CHAPTER III. LITERATURE REVIEW

3.1 Vitiligo

Vitiligo also known as Leukoderma is a chronic skin disease. This is caused by the loss of pigment, resulting in irregular pale patches of skin. There is some evidence suggesting it is caused by a combination of auto-immune, genetic, and environmental factors Vitiligo develop patches of de-pigmented skin appearing on extremities.

It is reported that Vitiligo is a disorder in which the body destroys its own pigment cells, melanocytes (autoimmune) in various parts of the skin. In affected areas, the pigment gradually disappears. Small or larger areas of skin then become white with sharp margins where they adjoin unaffected parts of skin. If hairs are involved in this area, they too also grow white rather than pigmented. Patches often occur symmetrically across both sides on the body. Occasionally small areas may repigment as they are decolonized by melanocytes.

Vitiligo affected skin changes over time, with some patches re-pigmenting and others becoming affected. In some cases, mild trauma to an area of skin seems to cause new patches - for example around the ankles. Vitiligo may also be caused by stress that affects the immune system, leading the body to react and start eliminating skin pigment. The condition is most noticeable in summer, when normal skin darkens. Because of the lack of protective pigment, the affected areas are also likely to burn and blister in the sun. Vitiligo may also occur at sites of injury to the skin and therefore may at first show itself in scars or burns.

Figure 1: Life style, richness and sex don’t have anything to do with Vitiligo (Adapted from: www.vithappens.com)[20]
In Vitiligo, white blood cells attack and destroy pigment cells in discrete areas of the skin. It is called an auto-immune disorder, because the pigment cells become regarded by the body as "foreign" and are therefore rejected. If the affected areas of skin are examined under the microscope, white blood cells called lymphocytes can be seen clustering around the pigment cells. These are eventually destroyed and the skin loses its pigment as shown in figure 1. Vitiligo on the scalp may affect the color of the hair (though not always), leaving white patches or streaks. It will similarly affect facial and body hair [19]

3.1.1 Classification of vitiligo

Vitiligo is classified according to the distribution, pattern and extent of depigmentation. There are many reports on classification. However, most investigators distinguished two large subtypes of Vitiligo, segmental Vitiligo(S) and non-segmental Vitiligo(NS) [21].

According to another classification proposed by Norlund and Lerner, three types are identified i.e., localized, generalized and universal Vitiligo. Localized Vitiligo is further classified into focal and segmental: generalized into acrofacial, vulgaris and mixed subtypes [22].

i. Focal Vitiligo

one or more macules in one area but not clearly in a segmental distribution. In general terms Focal Vitiligo is localized to a single area, if the patient has a single patch on hand, face or leg with no other occurrences of disease on other body parts. Focal Vitiligo if not active is generally more suited for localized treatments .

ii. Segmental Vitiligo

When one or more macules are in a quasi-dermatomal pattern. In common man’s language here the patches are unilateral and asymmetric in distribution. One or more
patches are distributed within the same segment (Anatomical distribution of the body) of the body. Segmental Vitiligo limited to lips and fingers tips is often called “lip-tip Vitiligo”.

**iii. Acrofacial Vitiligo**

Affecting distal extremities and face some on other body parts, but not all over the body. In common terms, Acrofacial Vitiligo is the term used for a very common distribution of this disease in which the disease is limited to parts away from the centre of the body.

**iv. Vitiligo vulgaris**

In Vitiligo vulgaris small patches of de-pigmentation are spread in a shower pattern admixed with normal skin in segmental or acrofacial pattern. This is most common type with bilaterally lesions, predominantly distributed over face, torso, fingers, toes, palms, soles and facial orifices.

**v. Universal Vitiligo**

Universal Vitiligo or Vitiligo totalis is the complete or nearly complete loss of skin pigment. The pigment is usually lost from entire body surface except a few small islands of normally pigmented skin. Permanent medical bleaching of the normal skin is usually the only option. According to clinical subtypes they are classified into two types; they are segmental and non-segmental Vitiligo.

### 3.1.2. Mechanism of Vitiligo

Literature review enlightens that Vitiligo is associated with autoimmune and inflammatory diseases, commonly thyroid over expression and under expression. Immune mediated points humoral response (antibodies belonging to IgG class)[23] and cell mediated response (Infiltrating T cells)[24]. A study comparing 656 people with and without vitiligo in 114 families which found several mutations NALP gene. The NALP1 gene, which is on chromosome 17 located at 17p13, is on a cascade that regulates inflammation and cell death including myeloid and lymphoid cells, which are white cells that are part of the
immune response. NALP1 is expressed at high levels in T cells and Langerhan’s cells, white cells that are involved in skin autoimmunity[25,26].

Among the inflammatory products of NALP1 are caspase1 and caspase5, which activate the inflammatory cytokine, interleukin 1β. Interleukin 1β is expressed at high levels in patients with Vitiligo. There are compounds which inhibit caspase and interleukin 1β and so might be useful drugs for Vitiligo and associated autoimmune diseases. In one of the mutations, the amino acid leucine in the NALP1 protein was replaced by histidine. The original protein and sequence is highly conserved in evolution and found in humans, chimpanzee, rhesus monkey and bush baby, which means that it’s an important protein and an alteration is likely to be harmful.

The following is the normal DNA and protein sequence in the NALP1 gene:

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In some case of Vitiligo, the first leucine is altered to histidine, by a Leu 155 His mutation.

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Leucine is non-polar and hydrophobic; histidine is positively charged and hydrophobic, so it is unlikely both serve the same function. The normal sequence of the DNA code for NALP1 of TCACTCCTCTACCAA is replaced in some of these Vitiligo families by the sequence TCACACCTCTACCAA, which respectively code for the amino acid sequence of the normal NALP1, protein SLYQ being replaced by SLHYQ[27].

Factors affecting the production of melanin are UV rays, chemicals and drugs antihistamines and antibiotics. There are two major classes of melanin pigments, eumelanin and pheomelanin. Eumelanin is dark-brown to black in colour and pheomelanin is yellow reddish-brown and posses nitrogen (8-11%) and sulphur (9-12%) [28]. Melanin content in melanogenic tissues is some 103 times higher than the levels of these, melanin precursor[29].

The isolation of melanin from melanogenic tissues is time consuming and may involve the risk of structural alteration due to the drastic isolation procedures employed[30,31]. The major function of melanin is to confer photo protection to the skin from ionizing radiations[31].
3.1.3. Itiopathogenesis of Vitiligo

Ironically etiology of vitiligo is still a debate although it has been addressed for the past five decades. Generally it is found that Vitiligo may be precipitated by congenital, tuberous sclerosis, partial albinism, Piebaldism and Warrensburg’s syndrome, immunological, vitiligo halo mole, post-inflammatory, thermal burns, dermatitis (eczema), psoriasis, cutaneous lupus erythematosus, pityriasis versicolor, leprosy, lichen planus, syphilis, occupational/chemical, exposure to depigmentation agents, e.g. p-tertiary butyl phenol. Depigmented areas may continue to arise even after the patient is no longer in contact with the chemicals and to make situation worse depigmentation may occur to other parts which have not been exposed to chemicals.

The main three supportive hypotheses which orient the pathogenesis of Vitiligo are neurochemical, autoimmune and oxidative stress[32-35]. Figure 2 demonstrates the pathway involved.

Neurochemical hypothesis

Nerve endings secrete neurotransmitters (nor epinephrine and acetylcholine) which consequently damage the melanocytes. It is presumed that the high level of catecholamines and its metabolites in the plasma and urine at early stage vitiligo patients are due to the reduction of phenylethanolamine N methyl transferase and tyrosine hydroxylase (figure 3).

Autoimmune hypothesis

This correlates vitiligo with other autoimmune diseases like diabetes, pernicious anemia, thyroid malfunctions, Addison’s disease, alopecia areata and along with circulating antimelanocyte, antikeratinocyte antibodies in the sera of Vitiligo patients.

Oxidative stress hypothesis

The initial pathogenic even in melanocytic destruction is oxidative stress as it is reported that H2O2 accumulates in the epidermis of vitiligo patients due to the defective recycling cycle of tetrahydroprotopien. Additionally it is observed that antioxidant system alteration and drastic
reduction in catalase activity in both lesional and non-lesional epidermis of vitiligo patients. Surplus oxygen release is due to mitochondrial impairment.

Figure 2: Schematic representation of melanogenic pathway showing major intermediates and enzymes (tyrosinase and tyrocinase related proteins, TRP1, TRP2) involved in the biogenesis of melanin.[48]
Figure 3: Flow chart illustrating neurochemical melanocytic degradation leading to vitiligo due to accumulation of norepinephrine, defective recycling of 6BH4, concomitant release of H₂O₂ along with other factors[49].
3.1.4. **Factors affecting speed of re-pigmentation**

The benefits may be visible in a fortnight in quick responders, but generally it takes 2-6 months. The process is faster in disease of recent onset and slower if the disease is longstanding. Another factor that affects the rate of pigmentation is the site of involvement. The process is faster if the disease affects face, neck, chest, abdomen, upper arms and thighs. It is generally slow in case of hands, feet, calves, inguinal and perianal regions; where it may take as long as six months to be visible. It has also been observed that the process of healing is faster in people with a darker complexion than fairer individuals. Bee venom is being studied for its effects on the migration of melanocytes; it may one day be developed into a treatment for Vitiligo [29].

3.1.5. **Statistics of vitiligo and its occurrence**

About 0.5 to 1 percent of the world’s population, or as many as 65 million people, have Vitiligo. In the United States, one to two million people has the disorder. Half the people who have Vitiligo develop it before age twenty. Most develop it before their fortieth birthday. The disorder affects both sexes and all races equally. However, it is more noticeable in people with dark skin [36]. The patches may grow or remain constant in size out of the total vitiligo patients, as many as 25%, 50% and majority of patients have developed symptoms before the age of 10 years, 20 years and 40 years respectively [37]. Its prevalence is varying from 0.46 - 8.8% in India. Gujarat and Rajasthan scores the highest [38,39]. Genetically insight further reveals that prevalence of family cases varies from 6.25-38%. First degree relatives bear a relative risk of getting Vitiligo by 7-10 folds. [40]

3.1.6. **Signs, Symptoms and diagnosis [41-43]**

People who develop Vitiligo usually first notice white patches (depigmentation) on their skin. These patches are more commonly found on sun-exposed areas of the body, including the hands, feet, arms, face, and lips. Other common areas where these white patches appear are the armpits and groin, and around the mouth, eye lids, nostrils, navel, genitals and rectal areas.
In addition to white patches on the skin, people with Vitiligo may have premature graying of the scalp hair, eyelashes, eyebrows, and beard. People with dark skin may notice a loss of color inside their mouths.

The diagnosis of Vitiligo is made based on a physical examination, medical history, and laboratory tests. A doctor will likely suspect vitiligo if the physical examination reveals white patches of skin on the body particularly on sun-exposed areas, including the hands, feet, arms, face, and lips. If vitiligo is suspected, the doctor will take a complete medical history. Important factors in the diagnosis include family history of Vitiligo, rash, sunburn, or other skin trauma at the site of Vitiligo two to three months before depigmentation started, Stress or physical illness, premature (before age 35) graying of the hair, family history of any autoimmune diseases.

3.1.7. Conventional Treatment for vitiligo

The treatment of choice is corticosteroid (cortisone-like) ointment or cream. The appropriate strength will be used, depending on the site involved. A mild steroid would be used on the face and a stronger steroid for the trunk and limbs. Some studies indicate Vitiligo disease patients exhibit a positive response by taking vitamin B complex, Folic Acid (1mg), Vitamin E (600 IU), Vitamin D, ascorbic acid (1000 mg) everyday. Many doctors prescribe a tacrolimus ointment 1% for 4 to 6 months to help treat Vitiligo. Topical cortisone Vitiligo treatments were more common in the 80s. While it has been shown to help in many Vitiligo disease cases, it also has exhibited some consistent side-effects. Vitiligo Treatments are currently a hodgepodge of experiments and cover-ups. For the time being, some of the best and most inexpensive treatments address the appearance without addressing the underlying problem. Phototherapy is also beneficial. Exposure to long-wave ultraviolet (UVA) light from the sun or from UVA lamps, together with Psoralen, called "PUVA", can help in many cases. Psoralen can be taken in a pill 1-2 hours before the exposure or as a Psoralen bath or soaking the area before the exposure. Psoralen photo chemotherapy involves the use of psoralens combined with UV-A light (usually 0.1-0.3 J/cm² UV-A). Treatment with 8-methoxypsoralen, 5-methoxypsoralen, and trimethylpsoralen plus UV-A (PUVA) has often been the most practical choice for treatment, especially in patients with skin types IV-VI who have widespread Vitiligo. Psoralens can be
applied either topically or orally, followed by exposure to artificial UV light or natural sunlight. Vitiligo on the back of the hands and feet is highly resistant to therapy.

The best results from PUVA can be obtained on the face, trunk, and proximal parts of the extremities. However, 2-3 treatments per week for many months are required before repigmentation from perifollicular openings merges to produce confluent repigmentation. The total number of PUVA treatments required is 50-300. Repigmentation occurs in a perifollicular pattern.

