BIOACTIVE AGENT CARRYING PLGA NANOPARTICLES IN THE TREATMENT OF SKIN DISEASES

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ABSTRACT

BIOACTIVE AGENT CARRYING PLGA
NANOPARTICLES IN THE TREATMENT OF SKIN DISEASES

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The aim of this study was to develop drug delivery system based on poly(lactic acid-co-glycolic acid) (PLGA) nanoparticles (NPs) to achieve personalized treatment of selected skin disorders, like photo-aging, psoriasis and atopic dermatitis. Dead Sea Water (DSW) and Retinyl Palmitate (RP) were used as active agents and they were loaded in PLGA NPs prepared either as spheres or capsules by o/w or w/o/w methods. $MgCl_2$ and bovine serum albumin (BSA) served as model active compounds.

The diameter of the NPs was found to be in the range of 280 - 550 nm. The entrapment efficiency (E.E.) was less than 1% for RP, DSW and $MgCl_2$, and 41% for BSA. Loading of Cl^- together with BSA doubled the E.E. value of Cl^- .

In situ release studies showed a burst in the first day and more than 85% of the chloride content was released within a week. When the macromolecule BSA was encapsulated, a much slower and triphasic release profile was observed which continued for up to 80 days.

In vitro tests were performed using L929 fibroblast cells. Results of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test revealed that

none of the NPs were cytotoxic. Additionally, all particles were hemocompatible with hemolytic activity <1.5%. L929 fibroblast and Saos 2 human osteosarcoma cells were used to study the uptake of NPs by the cells. Particles accumulate near the nucleus.

The characterization and cell viability tests, and drug release behavior indicate the suitability of these NPs for further testing to develop a patient specific skin diseases treatment approach.

Keywords: Drug delivery systems, PLGA, nanoparticles, skin therapy, retinyl palmitate, Dead Sea Water.

ÖZ

BİYOAKTİF AJAN TAŞIYAN PLGA NANOTANECİKLERİN DERİ HASTALIKLARININ TEDAVİSİNDE KULLANIMI

Küçüktuhan, Aysu

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Bu çalışmanın amacı, poli(laktik asit-ko-glikolik asit) (PLGA) bazlı nanotaneciklerin (NT), güneşin zararlı etkilerine bağlı yaşlanma, sedef hastalığı ve atopik derrmatit gibi hastalıklarda, kişiye özel tedavi sağlaması amacıyla ilaç salım sistemleri olarak geliştirilmesidir. Ölü Deniz Suyu (DSW) ve Retinyl Palmitat (RP) aktif ajanlar olarak kullanılmış ve küre biçimi için o/w yöntemi ile ve kapsül biçimi için w/o/w yöntemi ile hazırlanan PLGA NT içerisine yüklenmiştir. Magnezyum Klorür ($MgCl_2$) ve Bovin Serum Albümini (BSA) ise model aktif bileşenler olarak kullanılmıştır.

NT çapları 280 - 550 nm arasında bulunmuştur. RP, DSW ve $MgCl_2$ için yükleme verimliliği %1'den az ve BSA için %41'dir. Cl^-'un BSA ile birlikte yüklenmesi Cl^- yükleme verimliliği değerini iki katına çıkarmıştır.

In situ salım çalışmaları, Cl^- içeriğinin %85'inden fazlasının birinci gün sonunda ani bir patlama ile çıkışını ve bir hafta içerisinde de tamamen salındığını göstermektedir. Makromolekül BSA enkapsüle edildiğinde, 80 güne kadar devam eden, daha yavaş ve trifazik bir salım profili gözlenmiştir.

In vitro testleri L929 fibroblastik hücreleri kullanılarak gerçekleştirilmiştir. MTT (3-(4,5-dimetilthiazol-2-yl)-2,5-difeniltetrazolyum bromid) test sonuçları tüm NT'in sitotoksik olmadığını açığa çıkarmaktadır. Ayrıca, tüm tanecikler %1.5'dan az hemolitik aktivite ile kanla biyouyumludurlar. Hücreler aracılığıyla NT alınımının çalışmasında L929 fibroblastik ve Saos 2 insan osteosarkoma hücreleri kullanılmıştır. Tanecikler hücre çekirdeğinin yakınında birikmektedir.

Tüm bu karakterizasyon ve hücre sitotoksisite deneyleri ile ilaç salım davranışları, ileri testler ile hastaya özel deri hastalıkları tedavisi yaklaşımının geliştirilmesi için NT'in uygunluğunu göstermektedir.

Anahtar Kelimeler: İlaç taşıyıcı sistemler, PLGA, nanotanecikler, deri terapisi, hücre içine alınma, retinly palmitate, Ölü Deniz Suyu.

Dedicated to Nesrin and Mehmet Kazım Küçükturhan

for having chance to be their daughter…

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AD	Atopic Dermatitis
AFM	Atomic Force Microscopy
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscope
CRS	Controlled Release Systems
DDS	Drug Delivery Systems
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DSW	Dead Sea Water
E.E.	Encapsulation Efficiency
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
GA	Glycolic acid
LA	Lactic acid
MW	Molecular Weight
$MgCl_2$	Magnesium Chloride
NLC	Nanostructured Lipid Carriers
NC	Nanocapsules
NS	Nanospheres
NP	Nanoparticles
PBS	Phosphate Buffered Saline
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PVA	Polyvinyl Alcohol
PLGA	Poly(lactic acid-*co*-glycolic acid)
RP	Retinyl Palmitate
RPMI	Roswell Park Memorial Institute

RT	Room Temperature
SEM	Scanning Electron Microscope
SC	Stratum Corneum
SLN	Solid Lipid Nanoparticles
TCPS	Tissue Culture Polystyrene
TEM	Transmission Electron Microscope
TEWL	Transepidermal Water Loss
UV	Ultraviolet
UVR	Ultraviolet Radiation
UV-VIS	Ultraviolet – Visible

CHAPTER 1

INTRODUCTION

1.1. The Skin

Skin, in Latin *cutis*, is the largest and the outermost organ in the body and the major component of the integumentary system, besides sweat glands, oil glands, hair, nails, blood vessels and nerves. Skin has 1.5 - 2 m^2 surface area and this surface is in constant interaction with the external environment (e.g. continuously abraded, irradiated by sunlight, attacked by microorganisms, exposed to environmental particulates and chemicals). As a result of this close interaction, it plays a major role as a protective and supportive interface between the organism and the environment (Light, 2004, Lee *et al.*, 2006, Martini and Nath, 2009).

1.1.1. Skin Structure and Function

The skin is mostly able to overcome the challenges posed by the environment without the occurrence of apparent damage or disease (Light, 2004, Lee *et al*., 2006, Eckes *et al.*, 2010).

