CHAPTER II

LITERATURE REVIEW

1. Transdermal Vaccine Delivery

Following the first success in human prevention from a deadly human disease, small pox by Edward Jenner who had deliberately exposed people to virus that causes cowpox in order to prevent infection by the related small pox in 1798 (Bourgaize, Jewell, and Buiser, 2000), numerous attempts have been made at developing safer and more efficacious vaccines. Jenner was the first to take a rational, scientific approach to the manipulation of human immune responses with live cell immunization. His approach was to develop active immunity which is the patients' immune system responded to the potential pathogen to bring about protective immunity. This had led to develop the passive immunization which involves the administration of pre-formed antibodies to a patient who has been exposed, or is at risk of exposure to an infectious pathogen in the later (Hannigan, and Pallister, 2000). In the 1970s, development in molecular biology heralded the era of recombinant DNA technology to produce a new generation of vaccines. These recombinant subunit vaccines are considered to be safer than conventional vaccine; killed vaccines, subunit vaccines and live vaccines. The killed and subunit vaccines possess low potency to elicit effective immune responses. They need to be administered with adjuvants which unfortunately cause tissue reactions, thus, currently only alum is licensed for human use (Babiuk et al., 2000). Also, the live vaccine may not be as safe due to the potential risk of reversion to virulence.

Immunization of animals using plasmid encoding protective antigens is one of the most recent vaccination methods. The basis behind DNA immunization is that an antigen encoded in a plasmid, with the proper regulatory sequences, transfects cells *in vivo* resulting in expression of the antigen and induction of immune response to the expressed protein. Since the protein antigen is produced *in vivo* in a manner similar to what occurs in natural pathogen infections, the post-translational and intracellular processing of the protein is considered to be authentic and therefore both protective cellular and humoral immune responses are induced. The universality of this approach has been shown in various species and with numerous genes from different pathogens/cells. Furthermore, DNA immunization has been shown to be effective in eliciting an immune response by many kinds of administration routes such as intramuscular (im), intradermal

(id), intraperitoneal (ip), intravenous (iv), oral, intranasal, ocular and the recent recognition one, transdermal route (Mumper, and Roland, 1998; Babiuk et al., 2000; Watabe et al., 2001; Wu et al., 2001; Cui, Baizer, and Mumper, 2003; Peachman, Rao, and Alving, 2003; Prather, Sagar, Murphy, and Chartrain, 2003; Bergmann-Leitner, and Leitner, 2004).

1.1 Anatomy of Skin and Its Function

The skin is the outermost layer of the body. The function of the skin is to protect against water loss, ultraviolet light and as the first line to defense prevent the entry of pathogens into the body. The structure of the skin depicted in Figure 2.1 shows three layers of skin including epidermis, dermis and hypodermis/subcutaneous (Babiuk et al, 2000).

The hypodermis forms the lowest layer of the skin and is composed of fibroblasts and adipocytes. Above the hypodermis is the dermis, which is 2-3 mm thick and composed of mostly fibroblasts. Between the dermis and epidermis is the basal lamina, which serves as a basement membrane for the cells of the epidermis, the outermost layer of the skin. The viable epidermis is composed of approximately 90% keratinocytes.

Throughout the viable epidermis, immune competent dendritic cells called Langerhans cells are found. These Langerhans cells, despite only composing 1% of the cell population, cover nearly 20% of the surface area through their horizontal orientation and long protrusions which form a meshwork that allows them to uptake antigens that they encounter. This network of Langerhans cells represents the second line of defense and initiates specific immune responses by presenting the processed antigens to other cells of the immune system. Langerhans cells are bone marrow-derived dendritic cells residing in the epidermis. They play a dual role of immunosurveillance in the skin and, upon activation by microorganisms or their products, crawl out of the skin to the draining lymph nodes, where they orchestrate a specific immune response. Activated, mature Langerhans cells are antigen-presenting cells (APCs), express high level of immunogenic molecule, and secrete cytokines, resulting in strong immune effector responses by B and T lymphocytes (Guebre-Xabier et al., 2003).