Systemic phototherapy induces cosmetically satisfactory repigmentation in up to 70% of patients with early or localized disease. Narrow-band UV-B phototherapy is widely used and produces good clinical results. Narrow-band fluorescent tubes (Philips TL-01/100W) with an emission spectrum of 310-315 nm and a maximum wavelength of 311 nm are used. Treatment frequency is 2-3 times weekly, but never on consecutive days. This treatment can be safely used in children, pregnant women, and lactating women. Short-term adverse effects include pruritus and xerosis. Several studies have demonstrated the effectiveness of narrow-band UV-B therapy as monotherapy.

UV-B narrow-band micro phototherapy is therapy targeting the specific small lesions. Selective narrow-band UV-B (311 nm) is used with a fiber optic system to direct radiation to specific areas of skin. Narrow-band UV-B has become the first choice of therapy for adults and children with generalized Vitiligo.

The advantages of narrow-band UV-B over PUVA include shorter treatment times, no drug costs, no adverse GI effects (eg, nausea), and no need for subsequent photo protection [41-44].

3.2. Pepper, piperine and Vitiligo

Scientists at King’s College London have discovered that Piperine the compound that gives black pepper its spicy, pungent flavor and its synthetic derivatives can stimulate pigmentation in the skin, especially when combined with UVR treatment[48]. Black pepper could provide a new treatment for the skin disease Vitiligo, groundbreaking new research in the British Journal of Dermatology will reveal. Current
treatments include corticosteroids applied to the skin, and phototherapy using UV radiation (UVR) to re-pigment the skin. Both, however, carry possible long-term side effects and are not always effective. In particular, less than a quarter of patients respond successfully to corticosteroids, while UVR causes a re-pigmentation that is spotted and patchy and in the long-term could lead to a higher risk of skin cancer. The researchers compared the effect of Piperine and its analogues tetrahydro Piperine (THP), cyclohexyl analogue of Piperine (CHP) and reduced CHP (rCHP) when applied to the skin of mice, either alone or followed by UVR. Treatment was also interrupted in certain groups to see how long-term effects would be.

While CHP did not show significant results, Piperine, THP and rCHP did induce pigmentation in the skin. Used alone, the compounds stimulated pigmentation to an even, light brown colour within six weeks. However, by accompanying use of Piperine or THP with UVR, the skin became significantly darker, and within only seven weeks. Furthermore, the pigmentation was even, compared to the patchy pigmentation caused by UVR treatment alone. The researchers also found that for skin treated with a Piperine compound, just four exposures of UVR were sufficient to significantly darken the skin.

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The researchers also found that for skin treated with a Piperine compound, just four exposures of UVR were sufficient to significantly darken the skin. However, when using UVR alone, more than 10 exposures were needed to produce a similar but less even response. The
results also took longer to fade in those treated with both a Piperine compound and UVR, and did not disappear completely. By contrast, there was no remaining detectable pigmentation within the same timeframe for skin treated only with UVR.

Additionally, when treatment was resumed, results were noticeable faster in the group treated with Piperine compounds. The team believes that their remarkable findings are due to Piperine stimulating the production of the skin’s pigment cells, called melanocytes.

Lead investigator and Associate Professor, Oregon Health & Science University, USA, Dr Amala Soumyanath said: “This is an important step in a multidisciplinary drug discovery program. We have moved from testing plant extracts in pigment cell cultures, to identifying active natural compounds and designing novel chemical analogues, and now confirmed activity of these compounds in whole animals. Our next goal is to move the work to humans through safety studies and clinical trials.”

Professor Antony Young, Photo biologist at St John’s Institute of Dermatology, King’s College London, and another of the study’s authors, said: “We have shown that topical treatment with Piperine stimulates even pigmentation in the skin. Combining this with UVR significantly enhances the pigmentation with results that are cosmetically better than conventional Vitiligo therapies. This provides strong support for the future clinical evaluation of Piperine and its derivatives as novel treatments for Vitiligo.”

Nina Goad of the British Association of Dermatologists said: “These findings could potentially lead to the development of treatments that not only provide improved results, but could also reduce the need for UV radiation in Vitiligo treatment, in turn lowering the risk of skin cancer.

Stimulation of mouse melanocyte proliferation by *Piper nigrum* fruit extract and it’s main alkaloid, Piperine was found to posses growth-stimulatory activity towards cultured melanocytes during a herbal screening programme for the treatment of Vitiligo. It’s aqueous extract at 0.1 mg/ml was observed to cause nearly 300% stimulation of the growth of a cultured mouse melanocyte line, melan-a, in 8 days (p < 0.01) [49].
3.3. Anatomy and physiology of skin

Skin along with having the credit of being the largest organ, it also accounts for functioning as an immune organ by accommodating T-cells, Langerhans cells, monocytes, granulocytes and mast cells. In adults, the skin covers an area of about 2 sq m (22 sq feet), weighs 4 ½ to 5 kg (10-11 lb). Its thickness is 0.5-4 mm, depending on location. pH of skin is 4-5.6 [51].

3.3.1. Anatomy of skin

As chartered in figure 4, structurally the skin consists of two important parts, the superficial, thinner portion, which is composed of epithelial tissue, is called as epidermis. The epidermis is attached to the deeper, thicker, connective tissue part called dermis. Deep to the dermis is a subcutaneous layer which is also called superficial fascia or hypodermis consists of areolar and adipose tissues.
3.3.2. Physiology

1. Regulation of body temperature: In response to high environmental temperature or strenuous exercise, the evaporation of sweat from the skin surface helps lower an elevated body temperature to normal. In response to low environmental temperature, production of sweat is decreased which helps conserve heat. Changes in flow of blood to the skin also help regulate body temperature.

2. Protection: The skin covers the body and provides barrier that protects underlying tissues from physical abrasion, dehydration and UV radiation. Hair and nails also have protective functions.

3. Sensation: The skin contains abundant nerve endings and receptors that detect stimuli related to temperature, touch, pressure and pain.

4. Excretion: Besides removing heat and some water from the body sweat also is the vehicle for the loss of a small quantity of ions and several organic compounds.

5. Immunity: Certain cells of the epidermis are important components of the immune system which fend off foreign invaders.

6. Blood reservoir: The dermis consists of extensive networks of blood vessels that carry 8-10% of the total blood flow in a resting adult. In moderate exercise, skin blood flow may increase which helps dissipate heat from the body.

7. Synthesis of Vitamin D: Synthesis of Vitamin D begins with activation of a precursor molecule in the skin by UV rays in sunlight.

3.3.3. Epidermis

The epidermis is the outer layer of skin. The thickness of the epidermis varies in different types of skin. It is the thinnest on the eyelids at 0.05mm and the thickest on the palms and soles at 1.5mm.

The epidermis contains 5 layers. From bottom to top the layers are named:

- Stratum basale
- Stratum spinosum
- Stratum granulosum
- **Stratum llicitum**
- **Stratum corneum**

  The bottom layer, the stratum basale, has cells that are shaped like columns. In the layer the cells divide and push already formed cells move into the higher layers, they flatten and eventually die. The top layer of the epidermis, the stratum corneum, is made of dead, flat skin cells that shed for every 2 weeks.

  Illustration of maturation of epidermal cells

### 3.3.5. Specialized Epidermal cells

There are three types of specialized cells in the epidermis.

- The melanocyte produces pigment (melanin)
- The langerhan’s cell is the frontline defense of the immune system in the skin.
- The Merkel’s cell’s function is not clearly known.

**Dermis**

The dermis also varies in thickness depending on the location of the skin. It is 0.3mm on the eyelid and 3.0mm on the back. The dermis is composed of three types of tissue that are present throughout the body but not in layers. The types of tissue are:

- Collagen
- Elastic tissue
- Reticular fibres

Layers of the dermis are the papillary and reticular layers.

- The upper, papillary layer contains a thin arrangement of collagen fibres.
- The lower, reticular layer, is thicker and made of thick collagen fibres that are arranged parallel to the surface of the skin,
3.3.6. Specialized Dermal cells

The dermis contains many specialized cells and structures.

- The hair follicles are situated here with the erector pill muscle that attaches to each follicle.
- Sebaceous (oil) glands and apocrine (scent) glands are associated with the follicle.
- This layer also contains eccrine (sweat) glands, but they are not associated with hair follicles.
- Blood vessels and nerves course through this layer. The nerves transmit sensations of pain, itch and temperature.

There are also specialized nerve cells called Meissner’s and vater-Pacini corpuscles that transmit the sensations of touch and pressure.

3.3.7. Subcutaneous Tissue

The subcutaneous tissue is a layer of fat and connective tissue that houses larger blood vessels and nerves. This layer is important in the regulation of temperature of the skin itself and the body. The size of this layer varies throughout the body and from person to person.

The skin is a complicated structure with many functions. If any of the structures in the skin are not working properly, a rash or abnormal sensation is the result. The whole specialty of dermatology is devoted to understanding the skin, what can go wrong and what to do if something goes wrong.

Melanin-Melanocytes synthesize melanin from the amino acid tyrosine in the presence of an enzyme called tyrosinase, synthesis occur in an organelle called a melanosome and secreted by melanocytes in the deep germinating layer, is absorbed by surrounding epithelial cells. The amount is genetically determined and varies between different parts of the body between members of the same race and between races. The number of melanocytes is fairly constant so the differences in colour depend on the amount of melanin secreted. It protects the skin from the harmful effects of sunlight. Exposure to UV light increases the enzymatic activity within melanosomes and thus increases melanin production. Both the amount and darkness of melanin increase up on UV exposure which gives the skin a tanned appearance and helps to protect the
body against further UV radiation. Thus within limits melanin serves as a protective function. A
tan is lost when the melanin containing keratinocytes are shed from the stratum corneum. The
level of oxygenation of haemoglobin and the amount of blood circulating in the dermis gives the
skin its pink colour.

3.3.8. Factors affecting permeability

There are many factors affecting permeability of the skin for drugs and vehicles:

1. Factors associated with skin, which include hydration and thickness of horny layer and
skin condition. High water content in keratinized cells and thickness of the horny layer
improves the rate of penetration of drugs. Skin conditions like age, disease, climate and
injury also affect the permeability.

2. Factors associated with the medicament, which include many physico-chemical
properties of the drugs e.g.,

(a) Solubility: High lipid solubility of drugs favours rapid absorption through follicles which
through the epidermis by more or less balanced lipid and water solubility.

(b) Dissociation constant: Penetration of ionic medicament is influenced by its dissociation
constant and the pH of its surroundings, since it has come under the effect of electrostatic
charges in the layers of skin. It forms an electrostatic barrier for the absorption of certain drugs.
Esterification of drug molecules has been suggested to overcome this barrier.

   e.g., methyl salicylate and methyl nicotinate have been found to penetration much more readily
than the corresponding un esterified acids.

(c) Particle size: Reduction in particle size of poorly soluble drug enhances dissociation rate and
that release from the vehicles.
3.3.9. Membrane transport mechanisms

As mentioned in the flow chart (figure 5) various transport mechanisms are proposed. Many drugs need to pass through one or more cell membranes to reach their site of action. A common feature of all cell membranes is a phospholipid bilayer, about 10 nm thick, arranged with the hydrophilic heads on the outside and the lipophilic chains facing inwards. This gives a sandwich effect, with two hydrophilic layers surrounding the central hydrophobic one. Spanning this bilayer or attached to the outer or inner leaflets are glycoproteins, which may act as ion channels, receptors, intermediate messengers (G-proteins) or enzymes [53].

The cell membrane has been described as a ‘fluid mosaic’ as the positions of individual phosphoglycerides and glycoproteins are by no means fixed. An exception to this is a specialized
membrane area such as the neuromuscular junction, where the array of post-synaptic receptors is found opposite a motor nerve ending.

The general cell membrane structure is modified in certain tissues to allow more specialized functions. Capillary endothelial cells have fenestrae, which are regions of the endothelial cell where the outer and inner membranes are fused together, with no intervening cytosol. These make the endothelium of the capillary relatively permeable; fluid in particular can pass rapidly through the cell by this route. In the case of the renal glomerular endothelium, gaps or clefts exist between cells to allow the passage of larger molecules as part of filtration. Tight junctions exist between endothelial cells of brain blood vessels, forming the blood–brain barrier (BBB), intestinal mucosa and renal tubules. These limit the passage of polar molecules and also prevent the lateral movement of glycoproteins within the cell membrane, which may help to keep specialized glycoproteins at their site of action (e.g. transport glycoproteins on the luminal surface of intestinal mucosa).

(i) Para cellular and transcellular transport:

Permeation of peptide-based compounds across cellular barriers can occur by several routes. One needs to be mindful that each of these routes of permeation can apply to different peptide-based compounds; however, the physicochemical properties of the compound and the physiological significance of each route will determine the relative importance in controlling the compound’s net absorption. In screening compound permeation across cellular barriers, delineation of the key rate-determining steps for controlling absorption needs to be identified. Methods to delineate the functional relevancy of each route are now being enhanced further by focusing the kinetics on potential para cellular and transcellular events.