Skin is essential for homeostasis, the ability to maintain a relatively constant environment within the body. This is achieved mainly by the maintenance of constant body temperature by the vasculature and assistance of the sweat glands, subcutaneous fat and the hair. The skin has a significant role in the preservation of hydration of the tissues and its permeability has a critical role in slowing down the water loss by evaporation. Thus the control of the transepidermal water loss (TEWL) is very critical. In an intact skin the TEWL is low. On the other hand, increased TEWL is a sign of skin barrier abnormalities

such as atopic dermatitis, ichthyosis vulgaris, and psoriasis (Marks, 2003, Angelova-Fischer *et al.*, 2005, Rim *et al.*, 2005, Fluhr *et al.* 2006, Endo *et al.*, 2007, Atrux-Tallau *et al.*, 2008, Elias, 2008, Darlenski *et al*., 2009).

Due to its barrier properties, the skin membrane is capable of limiting the molecular transport from and into the body in addition to water. Overcoming this barrier is the purpose of transdermal drug delivery as planned in this study. In order to devise approaches to overcome the barrier, the structure of the skin should be examined. Anatomically, the skin consists of 4 basic layers: the stratum corneum, epidermis, dermis and subcutaneous tissues (Figure 1.1). In addition, there are hair follicles, sweat and oil glands, and capillaries. The subcutaneous tissues, the innermost layer, is a fibrous connective structure which acts as an insulator, shock absorber, reserve depot of calories and supplier of nutrients for the upper skin layers. The dermis gives the skin its mechanical strength, and is the thickest structure of the skin which consists of collagen fibers and glycoprotein filaments embedded in amorphous connective tissue. Epidermis is the outermost layer and serves as a barrier between the body and the environment, and consists of 5 different layers. The stratum corneum is the outermost layer of epidermis.

1.1.1.1. Dermis and Subcutaneous Tissues

The dermis is located between epidermis and the subcutaneous tissues. It is a fibrous layer, whose thickness varies from 0.3 - 3 mm and supports and strengthens the epidermis. It consists of a matrix of loose connective tissue composed mainly of collagen. This matrix holds the cells and allows the oxygen and nutrients to diffuse to the epidermal cells. It contains an extensive blood vessel and nervous network, as well as hair follicles, sebum and sweat glands. The layer adjacent to the dermis, is called the papillary layer, provides nutritional support and has a role in the regulation of temperature, pressure and pain. In addition, it contains melanocytes and is involved in the production of melanin for the skin color under solar radiation (Contreras, 2007).

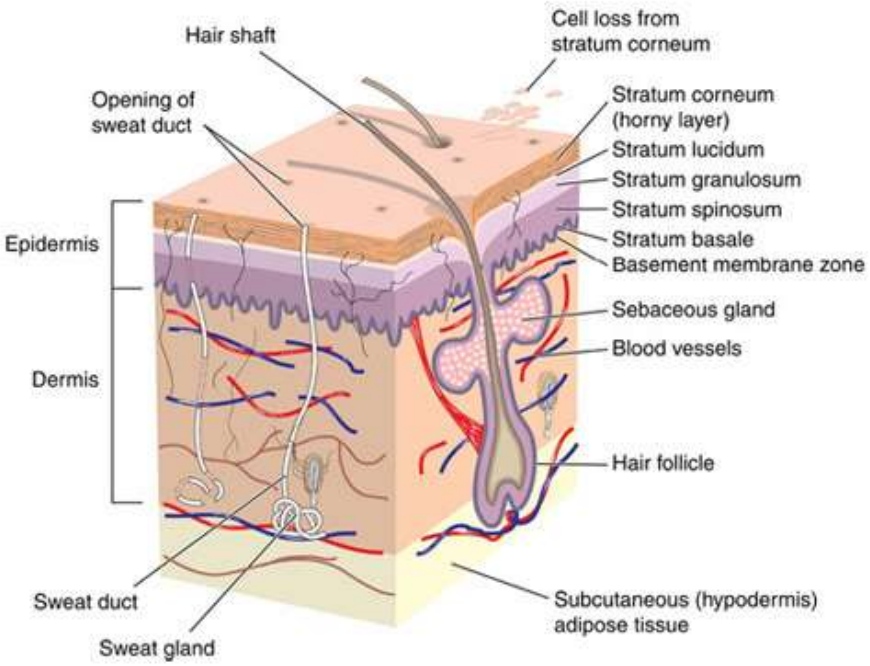


Figure 1. 1 The anatomy of the skin (http://www.azurlis.co.nz/anatomy_of_our_skin_and_some_of_its_properties).

The subcutaneous tissues contain blood vessels that provide nutrition and control the body temperature, while the nerves in subcutaneous tissues provide sensations. This layer acts as an insulator, shock absorber, and reserve depot of calories as well as supplier of nutrients for the more superficial skin layers. The base of the hair follicles, the secretory portion of the sweat glands, the cutaneous nerves as well as networks of lymph and blood vessels are found inside of this layer (Contreras, 2007, Martini and Nath, 2009).

1.1.1.2. Epidermis

The epidermis is the outermost part of the skin. It is a multiple layered structure which is made up of stratified squamous epithelium, and its thickness varies between 0.05 mm - 1.5 mm. From the outside to the inside stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spinosum, and

stratum basale (or stratum germinativum) could be seen (Figure 1.1). Epidermis does not contain any blood vessels, and therefore, in order to maintain its integrity, nutrients and waste products are transported through diffusion by blood vessels in the dermis across the dermis-epidermis junction. Basically, almost all epidermal functions can be considered protective and defensive, and most of these critical protective functions are performed by the outermost part of the epidermis, the stratum corneum (Williams, 2003, Elias, 2008, Martini and Nath, 2009, Habif, 2010).

The stratum corneum (SC) plays the major role in the transdermal and topical drug delivery. Therefore, it is often treated as a separate part of the skin.

1.1.1.3. Stratum Corneum (SC)

The stratum corneum (SC), is a 10 - 30 μm thick layer composed of two different structural components derived from keratinocytes: corneocytes and intercorneocyte lipids. About two decades ago, Elias (1983) proposed the "brick and mortar model", according to which the SC is built up of flat cells (bricks, the corneocytes) surrounded by a lipid matrix (mortar) (Figure 1.2). The corneocytes, provide the structural support for the SC, serve as a barrier against UV and mechanical and keep the SC hydrated. The intercellular lipids on the other hand serve as the antimicrobial, antioxidant and permeability barrier (Wolf *et al.*, 2008, Johnson, 2009).

This brick-mortar barrier has other components such as hair (with its shaft and follicles), sweat ducts and glands (and nail in the fingers). These cover less than 1% of the total surface area of the skin but serve as the main route of entry for nanoparticles through the topical drug delivery applications (Wosicka, 2010).