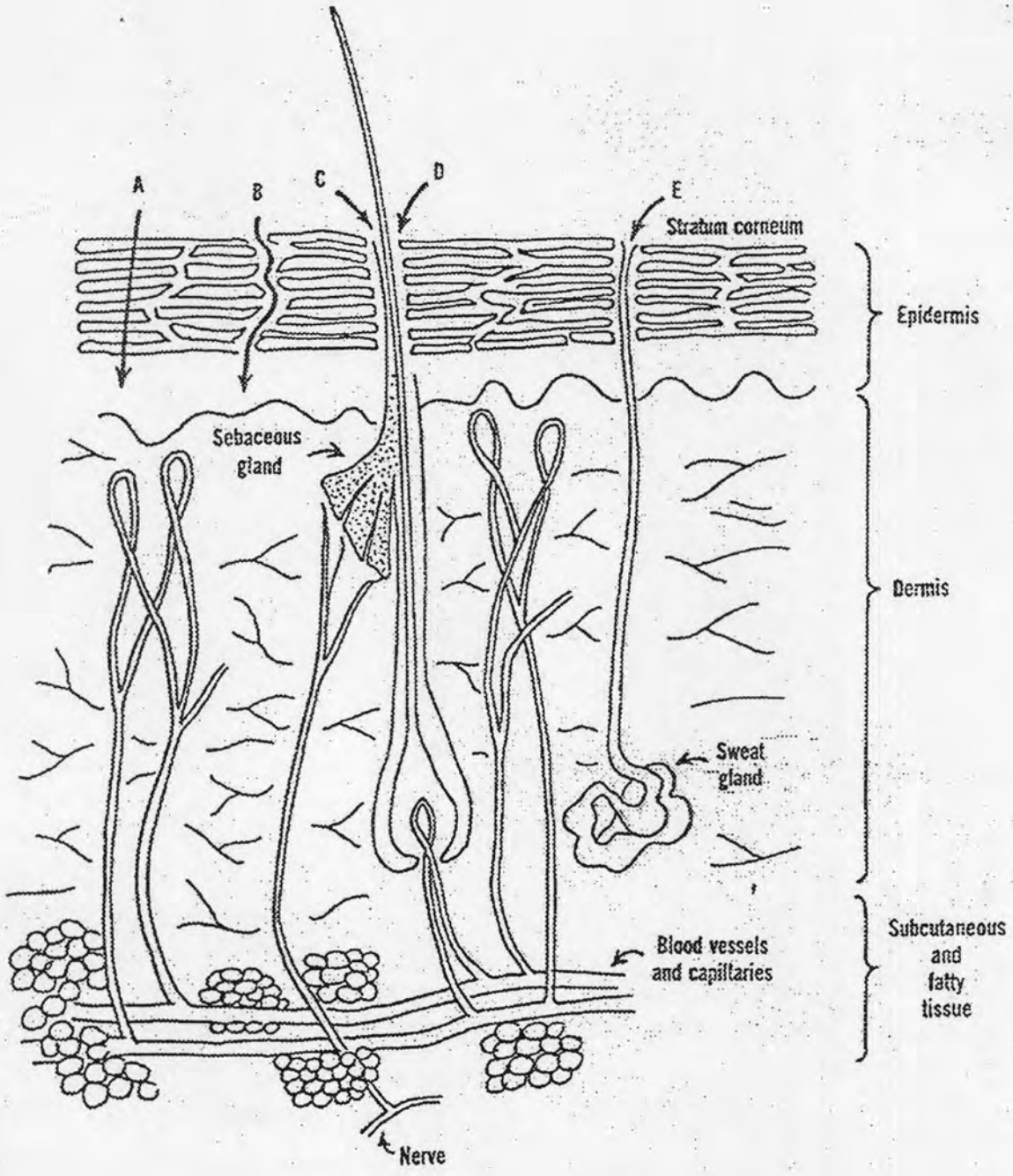


Figure 2.1 Anatomy of skin and skin penetration pathway, (A) transcellular; (B) diffusion through channels between cells; (C) through sebaceous ducts; (D) transfollicular; (E) through sweat duct (Martin, 1993).

The remaining cells include the melanocytes, whose function is to protect all of the lower cells from ultraviolet light by their pigmentation, epidermotropic lymphocytes and Merkel cells, involved in sensation. Keratinocytes in the epidermis undergo a programmed process of differentiated to form different cell layers. These cell layers are the basal, spinous, granular, clear and horny layer.

The basal cell layer consists of undifferentiated kerationcytes and stem cells on top the basal lamina. Above the basal cell layer is the spinous layer of cells, named spinous because of the presence of many desmosomes that appear spine like. Above the spinous layer is the granular layer of cells. The transition zone clear layer occurs above the granular zone and consists of both living and dead cells. The cells in the transition zone undergo major continuous cellular changes. Some of these changes include remodeling of keratin filaments into more structurally stable bundles and release of their lipid containing granules into the extracellular matrix. Above the transition zone lines the stratum corneum. The stratum corneum is 10-20 μm thick and consists of terminally differentiated keratinocytes called corneocytes. Corneocytes are flatted cells, which contain intracellular cross-linked macrofibrillar bundles of keratin giving the corneocyte a rigid structure. Desmosomes connect corneocyte into the ordered superstructure of the stratum corneum. The stratum corneum also contains multilamellar arrays of lipid found extracellularly between the corneocytes. These multilamellar arrays are composed of primarily cholesterol, free fatty acids, and ceramides. The stratum corneum is constantly being sloughed of and replaced. When the skin is breached either physically, chemically or biologically, many metabolic responses such as the synthesis of fatty acids, cholesterol and ceramides occur, and preformed lamellar bodies are secreted to return the barrier to normal. For these reasons the stratum corneum is not a stagnant structure, but is continuously being altered due to the interaction with the environment preventing barrier disruption for a prolonged period of time (Roland, 1993).

Owing to its multilayered structure, the skin represents a complex and relatively efficient barrier to the penetration of drugs. The stratum corneum provides a rate-limiting step in the process of percutaneous absorption. This hydrophobic membrane is thus the major obstacle to the epidermal transfer of water soluble drugs, as illustrated by the fact that their percutaneous absorption may be 1,000 times more rapid when the stratum corneum is absent or damaged than when it is present and intact.

Mikszta et al. (2003) proposed the optimized methods for topical delivery of macromolecule substances, particularly nucleic acids, amino acids, amino acid derivatives, peptides or polypeptides. One of their successes is eliciting the antibody response to topical delivery of pDNA. They administered pDNA encoding the Hepatitis B surface antigen (HbsAg) to administer to anaesthetized Balb/c mice topically using abrasion with a 200 μm silicon solid microneedle array. The results indicated that the abrasion protocol induced strong serum antibody responses in vivo. The magnitudes of such responses were significantly greater than those induced via either im or id injections. In addition, the responses following abrasion protocol were considerably less variable than those observed following standard needle-based injection route. Because the design of microneedles which painlessly permeabilize skin is based on an understanding of skin anatomy, microneedles which penetrate the skin just a little more than 10-15 μm can provide transport pathways across the stratum corneum, but do so painlessly since the microneedles do not reach nerves found in deeper tissue (Henry et al., 1998; Mikszta et al., 2002).

However, the stratum corneum is not a totally impermeable barrier hindering hydrophilic molecules from reaching the viable tissue since polar channels exist within the intercorneocyte space, and more precisely in between the lipid bilayers. There are indeed two possible routes of drug penetration through the stratum corneum: the transcellular and the intercellular pathway. The relative importance of one of these penetration pathways will be mainly dependent on the physicochemical characteristics of the drug molecules, particularly the partition and diffusion coefficients into the protein or lipid region. The main transport pathway across the stratum corneum involves the tortuous, extracellular lipid domain; for polar compounds, a "polar" route or pore pathway has been proposed.

However, recent evidence suggests that aqueous channels across the stratum corneum are not necessary to explain skin permeability. If a drug penetrates and does not form a reservoir within the stratum corneum, the next stage will be its partitioning, relative to its physicochemical properties, from a primarily lipid-rich domain into the viable epidermis, a predominantly aqueous-rich domain environment. Drugs with a suitable octanol-water partition relatively well into the epidermis after diffusion into the stratum corneum. However, too hydrophilic or lipophilic compounds will not easily be partitioned into the stratum corneum or the underlying viable tissues, respectively. Drugs that reach the epidermis will be able to diffuse further into the

dermis and consequently enter the bloodstream. Besides, in either local or transdermal therapy, drug metabolism within the skin is of major importance, since some drugs can be designed for enhancing penetration into the skin and for delivering to the viable tissues a pharmacologically active molecule. In addition to the transepidermal pathway, a shunt pathway (passage through the skin appendage) may be significance in several cases. The transfollicular pathway in particular was shown to play an important role in the percutaneous absorption of some drugs such as hydrocortisone, niflumic acid and caffeine. A significant appendageal diffusion may occur in spite of the small area occupied by the pilosebaceous units at the surface of the skin (Roland, 1993).