(ii) Passive diffusion:

This is the commonest method for crossing the cell membrane. Drug molecules move down a concentration gradient, from an area of high concentration to one of low concentration, and the process requires no energy to proceed. Many drugs are weak acids or weak bases and can exist in either the unionized or ionized form, depending on the pH. The unionized form of a drug
is lipid-soluble and diffuses easily by dissolution in the lipid bilayer. Thus the rate at which transfer occurs depends on the pKa of the drug in question.

In addition, there are specialized ion channels in the membrane that allow intermittent passive movement of selected ions down a concentration gradient. When opened, ion channels allow rapid ion flux for a short time (a few milliseconds) down relatively large concentration and electrical gradients, which makes them suitable to propagate either ligand- or voltage-gated action potentials in nerve and muscle membranes.

(iii) Active transport:

Active transport is an energy-requiring process. The molecule is transported against its concentration gradient by a molecular pump, which requires energy to function. Energy can be supplied either directly to the ion pump, or indirectly by coupling pump-action to an ionic gradient that is actively maintained. Active transport is encountered commonly in gut mucosa, the liver, renal tubules and the BBB.

(iii) Facilitated diffusion:

Facilitated diffusion refers to the process where molecules combine with membrane bound carrier proteins to cross the membrane. The rate of diffusion of the molecule–protein complex is still down a concentration gradient but is faster than would be expected by diffusion alone. Examples of this process include the absorption of steroids and amino acids from the gut lumen. The absorption of glucose, a very polar molecule, would be relatively slow if it occurred by diffusion alone and requires facilitated diffusion to cross membranes (including the BBB) rapidly.

(iv) Pinocytosis:

Pinocytosis is the process by which an area of the cell membrane invaginates around the (usually large) target molecule and moves it into the cell. The molecule may then be released into the cell or may remain in the vacuole so created, until the reverse process occurs on the opposite side of the cell. The process is usually used for molecules that are too large to traverse the membrane easily via another mechanism.
(v) Vesicular transport:

Vesicular transport is an active process in which materials move into or out of the cell enclosed as vesicles. Vesicles are bubble-like structures surrounded by a membrane. They can form at the cell membrane or can fuse with the membrane. Solid particles, droplets of fluid or many molecules at a time can be moved across the membrane in vesicles. Vesicular transport is also known as bulk transport because large quantities of materials can be transported in this way.

There are two basic types of vesicular transport—endocytosis and exocytosis. Permeation of peptide-based compounds across cellular barriers can occur by several routes. One needs to be mindful that each of these routes of permeation can apply to different peptide-based compounds; however, the physicochemical properties of the compound and the physiological significance of each route will determine the relative importance in controlling the compound’s net absorption. In screening compound permeation across cellular barriers, delineation of the key rate-determining steps for controlling absorption needs to be identified. Methods to delineate the functional relevancy of each route are now being enhanced further by focusing the kinetics on potential paracellular and transcellular events. Adson et al. demonstrated that the delineation of the paracellular and transcellular mass transfer resistances from observed cell-based assay permeability studies was possible. Several studies conducted by Burton and colleagues further demonstrated that hydrogen-bonding potential may provide a better means for predicting the potential passive transcellular diffusion of peptides.

Based on these observations, several studies conducted with larger oligopeptides demonstrated that conformation and lipophilicity may dictate the extent of passive paracellular and transcellular peptide transport across bovine brain micro vessel endothelial cell (BBMEC) barriers[55-60].

3.3.10. Factors to consider when choosing a topical preparation

1. Always consider the effect of the vehicle. An occlusive vehicle enhances penetration of the active ingredient and improves efficacy. The vehicle itself may have a cooling,
drying, emollient, or protective action. It can also cause side effects by being excessively drying or occlusive.

2. Match the type of preparation with the type of lesions. For example, avoid greasy ointments for acute weepy dermatitis.

3. Match the type of preparation with the site (e.g., gel or lotion for hairy areas).

4. Consider irritation or sensitization potential. Generally, ointments and w/o creams are less irritating, while gels are irritating. Ointments do not contain preservatives or emulsifiers if allergy to these agents is a concern.

5. Presence of permeation enhancers also alters drug absorption (table 1).

| Table 1: classification of permeation enhancers [61,62] |
|---|---|
| **Class** | **Example** |
| Surfactants | SLS, tween 80, polysorbates |
| Bile salts and derivatives | Sodium glycolate, sodium deoxycholate |
| Fatty acids and derivatives | Oleic acid, caprylic acid |
| Chelating agents | EDTA, citric acid |
| Sulphoxides | DMSO, DMA, DMF |
| polyols | PG, PEG, Glycerol |
| Monohydric alcohols | Ethanol, 2-propanal |
| Urea and its derivatives | |
| Terpenes and terpenoids | 1-menthol, nerolidol |
3.4. Topical formulations

3.4.1. Cream

Creams are semisolid dosage forms containing one or more drug substances dissolved or dispersed in a suitable base. This term has traditionally been applied to semisolids that possess a relatively fluid consistency formulated as either water-in-oil (e.g., Cold Cream) or oil-in-water (e.g., Fluocinolone Acetonide Cream) emulsions. However, more recently the term has been restricted to products consisting of oil-in-water emulsions or aqueous microcrystalline dispersions of long-chain fatty acids or alcohols that are water washable and more cosmetically and aesthetically acceptable. Creams can be used for administering drugs via the vaginal route (e.g., Triple Sulfa Vaginal Cream).

Some salient features of creams:

- Emulsion of water and oil.
- Classified as oil in water (o/w) or water in oil (w/o) emulsions.
- O/W Creams (e.g. vanishing creams) spread easily and do not leave the skin greasy and sticky.
- W/O Creams (e.g. cold cream) are more greasy and more emollient.
- Creams contain emulsifiers and preservatives which may cause contact allergy.

A cream is a topical preparation usually for application to the skin. Creams for application to mucus membranes such as those of the rectum or vagina are also used. Creams may be considered pharmaceutical products as even cosmetic creams are based on techniques developed by pharmacy and un medicated creams are highly used in a variety of skin conditions (dermatoses).

Creams are semi-solid emulsions, that is mixtures of oil and water. They are divided into two types: oil-in-water (O/W) creams which are composed of small droplets of oil dispersed in a continuous aqueous phase, and water-in-oil (W/O) creams which are composed of small droplets of water dispersed in a continuous oily phase. Oil-in-water creams are more comfortable and cosmetically acceptable as they are less greasy and more easily washed off using water.
Water-in-oil creams are more difficult to handle but many drugs which are incorporated into creams are hydrophobic and will be released more readily from a water-in-oil cream than an oil-in-water cream. Water-in-oil creams are also more moisturizing as they provide an oily barrier which reduces water loss from the stratum corneum, the outmost layer of the skin. Presence of permeation enhancers also alters drug absorption (table 1).

3.4.1.1. Uses of creams

- The provision of a barrier to protect the skin. This may be a physical barrier or a chemical barrier as with sunscreens.
- To aid in the retention of moisture (especially water-in-oil creams)
- Cleansing.
- Emollient effects.
- As a vehicle for drug substances such as local anesthetics, anti-inflammatories. (NSAIDs or corticosteroids), hormones, antibiotics, antifungals or counter-irritants.

3.4.2. Ointment

- Semi-Solid preparations of hydrocarbons (petrolatum, mineral oil, paraffins, synthetic hydrocarbons)
- Strong emollient effect makes it useful in dry skin conditions.
- Occlusive effect enhances penetration of active drug and improves efficacy (especially in thickened, lichenified skin).
- Provides a protective film on the skin (e.g., useful in housewife’s hands, irritant dermatitis).
- Greasy, sticky, retains sweat (therefore, not suitable in wet weepy dermatitis, hairy areas, skin prone to folliculitis, or hot weather conditions).
- Contains no water and does not require a preservative.
3.5. Theories and Approximations In Micelle and Vesicle Formations

3.5.1. Vesicles and its role in drug delivery

In the last few years, the vesicular systems have been promoted as a mean of sustained or controlled release of drugs. These vesicles are preferred over other formulations because of their specific characteristics such as lack of toxicity, biodegradation, capacity of encapsulating both hydrophilic and lipophilic molecules, capacity of prolonging the existence of the drug in the systemic circulation by encapsulation in vesicular structures, capacity of targeting the organs and tissues, capacity of reducing the drug toxicity and increasing its bioavailability. Vesicular systems such as liposomal formulations have been used as drug delivery vehicles for sustained release of proteins and peptides. These formulations have been used as carriers of cytotoxic drugs with the strategy based on reduction of toxicity and passive delivery to tumors. The propensity of the reticuloendothelial system (RES) uptake to liposomes from the circulation has thus far limited the prospect of targeting liposomes to tissues other than liver, spleen, and lung. Sterically stabilized liposomes, in particular, are considered promising carriers for therapeutic agents because they can facilitate controlled release and targeted delivery of drugs, thereby reducing drug-related toxicity [63-68].

Vesicles are water-filled colloidal particles. The walls of these capsules consist of amphiphilic molecules (lipids and surfactants) in a bilayer conformation. In an excess of water these amphiphilic molecules can form one (unilamellar vesicles) or more (multilamellar vesicles) concentric bilayers. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic, lipophilic and charged hydrophilic drugs can be associated with the vesicle bilayer by hydrophobic and/or electrostatic interactions. Most commonly, the vesicles are composed of phospholipids or non-ionic surfactants. The reason for using vesicles in transdermal drug delivery is based on the fact that they act as drug carriers to deliver entrapped drug molecules across the skin, as well as penetration enhancers because of their composition. In addition, these vesicles serve as a depot for the sustained release of active compounds in the case of topical formulations, as well as rate-limiting membrane barrier for the modulation of systemic absorption in the case of transdermal formulations [69].
3.5.2. Micelle formation: Law of Mass Action: We consider an aqueous solution of neutral amphiphilic molecules (i.e., non-ionic surfactants), each of which has a single alkyl chain as its hydrophobic tail. In general, amphiphiles can form aggregates of various sizes and shapes. We will assume each micelle is spherical and neglect the effects of fluctuations in micelle size and shape. Thus, we imagine that each surfactant molecule exists either as a monomer or as part of a spherical n-mer. We denote the number densities of the monomers and n-mers by $\rho_1$ and $\rho_n$, respectively, so that the total surfactant concentration is given by

$$\rho = \rho_1 + n \rho_n$$

(1)

The concentrations of monomers and micelles are related by the law of mass action $^{[9]}$

$$\rho_n a^3 = (\rho_1 a^3) \exp (-\beta \Delta G)$$

(2)

where $\beta$ denotes inverse temperature (i.e., $\beta^{-1} = k_B T$), ‘$a$’ is a microscopic length that specifies the standard state convention, and is the driving force for assembly, namely, the free energy of the n-mer, $f_n$, relative to that of n monomers, $nf_1$. We take ‘$a$’ to be approximately the girth of a surfactant molecule.

$$\Delta G = f_n - nf_1$$

(3)

For large $n$, equation 3 implies the existence of a threshold concentration of surfactant molecules $\rho_{\text{cmc}}$, at which the density of aggregates becomes significant. Because this crossover is precipitous, its location is almost independent of the specific definition of the threshold as long as it is physically sensible. Specifically, to within corrections of order $n^{-1} \ln n$.

$$\ln \beta_{\text{cmc}} a^3 = \beta \Delta G \ln^*$$

(4)

The driving force per surfactant, $\Delta G/ n$, is a function of $n$, and it is to be evaluated at the most probable aggregation number, $n^*$. This number is the value of $n$ that minimizes $\Delta G/n$.

Driving Force: The contributions to $\Delta G$ can be found in three steps,

(1) Creation of a Cavity: A micelle will fill a region vacated by water. Assuming the extent of the surface is at least 1 nm$^2$, the free energy to create this cavity is

$$\Delta G_1 = -A$$

(5)
Where \( A \) denotes the surface area of the cavity and \( \delta \) is the water-vapour surface tension. In general, there is also pressure volume work for forming a cavity in a liquid. For water at standard conditions, pressure is sufficiently small that this contribution is negligible for cavities with diameters less than 5 nm. We will limit our consideration to sizes within this range.

(2) **Filling the Hydrophobic Core:** Imagine disconnecting each hydrophobic tail in a surfactant from its respective hydrophilic head group and moving the hydrophobic tail from water into the micelle core.

A total of \( n \) tails must be moved to fill the cavity formed in step 1. As such, one part of the free energy to fill the cavity is \(-n\Delta \mu\), where \(-\Delta \mu\) is the free energy change in transferring the hydrophobic tail (e.g., an alkane chain) from water into the oily hydrophobic core. An additional part of the free energy for filling the cavity is an interfacial contribution due to the presence of Vander Waals attractions between oil and water. These interactions cause the oil-water surface tension, \( \gamma_{ow} \), to be lower than the water-vapour surface tension, \( \gamma \). [71]

Thus, the free energy for filling the cavity is

\[
\Delta G_2 = -n\Delta \mu - \Delta \gamma A \quad \text{........} \quad \text{.................................} (6)
\]

Where: \( \Delta \gamma = \gamma - \gamma_{ow} \)

The interior of a micelle is densely packed and much likes a hydrocarbon liquid. Thus, \( \Delta \mu \) is close to the transfer free energy for moving the associated alkenes chain from oil into water. However, it is slightly smaller than this value because the environment of an alkane chain in a micelle interior is more confining than that in bulk oil [71].