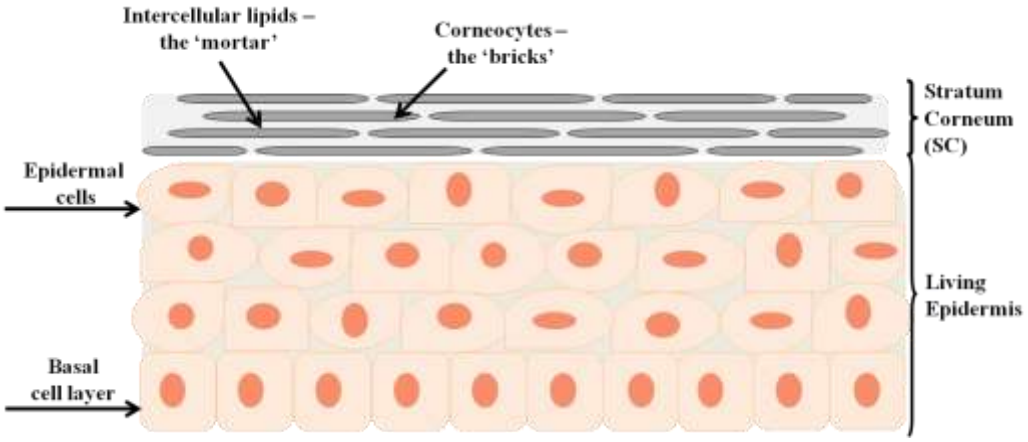


Figure 1. 2 Composition and functions of epidermis and stratum corneum.
A typical "bricks and mortar" representation of skin cells (adopted from Johnson, 2009).

1.2. Skin Diseases and Aging

Causes of skin disorders can be simply studied under two main types of headings congenital and acquired. Congenital conditions are those that are present at birth, which are usually the result of genetic inheritance. Some of these diseases may be treatable. Other conditions, such as vascular disorders, may remain with the patient throughout life. On the other hand, acquired conditions may be either a symptom of another disease, as in the case of AIDS-related Kaposi's sarcoma, or a symptom in itself a skin diseases such as skin cancer (Papadopoulos and Walker, 2003).

Millions of people suffer from some kind of skin disorder, skin disease or plain skin infections. Skin disorders are very common, so that 10 – 15% of the general practitioner's work is with skin disorders, and skin diseases are the second most common cause of loss of work. Atopic and other forms of eczema affect 15% of the population under the age of 12, psoriasis affects 1 – 2%, and viral warts, seborrhoeic warts and solar keratoses affect large segments of the population (Marks, 2003).

Skin diseases, basically affect the skin integrity and skin hydration (Watkinson *et al.*, 2001), and directly cause barrier dysfunction (Johnson, 2009).

Although many skin diseases are known, the most common inflammatory skin diseases such as psoriasis and atopic dermatitis, and UV damaged skin will be considered within the scope of this study.

1.2.1. Psoriasis

Psoriasis is a chronic inflammatory skin disorder with genetic predisposition, affecting 1 - 3% of the world population (more in men than women). The severity of the diseases is categorized as mild (< 10% of the body is affected) to severe (more than 10% of the body). Patients with psoriasis suffer significant loss in the quality of life depending on the disease severity. These include occurrence of disabilities or psychosocial alienation (Nevitt, 1996, Schmid-Ott, 2005, Turkington and Dover, 2007).

Psoriasis is neither a terminal disease nor contagious. It is diagnosed with production of silvery, scaly plaques on the skin and excess growth of skin cells. The most common type of psoriasis is called plaque psoriasis (*psoriasis vulgaris*), characterized by raised, inflamed lesions covered with silver-white scales (Figure 1.3).

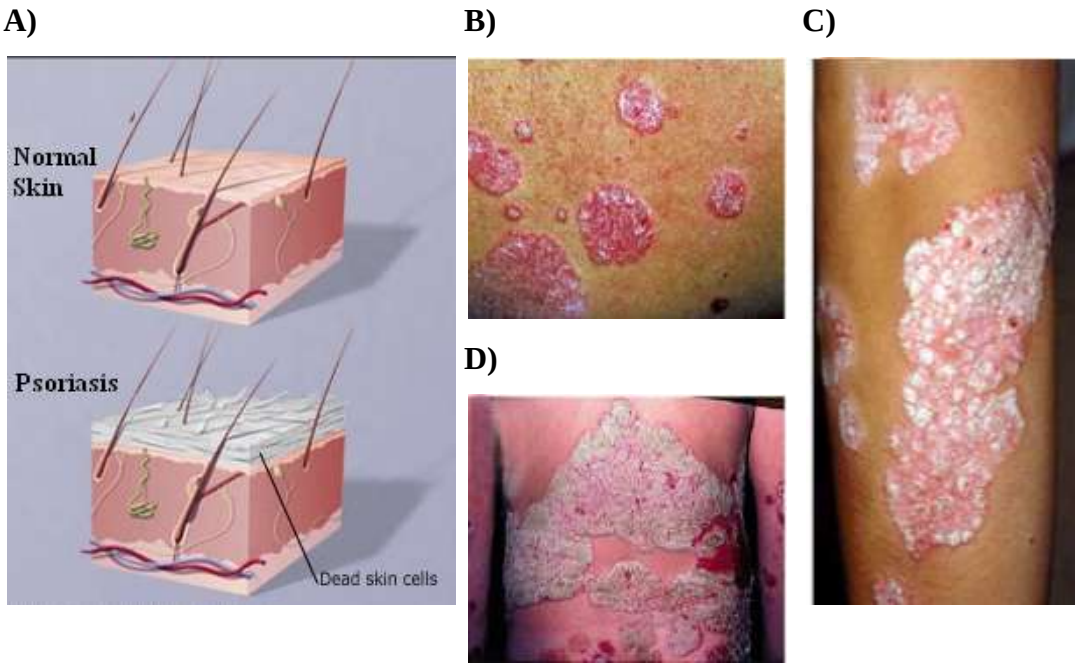


Figure 1. 3 Psoriatic skin. A) Cross-section of healthy and psoriatic skin (http://www.webmd.com/skin-problems-and-treatments/psoriasis/understanding-psoriasis-basics), B) Small plaque psoriasis, C) Localized thick plaque type psoriasis, D) Large plaque psoriasis (Menter *et al.*, 2008).

Several factors such as endocrine factors, exposure to sun, stress, alcohol consumption, traumas, infection and medications can trigger the psoriasis and have a major impact on the effectiveness of the treatments.

The extent of disease, its effect on a patient's life, and the patient's perception of their illness are the major factors influence the choice of treatment. Treatment generally starts with topical and continues with the systemic therapy. Frequently used systemic agents are given in the Table 1.1 (Lowes *et al.*, 2007, Fox, 2010).