When the skin becomes damaged, kerationcytes and Langerhans cells become activated. The activated kertinocytes can synthesize a large number of cytokines involved in modulating the immune response. In addition, keratinocytes can express intracellular adhesion molecules and other adhesion molecules for various immune cells. These features make keratinocytes immunologically active cells as well as providing structure to the skin. Activated Langerhans cells increase their phagocytic activity and move from the skin into draining lymph nodes (DLNs) where they encounter foreign antigens and initiate immune responses (Babiuk et al., 2000). Guebre-Xabier et al. (2003) investigated the track of Langerhans cells in the afferent immune response. They immunized female C57BL/6 mice 6 to 8 weeks of age with intradermally injected pDNA with topical adjuvant, cholera toxin (CT). They used an aqueous solution of fluorescein isothiocyanate (FITC) to track Langerhans cells, which minimized the activating effect of actone-solubilized FITC. Following topical FITC-CT application, the number of $FITC^+$ cells increase in DLNs, and the $FITC^+$ cells derived from CT-treated skin demonstrated a strong up-regulation of CD86 and modest increase in major histcompatibility complex (MHC) class II. Similar results were seen in studies with direct labels of the adjuvant or antigen, suggesting that CT coadministered with antigen promotes the activation, maturation, and migration of activated Langerhans cells from skin to DLNs.

1.2 Transdermal Vaccine Delivery System

In order to deliver vaccines, the ideal delivery vehicle would exhibit several attributes. It would be non-toxic as well as non-immunogenic. It would be economical, allowing widespread use and also be effective and reproducible at inducing the proper immune responses to the

delivered antigen. In addition, the delivery vehicle would be pharmaceutically acceptable, being stable and biocompatible. Moreover, the ideal delivery vehicle would allow non-invasive vaccination with the possibility of controlled release increasing compliance (Babiuk et al., 2000).

Alving and Glenn (1999) prepared liposomes for transdermal CT delivery. The liposomes containing dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, cholesterol and lipid A were mixed with CT. Balb/c mice were immunized topically and repeated using the same formulation and technique three weeks after primary immunization. It was found that the system used liposomes could elicit significantly an antigen-specific immune response after topical application of a formulation containing liposomes and antigen to intact skin. Niemiec et al. (1997) showed that the expression pDNA for the human interleukin-1 receptor antagonist (IL-1ra) protein formulated with nonionic:cationic (NC) liposomes were potential formulations for stimulation of biological responses. The authors reported that these NC liposomal-DNA formulations could deliver pDNA into the hair follicles of intact hamster ear skin but controls animals treated with an aqueous formulation containing an equivalent dose of the fluorescently labeled plasmid DNA failed to show evidence of DNA beyond the superficial epidermis 24 hours after topical application. The result indicated that perifollicular delivery is a physicochemical property specific to NC liposomal formulations. This finding was consistent to the study performed by Birchall et al. (2000). They found that the main route of small partitioned to skin was the pilocebaceous units which were approximately 70 μm in diameter. However, the exact mechanism of transdermal liposome delivery system has been argued. Cevc, Schatzlein, and Blume (1995) prepared lipid vesicles called "Transferosomes™". Transferosomes have a property of self-optimizing deformability. Their efficiency of passage for 500-nm Transferosomes through pores of 100 nm diameter is as high as that of pure water, 1,500 times smaller than the former. When applied onto the intact skin surface non-occlusively, Transferosomes penetrated the skin permeability barrier spontaneously. Subsequently, they were distributed, probably via the lymphatic system, throughout the whole body. Combinations of pDNA or peptides and Transferosomes provided very successful mean in drug delivery, especially Transferosomes-associated insulin was carried across the skin with an efficacy of more than 50%-80%.

Baca-Estrada et al. (2000) studied the immune responses induced by transcutaneous immunization with antigens formulated in a novel lipid-based biphasic delivery system. They

used recombinant *Pasteurella haemolytica* leukotoxin (Lkt) and hen egg lysozyme (HEL) as model antigens to investigate. Mice were immunized by the transdermal route with Lkt or HEL formulated in a novel lipid-based biphasic delivery system. The formulations containing different doses of antigen were weighed onto a parafilm and placed on the skin with the parafilm covering the formulation; this was further secured with an adhesive membrane and tape. The patches were removed 24-48 hours later and the site of application was thoroughly cleaned. The immunization procedure was repeated 3 weeks later. It was found that a novel lipid-based biphasic transdermal delivery system of Lkt or HEL induced strong polarized Th2 responses and predominant induction of antigen specific IL-4 over IFN-γ in spleen and draining lymph nodes cells

Glenn et al. (1998) pointed out that when cholera toxin (CT) was applied to the surface of the skin, it could stimulate an immune response. This might due to transcutaneous immunization was achieved using CT by itself. When given alone by the oral route, CT stimulates a potent immune response in the form of anti-CT antibodies. The investigators proved the capacity of CT to induce a similar response through the skin by applying a saline solution of CT to the bare skin of the shaved mouse. This application induced high levels of IgG antibodies specific for CT. This suggested that immunization could thus be achieved by the simple application of a mixture of CT and vaccine components without disruption of the skin.

In a human study, a graded-dose phase I trial was conducted in 18 volunteers to evaluate the safety and immunogenicity of heat-labile enterotoxin (ET) delivered in a patch (Glenn et al., 2000). The volunteers received variety doses of ET. The ET solution was added to a gauze pad under an adhesive patch and applied to the upper arm for six hours. Volunteers were monitored for systemic or local reactions at one, two, three and seven days post vaccination. All volunteers were boosted at 12 weeks. No serious vaccine-related adverse reactions were observed either systemically or at the site of immunization. All individuals in the 500-μg group had detectable IgG or IgA antibody against ET in either the urine or stool. These may be the trancutaneous immunization seems to allow passage of a large antigen such as ET (86 kDa) through the stratum corneum into the epidermis in humans, as it does in mice. Although the mechanisms of stratum corneum penetration in transcutaneous immunization are not well understood, the importance of hydration for humans is reinforced by the observation the transcutaneous immunization using hydration is effective in other mammalian species. These findings indicated that transcutaneous immunization is feasible for human immunization.

1.3 Solid Lipid Nanoparticles (SLN) and Chitosan Nanoparticles (CSN) as transdermal pDNA vaccine delivery system

Although liposomes have extensively been studied for using as transdermal vaccine delivery system, a few research groups have also investigated the potential of other nanoparticles such as solid lipid nanoparticles (SLN) and chitosan nanoparticles (CSN) for this purpose and reported their success.

1.3.1 SLN

Nanoparticles based on solid lipid have been proposed as an alternative colloidal drug delivery system to polymer nanoparticles, emulsions and liposomes. They may be described as parenteral emulsion in which the liquid lipid (oil) was replaced by a solid lipid (Tabatt, Kneuer et al., 2004). SLN are in the submicron size range and are composed of solid lipid such as triacylglycerol, waxes, and paraffins. Depending on the intended type of application, different pharmaceutical and cosmetic surfactants and surfactant blends, eg. poloxamer, bile salts and polysorbates, can be used as stabilizers (Mehnert and Mäder, 2001). A standard manufacturing procedure of SLN is the melt-emulsification by high pressure homogenization avoiding organic solvents and allowing large-scale production. Drug incorporation is accomplished by dissolving/dispersing the drug in the molten lipid prior to high pressure homogenization (Müller, Mäder, and Gohla, 2000). Depending on drug solubility in the lipid matrix, SLN drug load related to the lipid matrix varies from less than 1% for iotrolan up to 50% as in the case of ubidecarenone. So far, a variety of drug like retinol, prednisolone, doxorubicin, etc. have been successfully incorporated into SLN.

