries. As cytology screening is not feasible in most low-resource settings, WHO recommend visual cervical inspection and cryotherapy for lesions. Access to these services in developing countries is limited, partly because preventive care for women (other than family planning) is rare.

iv) Access to diagnosis, treatment and palliative care for cervical cancer and other HPV-related cancers is limited in many low and middle-income countries. Consequently, individuals with cancer often suffer prolonged, painful deaths that may isolate them from family and friends.

v) Abstinence and condom use can reduce the risk of acquiring warts, but limited use of these methods reduces their impact at a population level. Condoms cannot prevent skin-to-skin HPV transmission in genital areas not covered by the condom or during non-penetrative intercourse.

vi) Anogenital warts can be treated with patient or provider-applied medication, ablation and surgery. Access to these therapies is limited in many countries.

**Current strategy in preventive vaccines for HPV:** Current strategy utilizes the capsid proteins L1 and L2 as target antigens, inducing antibodies to neutralize and prevent entry of HPV into cells. Expression of recombinant L1, the major component of the capsid, in various cell types, results in spontaneous assembly of virus-like particles (VLPs), which are immunologically and morphologically similar to HPV virions (Rose *et al.*, 1994; Zhou *et al.*, 1991; Kirnbauer *et al.*, 1992). Vaccination of animal models with L1 VLPs protects them against subsequent exposure to the homologous virus. The main focus of preventive
vaccines has been on HPV types 16 and 18 which together account for around 70% of cervical cancers (Bosch et al., 2002). Clinical trials of L1 VLP vaccines in seronegative healthy volunteers have proven their immunogenicity and safety, producing high titers of neutralizing IgG antibodies, up to 40 times those found in natural infection with HPV-16 (Roden et al., 2004).

**Characteristics of marketed HPV vaccines:** Two prophylactic HPV vaccines are currently marketed. Both vaccines are designed to prevent HPV infection and HPV-related disease; they are not designed to treat women with current HPV infection or HPV-related disease. Both vaccines are made with recombinant technology in which proteins form virus-like particles (VLPs). They are non-infectious and lack live biological products and genetic material.

- **Cervarix®,** a bivalent vaccine, is manufactured by GlaxoSmithKline (GSK). It contains VLP antigens for HPV 16 and 18 reassembled from L1 proteins of HPV 16 and 18, and is designed to protect against infection and disease due to these types. It is produced using a novel recombinant baculovirus expression system and a cell line derived from *Trichoplusiani*cells. It contains the adjuvant AS04, which includes monophosphoryl lipid A (MPL). It is given as three intramuscular injections at 0, 1 and 6 months.

- **Gardasil® (also marketed as Silgard®),** a quadrivalent vaccine, is manufactured by Merck. It contains VLP antigens for HPV 6, 11, 16 and 18, reassembled from L1 proteins of HPV 6, 11, 16 and 18, and is designed to protect against infection and disease due to these types. It is produced using yeast substrate, and contains the adjuvant amorphous aluminium hydroxyphosphatesulphate. It is given as three intramuscular injections at 0, 2 and 6 months.

- Neither vaccine contains thimerosal, preservatives or antibiotics.
- Both vaccines are currently marketed as single dose vials or prefilled syringes.
- Both vaccines require storage and transport in a cold-chain system.

**Disadvantages of using Aluminium adjuvants:** Although, until recently only the aluminium based adjuvants had been approved for use in humans, they did not fulfil all the requirements of new generation vaccines due to a number of limitations, and therefore a number of newer adjuvants are currently undergoing investigation and clinical trials. These
adjuvants comprise a vastly diverse group of compounds and indeed their adjuvanticity may be their only common functional characteristic (Kersten and Crommelin, 1995).

However, although Alum is particularly potent at inducing such humoral immune responses, it is a poor stimulator of classical cell mediated immunity (CMI). Induction of cellular and humoral immune responses has been attributed to mutually antagonistic subsets of CD4+ T helper (Th) lymphocytes. Activation of the Th1 subset is associated with the production of Interleukin-2 (IL-2) and Interferon-g (IFNg) which induces B cell production of the IgG2a subclass of antibodies and results in the development of a classical cell mediated immune response (Mosmann and Cherwinski, 1986; Cher and Mosmann, 1987). Conversely, activation of the Th2 subset and the subsequent production of cytokines such as IL-4, IL-5, IL-6 and IL-10, induces B cell IgG1 and IgE production and is associated exclusively with the development of classical humoral immune responses (Boom and Liano, 1988; Mosmann and Coffman, 1989).

Accordingly, the generation of a predominantly Th1 response is essential for the development of a protective immune response against obligate intracellular organisms such as *Mycobacterium tuberculosis* and *Leishmania major* (Cox and Liew, 1992) while the induction of a predominately Th2 response is more appropriate for the effective of certain helminth infections (Finkelman and Pearce, 1991). A number of studies (Hay Glass and Gieni, 1991, Brewer and Conacher, 1996, Brewer and Roberts, 1996) have characterized the antibody response induced by Alum adjuvants as being mainly IgE and IgG1 with no production of IgG2a evident. Similarly, T cell cytokine production induced by Alum adjuvant is characterized as being predominantly IL-4 and IL-5 with no IL-2 or IFNg production, strongly indicating that Alum acts as an adjuvant which produces a polarised Th2 response (Grun and Maurer, 1989; Brewer and Conacher, 1996; Brewer and Roberts, 1996). This activity significantly limits the continued application of Alum adjuvants in new vaccines directed against intracellular pathogens (Bomford, 1989, Kersten and Crommelin, 1995). Furthermore, it has also been demonstrated that Alum has little adjuvant activity when used in synthetic peptide-based vaccines.