Reducing the number of pathogenic T cells and blockade of the activation and/or migration of them (Singri *et al.*, 2002, Gordon *et al.*, 2004), anti-TNF drugs (TNFα appears to be a critical cytokine for many of the clinical features of psoriasis) (Gottlieb *et al.*, 2003, Leonardi *et al.*, 2003), retinoids (Lowe *et al.*, 1988) and combinational therapies with phototherapy (Lebwohl *et al.*, 2005) are all the preferred treatments in psoriasis.

Table 1. 1 Examples of systemic therapeutics for psoriasis vulgaris (adopted from Lowes *et al.*, 2007).

Trade name	Active agent	Status
Agents used in early studies to establish immunological basis of psoriasis		
Ontak	Denileukin diftitox*/DAB389IL-2	
Orencia	Abatacept/CTLA4lg	
Prograf	Tacrolimus/FK506	
Zenapax	Daclimuzab*†	
Simulect	Basiliximab*†	
Widely used systemic agents approved to use (immunosuppressives)		
Neoral, Gengraf	Cyclosporine*	Widely used
Rheumatrex, Trexall	Methotrexate	Widely used
	Fumarates*	Widely used in Germany
Approved biological agents		
Amevive	Alefacept*	FDA approved
Raptiva	Efalizumab*†	FDA and EMEA approved
Remicade	Infliximab†‡	FDA and EMEA approved
Enbrel	Etanercept‡	FDA and EMEA approved
Drugs/biological agents under investigation (human or murine trials)		
Humira	Adalimumab†‡	In clinical trials (FDA approved for psoriatic arthritis)
	Pimecrolimus, Cent-1275†‡, ABT-874†‡, and 146B7†‡	In clinical trials
*** T-cell targeted, † Monoclonal antibodies, ‡ Cytokine inhibitors**		

1.2.2. Atopic Eczema (Dermatitis)

Eczema or dermatitis is an inflammatory condition of the skin and is a name that covers many types of disorders such as atopic, contact, seborrheic and hand eczema (Table 1.2). Atopic dermatitis (AD) is a very common, chronic, extremely itchy disorder starting from infancy. It usually affects the face and the flexures, and is strongly associated with asthma and hay fever. Any substance

(e.g. dust mites, pollen, animal dander, mold, foods) responsible of the occurrence of allergy can also cause AD. Atopic skin is characterized by papules, itching attacks, and lichenification (leathery induration and thickening of the skin) (Figure 1.4) (Leung *et al.*, 2008, Anderson, 2008).

Table 1. 2 Types of eczema disease (adopted from Anderson, 2008).

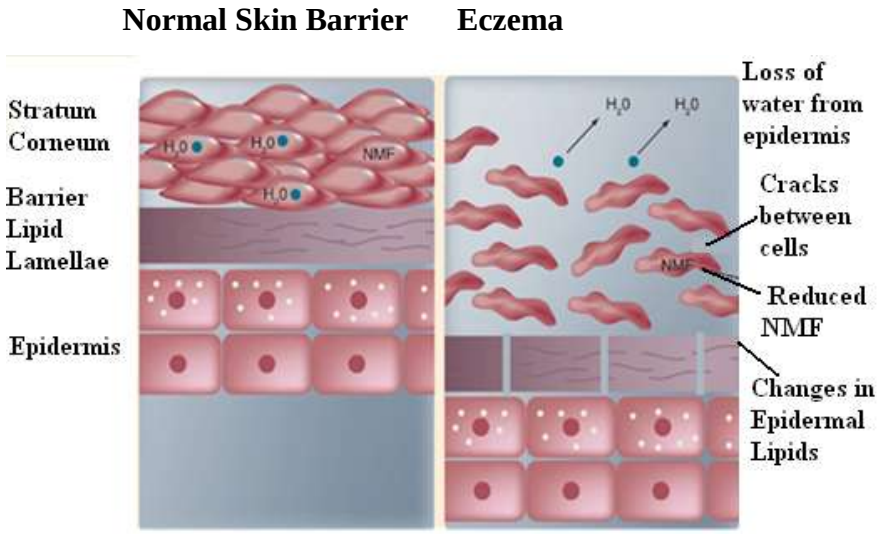
Contact dermatitis	Discoid (nummular)
Atopic	Pityriasis alba (pityriasis sicca)
Seborrheic	Intertriginous eczema
Venous/gravitational eczema	Hand eczema
Lichen simplex chronicus (neurodermatitis)	Infective/microbial eczema
Asteatotic eczema	Generalized eczema

In addition to allergens, microbial organisms, sex hormones, stress, sweating and environmental (e.g. climatologic) conditions can trigger occurrence of AD. These factors cause the immune system to overreact, and activate the cells that produce inflammation in the skin (Morren, 1994).

Increased basal transepidermal water loss (TEWL) and differences in SC lipid composition were reported as evidences for an impaired skin in AD. Skin barrier dysfunction in AD has also been demonstrated by increased SC permeability to theophylline and increased erythematic response to dimethyl sulfoxide (Yoshiike *et al.*, 1993, Pilgram *et al.*, 2001, Holm *et al.*, 2006).

The treatments for eczema include topical emollients and steroids, oral antihistamines and antibiotics, and PUVA therapy (a combination of psoralens and exposure to UVA light) (Habif, 2010).

A)



B)

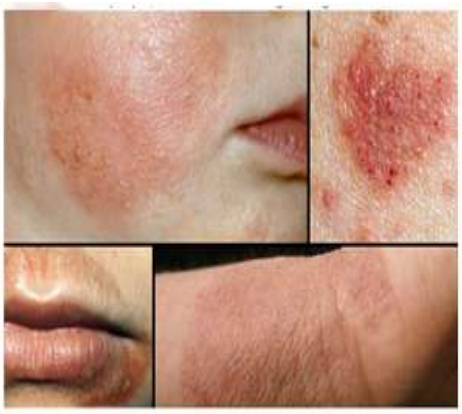


Figure 1. 4 Atopic Dermatitis (AD). A) AD formation in the skin (http://www.medscape.com/viewarticle/584252_11), B) AD in different parts of the skin (http://otorhinolarygology.blogspot.com/2011/02/eczema-webmd.html).

1.2.3. UV Damaged Skin

UV radiation is 100 - 400 nm range within the wide range of wavelengths that the sun emits (Figure 1.5).