Considering incorporation of shear and temperature sensitive compounds, the melted-emulsion by high pressure homogenization is not suitable, and therefore, other preparation techniques such as cold high pressure homogenization or precipitation from microemulsions have to be applied. As an interesting alternative, subsequent adsorptive loading with the respective drug after the preparation process, avoiding thermal and mechanical stress completely might be a promising approach, especially if drug deterioration has to be minimized and/or drug encapsulation within the lipid matrix is not necessary (Schubert and Müller-Goymann, 2005).

Recently, A few research groups showed the potential of SLN for using as pDNA delivery system. Tabatt, Sameti et al. (2004) investigated the enhancement of *in vitro* transfection

activity by optimizing cationic lipid and matrix lipid composition of SLN. SLN were formulated by using two different matrix lipids; compritol and cetylpalmitate with six different cationic detergents; esterquat 1, benzalkonium chloride, cetrimide, DOTAP, DDAB and CPC. The transfection activity study performed in mammalian cell, COS-1 cells showed that the combination of cetylpalmitate and DOTAP possessed the highest transfection activity with low cytotoxicity. This suggested that SLN could be used as pDNA vector for transfection whenever good combinations of safety cationic lipid and matrix lipid are selected.

Cui and Mumper (2002) developed a method for nano-engineered genetic vaccines delivery. They used expressed β-galactosidase as a model antigen. Plasmid DNA was coated on the surface of preformed cationic nanoparticles engineered directly from warm oil in water microemulsion precursors comprised of emulsifying wax as the oil phase and hexadecyltrimethyl-ammonium bromide as a cationic surfactant. Mannan, a dendritic cell ligand, was coated on the nano-particles with and without entrapped endosomolytic agents, cholesterol and DOPE. *In vitro* cell transfection studies were performed to confirm transgene expression with these pDNA coated nanoparticles. The humoral and proliferative immune responses were assessed after topical application of these nano-engineered systems to the skin of shaved Balb/c mice. All pDNA coated nanoparticles, especially the mannan-coated pDNA with DOPE, resulted in significant enhancement in both antigen-specific IgG titers and splenocyte proliferation over naked pDNA alone.

Tabatt, Kneuer et al. (2004) found that cationic SLN and cationic liposome of comparable composition (with regard cationic lipids) were similar in terms of *in vitro* DNA transfection efficacy. They proposed that as SLN could be produced in large-scale and under favorable technological parameters, they may become a valuable addition to the well-established repertoire of non-viral transfection agents lead by cationic liposome.

1.3.2 CSN

The chitosan-based nanoparticles are one of interesting systems that have been studied by researchers for drug delivery. Chitosan is a polysaccharide, similar in structure to cellulose. Both are made by linear β-(1-4)-linked monosaccharides. However, an important difference to cellulose is that chitosan is composed of 2-amino-2-deoxy-β-D-glucan combined with glycosidic linkages. The primary amine groups render special properties that make chitosan very useful in

pharmaceutical applications. Compared to many other natural polymers, chitosan has a positive charge and is mucoadhesive. Therefore, it is used extensively in drug delivery applications (Aktas et al., 2005). Chitosan can be obtained from the deacetylation of chitin, a naturally occurring and abundantly available (in marine crustaceans) biocompatible polysaccharide. However, applications of chitin are limited compared to chitosan because chitin is structurally similar to cellulose, but chemically inert. Acetamide group of chitin can be converted into amino group to give chitosan, which is carried out by treating chitin with concentrated alkali solution. Chitosan is relatively reactive and can be produced in various forms such as powder, paste, film, fiber, etc. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Solubility of chitosan depends upon the distribution of free amino and N-acetyl groups. Usually 1-3% aqueous acetic acid solutions are used to solubilize chitosan (Agnihotri, Mallikarjuna, and Aminabhavi, 2004).

Chitosan has been extensively investigated as carrier and delivery systems for therapeutic macrosolutes, particularly genes and protein molecules. This is primarily because positively charged chitosan can be easily complexed with negatively charged DNAs and proteins. Chitosan can effectively bind with DNA and protect it from nuclease degradation. It has advantages of not necessitating sonication and organic solvents for its preparation, therefore minimizing possible damage to DNA during the complexation process. Furthermore, there is evidence demonstrating that cationic polymers play an important role in both membrane adhesion and lysosome escape of the encapsulated DNA, providing a potential explanation for the superiority of polymer-mediated gene transfer relative to naked DNA administration in many applications.

These hybrid DNA-chitosan systems can be classified into two categories which differ in their mechanism of formation and morphology; complexes and nanospheres (Gan et al., 2005). Two different techniques are usually employed to obtain CSN: on the one hand, chitosan chains can be chemically cross-linked leading to quite stable matrixes, where the strength of the covalent bonds stands out. Glutaraldehyde is broadly used as a cross-linking molecule in covalent formulations. On the other hand, chitosan hydrogel can be also obtained by ionic gelation, where nanoparticles are formed by means of electrostatic interactions between the positively charged chitosan chains and polyanions employed as cross-linkers. The most extensively used polyanion is the tripolyphosphate. Due to the proved toxicity of glutaldehyde and other organic molecules

used in the synthesis of gels covalently stabilized, only the ionic gelation can be used for pharmaceutical applications.

Gan et al. (2005) demonstrated that ionic gelation of cationic chitosan molecules offers a flexible and easily controllable process for systematically and predictably manipulating particle size and surface charge which are important properties in determining gene transfection efficacy if the nanoparticles are used as non-viral vectors for gene delivery, or as delivery carriers for protein molecules. Numerous studies have been reported on prophylactic and therapeutic use of genetic vaccine for combating a variety of infectious deseases in animal models. Recent human clinical studies with the gene gun have validated the concept of direct targeting of dendritic cells in the viable epidermis of the skin (Cui and Mumper, 2001; Agnihotri, Mallikarjuna, and Aminabhavi, 2004).

Cui and Mumper (2001) investigated the topical application of chitosan-based nanoparticles containing pDNA as a potential approach to genetic immunization. There were two types of nanoparticles were investigated: (i) pDNA-condensed chitosan nanoparticles, and (ii) pDNA-coated on pre-formed cationic chitosan/carboxymethylcellulose nanoparticles. These studies showed that both chitosan and a chitosan oligomer can complex carboxymethylcellulose to form stable cationic nanoparticles for subsequent pDNA coating. Selected pDNA-coated nanoparticles (with pDNA up to 400 μg/ml) were stable to challenge with serum. Several different chitosan-based nanoparticles containing pDNA resulted in both detectable and quantifiable levels of luciferase expression in mouse skin 24 hours after topical application. However, only chitosan oligomer/carboxymethylcellulose nanoparticles could elicit more antigen-specific IgG titer to expressed β-galactosidase significantly at day 28 than those of naked pDNA.