To the extent that the micelle is spherical, \( A=4\pi L^2 \), where \( L \) is the micelle radius. Since the interior is densely packed, \( L \) is given by \( 4\pi L^3/3 = n\delta \alpha^2 \), where \( \alpha \) is the mean length over which a polar head group is separated from an alkyl group within a surfactant molecule. From these considerations.

\[
\Delta G_1 + \Delta G_2 = -n\Delta \mu + \mu g n^{2/3} \quad \text{.................................} \quad \text{........}(7)
\]

\[
G = g = (36\pi)^{1/3} (\gamma_{ow}\alpha^2) = 4.8 \times (\gamma_{ow} \alpha^2) (\gamma/\alpha)^{2/3} \quad \text{.................................} \quad \text{........}(8)
\]
The right-hand side of equation is essentially the free energy for nucleating oil clusters in water\[72\]. It is the hydrophobic driving force identified in the Lum-Chandler-Weeks theory \[73\].

The first term is proportional to the volume of hydrophobic units. The second term is proportional to the area of the interface. The first term is extensive in \(n\) and dominates at large \(n\). Thus, if only \(\Delta G_1\) and \(\Delta G_2\) were significant, the strength of the driving force would grow without bound leading to macroscopic clusters.

Placing Hydrophilic Head groups on the Micelle Surface: In the final step, the hydrophilic head groups are reconnected to the hydrophobic tails, placing them at the water-oil interface so as to maintain favorable solvation energy. This positioning is to be done while simultaneously enforcing the connectivity between heads and tails and while also maintaining the densely packed interior. These conditions result in an entropic cost that increases super-extensively with aggregate size. The form of this third contribution to the driving force is conveniently estimated from the electrostatic analogy of stoichiometric constraints \[74,75\].

The result is \(\Delta G_3 = hn^{5/3}/\beta \) ……………………………………………. (8)

Where: \(h = \frac{3}{(4\pi)^{2/3}(96/49)}\left(\frac{\alpha}{\delta}\right)^{4/3} X 0.75 \times \left(\frac{\alpha}{\delta}\right)^{4/3} \) …………………………………………… (9)

In employing this analogy, it is important to note that the micelle volume is essentially that of the densely packed alkyl chains.

3.5.3. Micelle Size and Critical Micelle Concentration: Combining the three contributions discussed above gives the driving force in units of \(k_B T\).

\[\beta \Delta G \sim n \beta \Delta \mu + \beta gn^{2/3} + hn^{5/3} \] ……………………………………………… (9)

Minimization of \(\Delta G/n\) therefore gives

\[N^* \sim \frac{\beta \gamma}{2h} = \frac{(49\pi/48)}{\beta \gamma} \frac{\delta^2}{2} \] …………………………………………… (10)

With this aggregation number,

\[\ln \rho_{\text{cmc}} = c\left(\frac{\Delta \mu}{\rho_{\text{ow}} \alpha^2}\right)^{2/3} - \beta \Delta \mu \] ……………………………………………… (11)
Where \( c = \left(\frac{5832}{49}\right)^{2/3} \cdot 4.9 \)

The thermodynamic cycle of micelle formation can be observed in figure 6. Correlation of the amphiphile structure with its phase behavior could be understood with a simple geometric model, which defines a dimensionless critical packing parameter (CPP) to describe the relative bulkiness of the hydrophobic part and the hydrophilic part in an amphiphile. With the CPP increasing from a small value to a high value the amphiphile changes from hydrophilic to hydrophobic, its preferred phase structure from direct structures via lamellar structure to reverse structures. This model provides a basis for the molecular design of amphiphiles.

Figure 6: Thermodynamic cycle of micelle formation. The process of assembling \( n \) separated amphiphiles (a) to a micelle (d) can be performed in three steps: (1) Creating a cavity in the solvent (light gray) (b); (2) Transferring the hydrophobic chains (dark gray) from the aqueous solution into the cavity (c); (3) Distributing the polar units (gray) over the surface of the cavity and reconnecting them to the hydrophobic groups (d).

Molecules on the surface feels curvature when the size is small and molecules experience stress. The stress gets significant role when the size is below 100 nm. For bilayer formation, the molecules must be amphiphilic. Typical geometry for a vesicle formation is,
\[ P = \frac{V}{a_0 I_c} \quad \text{.................. (12)} \]

Were \( P \) = critical packing parameter; \( I_c \) = hydrophilic chain length; \( V \) = volume of hydrophilic part; \( a_0 \) = optimum surface area/molecule at interface\(^{76}\).

A pictorial representation of geometry and variants of packing arrangements are shown in figure 7 and 8 respectively\(^{77}\) and its formation is described in figure 9.

![Figure 7: Representation of typical geometry for a vesicle formation, \( P = \frac{V}{a_0 I_c} \)](image)

![Figure 8: A) \( P = \frac{1}{3} \), micelle formation B) \( P = \frac{1}{2} \), cylindrical micelle C) \( P = 1 \), planar bilayer D) \( P > 1 \), inverted micelle.](image)
3.5.4. Vesicle formation:

*Formation of Large Vesicles from Small, Preformed Vesicles:*
Small, uniform-sized egg lecithin vesicles were described. Any solute to be entrapped was added to the vesicle solution at this point and the vesicles were adjusted to 20mM phospholipids. The solution was then warmed to 25°C and an aliquot of 250mM sodium deoxycholate was rapidly added and mixed to give a final mixture containing a ratio of deoxycholate to phospholipids of 1:2. The large vesicles began to form almost immediately, as indicated by the increase in the light scattering of the solution, a change from nearly clear for the small vesicles to a transparent opalescence for the large vesicles. Vesicle formation was complete within 5-10 min at 250°C (vesicles could also be formed at 40°C and required 15-30 min). The bulk of the detergent (96-98%) was then removed by passage of the sample over 60 vol of Sephadex G-25 (medium porosity). The residual deoxycholate may represent detergent trapped within the vesicle which re-equilibrates with the outside volume after the removal of the bulk of the detergent; it was very readily removed by a second gel filtration column of 20-30 vol. The final preparation contained less than 1 deoxycholate molecule per 1000 phospholipid molecules. The vesicles are stable at this stage for several weeks, as indicated by the lack of change in the turbidity or the size when examined by electron microscopy. The stability appears to be limited by the chemical stability of the unsaturated phospholipid component [78].
3.5.5. Formation of large vesicles from a phospholipid film

Egg lecithin (20µmol) in chloroform was dried under reduced pressure to a thin film in a 15ml Cortex tube. One millilitre of buffer containing 10mM sodium deoxycholate plus any solute to be entrapped in the vesicles was added and the tube was swirled to suspend the lipid. This suspension was sonicated briefly in a bran sonic 12 sonifier bath at 25°C under N₂. After 1-2 min of sonication, the solution changed from an opaque, milky colour to a transparent opalescence. Continued sonication for 15 min brought no further change in the appearance of the solution and no further decrease in the turbidity. At this point deoxycholate was removed.

Vesicles are classified into multi lamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs). General procedures for vesicular preparations are

- Direct hydration,
- Hydration from organic solvent, and
- Detergent removal.

Hydration from organic solvent method is used for producing multi lamellar vesicles (MLVs). MLVs extrusion through 0.1µm polycarbonate filter and plus freeze-thaw technique, reverse phase evaporation and detergent removal procedures are used for producing large unilamellar vesicles (LUVs.). Sonication is used for producing small unilamellar vesicles (SUVs).

Direct hydration procedure has certain advantages such as it is fast procedure. Its disadvantages include low trapped volume, low trapping efficiency and unequal distribution of solute. The advantage of hydration from organic solvent procedure is high trapping efficiency and its disadvantages include technically complex and limited by lipid solubility in organic phase.

Detergent removal procedure has advantages such as reconstitution of proteins possible, high trapped volume and its disadvantages are its difficult to remove detergents completely, low trapping efficiency.

3.5.6. Vesicles and its hybrids

Liposomal formulations can be classified in two categories:
• Rigid vesicles – liposomes and niosomes
• Elastic or ultra deformable vesicles – transfersomes and ethosomes.

Depending on the structure, composition and methods vesicles can be modulated into several categories which have been described in table no. 2.

Characterization and evaluation of vesicles include entrapment efficiency, drug loading, surface morphology (Transmission Electron Microscopy, Scanning Electron Microscopy, Atomic Force Microscopy), size distribution (Dynamic Light Scattering), tissue vesicular transport mechanism [Confocal Scanning Light Microscopy] drug release etc. While degree of deformation is a specific test for transfersomes, Small angle X-ray scattering (SAXS) and NMR may be used for determining the nodal surface of cubosomes.

Table 2. Hybrids of vesicles and their composition

<table>
<thead>
<tr>
<th>Type</th>
<th>Sub-Type</th>
<th>Composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>Conventional</td>
<td>Neutral and or negatively charged phospholipids + cholesterol</td>
<td>Gregoriadis G., 1972. [80]</td>
</tr>
<tr>
<td></td>
<td>PH sensitive</td>
<td>Phospholipid such as phosphotidyl ethanolamine with CHEMS</td>
<td>Mathiowitz. Eds, 1999. [81]</td>
</tr>
<tr>
<td></td>
<td>Cationic</td>
<td>Cationic lipids</td>
<td>Felgner P.L., 1994. [82]</td>
</tr>
<tr>
<td></td>
<td>Long circulating</td>
<td>Neutral high transition temperature, lipid cholesterol + 5-10% of PEG-DSPE, GMI, HPI.</td>
<td>Gregoriadis G., 1998. [83]</td>
</tr>
<tr>
<td></td>
<td>Immune-</td>
<td>Conventional or long</td>
<td>Plautz G.E.,</td>
</tr>
<tr>
<td>Type</td>
<td>Composition</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>liposomes</td>
<td>circulating liposomes with attached Ab or recognition sequence.</td>
<td>1993</td>
<td></td>
</tr>
<tr>
<td>Magnetic liposomes</td>
<td>Phosphotidylcholine, cholesterol, small amounts of a linear chain aldehyde and colloidal particles of magnetic iron oxide.</td>
<td>Elmi M.M., 2001</td>
<td></td>
</tr>
<tr>
<td>Temperature-sensitive liposomes</td>
<td>Dipalmitoylphosphatidylcholine</td>
<td>Sullivan S.M., 1986</td>
<td></td>
</tr>
<tr>
<td>Virosomes</td>
<td>Virus glycoprotein, incorporated into liposomal bilayers based on retro viruses derived lipids.</td>
<td>Huckriede A., 2003</td>
<td></td>
</tr>
<tr>
<td>Cryptosomes</td>
<td>Lipid vesicles</td>
<td>Blume G., 1993</td>
<td></td>
</tr>
<tr>
<td>Discomes</td>
<td>Niosomes with non-ionic surfactant</td>
<td>Vyas S.P., 1997</td>
<td></td>
</tr>
<tr>
<td>Aquasomes</td>
<td>Ceramic carbon nanocrystalline particulate core coated with glassy cellulose.</td>
<td>Khopade A.J., 2002</td>
<td></td>
</tr>
<tr>
<td>Ethosomes</td>
<td>Phospholipid, ethanol and water.</td>
<td>Godin B., 2003</td>
<td></td>
</tr>
<tr>
<td>Genosomes</td>
<td>Macromolecular complexes</td>
<td>Zhdanov R.I., 2002. [94]</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>Photosomes</td>
<td>Photolase encapsulated in liposomes</td>
<td>Petit-Frere C., 1998. [95]</td>
<td></td>
</tr>
<tr>
<td>Erythrosomes</td>
<td>Chemically cross linked human erythrocytes</td>
<td>Cuppoletti J., 1981. [96]</td>
<td></td>
</tr>
<tr>
<td>Hemosomes</td>
<td>Haemoglobin containing liposomes</td>
<td>Gornicki A., 2003. [97]</td>
<td></td>
</tr>
<tr>
<td>Phytosomes</td>
<td>Active ingredient is a herbal origin and is an integral part of the lipid membrane by chemical bond rather than occupying the centre cavity.</td>
<td>Vinod K.R. et al, 2010. [101]</td>
<td></td>
</tr>
</tbody>
</table>
3.5.7. Phytosomal technology

The “Somes” are the cell like formulations of novel drug delivery system. There are different types of somes like 

**Liposomes**, which encapsulate water and lipid-soluble pharmacologically and cosmetically active components. **Phytosomes** are standardized extracts or purified fractions complexed with phospholipids for a better bioavailability and enhanced activities. and **Cubosomes** are bicontinuous cubic phases, consisting of two separate, continuous, but nonintersecting hydrophilic regions divided by a lipid layer that is contorted into a periodic minimal surface with zero average curvature, and **Colloidosomes** are solid microcapsules formed by the self-assembly of colloidal particles at the interface of emulsion droplets. “Colloidosomes,” are hollow, elastic shells whose permeability and elasticity can be precisely controlled. **Ethosomes** are noninvasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation. Ethosomes contain phospholipids, alcohol (ethanol and isopropyl alcohol) in relatively high concentration and water. **Aquasomes** - these are spherical 60300nm particles used for drug and antigen delivery. The particle core is composed of noncrystalline calcium phosphate or ceramic diamond, and is covered by a polyhydroxyl oligomeric film. **Pharmacosomes** are the colloidal dispersions of drugs covalently bound to lipids and may exist as ultrafine vesicular, micellar, or hexagonal aggregates, depending on the chemical structure of the drug–lipid complex. **Niosomes** are non-ionic surfactant vesicles and, as liposomes, are bilayered structures. etc. A comprehensive hypothetical diagram is represented as figure 10.
Figure 10: The wide spectra of established “Somes”: (A) Organization of the Phytosome molecular complex (B) Colloidosomes (C) Ethosomes (D) Cubosomes (E) Liposomes (F) Niosomes (G) Differentiation of Phytosome and Liposome (Adapted from: www.chemistry-blog.com [113], www.pharmainfo.net [114], topnews.in [115], www.pharmainfo.net [116], www.phytosomes.info [117])
3.5.6.1. Background to the phytosome Technology

The Phytosome technology, developed by Indena S.p.A. of Italy, markedly enhances the bioavailability of select phytomedicines, by incorporating phospholipids into standardized extracts and so vastly improve their absorption and utilization [105].