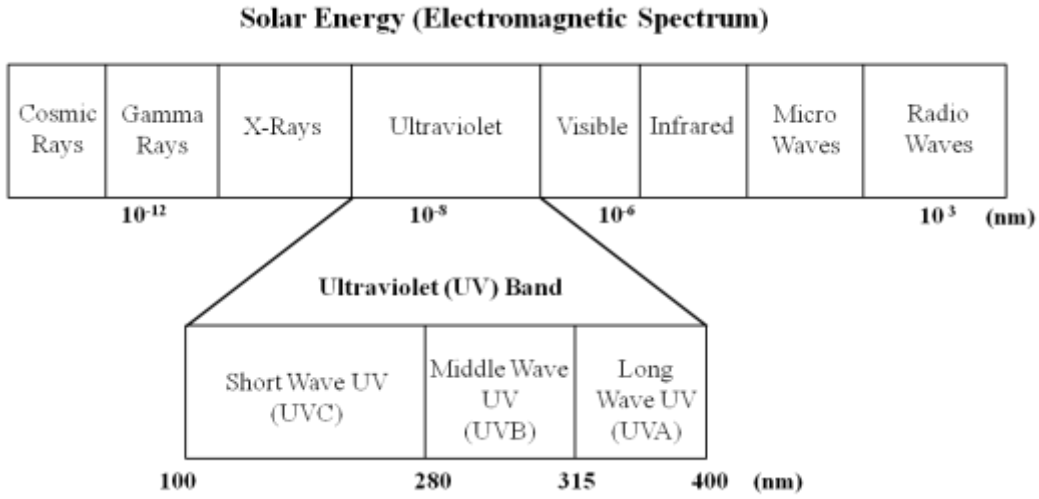


Figure 1. 5 Solar energy spectrum with the inset of UV band (adopted from http://www.drb-mattech.co.uk/uv%20spectrum.html).

UV radiation is divided into three parts: UVA (315 - 400 nm), UVB (280 - 315 nm), and UVC (100 - 280 nm). UVC is mostly filtered out by the ozone layer and it is not considered as a threat for biological systems. UVB is absorbed by the epidermis while UVA can penetrate the deeper regions of the dermis. Both of these UV radiations have hazardous effects on the skin, which are called the wavelength dependent long-term effects, such as skin photoaging, non-melanoma and malignant melanoma type skin cancers, and short-term effects (sunburn inflammation (erythema), tanning (enhanced melanogenesis), thickening of the skin (hyperkeratosis), edema, and occurrence of immunological responses) (Matsumura and Ananthaswamy, 2004, ISO 21348:2007) (Figure 1.6).

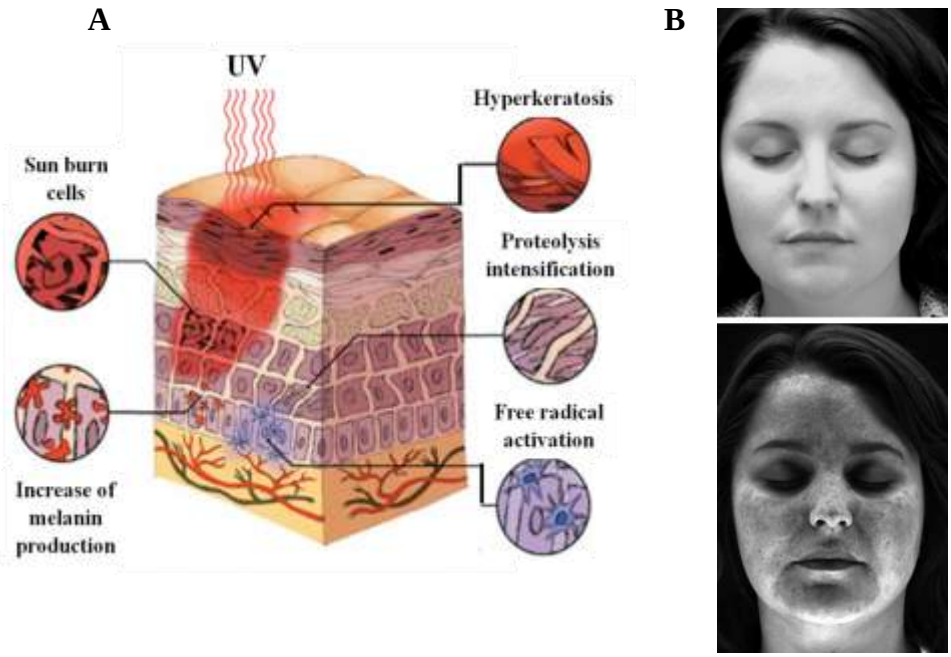


Figure 1. 6 UV damaged skin. A) Effect of UV radiation on the skin (adopted from http://www.solecrin.com/23,Skin-ageing.html).
B) Appearance of UV exposed skin; under normal and UV light (http://www.morethanamomma.com /2011/05/tingler-gingers-autobahn-to-skin-cancer.html).

The main reason for sunburn, suntan and skin cancer is UVB. It is down to the basal layer of the epidermis, causes the death of scattered keratinocytes (sunburn cells), damages their DNA and cause the release of cytokines and mediators. These produce edema, vasodilatation and a subepidermal inflammatory cell infiltration. Two days post-UV radiation (UVR), there is an increase in the rate of melanin synthesis, which stimulates a tan that is probably not possible without sustained UVR damage. Sunburn is easily recognized by the redness and, by swelling and blistering as well, depending on the severity. For some unexplained reason, it is quite sharply restricted to the area of skin exposed. Patient sensitivity to solar UVR depends on the degree of skin pigmentation and on inherent metabolic factors.

In contrast, UVA can penetrate deeper into skin, to dermis, and is 1000-fold less effective in causing erythema. UVA wavelengths are not significantly absorbed by DNA and thus DNA is not affected directly. However, UVA is

thought to play a role in causing a dermal degeneration known as solar elastosis, which is mainly responsible of ageing and initializing of skin cancer by producing reactive oxygen species (ROS) and oxidative damage. UVA induced ROS production is involved in photoaging that may accelerate the shortening of telomeres and cause earlier cell senescence. This could be the reason why various growth factors affect the speed and quality of wound healing. UVA is also the part of the spectrum that is mainly responsible for the photosensitivity reactions (Marks, 2003, Taylor, 2005, Von Thaler *et al*., 2010).

Interestingly, UVR, is used as a therapeutic agent for various skin diseases, specifically psoriasis, atopic dermatitis, and vitiligo. Since 1970's psoralens, which are phototoxic compounds, administered by oral or topical routes were shown to be effective when applied together with UVA radiation (PUVA therapy) (Hönigsmann *et al*, 1977). These phototherapies have acute (e.g. drug intolerance, UVA overdosage) and chronic side effects (e.g. melanoma, carcinogenesis) (Matsumura and Ananthaswamy, 2004, Hönigsmann, 2008).