1.4 Visualization of Skin Penetration (Alvarez-Román et al., 2004)

Light and electron microscopy have been important tools for the analysis of cellular structure, physiology and function of biological tissues. Although transmission electron microscopy (TEM), offers excellent resolution of ultrastructural details for skin visualization, it causes damage to the specimen and suffers from fixation and sectioning artifacts. Only small specimen areas can be directly visualized and quantification of a permeating drug molecule is

impossible. In addition, TEM provides static two-dimensional images that are difficult to reconstruct three-dimensionally from serial section.

Conventional light microscopy allows direct examination of viable as well as fixed cells and tissues and dynamic processes can be therefore observed and analyzed quantitatively. However, ultrastuctural details cannot be obtained because of the relatively low resolution; moreover, the specimen requires fixation and sectioning, which can lead to artifacts. Localization of a permeating molecules is possible, for example, with a fluorescent or radioactive tracer, but out of focus information can undermine the quality and clarity of the images, especially in thick specimens with overlapping structures. Video image processing microscopy increases contrast and improves detection, but does not completely circumvent this limitation. This problem can, however, be resolved by the use of confocal laser scanning microscopy (CLSM).

CLSM has become a well-established technique for obtaining high resolution images from biological and other specimens. The major technical advantages of CLSM include (i) the ability to obtain images of optical sections with reasonable time-resolution in a non-invasive manner both in *in vitro* and *in vivo* conditions, and, (ii) visualization of images parallel to the surface of the sample, at multiple depths, without mechanical sectional of the sample.

In conventional microscopy, much of the depth or volume of the specimen is uniformly and simultaneously illuminated as well as the plane in which the objective lens in focused. This leads to out of focus blur from areas above and below the focal plane of interest. Out of focus light reduces contrast and decreases resolution, making it difficult to discern cellular structures. In contrast, the illumination in confocal microscopy is not simultaneous, but sequential. The illumination is focused as a spot on one volume element of the specimen at a time. To achieve confocal imaging, excitation light from a laser scanning confocal microscope is directed towards the specimen. The beam of light passes through a scanning system and reaches the objective, which focuses the scanning beam as a spot on the specimen.

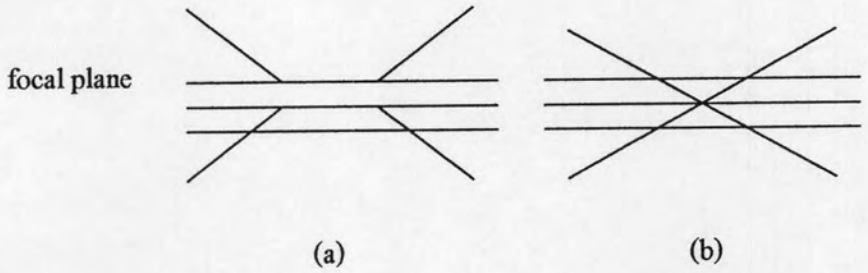


Figure 2.2 Focus on focal plane of (a) conventional microscopy and (b) CLSM (Shotton, 1989).

The main limitation of CLSM resides in the range of lasers for which efficient fluorophore excitations can be achieved. Also, exposure of the different lasers illumination can be highly destructive to both viable tissue and the fluorophore itself, the latter being manifested as photobleaching in the focal planes. Another possible problem of CLSM is the autofluorescence of biological samples. Ideally, therefore, CLSM optically sections thick tissues that are sufficiently transparent to the laser excitation and fluorescence emission wavelengths, that do not strongly scatter this light, and are relatively free of autofluorescence.

2. Human Immunodeficiency Virus (HIV)

In the late 1970s, young people were seen in hospitals and clinics in the USA with symptoms of infectious disease that was not normally seen in people of their age group, *pneumocystis carnii* pneumonia. The underlying defect that led to their symptoms was an immune deficiency resulting from infection with the virus now known as HIV causing the acquired immune deficiency syndrome (AIDS) (Hannigan, 2000). Since the first report of an outbreak in 1981, the AIDS pandemic has accounted for nearly 25 million deaths worldwide. In 2005, there were approximately 3 million deaths from AIDS, over 500,000 of which occurred in children (Grossman and Stawicki, 2006) and now, it has been estimate about 15,000 new infections every day (Balzarini and Damme, 2007).

2.1 HIV Particle

Efforts to control the AIDS epidemic have focused on studies of the biology, biochemistry, and structural biology of HIV and on interactions between viral components and new drug candidates. HIV is a member of the lentivirus genus, which includes retroviruses that possess complex genomes and exhibit cone-shaped capsid core particles. Like all retroviruses, HIV's genome is encoded by RNA which is reverse-transcribed to viral DNA by the viral reverse transcriptase upon entering a new host cell. All lentiviruses are enveloped by a lipid bilayer that is derived from the membrane of the host cell. Exposed surface glycoprotein (gp120) are anchored to the virus via interactions with the transmembrane protein (gp41). The lipid bilayer also contains several celluar membrane proteins derived from the host cell. A matrix shell comprising approximately 2,000 copies of the matrix protein (p17) lines the inner surface of the viral membrane, and a conical capsid core particle comprising about 2,000 copies of the capsid protein (p24) is located in the center of the virus. The capsid particle encapsidates two copies of the unspliced viral genome, which is stabilized as a ribonucleoprotein complex with about 2,000 copies of the nucleocapsid protein (p7), and also contains three essential virally encoded enzymes; protease, reverse transcriptase and integrase. Virus particles also package the accessory proteins, Nef, Vif and Vpr. Three additional accessory proteins that function in the host cell, Rev, Tat and Vpu, do not appear to be packaged (Turner and Summers, 1999).