For these reasons the development of new and more effective vaccine adjuvants is a key immunological objective which requires resolution before the full impact of new generation vaccines can be realized.
The use of liposomes as vaccine adjuvants: The ability of liposomes to act as adjuvants was first described in 1974 by Allison and Gregoriadis and subsequently they have been studied extensively. Liposomes are generated from phospholipids and other polar amphiphiles which, under certain physical conditions, form closed concentric bilayer membranes when in the presence of excess water. During this process, aqueous solutions can be entrapped within the liposome or, lipid soluble compounds and molecules conjugated to lipids, can be incorporated into the liposomal membrane. A great variety of substances can therefore be associated in liposomes regardless of solubility, charge, size or shape so long as they do not interfere with liposome formation (Gregoriadis and Gursel, 1996).

Liposomes can augment both humoral and cell-mediated immunity to a wide variety of antigens including, bacterial polysaccharide (Abraham and Shah, 1992), ovalbumin (Reddy and Zhou, 1992), bovine serum albumin (Aramaki and Fujii, 1994) and influenza subunit vaccine (De Haan and Geerligs, 1995). Macrophages function as the main antigen presenting cell (APC) for liposomes and, therefore, for liposome associated antigens (Su and van Rooijen, 1989). The adjuvanticity of liposomes appears to be dependent on structural characteristics such as, vesicle size, surface charge, and lipid to antigen ratio, the number of lamellae and the rigidity of the bilayer (Gregoriadis, 1990, Gupta and Relyvel, 1993). Although Alum is the only adjuvant widely used in human vaccines, recently a liposome based vaccine against Hepatitis A has been approved for clinical use (Epaxal-Berna, Ambrosch and Wiedermann, 1997). Furthermore, a number of clinical trials are currently underway using some other liposomal vaccines such as trivalent influenza, diphtheria and tetanus toxoid vaccines (Gregoriadis, 1995).

Disadvantages of liposomes as adjuvants: There are a number of problems associated with the use of liposomes as adjuvants. The relatively low adjuvanticity of liposomes, particularly evident with antigens of low immunogenicity, can be overcome by formulating liposomes in combination with other adjuvants such as lipid A, aluminium salts (Richards and Hayre, 1988), avridine (Pierce and Sacci, 1984), IL-2 (Tan and Gregoriadis, 1989) and muramyl dipeptide (MDP) (Nerome and Yoshioka, 1990), either in the bilayer or within the vesicle (Kersten and Crommelin, 1995). One of the most significant problems associated with the use of liposomes as adjuvants is the susceptibility of phospholipids to oxidative...
degradation in air. This requires that purified phospholipids and liposomes have to be stored and handled in an inert (e.g. nitrogen) atmosphere (Baillie and Florence, 1985). Phospholipid raw materials are naturally occurring substances and as such require extensive purification thus making them costly (Florence and Baillie, 1989).

**Niosomes as vaccine adjuvants:** Many existing and developmental adjuvants are water insoluble surfactants or possess the capacity to form a surface interface. The relationship between surface activity and adjuvant action was first noted in 1966 in studies of aliphatic nitrogenous compounds (Gall, 1966) and many adjuvants described subsequently, such as liposomes, ISCOMs (Morein and Sundquist, 1984) or pluronic block polymers are consistent with this observation (Morein and Lovgren-Bengtsson, 1986). Studies performed by Hunter and colleagues further defined the relationship between surfactant HLB and adjuvant activity (Hunter and Strickland, 1981; Hunter and Bennett 1984). Using simple block copolymers of hydrophobic polyoxypropylene (POP) and hydrophilic polyoxyethylene (POE), a series of different Pluronic polyols were produced which exhibited a range of surface-active properties as well as adjuvant activities (Hunter, Strickland et al., 1981; Hunter and Bennett, 1984). One of these block copolymers, L-121 possessed potent adjuvant activity when formulated on the surface of oil in water droplets. In studies using trehalosedimycolate (TDM), a naturally occurring non-ionic surfactant, adjuvanticity could be enhanced by preparing TDM in oil in water emulsions (Retzinger and Meredith, 1981).