1.2.4. Aging

Aging is the total outcome of the accumulation of alterations in cells and tissues as a result of the disorderliness of regulatory mechanisms caused by environmental conditions, stress and diseases which reduce the organization and strength of the organism. Disorderliness of the regulatory mechanism involves the deterioration of controls on the secretion of various hormones (luteinizing hormone (LH), follicle stimulating hormone (FSH), adrenocorticotropic hormone (ACTH) and growth hormone (GH) as a result of the adulthood hormonal changes due to menopause, andropause, and adrenopause (Quiroga, 2005).

Skin aging is a combination of intrinsic aging (due to inherent genetics) and extrinsic aging (due to environmental conditions, solar exposure). As a result of aging, the skin loses its elasticity, becomes thinner, wrinkled and saggy with graying of hair. Level of dendritic (Langerhans) cells decrease at maturity, and as a result of this decrease the sensitivity of the immune system may reduce and cause further skin damage and infection. Repair mechanism of the skin proceeds

more slowly; the time required to turnover an uninfected wound at age 70 is twice as high as that for a young adult (Quiroga, 2005, Martini and Nath, 2008, Thakur *et al.*, 2008) (Figure 1.7).

A) **B)**

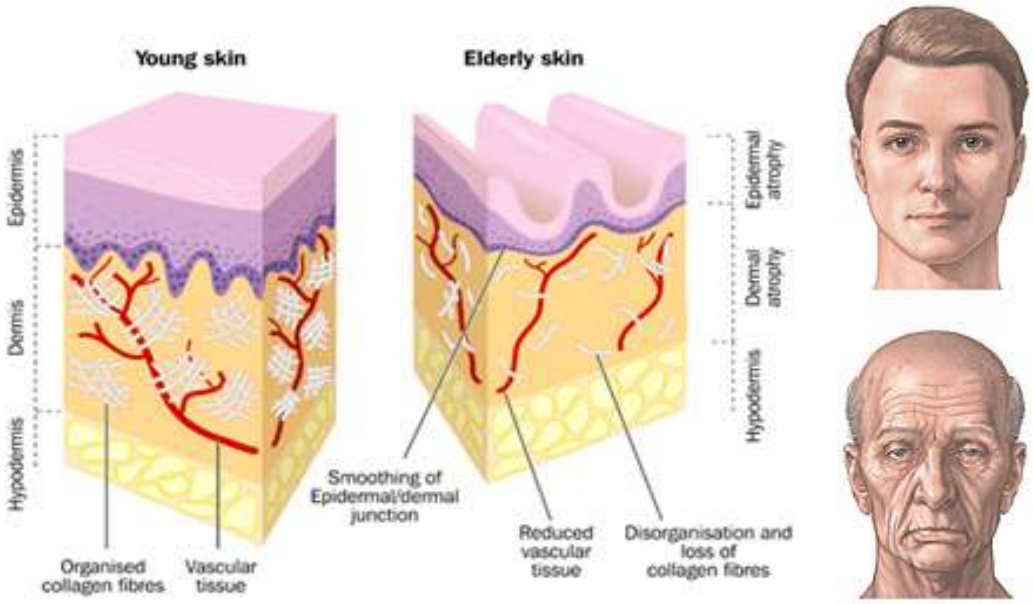


Figure 1. 7 Aging of the skin. A) Illustration of skin aging showing structural alterations (http://www.rejuvenateyourskin.co.uk/skin-ageing-process/),
B) Appearance of skin deformations as a result of aging
(http://www.nlm.nih.gov/ medlineplus/ skinaging.html).

Cellular senescence is caused by continuous genetic and epigenetic changes over time, which limits cell divisions in mitotically active somatic cells, and may play a major role in numerous age-related diseases (Quiroga, 2005, Dellambra and Dimr, 2008).

Extrinsic aging, due to the environmental factors, occurs as result of stress, smoking and exposure of solar radiation (UV exposure) (Farage *et al.*, 2008). Since the major cause of extrinsic aging is UV induced damage, it is also known as photoaging (Dellambra and Dimr, 2008).

1.3. Treatment of Skin Diseases

Today, skin diseases are considered as critical health problems, because of the growing frequency and type of skin diseases: 0.5 – 5% among the population and around 3000 types.

Most dermatological disorders can be categorized as skin diseases; however, they differ in their cause, phenotype and severity. Most frequently viewed and severe skin diseases are inflammatory, infectious and neoplastic pathologies. Although the genetic factors are important, the changes in the immune system, exposure to carcinogenic and external factors and other diseases affect the development of these diseases (Elishmereni and Levi-Schaffer, 2008).

Most of the skin diseases have common characteristics such as consistent itching, irritation, swelling, redness, pain and deformation of cutaneous tissues.

When the skin fails to respond to topical therapy, topical therapy is not tolerated by the patient or is impractical given the extent of cutaneous involvement, the skin disease is associated with other diseases of internal organs that cannot be treated with topical therapy alone, or the skin disease alone may potentially lead to significant morbidity and/or mortality if left untreated then systemic therapy is started (Fox, 2010).

Topical formulations have 2 main components: the active ingredient or drug, and the vehicle systems, the inactive carrier. The vehicle is very important for the texture of the product (e.g. thick and greasy or light and watery) and the bioactivity (MacNeal, 2006). Solubility, stability, rate of release of the active therapeutic agents in the vehicle, and chemical and physical interactions of the vehicle with both SC and therapeutic agent are important considerations (Anigbogu and Maibach, 2005). Table 1.3 shows the different types of topical formulations, used in the treatment of skin diseases.

Table 1. 3 Topical formulations used in the treatment of skin diseases (adopted from Anigbogu and Maibach, 2005, MacNeal, 2006, Oakley, 2010).

Type	State and Properties
Lotion	Highly aqueous, oil-in-water emulsion with a high water content or hydro-alcoholic systems
Gel	Transparent preparations containing cellulose ethers or water-alcohol mixtures
Paste	Mixture of powder and ointment
Cream	Emulsion of water and oil
Ointment	Semisolid preparations of hydrocarbons
Aerosols/Sprays	Solutions with pressurized propellant
Powders (Solids)	Dried forms of substances
Transdermal Patches	Drug delivery systems that allow precise dosing
Nanosystems	Drug loaded lipoid or polymeric nanoparticulates

Topical treatments are used for most psoriasis cases. The ointments are more effective than creams; however, are less cosmetically acceptable because of their thickness and dense structure. Therefore, in general, patients prefer to use both formulations selectively: creams in the morning and ointments at night. Meanwhile, topical corticosteroids are commonly first choice in mild to moderate psoriasis and specifically at flexures and genitalia where other topical treatments can induce irritation. The use of topical corticosteroids has to be considered carefully because their long term use may cause striae and adrenal suppression (Gudjonson and Elder, 2008).