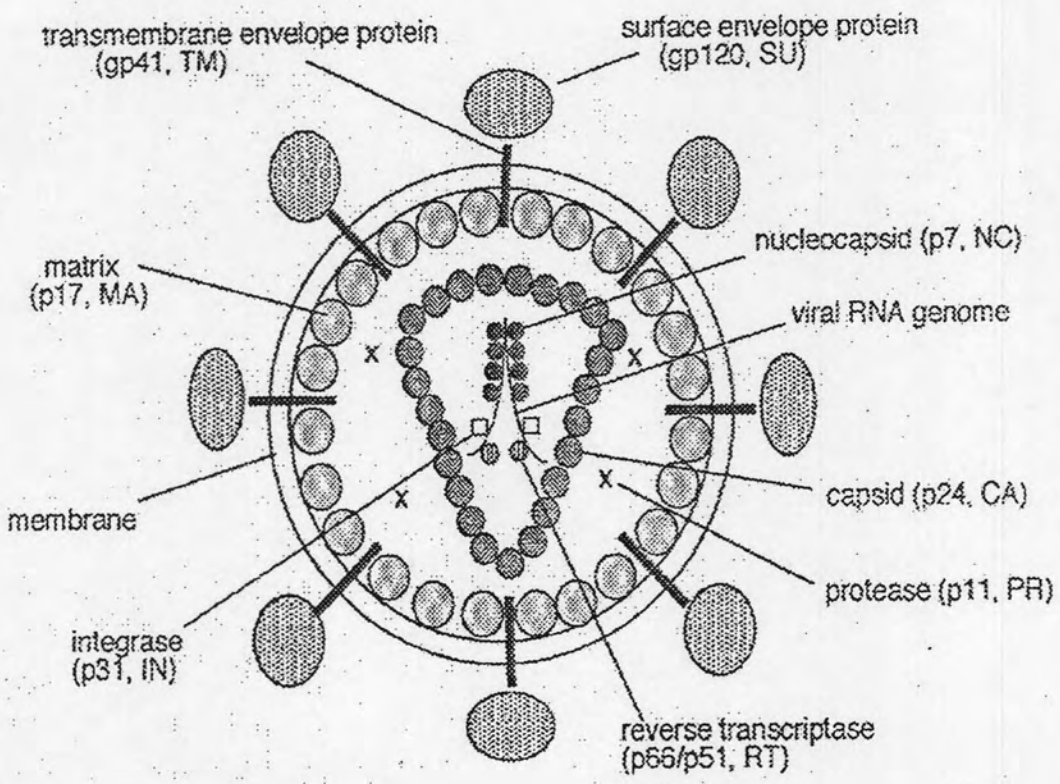


Figure 2.3 HIV-1 virion (Freed, 1998).

2.2 Replication Cycle of HIV

HIV-1 particles can bind specifically to cells bearing CD4, a protein that normally functions in immune recognition. Binding occurs via specific interactions between the viral envelope glycoprotein gp120 and the amino-terminal immunoglobulin domain of CD4. These interactions are sufficient for binding but not for infection. Unlike other retroviruses, the primate lentiviruses require additional cell-surface proteins to promote fusion of the viral and cellular membranes. For HIV-1, membrane fusion can be triggered by one of several chemokine receptors, including CXCR4 and CCR5. Membrane fusion is followed by a poorly understood event that affords an intracellular reverse transcription complex. Reverse transcription is catalyzed in the cytosol by reverse transcriptase. The accessory protein Vif appears to be important during one or more of these early events, perhaps by facilitating the initial stages of reverse transcription. Reverse transcriptase-depentdent DNA synthesis is also dependent on the viral nucleocapsid proteins, and is initiated by the binding of a cellular tRNA primer.

Once synthesized, the viral DNA is transported to the nucleus as part of a preintegration complex. Nuclear localization of the preintegration complex is directed by the accessory protein Vpr, which does not contain a nuclear localization signal but appears to function by connecting the preintegration complex to the cellular import substance. After active transport to the nucleus, the viral DNA is covalently integrated into the host genome by the catalytic activity of integrase.

The late phase of the virus life cycle begins with the synthesis of unspliced and spliced mRNA transcripts, which are transported out the nucleus for translation. Initially, short spliced RNA species that encode the regulatory proteins Tat, Rev and Nef are synthesized. Ordinarily, unspliced cellular mRNAs are retained in the nucleus where they can be further processed or degraded. However, full length and singly spliced HIV mRNA transcripts that contain functional introns are needed in the cytoplasm for gag and gag-pol synthesis and packaging, and their export is mediated by forming complex with the essential HIV accessory protein, Rev. This complex is then transported through the nuclear pore to the cytosol. In this manner, Rev functions as a switch between the early synthesis of highly spliced mRNAs (encoding Tat, Rev and Nef) and the later synthesis of unsliced (encoding gag and gag-pol proteins) and singly spliced (encoding Env, Vpu, Vif and Vpr) mRNAs.

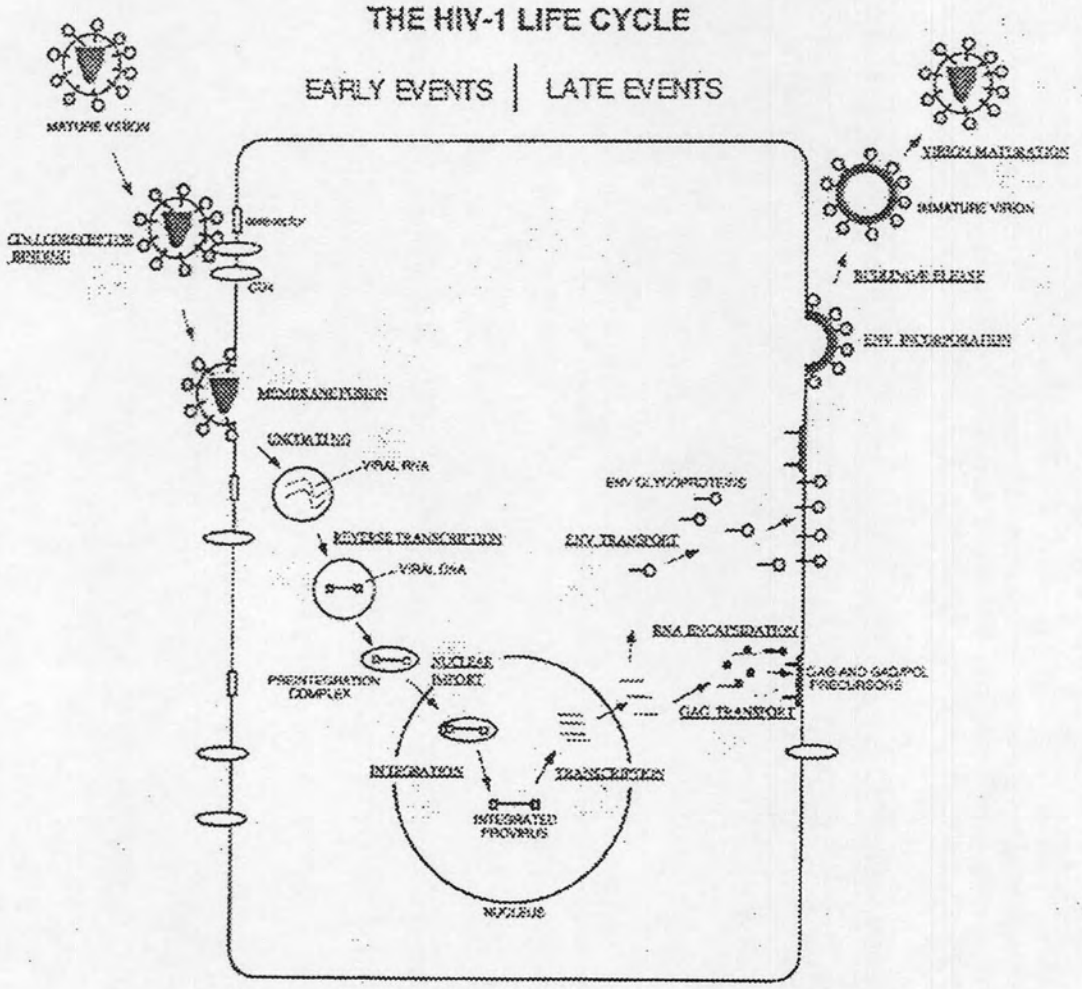


Figure 2.4 HIV-1 life cycle (Freed, 1998).

The Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum using the spliced Env mRNA gene as the message. The protein appears to oligomerize to a trimeric structure in the endoplasmic reticulum, and is glycosylated. Env is post translationally modified in the endoplasmic reticulum and golgi apparatus and is cleaved to produce the non-covalently associated trimeric glycoprotein complex. The heterogeneously glycosylated complex trimer is then transported to the cell membrane for virus assembly. The gag polyprotein is synthesized in the ribosomes from unspliced mRNA. A translational frameshift results in the generation of smaller amounts of gag-pol precursor proteins, which associate with the gag polyprotein at the cellular membrane. Approximately 1,200 to 2,000 copies of gag bud to form an immature particle, which encapsidates two copies of the unspliced viral genome. Subsequent to budding, the polyproteins are cleaved by protease to produce the matrix, capsid and nucleocapsid structural proteins. The structural proteins rearrange via a process called maturation to form the infectious virus particle.

2.3 HIV Infection Control

While the discovery of the relationship between a high level of plasma viral RNA and an increased rate of disease progression, therapeutic emphasis has been made to reduce plasma viraemia completely. Theoretically, all steps in the viral life cycle represent targets for antiretroviral therapy. However, to date the three major groups of drugs being used in the clinical practice are inhibitors of the reverse transcriptase (nucleoside/nucleotide and non-nucleoside) and the protease inhibitor. Recently, the fourth class of antiretrovirals started to be used clinically, with introduction of enfuvirtide, the first fusion inhibitor. The current therapeutical strategy, the so-called highly active antiretroviral therapy (HAART), involves the use of agents from at least two distinct classes of antiretrovirals. In the majority of HIV-1 infected individuals, HAART resulted in increased $CD4^+$ T cell counts and reductions of plasma viraemia to undetectable levels that can be maintained for years (Sierra, Kupfer, and Kaiser, 2005).

2.4 HIV Vaccine

A safe and effective prophylactic vaccine will likely be the most cost-effective means of controlling HIV. Several strategies for developing HIV vaccines have been employed, including subunit vaccines, killed virus vaccines, live attenuated vaccines, virus like particles and DNA

vaccine. Varying vaccination strategies induce variable levels of cellular and humoral immunity (Baliga et al., 2006). Of the various approaches being developed as prophylactic HIV vaccines, those based on a heterologous pDNA vaccination regimen appear especially promising in the non-human primate/simian-human immunodeficiency virus challenge model. It was found that a series of im immunizations with a SIV gag/IL-12 pDNA vaccine could effectively enhance the immunogenicity and post-challenge efficacy of recombinant vesicular stomatitis virus (rVSV) based vectors expressing HIV-1 Env 89.6P gp160 and SIVmac239 gagp55 in rhesus macaques. The addition of a SIV gag pDNA regimen also tended to increase the preservation of peripheral blood $CD4^+$ cells and reduce the incidence of AIDS-like disease symptoms and death associated with SHIV89.6P infection. An analysis of immune correlates of protection after SHIV89.6P challenge in this study revealed that the SHIV-specific IFN-γ ELISpot responses elicited by vaccination correlated with post-challenge clinical outcome. The higher SHIV-specific cell-mediated immune responses elicted by the IL-12 supplemented SIV gag DNA prime and rVSV boost vaccination regimen was associated with an increased maintenance of $CD4^+$ cells and a reduction in plasma SHIV89.6P viral loads (Egan et al., 2006).

Microparticles and nanoparticles prepared with poly(D, L-lactide-co-glycolide: PLGA) or poly(D, L-lactide: PLA) polymers represent a promising method for *in vivo* delivery of encapsulated peptide, protein antigens or DNA (Ataman-Önal et al., 2006). However, one major issue that limits the potential of these delivery systems is the instability or the degradation of the entrapped antigen. Charged microparticles carrying surface adsorbed antigen were developed to resolve this problem and appear more suitable for vaccine applications. The authors showed that new anionic PLA nanoparticles obtained by the dialysis method that are absolutely surfactant-free, which makes them more appropriate for use in humans. The potency of this delivery system as a vaccine carrier was tested in various animal models using HIV-1 p24 protein. p24-coated PLA nanoparticles (p24/PLA) induced high antibody titres in mice, rabbits and macaques immunized by sc injection. Moreover, p24/PLA nanoparticles elicited strong immune responses in mice. The p24 protein seemed to generate a more immune response when administered coated onto the surface of PLA nanoparticles than adjuvanted with Freund's adjuvant. Most importantly, the ability of p24/PLA particles to induce immune responses was also confirmed in the macaque model, since high levels of IFN-γ producing $CD4^+$ T cells and $CD8^+$ T cells could be detected by

the ELISpot assay. This protein delivery system confirmed the potential of charged nanoparticles in the field of vaccine development.