The poor absorption of flavonoid nutrients is likely due to two main factors. First, these are multiple-ring molecules not quite small enough to be absorbed from the intestine into the blood by simple diffusion. Nor does the intestinal lining actively absorb them, as occurs with some vitamins and minerals. Second, flavonoid molecules typically have poor miscibility with oils and other lipids. This severely limits their ability to pass across the lipid-rich outer membranes of the enterocytes, the cells that line the small intestine. The Phytosome technology meets this challenge. Certain of the water – phase flavonoid molecules can be converted into lipid – compatible molecular complexes, aptly called phytosomes. These are better able to transition from the water phase external to the enterocyte, into the lipid phase of its outer cell membrane and from there into the cell, finally reaching the blood.

The lipid - phase substances that successfully employed to make flavonoids lipid - compatible are phospholipids from soy, mainly phosphatidylcholine (PC). Phospholipids are small lipid molecules where glycerol is bonded to two fatty acids, while the third hydroxyl, normally one of the two primary methylenes, bears a phosphate group bound to a biogenic amino or to an amino acid thus making phytosomes different from liposomes. PC is the principal molecular building block for cell membranes, and the molecular properties that suit PC for this role also render it close to ideal for its phytosome role. PC is miscible both in the water phase and in oil/lipid phases, and is excellently absorbed when taken by mouth, and has the potential to act as a chaperon for polyphenolics, shuttling them through biological membranes. Precise chemical analysis indicates the unit phytosome is usually a flavonoid molecule linked with at least one PC molecule. A bond is formed between the two molecules to create a hybrid molecule. This hybrid is highly lipid - miscible, better suited to merge into the lipid phase of the enterocyte’s outer cell membrane. Once there, it can cross the enterocyte and reach the circulating blood [106,107].
The phytosome is a unit of a few molecules bonded together, while the liposome is an aggregate of many phospholipid molecules that can enclose other phytoactive molecules but without specifically bonding to them. This difference results in phytosome being much better absorbed than liposomes showing better bioavailability. Phytosomes have also been found superior to liposomes in topical and skin care (cosmetic) products [108].

In liposomes, the active principles are water soluble and are hosted in the inner cavity, with little, if any, interaction taking place between the hydrophilic principle and the surrounding lipid core. Conversely, Phytosomes host their polyphenolic guest, generally little soluble both in water and in lipids, at their surface where the polar functionalities of the lipophilic guest interact via hydrogen bonds and polar interactions with the charged phosphate head of phospholipids, forming a unique arrangement that can be evidenced by spectroscopy.

The Phytosome formulation also increases the absorption of active ingredients when topically applied on the skin, and improves systemic bioavailability when administered orally. In water medium, a Phytosome will assume a micellar shape, forming a spherical structure, overall similar to a liposome, but with a different guest localization.

3.5.6.2. Physico-chemical characterization of phytosomes

Phytosome is a complex between a natural product and natural phospholipids, like soy phospholipids. Such a complex is obtained by reaction of stoichiometric amounts of phospholipid and the substrate in an appropriate solvent. Their sizes vary between 50 nm to a few hundred μm. On the basis of spectroscopic data it has been shown that the main phospholipid-substrate interaction is due to the formation of hydrogen bonds between the polar head of phospholipids (i.e. phosphate and ammonium groups) and the polar functionalities of the substrate. These phyto- phospholipid complexes are often freely soluble in aprotic solvents, moderately soluble in fats, insoluble in water and relatively unstable in alcohol. When treated with water, phytosomes assume a micellar shape forming liposome-like structures. In liposome the active principle is dissolved in the internal pocket or it is floating in the layer membrane, while in phytosomes the active principle is anchored to the polar head of phospholipids, becoming an integral part of the membrane for example in the case of the catechindistearoylphosphatidylcholine complex, in this there is the formation of H-bonds...
between the phenolic hydroxyl ends of the flavonoid moiety and the phosphate ion on the phosphatidylcholine moiety. Phosphatidylcholine can be deduced from the comparison of the 1H-NMR and 13C-NMR spectra of the complex with those of the pure precursors. The signals of the fatty chain remain almost unchanged. Such evidences inferred that the two long aliphatic chains are wrapped around the active principle, producing a lipophilic envelope, which shields the polar head of the phospholipid and the flavonoid molecule and enables the complex to dissolve in low polarity solvents [109].

The behavior of phytosomes in both physical and biological system is governed by the factors such as physical size membrane permeability, percent entrapped solutes, chemical composition as well as the quantity and purity of the starting materials. Therefore, the phytosomes are evaluated for their organoleptic properties i.e. shape, size, its distribution and physico-chemically characterized by UV, IR, NMR, DSC, SEM, XRPD(Xray powder diffraction) etc. Percentage drug entrapment, percentage drug release profile are also studied accordingly [110].

3.5.6.3. Pharmaceutical scope of phytosomes:

1. It enhances the absorption of lipid insoluble polar phytoconstituents through oral as well as topical route showing better bioavailability, hence significantly greater therapeutic benefit.
2. Appreciable drug entrapment.
3. As the absorption of active constituent(s) is improved, its dose requirement is also reduced.
4. Phosphatidylcholine used in preparation of phytosomes, besides acting as a carrier also acts as a hepatoprotective, hence giving the synergistic effect when hepatoprotective substances are employed.
5. Chemical bonds are formed between phosphatidylcholine molecule and phytoconstituent, so the phytosomes show better stability profile.
6. Application of phytoconstituents in form of phytosome improve their percutaneous absorption and act as functional cosmetics.
7. Added nutritional benefit of phospholipids.
8. Phytosomes have improved pharmacokinetic and pharmacological parameters, which in result can be advantageously used in treatment of acute liver disease, either metabolic or infective origin.

9. Absorption of phytosomes in gastro-intestinal tract is appreciably greater resulting in increased plasma level than the individual component.

10. After screening and selection for phytoconstituents for therapeutics use, phytosomal drug delivery can be developed for various categories like anticancer, cardiovascular and anti-inflammatory activities [111,112]

3.5.7. Transfersomal technology

Transfersome is a term registered as a trademark by the German company IDEA AG, and used by it to refer to its proprietary drug delivery technology. The name means “carrying body”, and is derived from the Latin word 'transfere', meaning ‘to carry across’, and the Greek word ‘soma’, for a ‘body’. A Transfersome carrier is an artificial vesicle designed to be like a cell vesicle or a cell engaged in exocytosis, and thus suitable for controlled and, potentially targeted, drug delivery. Transfersomes are a special type of liposomes, consisting of phosphatidylcholine and a surfactant which act as an edge activator. The concept of transfersome was introduced in 1992 by Cevc and co-workers. These vesicular transfersomes are more elastic than the standard liposomes and thus well suited for the skin penetration [118,119].

Transfersomes were developed in order to take the advantage of phospholipid vesicles as transdermal drug carrier. These self-optimized aggregates, with the ultra flexible membrane, are able to deliver the drug reproducibly either into or through the skin, depending on the choice of administration or application, with high efficiency. Transfersomes overcome the skin penetration difficulty by squeezing themselves along the intracellular sealing lipid of the stratum corneum. There is provision for this, because of the high vesicle deformability, which permits the entry due to the mechanical stress of surrounding, in a self-adapting manner. Flexibility of transfersomes membrane is achieved by mixing suitable surface-active components in the proper ratios. The resulting flexibility of transfersome membrane minimizes the risk of complete vesicle rupture in the skin and allows transfersomes to follow the natural water gradient across the epidermis, when applied under non-occlusive condition. Transfersomes can penetrate the intact
The high and self-optimizing deformability of typical composite transfersomes membrane, which are adaptable to ambient stress allow the ultra deformable transfersomes to change its membrane composition locally and reversibly, when it is pressed against or attracted into narrow pore. The transfersomes components that sustain strong membrane deformation preferentially accumulate, while the less adaptable molecules are diluted at sites of great stress. This dramatically lowers the energetic cost of membrane deformation and permits the resulting, highly flexible particles, first to enter and then to pass through the pores rapidly and efficiently. This behavior is not limited to one type of pore and has been observed in natural barriers such as in intact skin. The typical diagram of transfersome is shown in Figure 4.

Transfersomes in the thermodynamic equilibrium are normally large, \( r_v > 40 \text{nm} \). Lipids mostly hydrophobic in nature accumulate in the membrane region with a positive surface curvature or at the site with a quasi planar geometry. Flexibility of transfersomes depends on the type of lipids, edge active substances or different lipid concentrations. The proviso is that the transfersome becomes ultraflexible mixed lipid vesicles in a (quasi) metastable state by which it facilitates squeezing easily through pores of biological barriers much smaller than its own size (one-tenth of its own diameter) \([122,123]\).

### 3.5.7.1. Scope of Transfersomes

Transfersome technology is best suited for non-invasive delivery of therapeutic molecules across open biological barriers. The Transfersome vesicles can transport across the skin, for example, molecules that are too big to diffuse through the barrier. Examples include systemic delivery of therapeutically meaningful amounts of macromolecules, such as insulin or interferon, across intact mammalian skin. Other applications include the transport of small molecule drugs which have certain physicochemical properties which would otherwise prevent them from diffusing across the barrier.

Another attraction of the Transfersome technology is the carrier’s ability to target peripheral, subcutaneous tissue. This ability relies on minimisation of the carrier-associated drug...
clearance through cutaneous blood vessels plexus: the non-fenestrated blood capillary walls in the skin together with the tight junctions between endothelial cells preclude vesicles getting directly into blood, thus maximizing local drug retention and propensity to reach the peripheral tissue targets.

### 3.5.7.2. Salient Features of Transfersomes

At first glance, transfersomes appear to be remotely related to lipid bilayered vesicle, liposomes. However in functional terms, transfersomes differ vastly from commonly used liposomes in that they are much more flexible and adaptable. The extremely high flexibility of their membrane permits transfersomes to squeeze themselves even through pores much smaller than their own diameter.

Transfersomes possess an infrastructure consisting of hydrophobic and hydrophilic moieties together and as a result, can accommodate drug molecules with wide range of solubility. Transfersomes can deform and pass through narrow constriction (from 5 to 10 times less than their own diameter) without measurable loss. This high deformability gives better penetration of intact vesicles through tight junctions. They can act as a carrier for low as well as high molecular weight drugs e.g. analgesic, anesthetic, corticosteroids, sex hormone, anticancer, insulin, gap junction protein, and albumin. They are biocompatible and biodegradable as they are made from natural phospholipids similar to liposomes. They have high entrapment efficiency, in case of lipophilic drug near to 90%. They protect the encapsulated drug from metabolic degradation. They act as depot, releasing their contents slowly and gradually. They can be used for both systemic as well as topical delivery of drug. Easy to scale up, as procedure is simple, do not involve lengthy procedure and unnecessary use or pharmaceutically unacceptable additives [124].
3.5.7.3. Product Development of Transfersomes

Scope of transfersomes is mainly intended for topical application although other routes may be considered for further investigations. Drug should be selected in such a way that it fits in the criteria of topical delivery. It should have ideal limits for aqueous solubility, lipophilicity, molecular size, melting point and pH of the aqueous saturated solution. The most commonly used materials in the preparation of transfersomes are phospholipids, surfactants, alcohol, and buffering agents. Here, each material has its own importance. Phospholipids such as Soya phosphatidyl choline, Dipalmitoyl phosphatidyl choline, Distearoyl phosphatidyl choline are vesicle forming components. Surfactants act as edge activators which are responsible for the flexibility of transfersomes. Sodium cholate, Sodium deoxycholate, tween-80, Span-80 are used as surfactants. Alcohol is used as solvent. Dyes are used for confocal scanning microscopy study. Here buffering agent (saline phosphate buffer) is used as hydrating medium.