Formulation of TDM in this fashion is thought to enable this surfactant to bind antigen and host proteins such as complement, a feature thought to be responsible for adjuvant activity (Hunter, Strickland et al., 1981). However no adjuvant activity could be attributed to TD Min the micellar form (Retzinger and Meredith, 1981). Thus many studies of other adjuvant active non-ionic surfactants such as sorbitantriololate (Woodard, 1989) or glycerol trioleate, have utilized this type of emulsion, although it has been shown that the micellar forms of certain surfactants also have adjuvant activity (Snippe and DeReuver, 1981; Zigterman and Schotanus, 1989). Another block copolymer, CRL1005, was designed to be soluble in aqueous isotonic buffers and was found to have superior adjuvant activity compared to Alum and Quil A when formulated with ovalbumin (Todd and Pozzi, 1997). One of the features of non-ionic surfactants which make them desirable adjuvants is
their apparent lack of toxicity (Hunter and Strickland, 1981), especially when compared with cationic or anionic surfactants (Baillie, 1981).

However, the choice of mineral oil emulsions as carrier systems has provoked certain fears over the toxicity of the oil component used (Warren and Chedid, 1988). Thus a great deal of attention has focused on the identification of low toxicity metabolisable oils such as squalane (Allison and Byars, 1987), hexadecane (Woodard, 1989) or vegetable oils (Edelman, 1980; Warren and Chedid, 1988) for use in these systems. Alternatively, the use of vesicular or micellar preparations of non-ionic surfactants may be able to dispense with the requirement of an oil phase altogether. Thus studies have shown that the addition of non-ionic surfactants to liposomes greatly enhances the adjuvanticity of these preparations (Zigterman and Snippe, 1987, Zigterman and Snippe, 1988). Other advantages of synthetic non-ionic surfactants are that they are readily available and this makes them significantly cheaper than their phospholipid analogues. Furthermore, they are also stable in air and do not require any special handling or storage conditions. Non-ionic surfactant vesicles (NISV) are liposome-like vesicles but their increased chemical stability gives them a significant advantage over liposomes (Baillie and Florence, 1985). Like liposomes, NISV form a wide spectrum of small unilamellar to large multi lamellar structures and several different classes of non-ionic surfactants have been demonstrated to assemble into vesicles (Florence and Baillie, 1989).

The future generations of preventive vaccines must address two main issues: (1) lowering the cost in order to increase availability of the vaccine to developing countries, (2) to increase the number of HPV types covered in order to maximize protection against HPV-associated malignancies and (3) a possible needle free immunization. An attractive approach to substantially reduce the cost of producing L1 vaccines is the employment of L1 capsomers. The current L1 vaccines, Cervarix and Gardasil, are produced in insect cells and yeast respectively. Production of the vaccine in *Escherichia coli* may be a cheaper option. Use of recombinant *E. coli* to produce these L1 capsomers has demonstrated success in inducing protective antibodies in animal models (Chen *et al.*, 2000; Rose *et al.*, 1998; Li *et al.*, 1997). Additionally, L1 capsomer vaccines are stable at room temperature, negating the need for refrigeration. Trials with VLP vaccines have investigated needle-free administration routes such as transdermal application (Rechtsteiner *et al.*, 2005) and nasal

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Table 1a: Comparison between liposomes & Niosomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Liposomes</th>
<th>Niosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td>Natural Phospholipids, cholesterol</td>
<td>Non-ionic surfactant with cholesterol</td>
</tr>
<tr>
<td>Chemical stability</td>
<td>Phospholipids undergo the oxidative degradation</td>
<td>Stable</td>
</tr>
<tr>
<td>GIT stability</td>
<td>Unstable</td>
<td>Unstable</td>
</tr>
<tr>
<td>Antigen dose</td>
<td>Comparatively high</td>
<td>Comparatively high</td>
</tr>
<tr>
<td>Storage and handling condition</td>
<td>Required special conditions (liquid nitrogen storage)</td>
<td>Do not required special conditions</td>
</tr>
</tbody>
</table>

**Novel methods of delivery:** Potential approaches to reducing costs may also include delivery of a single dose and removing the need for cold storage. Currently, the VLP-based vaccines require storage at 2 to 8 °C for conformational stability. Developing vaccines that are lyophilized to a powder allows for easier storage and delivery.

Mucosal delivery vaccines may be advantageous for several reasons. Needle-free vaccines may reduce the cost of vaccine delivery and decrease the risk of exposure to unclean needles. Mucosal delivery would hopefully induce mucosal and systemic immune responses. There are data that show that the induction of mucosal immune responses, such as at the nasal mucous membranes, can “cross talk” to other mucosal sites (i.e., the vagina) (Manuri et al., 2007). Human papilloma virus (HPV) vaccines based on L1 virus-like particle (VLP) can prevent genital HPV infection and associated lesions after three intramuscular injections. Needle-free administration might facilitate vaccine implementation, especially in developing countries.

This research investigated the subcutaneous & nasal administrations of HPV16 L1 VLPs formulated in Niosomes, in mice and their ability to induce anti-VLP antibodies in serum and in mucosal (vaginal) fluid (sIgA).
Objectives of the current study

- To characterize the vaccine antigen (protein stability profile).
- To formulate HPV Antigen using Aluminium adjuvants & immunogenicity studies.
- To formulate HPV Antigen into Niosomes & immunogenicity studies in different routes of administration (subcutaneous & intranasal) and study the systemic & mucosal immune response (with and without boosters).
- To compare the immunological response between different preparations and study the effectiveness of the Niosome formulations.
- To study the stability of the Aluminium and Niosome formulations.