3. Central Composite Design (CCD)

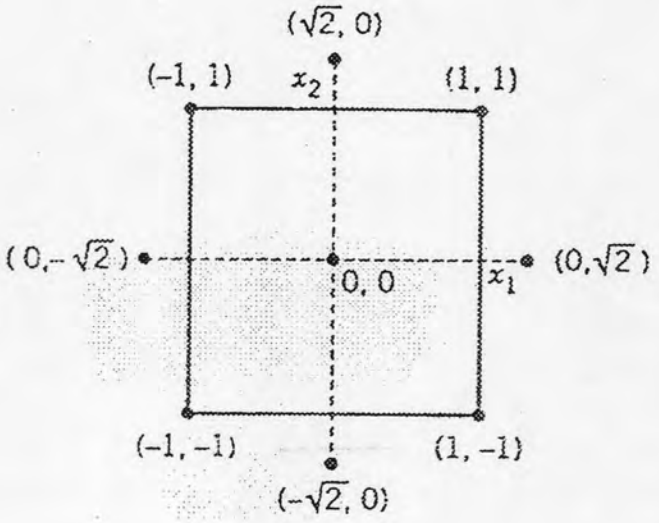


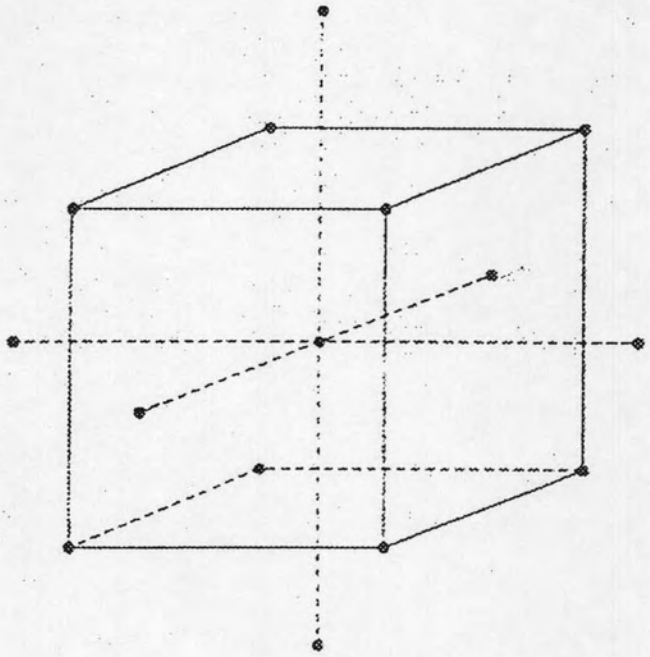
Figure 2.5 Central composite design for k = 2 and $\alpha = (2)^{1/2}$ (Myers and Montgomery, 2002).

The CCD introduced by Box and Wilson in 1951 is an efficient design. It is ideal for sequential experimentation and allows a reasonable amount of information for testing lack of fit while not involving an unusually large number of design points. Thus, it is the most popular class of designs used for fitting a second-order model. Generally, the CCD consists of a p^k factorial runs with *p*k axial or star runs and center points. The practical deployment of a CCD often arises through sequential experimentation. For example (Figure 2.5), a 2^k has been used to fit a first-order model, then, this model has exhibited lack of fit, and the axial runs are added to allow the quadratic terms to be incorporated into the model. There are two parameters in the design that must be specified; the distance α of the axial runs from the design center and the number of center points.

It is important for the second-order model to provide good predictions throughout the region of interest. One way to define "good" is to require that the model have a reasonably consistent and stable variance of the predicted response at points of interest x. It was suggested that a second-order response surface design should be rotatable. This means that the variance of the predicted response at some point x is the same at all points x that are the same distance from the design center. That is the variance of predicted response is constant on a sphere. A CCD is made rotatable by the choice of α. For a spherical region of interest, the best choice of α from prediction variance viewpoint for the CCD is to set $\alpha = (k)^{1/2}$. This design, called a spherical CCD, puts all the factorial and axial design points on the surface of a sphere of radius $(k)^{1/2}$. When the region is a sphere, the design must include center runs to provide reasonably stable variance of predicted response. Generally, three to five center runs are recommended.

Nevertheless, there are many situations where the region of interest is cuboidal rather than spherical. In these cases, a useful variation of the CCD is the face-centered CCD or the face-centered cube, in which $\alpha = 1$. This design locates the star or axial points on the centers of the faces of the cube. This variation of CCD is also sometimes used because it requires only three levels of each factor, and in practice, it is frequently difficult to change factor levels. However, the face-centered CCD is not rotatable. It does not require as many center points as the spherical CCD. In practice, two or three is sufficient to provide good variance of prediction throughout the experiment region. However, it should be noted that sometimes more center runs will be employed to give a reasonable estimate of experimental error (Myers and Montgomery, 2002).

(a)



(b)

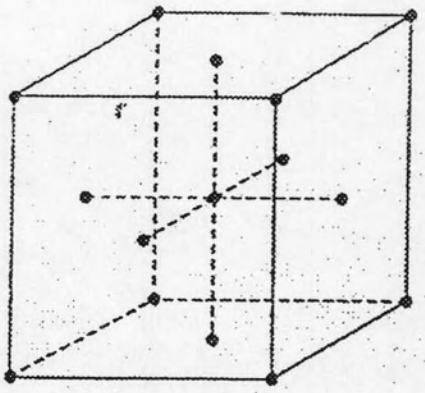


Figure 2.6 Central composite design, (a) for k = 3 and $\alpha = (3)^{1/2}$; (b) for k = 3 and $\alpha = 1$ (face-centered central composite design) (Myers and Montgomery, 2002).

The obtained model equations from the experimental design are very useful for prediction of the responses and product optimization. Chacón et al. (1996) used rotatable CCD for studying in the influence of needle gauge, polymer amount and the injection rates on the mean particle size, relative standard deviation, and drug encapsulation in cyclosporine-loaded poly D, L(lactide-glycolide) (PLAGA) nanoparticles. From the statistical analysis of data, polynomial equations were generated. They found very good correlations between these factors and particles size ($r^2 = 0.9443$), relative standard deviation ($r^2 = 0.8034$), and drug encapsulation ($r^2 = 0.9016$). By using response surface diagrams and the mathematical models proposed, the cyclosporine-loaded PLAGA was possible to easily deduce experimental conditions to prepare with the desired properties.

The sequential development of poly(lactic-co-glycolic-acid) nanoparticles produced by using the w/o/w emulsification solvent evaporation method was performed by Vandervoort and Ludwig (2002). They started their experiment with 2^2 full factorial design to investigate the effect of polymers for combination with poly(vinyl alcohol) (PVA) as stabilizer of the emulsion, and expanded to CCD in order to fit the measured data to a quadratic model and to calculate response surface. It was found that nanoparticles were obtained with most polymers when they were used in combination with PVA. Leaving PVA out of the formulation increased the size of the particles over 1 μm, excepted for poloxamer and carbopol. Therefore, these polymers were selected for the further study performed by using CCD to depict their effect on physicochemical properties of nanoparticles as response surfaces.

Senthilkumar et al. (2005) used CCD to optimize a fermentation medium for the production of alkali-stable cellulose-free xylanase by *Aspergillus fischeri* in solid-state fermentation at pH 9.0 with wheat bran as substrate. They proposed the optimized formulation containing (in g/l) $NaNO_2$-7.0, K_2HPO_4-1.0, $MgSO_4$-0.5 and yeast extract-5.0 resulted in 1.9-fold increased level of alkali-stable xylanase (1,024 U/g wheat bran). Rekhi et al. (1999) studied the influence of critical formulation and processing variables as described in the AAPS/FDA Workshop II report on scale-up of oral extended-release dosage forms, using a hydrophilic polymer hydroxypropyl methylcellulose. The face-centered CCD (26 runs with 3 center points) was selected for investigation of the effect of filler ratio, polymer levels, magnesium stearate levels, lubricant blend time and compression force on percent drug released at 1, 4, 6, and 12 hours. The authors found that the change in polymer level was the most significant factor

affecting drug dissolution time points. Some interaction effects between the variables studied were also found. Response surface plot and regression models were developed which adequately described the experimental space. Finally, they suggested three formulations providing required characteristics for a future bioavailability/bioequivalency study.

This suggested that the CCD is useful not only for the additional knowledge supplied about the process and formulation, but also for process control.