All the methods of preparation of transfersomes are comprised of two steps. First, a thin film is prepared hydrated and then brought to the desired size by sonication; and secondly, sonicated vesicles are homogenized by extrusion through a polycarbonate membrane. The mixture of vesicles forming ingredients, that is phospholipids and surfactant were dissolved in volatile organic solvent (chloroform-methanol), organic solvent evaporated above the lipid transition temperature (room temp. for pure PC vesicles, or 500C for dipalmitoyl phosphatidylcholine) using rotary evaporator. Final traces of solvent were removed under vacuum for overnight. The deposited lipid films were hydrated with buffer (pH 6.5) by rotation at 60 rpm at the corresponding temperature. The resulting vesicles were swollen for 2hr at room temperature. To prepare small vesicles, resulting LMVs were sonicated at room temperature or 500C for 30 min. using a B-12 FTZ bath sonicator or probe sonicated at 40C for 30 min (titanium micro tip, Heat Systems W 380). The sonicated vesicles were homogenized by manual extrusion 10 times through a sandwich of 200 and 100nm polycarbonate membrane[125].

3.5.7.4. In Vivo fate of transfersomes and kinetics of penetration

After having penetrated through the outermost skin layers, transfersomes reach the deeper skin layer, the dermis. From this latter skin region they are normally washed out, via the lymph,
into the blood circulation and through the latter throughout the body, if applied under suitable conditions. The kinetics of action of an epicutaneously applied agent depends on the velocity of carrier penetration as well as on the speed of drug redistribution and the action after this passage.

The most important single factors in this process are:

1. Carrier in-flow,
2. Carrier accumulation at the targets site,
3. Carrier elimination.

The onset of penetration-driving force depends on the volume of the suspension medium that must evaporate from the skin surface before the sufficiently strong trans-cutaneous chemical potential chemical potential or water activity gradient is established. Using less solvent is favorable in this respect. The rate of carrier passage across the skin is chiefly determined by the activation energy for the carrier deformation. The magnitude of the penetration driving force also plays a big role. This explains, for example, why the occlusion of an application site or the use of too strongly diluted suspension hampers the penetration process. Carrier elimination from the sub cutis is primarily affected by the lymphatic flow, general anesthesia or any other factor that affects this flow, consequently, is prone to modify the rate of trans-cutaneous carrier transport. While it has been estimated that approximately 10% of the cardiac blood flow pass through each gram of living skin tissue, no comparable quotation is available for the lymph. Further, drug distribution is also sensitive to the number of carrier used, as this may affect the rate of vehicle degradation and/or filtration in the lymph nodes.

The lag between the time of application and the time of drug appearance in the body, therefore, is always quite long, complex and strongly sensitive to the type of drug and formulation administration. In the best case, the skin penetration lag amounts to approximately 15 min. if rapidly exchanging agents such as local analgesics are detected right under the skin permeability barrier. Less rapidly exchanging molecules or molecules measured in the blood compartment are typically detected with a lag time between 2 and 6 hr. depending on the details of drug formulation. Molecules that do not diffuse readily from the carriers or agents delivered with the suboptimal carriers normally fall in this category. The kinetics of vesicle penetration into and across the skin can be controlled to a large extent by fixing the physicochemical characteristics of the drug carrier suspension. Kinetics of the transfersomes penetration through
the intact skin is best studied in the direct biological assays in which the vesicle associated drugs exert their action directly under the skin surface. Local analgesics are useful for this purpose, for determining the kinetics of penetration, various Lidocaine loaded vesicles were left to dry out on the intact skin.

Corresponding subcutaneous injection is used as control. The animal's sensitivity to pain at the treated site after each application was then measured as a function of time. Dermally applied standard drug carrying liposomes or simple Lidocaine solution have never caused any analgesic effect. It was necessary to inject such agent preparations to achieve significant pain suppression. In contrast to this, the lidocaine-loaded transfersomes were analgesically active even when applied dermally. Maximum analgesic effect with the latter type of drug application was typically observed 15 minutes after the drug application. A marked analgesic effect was still noticeable after very long time. The precise reach as well as kinetics of transfersomes penetration through the skin are affected by: drug carrier interaction, application condition or form, skin characteristics, applied dose [126].

3.5.7.5. Applications of Transfersomes

Transfersomes have been widely used as a carrier for the transport of proteins and peptides. Proteins and peptide are large biogenic molecules which are very difficult to transport into the body, when given orally they are completely degraded in the GI tract. These are the reasons why these peptides and proteins still have to be introduced into the body through injections. Various approaches have been developed to improve these situations. The bioavailability obtained from transfersomes is somewhat similar to that resulting from subcutaneous injection of the same protein suspension. The transfersomal preparations of this protein also induced strong immune response after the repeated epicutaneous application, for example the adjuvant immunogenic bovine serum albumin in transfersomes, after several dermal challenges is as active immunologically as is the corresponding injected proteo - transfersomes preparations [127]-[135]. Information about the API, indication and justification is tabulated below (table 10).
Table 3. Drugs that can be accommodated in transfersomes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meloxicam</td>
<td>NSAID</td>
<td>Improved skin permeation</td>
<td>Sureewan et al., 2010.</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Anti cancer drug</td>
<td>Increasing skin permeation</td>
<td>Michele Trotta et al., 2004.</td>
</tr>
<tr>
<td>Curcumin</td>
<td>NSAID</td>
<td>Improved bioavailability and permeability</td>
<td>R.Patel et al., 2009.</td>
</tr>
<tr>
<td>Interlukin-2</td>
<td>Regulators of lymphocytes</td>
<td>Controlled release</td>
<td>Hafer C., 1999.</td>
</tr>
</tbody>
</table>
3.5.8. Cubosomes – Definition, History and Theory:

Cubosomes are discrete, submicron, nanostructured particles of bicontinuous cubic liquid crystalline phase, which can be localized in different body cavities such as skin or different mucosal surfaces and can incorporate large amounts of bioactive compounds of varying physico-chemical properties [147]. Cubic liquid crystalline phases play a significant role in the physiological processing of lipids which are of both dietary and formulation origin [148]. It has already been proposed that, in the intermediate stages of their digestion, common dietary triglycerides (TG) may assume cubic microstructures [149,150]. Cubic phases have provision for the modified release characteristics and additionally the solubility benefits make these systems interesting not only in understanding the process of lipid digestion but also their potential to manipulate the drug delivery kinetics[151]. Cubic liquid crystalline phases can entrap small molecules and proteins, which has been shown to be effective means of enhancing drug stabilization and delivery [152,153].

Cubosomes are nanoparticles, more accurately nanostructured particles of a liquid crystalline phase with cubic crystallographic symmetry formed by the self-assembly of amphiphilic or surfactant like molecules[154]. However, the cubic phases possess very high solid-like viscosities which is a unique property because of their intriguing bicontinuous structures which enclose two distinct regions of water separated by a controlled bilayer of surfactant [155]. As a result, the cubic phases can be fractured and dispersed to form particulate dispersions that are colloidal and/or thermodynamically stable for longer times.

The unique microstructure of cubosome confers some desirable characteristics as far as controlled release is concerned. Firstly, the drugs of varying polarity and size can be entrapped in the cubic phases [156]. Because of the dual polar/non-polar nature of the cubic phase, hydrophilic drugs are trapped in the aqueous channels while their hydrophobic counterparts will partition into the lipid
bilayer compartment [157,158]. Secondly, the unique microstructure of the cubic phase controls the degree of release of the drug. Thus, the drug must diffuse from the three-dimensional network in which both tortuosity and pore size of the aqueous nanochannels contribute to delayed/controlled release. Thirdly, the cubic phase exhibits bioadhesive properties [159] which makes it useful for gastrointestinal, lung, nasal, oral, buccal, rectal and vaginal drug delivery [160]. Finally, the cubic mesophases are stable *invitro* and may be subjected to lipolysis which facilitates eventual dissolution *invivo*.

### 3.5.8.1. History of Cubosomes:

The discovery of cubosomes is a unique story and spans the fields of food science, differential geometry, biological membranes, and digestive processes. Despite the early realization of their potential, the manufacture of cubosomes on a large scale is a difficult task because of their viscous properties and complex phase behavior. Although cubosomes are nanoparticles, they are not solids exactly. However, the cubic phases are unique in that they possess very high solid-like viscosities because of their intriguing bicontinuous structures which means a contorted bilayer of surfactant separates two distinct regions of water enclosed by them (Scriven, 1976). As a result cubic phases can be fractured and dispersed to form particulate dispersions that are colloidally and/or thermodynamically stable for longer times. Certain surfactants will spontaneously form cubic phases when mixed with water above a certain level and the first determination of their molecular structure was ingeniously carried out by Luzzati and Husson (1962), Luzzati et al. (1968), Larsson (1983) and Hyde et al. (1984) between 1960 and 1985. ‘Cubosomes’ were named by Larsson, who discovered them, to reflect their cubic molecular crystallographic symmetry and their similarity to liposomes (also known as vesicles: dispersed nanoparticles of lamellar liquid crystalline phase). Because cubosomes can be formed from biological lipids like monoglycerides, they can solubilize numerous biologically active molecules including proteins (Buchheim and Larsson, 1987), and possess a tortuous microstructure (Anderson and Wennerstro¨m, 1990), their application as drug delivery vehicles was pursued (Lawrence, 1994). Hypothetical graphical representation explaining internal structure as well as morphology is as below (figure 11).
Liquid crystals are formed when surfactants are added to water at higher concentrations, which self-assemble to form thick fluids. The most viscous liquid crystal is bicontinuous cubic phase, a unique material that is clear and resembles stiff gelatin. When the bulk cubic phase is fractured and dispersed into smaller particles, which are termed cubosomes. Discovered in the 1980s, cubosomes excited much research interest because their structures were thought to be excellent vehicles for delivering drugs. Their biodegradable nature, edibility, biological compatibility, and incorporation of proteins make them unique among the simple-to-make particles. Although there is no commercial product available in the market using cubosomes, the research scientists continues to make strides in understanding the equilibrium and dynamic aspects of cubosomes.

Although cubosomes have been around for a while, an effort to develop scalable processes to mass-produce these soft nanoparticles is under development. Several research scientists studied regarding the physical, chemical properties and rheology of cubosomes. The preparation of cubosomes based on different materials has been reported. It has also been reported that cubosomes transform into hexosomes, exhibiting a time-resolved behavior due to pH-induced lipid hydrolysis. The adjustment in lipid composition can control the internal and structural changes of cubosomes. The effect of vitamin E and polymer on cubosome structure, the cubic symmetry and ion-exchange properties, the bilayer phase transition, the instability of cubosomes in plasma due to interactions with lipoproteins (high-density lipoprotein and low-density lipoprotein) and albumin, transformation of vesicular into cubic nanoparticles by autoclaving of
aqueous monoolein/poloxamer dispersions [146], the influence of dipalmitoyl phosphatidylserine on phase behavior of cubosomes were also reported[147].

At present, most of the research related to cubosomes has been focused on the method of preparation, structural characteristics, stability, characterization and evaluation. For example, recently, few anticancer drugs have been successfully encapsulated in cubosomes and characterized physicochemically. Although the in vitro and in vivo pharmacological studies of cubosomes have not been widely conducted, the unique structure of this promising nanocarrier suggests its application in melanoma treatment. There are several publications concerning the release profile of cubosomes, application as a percutaneous delivery system and application as a formulation for skin hydration.

Cubosomes have great potential in drug nanoformulations owing to their potential advantages, including: 1) High drug payloads due to high internal surface area and cubic crystalline structures, 2) Relatively simple method of preparation, 3) Biodegradability of lipids, 4) Ability of encapsulating hydrophilic, hydrophobic and amphiphilic substances, 5) Targeted release and controlled release of bioactive agents.

3.5.8.2. Cubic Phase Structure and its Mechanism:

Cubosomes consist of honeycombed (cavernous) structures separating two internal aqueous channels and a large interfacial area (Fig 12). Self-assembled cubosomes as active drug delivery systems are receiving more and more attention and interest after the first discovery and nomination.
Figure 12: Theoretical diagram explaining the core of cubosomes lodging the drug
(adapted from: http://img.medscape.com) [164]

Cubosomes are nanoparticles whose size ranges from 10-500nm in diameter; they appear like dots square shaped, slightly spherical. Each dot corresponds to the presence of pore containing aqueous phase cubic phases in lipid water system in X-ray scattering technique was first identified by Luzzati and Husson. According to Fontell & Drew ternary systems of amphiphiles, oil & water, some mono glycerides will exhibits cubic phases. Monoglycerides are polar lipids, having poor water solubility that exhibits aqueous phase behaviour, which are structurally mimicking to non-ionic surfactants. Lutton results the monoglycerides whose hydrocarbon chain lengths between C12 and C22 of all the monoglycerides, particularly monoolein exhibits larger region of cubic phase.
3.5.8.3. Nodal surfaces of Cubosomes:

The bicontinuous cubic phase was first documented by Luzzati et al and the model of its geometric structure was given by scriven. Larsson found that cubic phases have continuous regions of both hydrophilic and hydrophobic nature using nuclear magnetic resonance (NMR) and X-ray, and concluded that cubic phases can be described using the concepts of differential geometry and periodic minimal surfaces.