In AD patients, one of the major problems is dryness due to the reduced water content of the skin. Application of emollients combined with hydration therapy helps to restore and preserve the SC barrier. Moisturizers are available in the form of lotions, creams, or ointments. The higher water content and lack of additives (e.g. solubilizers and fragrances) make lotions preferable by the patients. In addition to those, as in the case of psoriasis, topical glucocorticoid therapy, which in cream or ointment forms, is applicable for the anti-inflammatory eczematous skin lesions (Leung *et al.*, 2008).

Retinoid treatments for the acne, photoaged (UV induced skin aging) skin and psoriasis have been approved by the Food and Drug Administration (FDA) of USA. Topical tretinoin or tazarotene applications improve fine wrinkles and dyspigmentation of photoaged skin (Kang and Voorhees, 2008).

Novel treatments including nano-microsystems as topical and transdermal drug delivery methods have gained significant importance in the last two decades. Lipoid particulate technologies (e.g. liposomes, solid lipid nanoparticle (SLN)) in cosmeceutical and pharmaceutical formulations and polymeric nano-microparticulate delivery systems have shown promising developments in the treatment of skin diseases (Draelos, 2009, Venuganti and Perumal, 2009).

1.4. Drug Administration Routes

1.4.1. General Administration Routes and Forms

Drugs can be introduced into the human body through various anatomical routes, either for systemic effects or by targeting to specific organs and diseases. The choice of the administration route depends on the disease and the desired effects (Jain, 2008).

For a drug to be effective, it should be present at the site of the action for an adequate period of time. The effectiveness of the therapy influences is decided by the route and method of drug administration; these affect the kinetics, biodistribution, and elimination of the drug.

The most convenient and common drug administration route is oral. Administration directly into the body by injection or infusion is called parenteral drug delivery; these are intravenous, intramuscular, subcutaneous, intradermal and intraperitoneal depending on the site of administration. In order to avoid the skin barrier entry of drug into the body through mucosal membranes is another possibility (Perrie and Rades, 2010).

To sum up, the delivery of the drugs at the right time in a safe and reproducible manner to a specific target at the required level is the final aim of the drug delivery study. Delivery of drugs in a controlled manner to a target site is

superior to the conventional systems, and therefore, studies are concentrated on the design of controlled release systems (Orive *et al.*, 2003).

1.4.2. Controlled and Prolonged Release Systems (CRS)

A drug delivery system (DDS) is defined as a formulation or a device that enables the administration of a therapeutic agent into the body and improves its efficacy and safety by controlling the rate, time, and place of release of drugs in the body (Jain, 2008).

For the treatment of skin diseases, depending on the cause of the disease and the location of the target site the administration route could be either topical or oral (for systemic). In order to attain the correct pharmaceutics a number of delivery approaches could be employed.

In order to prolong the bioavailability and maintain the dose at a predetermined level various drug delivery approaches can be used.

Extended or sustained release systems allow for the drug to be released over prolonged time periods and the frequency of dosing can be reduced. This can be achieved using various dosage forms such as particulates, films or membranes or tablets. These systems achieve their aim mostly by the use of suitable synthetic or natural polymers that are generally used to construct the reservoir (encapsulate the drug) or the matrix (drug is dissolved or dispersed in the structure). Release of drug is obtained through diffusion of the drug in the polymer or by the degradation of the polymer over time, or by both.

Aim to deliver the drug more precisely at a constant rate, generally by employing a rate controlling membrane. They are therefore designed to maintain the plasma drug concentration in the therapeutic range regardless of the local environment (Kaparissides *et al.*, 2006, Jain, 2008, Perrie and Rades, 2010).

The efficiency of the DDS is judged by the maintenance of a sufficiently high concentration of the drug at the therapeutic site combined with a low systemic drug concentration. A number of mechanisms that can provide such a controlled drug release processes include transdermal patches, chemotherapeutic wafers, osmotic micropumps, bioadhesive systems, microencapsulations,

nanoparticles and microimplants (De la Heras Alarco´n *et al.*, 2004, Gardner, 2006).

1.4.2.1. Topical and Transdermal Drug Delivery

As it was mentioned above in Section 1.1.1.3 stratum corneum (SC) is the outermost desquamating layer of the skin, and it is the rate limiting barrier against percutaneous drug transport. Various products are designed for use in transporting across the skin. These include transdermal patches, gels, creams, ointments, lotions, and dermal vaccinations.

In transdermal drug delivery, bioactive agents have to be absorbed at an adequate rate to achieve and maintain uniform, systemic or local therapeutic levels. In general, once drug molecules pass the SC barrier, they travel into deeper dermal layers and systemic uptake occurs relatively quickly (Bickers, 2010).

In transdermal delivery in contrast to the traditional oral route, first pass metabolism is avoided; gastrointestinal bleeding or irritation caused by the drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) is prevented. The transdermal route has the convenience of termination on demand, specifically in the case of overdose or side reactions. Furthermore, it allows self-administration of drugs (Roberts *et al.*, 2002).

The application of the drugs to the skin within vehicles such as ointments, creams, gels and patches are classified as the passive transdermal delivery technologies. These dosage forms have been developed in order to improve the driving force of drug diffusion and/or increase the permeability of the SC. These systems may include penetration enhancers (Williams and Barry, 2004), and the drugs could be in the form of prodrugs (Tsai *et al.*, 1996), or carried in liposomes and other lipoid vesicles (Choi and Maibach, 2005), and polymeric structures (Valenta and Auner, 2004). The amount of drug delivered by passively could be limited unless the barrier properties of the skin are changed significantly by the external forces.

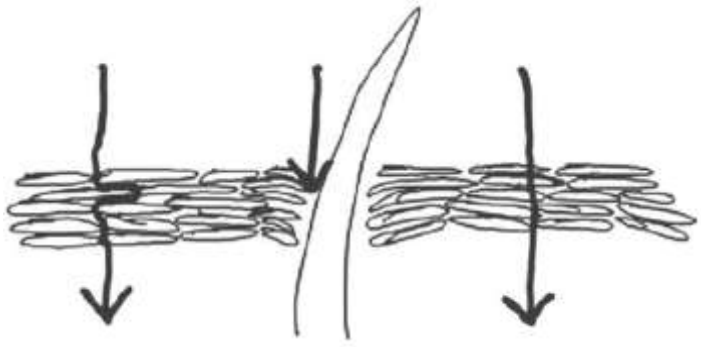
The active delivery methods, however, involve the use of external energy to improve the driving force for the crossing of drugs through the skin, and/or to decrease the barrier nature of the SC. The use of active enhancement methods is

of importance specifically for the high molecular weight (> 500 Da), polar and hydrophilic molecules. The skin permeability could be enhanced by use electrical methods (e.g. iontophoresis, electroporation), mechanical methods (e.g. micro needle based devices, skin abrasion, skin puncture and perforation, needleless injection), application of pressure (e.g. skin stretching), and other techniques such as ultrasound (e.g. sonophoresis and phonophoresis) (Brown *et al.*, 2006).