Lipid bilayer is the basic building block of bicontinuous cubic phase. In a cubic phase, lipid bilayers are arranged in periodic three-dimensional fashion by contorting the bilayers into the shape of infinite periodic minimal surfaces (IPMS). In such arrangement, the average curvature is maintained zero, at any place on the surface. Based on the nodal surfaces, three structures of cubosomes have been proposed: (i) Pn3m (D-surface) (Diamond surface), (ii) Ia3d (G-surface) (Gyroid surface), and (iii) Im3m (P-surface) (Primitive surface). The P-surface and the D-surface were discovered mathematically by Schwarz in 1890, whereas the G-surface was discovered by Schoen in the 1960s. The monolien-water system forms G-surface at lower hydration levels and D-surface at higher hydration levels (figure 13).

![P-surface, G-surface, D-surface](image)

**Figure 13:** Representation of nodal surface variations in cubosomes. (Courtesy: Current Opinion in Colloid and Interface Science)
3.5.8.4. Engineering of Cubosomes:

Bicontinuous cubic phases are found in natural lipids, cationic and nonionic surfactants and polymer systems, although the lipid most widely used to construct bicontinuous cubic phases is the monoglyceridemonoolein. Monoglycerides spontaneously form bicontinuous cubic phases upon the addition of water, are relatively insoluble (allowing the formation of colloidal dispersions of cubosomes), and are resistant to changes in temperature.

The main precursor of cubosome formation is monoolein. Monoolein or glycercylmonooleate is a mixture of the glycerides of oleic acid and other fatty acids, consisting mainly of the monooleate. The acyl chain is attached to the glycerol backbone by an ester bond. The two remaining carbons of the glycerol have active hydroxyl groups, giving polar characteristics to this portion of the molecule. The glycerol moiety may form hydrogen bonds with water in an aqueous environment and is commonly referred to as the head group. The hydrocarbon chain gives hydrophobic characteristics to monoolein and is often termed the tail.

Commercially available monoolein may be obtained in two forms, a mixed glyceride form or as distilled monoolein; the distilled monoolein is preferred for pharmaceutical applications because of its high purity. Monoolein occurs as a waxy yellow paste with a characteristic odor. It swells in water, giving rise to several lyotropic liquid crystalline structures. From a pharmaceutical standpoint the phase behavior of the system shows a number of interesting properties. Some phases may be in equilibrium with excess water solutions and temperature induced transition occurring between phases of different rheology.

Monoolein is a nontoxic, biodegradable and biocompatible material classified as GRAS (generally recognized as safe) and it is included in the FDA inactive ingredients guide and in non-parenteral medicines licensed in the United Kingdom. Monoolein show the mesomorphic phase, important in making more comprehensible the potential pharmaceutical application of the lipid.

A common feature of lipids is the existence of a temperature dependent phase transition, below the transition temperature, lipid exist in a gel state. An increase in temperature results in the transition to a fluid like state (liquid crystalline state) similar to the fusion of a crystalline
solid. When lipid molecule is heated, instead of melting directly convert into an isotropic liquid. The ability to exist in several different phases is an important property of pure lipids and lipid mixtures; it depends on temperature, hydration and lipid class. In general monoglycerides exhibit different phase behaviors when they exposed to water.

Surfactants, which are used in the production of cubosomes, are poloxamer 407 in a concentration range between 0% and 20% w/w with respect to the disperse phase. The tail concentration of the monoglyceride/surfactant mixture generally takes between 2.5% and 10% w/w total weight of the dispersion with respect to tail.

3.5.8.5. Methods of Preparation of Cubosomes:

Cubosomes can be manufactured by two distinct methods:

(1) Top down technique

(ii) Bottom up technique

(i) Top-down technique:

This approach was initially reported by Ljusberg-Wahren in 1996 [165]. The extreme viscous bulk phase is prepared by mixing structure-forming lipids with stabilizers and the resultant is dispersed into aqueous solution by the input of high energy (such as high-pressure homogenization [HPH], sonication or shearing) to form lyotropic liquid crystalline nanoparticles. HPH is the most extensively used technique in the preparation of cubosomes [166].

Worle et al. [167] investigated the parameters influencing the properties of glycercylmonoooleate (GMO)-based cubosomes and reported that the concentration of poloxamer and temperature during HPH were regarded as crucially important parameters. To fabricate lyotropic liquid crystalline nanoparticles, a novel approach of shearing was proposed using a laboratory-built shearing apparatus [168]. The shearing treatment could effectively result in more
stable and homogeneous cubosomes or hexosomes with high content of the hydrophobic phase (oil + lipophilic additives) within a short time (less than one minute) when compared to ultrasonication approach. The preparation procedure is very simple and can be used conveniently. The operation units in this procedure require several cycles to achieve the desired nanosize with appropriate characteristics, and the high-energy input is also regarded as a barrier to the temperature sensitive ingredients [166]. In addition, the cubosomes prepared using top-down approach are always observed to coexist with vesicles (dispersed nanoparticles of lamellar liquid crystalline phase) or vesicle-like structures, which will hamper the investigations on plain cubic mesophases (figure 14).

![Figure 14: Schematic diagram of preparation methods for cubosomes—Top-down approach](image)

(ii) **Bottom-up technique:**

In this technique, cubosomes are allowed to form or crystallize from precursors. Almgren et., al. discussed the formation of cubosomes by dispersing L2 or inverse micellar phase droplets in water at 80ºc, and allow them to cool slowly, gradually the formed droplets crystallizes to cubosomes. This is more robust in large scale production of cubosomes (figure 15).

The key factor in the bottom-up approach is hydrotrope, which can dissolve water-insoluble lipids to create liquid precursors and prevent the formation of liquid crystals at high concentration [169]. This dilution-based approach can produce cubosomes without laborious
fragmentation (less energy input) compared to top-down approach. Moreover, this technique is far more efficient in generating small particles which might be attributed to the forming mechanism of cubosomes.

The dilution-based approach can be regarded as a process in which small particles aggregate to form big particles, analogous to the precipitation processes used to produce nanoparticles. In addition, cubosomes prepared through dilution show long-term stability, which might be due to the homo-dispersion stabilizers onto the surface of cubosomes [166]. However, the use of hydro trope can simplify the preparation process and produce cubosomes possessing similar or better than those fabricated by the top-down approach. Since, this process via dilution is a pathway depending on the ternary phase diagram (lipid–water–hydro trope), which requires knowledge of the full phase behavior; hence, the extent of dilution is difficult to control precisely. The effects exerted by varying concentrations of hydro trope on the physicochemical properties of lyotropic liquid crystalline nanoparticles and the possible occurrence allergic response and irritation, when the mesophase formulations are administered were some of the challenges faced owing to the addition of hydro trope. Through cryo-TEM, many vesicles and vesicle-like structures were also observed to coexist with cubosomes [170].

Figure 15: Schematic diagram of preparation methods for cubosomes - Bottom-up approach
Spicer et al. developed cubosomes at room temperature by diluting monoolein-ethanol solution with aqueous poloxamer 407 solution. The cubosomes were spontaneously formed by emulsification and also from powdered precursors by spray drying technique. Spray dried powders comprising monoolein coated with starch or dextran, form cubosomes on simple hydration and were stabilized by the polymers.

Dispersion of the nanoparticles produced in the cubosome formation can be done by several techniques:

- Sonication
- High pressure homogenization
- Spontaneous emulsification
- Spray drying

Sonication and high-pressure homogenization suggests the formation of complex dispersions containing vesicles and cubosomes with time-dependent ratios of each particle type. Coarse cubosomes on the micron scale possess the same D-surface cubic structure as their originating bulk cubic phase but the P-surface dominates after homogenization, either because of the added polymer or other factors.

3.6. Conclusion of literature review

Vitiligo or leukoderma is an acquired, idiopathic, hypomelanotic disease characterized by circumscribed depigmented macules. This visible disorder may lead to many physiological malfunctions apart from social stigma. Conventional treatments may precipitate many side effects. Piperine, an alkaloid obtained from Pepper has shown appreciable capacity to repigment skin. Also side effects due to conventional treatments can be prevented by the use of piperine and sometimes can even avoid phototherapy. This shows a tremendous scope of formulation of piperine intended for vitiligo which can caste a limelight of hope for the 1-4% population suffering from this disorder. Conventional as well as novel drug delivery systems can be considered with this regard to active maximum tissue drug bio availability.
3.7. Plant, Drug and polymer profiles

3.7.1. Plant profile (Black pepper)

Botanical Name: *Piper nigrum*

Family Name: Piperaceae

Common Name: Black Pepper, Peppercorns

Part Used: Dried Unripe Fruits, Usually Known As Peppercorns

Habitat: Extensively cultivated in hotter and moist part of India.

Product offered: Whole plant, Fruits

The word “pimetto” is derived from the Spanish word pimiento for Black pepper as allspice resembles peppercorns. It is known as pepper in many languages. In Russian it is known as yamaiskiy pjerets-Jamaica pepper. In French poivre aromatique-aromatic pepper, In German clove pepper, In Swedish condiment pepper.

3.7.2. Vernacular names: Telugu Miriyalu, Tamil: Cheviyan, molagu, Sanskrit: Maricha, Hindi: Kalimirch, Bengali: Golmarich, English: Black pepper
Figure 16: (A) Pepper before ripening (B) High resolution picture of Peppercorn (C) Black and white peppercorns: Adapted from http://upload.wikimedia.org, http://upload.wikimedia.org, http://sdamji.arhamweb.com[171-173]

3.7.3. General Botanical Aspects

Piper nigrum L. belongs to the family Piperaceae. The family is dicotyledonous, but the stem has characteristic lines intermediate between those of the dicotyledons and the monocotyledons, i.e. the vascular bundles lie in two or more circles. The stem is usually swollen at the nodes. Numerous adventitious roots, known as clinging or climbing roots, are produced from the nodes. It is by means of these roots, the plants attach themselves to the standards and climb up by exposing the fresh leaves to maximum sunlight. The stem or the vines can be broadly classified into: (i) terminal stem, (ii) stolon or runners and (iii) lateral branches. The stem and branches bear alternate, shiny, dark green ovate leaves. Branches are produced from the dorsal buds. Each bud is accompanied by a single leaf lateral to the bud. The receme develops on the current year's flush opposite a leaf. Two successive primordials of a raceme are present within a single bud. Flowers are small, whitish and borne on hanging catkins or spikes. Humid tropical climate of 10°-40° C with annual rain of 250 cm is ideal for pepper cultivation. Seed and vegetative propagation is generally used although the later is least preferred (figure 16).
Figure 17: From left to right: ripened pepper, green pepper, white pepper, powdered pepper

- **White Pepper**: White pepper is prepared from the optimally mature peppercorns. The berries are kept under running water for 7 to 9 days to soften their pericarp. After removing the pericarp by scrubbing, the white peppercorns are washed and dried. White pepper is mainly used in the preparation of light-colored dishes, sauces and soups. West Europe constitutes the major market for white pepper (figure 17).

- **Ground Pepper**: Dried pepper berries today are commercially ground using various types of mills, depending on the users’ specifications (e.g. particle size, volatile-oil content, etc). Critical factors like grinding temperature, hygiene and packaging affect the quality of ground pepper. There are a number of spice grinders in producing countries who grind pepper and other spices, using mills from basic machines to sophisticated cryogenic systems.

**Green Pepper**: Green pepper is made from the fully developed but immature berries. They are preserved in brine, vinegar or citric acid and may be dried or kept in the preservative. Europeans are fascinated by the natural green colour and fresh flavor of green pepper.

**Canned Green Pepper**: The separated green pepper berries are washed and filled in cans containing a diluted solution of sodium chloride with or without added acidity. The filled cans are then sealed and sterilized by the autoclave process, and cooled under running water. Europe, USA and Australia use canned green pepper for flavoring food and garnishing meat dishes.
Green Pepper in Brine: Green pepper in brine is made from young, green pepper berries which are carefully detached from the stalks and preserved in a specially formulated solution of vinegar and brine, to retain the natural color and texture of the berries.

Dehydrated Green Pepper: Dehydrated green pepper has the green colour and the flavor of fresh pepper. On dehydration, the berries turn full and soft, but do not have the texture of green pepper in brine. Freeze-drying ensures better dehydration. Frozen green pepper is made by freezing the berries in a brass freezer. Europe is the major importer of frozen green pepper.

Pepper Oil: Pepper essential oil or volatile oil is a natural blend of mainly terpenes and their derivatives that form a clear yellowish green to bluish green colour. It is responsible for the characteristic aroma of pepper. Today, this essential oil is still commercially extracted from the pepper berries mainly by the process of steam distillation.

Pepper Oleoresin: Pepper oleoresin is a concentrated, resinous extract obtained by conventional solvent extraction or supercritical fluid extraction. As the name implies, pepper oleoresin consists of a blend of the essential oil, resinous matter of the spice and related compounds like the pungent alkaloid Piperine. Pepper oleoresin has a relatively full flavour profile characteristic of pepper as compared to pepper oil. In Malaysia, there is no commercially run oleoresin extraction.

Green Pepper Sauce: Green peppercorns are first ground to a puree and then blended with vinegar, salt, sugar or other ingredients. Distinctly piquant with a natural fresh flavour, green pepper sauce is also used as a dip for chips and fries. As of 2008, Vietnam is the world's largest producer and exporter of pepper, contributing 34% of the world's Piper nigrum crop. Other major producers include India (19%), Brazil (13%), Indonesia
(9%), Malaysia (8%), Sri Lanka (6%), China (6%) and Thailand (4%). Global pepper production peaked in 2003 with over 355,000 t (391,000 short tons), but has fallen to just over 271,000 t (299,000 short tons) by 2008 due to a series of issues including poor crop management, disease and weather. Vietnam dominates the export market, using almost none of its production domestically; however its 2007 crop fell by nearly 10% from the previous year to about 90,000 t (99,000 short tons). Similar crop yields occurred in 2007 across the other pepper producing nations as well. USA is the biggest importer of pepper [174,175]. Nutritional value of black pepper is tabulated in table 4.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount</th>
<th>DV (%)</th>
<th>Nutrient density</th>
<th>World’s healthiest foods rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium</td>
<td>0.24</td>
<td>12.0</td>
<td>19.9</td>
<td>Excellent</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>6.88</td>
<td>8.6</td>
<td>14.2</td>
<td>Very good</td>
</tr>
<tr>
<td>Iron</td>
<td>1.24</td>
<td>6.9</td>
<td>11.4</td>
<td>Very good</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>1.12</td>
<td>4.5</td>
<td>7.4</td>
<td>Good</td>
</tr>
</tbody>
</table>
### 3.7.4. Chemical constituents of black pepper

A pungent alkaloid Piperine, Piperidine, piperettine, piperolein A&B.

Propyl phenols viz., eugenol, myristicine, safrole

Volatile oil contains mainly 1-phellandrene and caryophyllene

Mono and sesquiterpenes for eg. 1, 8-Cineole,p-cymene,carvone.[178]

### 3.7.5. *Piper nigrum* dry extract [179,180]

Herb extract ratio : 7:1

Description : dark greenish yellow color

Odour and taste : pungent odour and taste
Identification: Presence of Piperine-positive on TLC in solvent system toluene–ethyl acetate

**Extractive values**

A) Water-soluble extractive value: minimum 80%

b) 50% v/v alcohol soluble extractive value: minimum 70%

pH of 1% solution: 5-7

**Ash value**

Total ash: maximum 10%

Moisture content: max 5.0%

Heavy metals: max 20ppm

Arsenic: max 1ppm

Lead: max 1ppm

**Microbiological test**

a) Total plate count =<1000 cfu/g

b) Yeast and moulds =<100 cfu/g

c) Coliforms = Negative
Powder microscopy

Identifying characters:

- Odour: characteristic aroma; with pungent taste.
- Colour: powder emits a yellow colouration on treating with chloral hydrate.
- Stone cells: groups of stone cells, nearly isodiametric with intercellular-space like-unthickened portions (simple pores) in surface view. In side view, from stone cells (from endocarp) appear as a row of ‘beaker’ shaped thick walled cells.
- Starch grains: numerous, mostly in polyhedral masses. One should not confuse these polyhedral starchy masses to the perisperm fragments of fructs cardomomi.

Table 5: Use of Black pepper all over the world

<table>
<thead>
<tr>
<th>Country</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mexico</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td></td>
<td>Anti-malarial Treatment</td>
</tr>
<tr>
<td></td>
<td>Cure for Stomach Ache</td>
</tr>
<tr>
<td>Morocco</td>
<td>Weight Loss Treatment</td>
</tr>
<tr>
<td></td>
<td>Anti-leukemia Treatment</td>
</tr>
<tr>
<td>Indonesia</td>
<td>Fever prevention/reducer</td>
</tr>
<tr>
<td></td>
<td>Treatment for Snake Venom Poisoning</td>
</tr>
<tr>
<td></td>
<td>Anti-epileptic Treatment</td>
</tr>
<tr>
<td>India</td>
<td>Condiment</td>
</tr>
<tr>
<td></td>
<td>Carminative</td>
</tr>
<tr>
<td></td>
<td>Digestive disorders</td>
</tr>
</tbody>
</table>
3.7.6. Reported extraction procedures of Piperine

Piperine can be extracted from the powdered black pepper by using 95% ethanol and heating it at reflux for 2hrs. Filter and concentrate the extract with the volume of 10-15ml. Add 10ml of 10% KOH solution and heat. Add water drop wise, yellow precipitate is formed which is filtered by suction filtration and recrystallized by using acetone. Piperine can also be extracted by using Calcium carbonate and IPA. Addition of Calcium carbonate prevents the extraction of acidic components of pepper. Methylene chloride can also be used for the isolation of Piperine. Microwave assisted extraction of Piperine has the mechanism of enhancing the extraction rates. The dielectric heating of the polar cellular matrix resulted in remark by swelling and coalescence of the oil cells and other constituents the resulting pressure, built up in the cell, breaks open the cell releasing the constituents and providing easy access for solvent penetration and subsequent solubilization of Piperine.

Another procedure is by using hydro tropes like sodium alkyl benzene sulfonates and sodium butyl mono glycol sulfate for extraction of Piperine by cell permeabilization of Piper nigrum fruits. Penetration of the hydro trope molecules into the cellular structures and subsequent cell permeabilization were hypothesized to explain the enhanced extraction rates of aq. hydro trope solutions[181-183].
3.7.2. Drug profile: Piperine

![Molecular Structure of Piperine](image)

**Figure 18: Molecular Structure of Piperine**

Molecular formula: $\text{C}_{17}\text{H}_{19}\text{NO}_3$

Molecular weight: $285.34 \text{ g mol}^{-1}$

Density: $1.193 \text{ g/cm}^3$

Melting point: $130^\circ\text{C}$

Solubility: Soluble in Chloroform, Ethanol and Acetone. Insoluble in water.

Description: Yellowish coloures powder having pungent odour.

Piperine is the alkaloid responsible for the pungency of black pepper and long pepper, along with chavicine (an isomer of Piperine). There are many structural features to Piperine. There is an acetal, an aromatic ring, a *trans, trans* diene and an amide. The *trans, trans* diene is, synthetically, the most interesting feature of the molecule. Since the natural product has the
trans, trans configuration, any reaction forming the diene must be stereo selective, and the diene must not isomerizes after the reaction has occurred.

Piperine is tasteless, but its stereoisomer, chavicine, is the active ingredient in black pepper that provides its characteristic taste. Loss of pungency during storage of black pepper is attributed to the slow isomerization of chavicine into Piperine [184].

3.7.2.1. Analytical works on Piperine

Thin layer chromatography of Piperine can be performed by using silica gel-G as stationary phase, mobile phase used is toluene: ethylacetate in the ratio of 70:3. Ethanolic extract is used as sample solution. \( R_f \) value corresponds to 0.55. The spot is observed under U.V. TLC can also be performed by using ethanolic extract. Apply the test sol on the Silica gel-G plates and elute the plate with toluene –diethyl ether-dioxane (9.4:3.2:2.4). Visualize the dried plate under U.V light of 254nm. Piperine appears as a violet colour spot at \( R_f \) value 0.48. If the TLC plate is sprayed with anisaldehyde-glacial acetic acid – ethanol-conc.sulphuric acid reagent (0.5:10:85:5) and heated at 110°C for 10mins .Piperine appears as the yellow spot. Estimation of Piperine in pepper corns can be determined by using spectrophotometer. Piperine extracted into ethylene di chloride and measured at maximal absorbance 342-345nm with a U.V light source. Piperine content is calculated by using an absorbance factor derived from Piperine. Estimation of Piperine by reverse phase HPLC method can be carried out by using –C\(_{18}\) as stationary phase and an acetonitrile-aq. acetic acid as mobile phase. Quantification based on peak areas is achieved by reference to purified Piperine as external standard. Quantitative determination of Piperine can be carried out by 2 methods.

Komarowsky reaction-a highly stable reagent consisting of Para methyl amino benzaldehyde or Para hydroxyl benzaldehyde or other cyclic aldehydes plus thio urea in concentrated sulphuric acid has been developed for use in determining Piperine the slightly yellow reagent if stored under refrigeration in a brown bottle with this reagent for 15mins at 100°C develops a red colour whose intensity is proportional to the amount of Piperine present.
The other method is by using 85% phosphoric acid which forms an unstable yellow colour, when heated for 8mins at 100ºC becomes a stable bluish green with absorption maximum at 635µm. This reaction was exploited for the direct determination of the Piperine content of pepper samples. Powdered pepper was treated with 85% phosphoric acid the resulting yellow colour was heated at 100ºC and cool absorbance was measured against a reagent blank at 635µm. Beer’s law was obeyed between the levels of 0.4 -0.28 PM of Piperine per ml of 85% phosphoric acid.

3.7.3. Polymer profiles
3.7.3.1. Phosphatidyl choline:[185]

Figure 19: Molecular structure of Phosphotidyl choline:

Appearance: Lyophilized sodium salt.

Molecular formula: C₄₂H₈₂NO₈P.

Molecular weight: 760.09

Solubility: Insoluble in water, soluble in ethanol and chloroform.

Description:

Phosphatidylcholine is a major phospholipid in the outer plasma membrane of mammalian cells. PC together with phosphatidylethanolamine is the substrate for PS synthase-1 and 2 to synthesize phosphatidylserine by a base-exchange reaction. It is used by Phospholipase D to generate the second messenger phosphatidic acid (PA). PC can be used in a lipid mix for PI3K lipid kinase activity assay together with PI, PIP, PIP2, PE, PS and SM.
3.7.3.2. Glyceryl monooleate:[186]

![Molecular structure of glyceryl monooleate](image)

**Figure 20: Molecular structure of glyceryl monooleate**

Synonyms: glycerol-1-oleate; glyceryl mono-oleate; GMO GE; Hodag GMO; Ligalub; monolein; mono-olein;

Molecular formula: C_{21}H_{40}O_{4}

Molecular weight: 356.55

Solubility: Insoluble in water, soluble in ethanol and chloroform.

Description: White or cream coloured hard fats of waxy appearance, plastic products or viscous liquids. It can be used as emulsifier.

Applications:

Glyceryl monooleate is a polar lipid which swells in water to give several phases with different rheological properties. It is available in both nonemulsifying (n/e) and self-emulsifying (s/e) grades, the self-emulsifying grade containing about 5% of an anionic surfactant. The nonemulsifying grade is used in topical formulations as an emollient and as an emulsifying agent for water-in-oil emulsions. It is also a stabilizer for oil-in-water emulsions. The self-emulsifying grade is used as a primary emulsifier for oil-in-water systems. Glyceryl monooleate gels in excess water, forming a highly ordered cubic phase which can be used to sustain the release of
various water-soluble drugs. Glyceryl monooleate is reported to enhance transdermal penetration.

3.7.3.3. Polaxamer 407:[187]

**Synonym:** Monolan; Pluronic; poloxalkol; polyethylene-propylene glycol copolymer; polyoxyethylene-polyoxypropylene copolymer;

![Figure 21: chemical structure of polaxamer 407](image)

(a=101, b=56)

Physical form: Solid

Description: Poloxamers generally occur as white-colored, waxy, free flowing prilled granules, or as cast solids. They are practically odorless and tasteless.

Molecular formula: \( \text{HO(C}_2\text{H}_4\text{O)}_{101}\text{(C}_3\text{H}_6\text{O)}_{56}\text{(C}_2\text{H}_4\text{O)}_{101}\text{H} \)

Solubility: Freely soluble in ethanol and water.

Applications:

Poloxamers are nonionic polyoxyethylene-polyoxypropylene copolymers used primarily in pharmaceutical formulations as emulsifying or solubilizing agents. The polyoxyethylene segment is hydrophilic while the polyoxypropylene segment is hydrophobic.

Poloxamers are used as emulsifying agents in intravenous fat emulsions, and as solubilizing and stabilizing agents to maintain the clarity of elixirs and syrups. Poloxamers may also be used as wetting agents, in ointments, suppository bases, gels, and as tablet binders and coatings.

3.7.3.4. Carbopol 934:[188]
Synonym: Acritamer; acrylic acid polymer; Carbopol; carboxyvinyl polymer; carboxy polymethylene; polyacrylic acid.

![Molecular structure of acrylic acid monomer in carbomer resin](image)

**Figure 22: Molecular structure of acrylic acid monomer in carbomer resin**

Description:

Carbopol is white-coloured, ‘fluffy’, acidic, hygroscopic powders with a slight characteristic odour. Carbopol is stable, hygroscopic materials that may be heated at temperatures below 104°C for up to 2 hours without affecting their thickening efficiency. However, exposure to excessive temperatures can result in discoloration and reduced stability.

Melting point: Decomposition occurs within 30 min at 260°C

Solubility: Soluble in water, and after neutralization, in ethanol (95%) and glycerin.

Applications:

It is used mainly in liquid or semisolid pharmaceutical formulations as suspending or viscosity-increasing agents. Formulations include creams, gels, and ointments for use in ophthalmic, rectal, and topical preparations. It is used in oral preparations, in suspensions, tablets, or sustained-release tablet formulations. It is also employed as emulsifying agents in the preparation of oil-in-water emulsions for external use. For this purpose, the carbopol is neutralized partly with sodium hydroxide and partly with a long-chain amine such as stearyl amine.