1.4.2.2. Mechanism of Penetration

The goal in transdermal systems is to maximize the drug absorption. The rate and extent of drug penetration into different layers of skin and into the systemic circulation are governed by the properties of the drug and the formulation characteristics (Venuganti and Perumal, 2009).

Under normal conditions, bioactive agents (e.g., free drug, DDS) for transdermal drug delivery have three pathways to cross the epidermis: intercellular (or paracellular, in between the corneocytes), intracellular (or transcellular, across the corneocytes), and through the appendageal shunt (transappendageal) (Figure 1.8) (Barry, 2001, Contreras, 2007, Venuganti and Perumal, 2009).



A) Intercellular **B)** Appendageal **C)** Intracellular

Figure 1. 8 Routes of skin penetration. A) Intercellular, B) Appendageal, C) Intracellular pathways (adopted from Venuganti and Perumal, 2009).

Intracellular pathway is longer (~500 μm) than the normal SC thickness (~20 μm), therefore, the most commonly preferred route for the application of the drugs is intercellular route. The carrier and proper route have to be determined by the drug characteristics (hydrophobic or hydrophilic), targeted zone and disease to be cured (Hadgraft, 2004). Various drugs and/or cosmetics groups and their target site in the skin are given in Table 1.4 to show which pathway is chosen for a specific treatment.

Table 1. 4 Effective target site for topical and transdermal delivery of drugs (adopted from Venuganti and Perumal, 2009).

Targeted Site	Drugs/Cosmetics
SC	Cosmetics, sunscreens, antimicrobials, skin protectants
Epidermis	Anti-inflammatory agents, antiproliferative agents, vaccines, antihistamines, gene therapy
Hair follicles	Antiacne agents, antimicrobials, depilatories, vaccines, gene therapy
Dermis	Local anesthetics, analgesics, drugs for systemic administration

In addition to those general routes and targets presented above, the majority of the transdermal target site may also be altered depending on the systems used in treatment of the diseases: when nanoparticulates are used then the appendageal route via hair follicles is selected. Hair follicles occupy only approximately 0.1% of the total skin surface, however, have an important role because the absorption area below the skin surface, being an invagination of the epidermis through the dermis increases both the skin surface and the depth penetrated (Lauer *et al.*, 1995).

For the particulate delivery systems, size is an important factor in the delivery and retention in the hair follicle. Different size solid nanoparticles may be used in follicular penetration to target different depths along the hair duct (Blume-Peytavi and Vogt, 2011). Wosicka and Cal (2010) stated that a particle

size of 0.04 – 6 μm is optimum for potential drug delivery carriers using the follicular route for transdermal delivery.

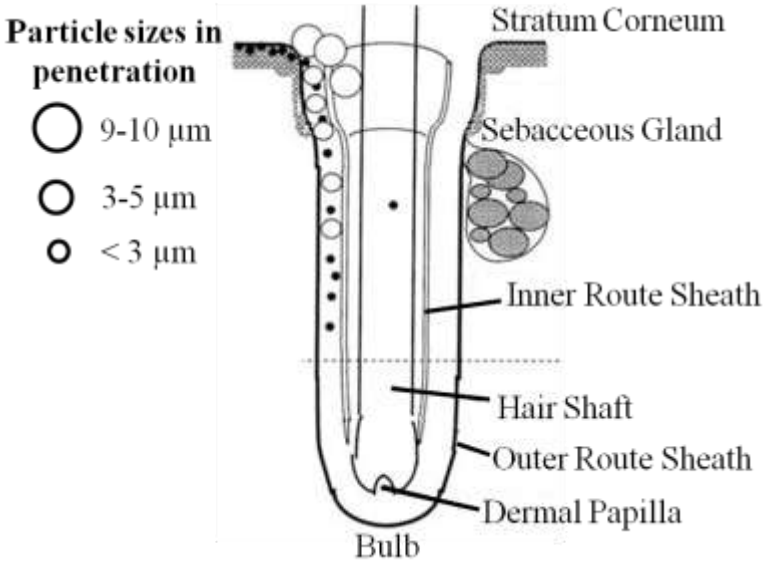


Figure 1. 9 Use of hair follicles in DDS transport (adopted from Roberts and Cross, 2002).

Figure 1.9 shows the relation between penetration depth and particulate size relation in transdermal delivery via hair follicles.

1.5. Nanoparticles for Drug Delivery

Nanotechnology is an interdisciplinary field that was using the principles of the traditional sciences such as chemistry, biology, materials sciences and physics (Vo-Dinh, 2007).

Nanoscale is generally considered as the range 1 - 100 nm even though it is the range 10^{-6} m – 10^{-9} m (Soppimath *et al.*, 2001, The Royal Society and The Royal Academy of Engineering, 2004).

Drug delivery is an important area of nanotechnology that makes a significant impact in nanomedicine especially in drug delivery systems (Pridgen *et al.*, 2007, Farokhzad and Langer, 2009). Nanoscale drug delivery vehicles have shown the ability to encapsulate a variety of therapeutic agents such as small molecules, peptides, protein-based drugs, and nucleic acids. By entrapping these molecules inside a nanocarrier, the solubility and stability of the drugs can be improved and this provides an opportunity to reevaluate drugs previously ignored because of poor pharmacokinetics (Langer, 1998).

Nano and micro-sized carriers are promising and important systems because of their small size which help them overcome biological barriers and achieve cellular uptake (Brigger *et al.*, 2002). The net result of these properties is to lower the systemic toxicity of the therapeutic agent while increasing the concentration of the agent in the area of interest, resulting in a higher therapeutic index for the therapeutic agent. In addition to therapeutics, imaging agents can also be incorporated into nanocarriers for use in tumor detection and imaging (Kim *et al.*, 2006, Montet *et al.*, 2006).

The first nanoscale drug delivery systems developed in the 1960s were lipid vesicles, known as liposomes (Bangham *et al.*, 1965). Langer and Folkman (1976) demonstrated the first controlled release polymer systems for the delivery of macromolecules. This was followed in the 1980s with the first application of targeted liposomes (Heath *et al.*, 1980, Leserman *et al.*, 1980). Afterwards, there has been considerable interest in developing biodegradable nanoparticles (NPs) as effective drug delivery devices (Figure 1.10). Currently, 70 clinical trials evaluating nanoparticle carriers, 208 evaluating drug conjugates, and 361 evaluating vesicle-based carriers are found in the literature (Alexis *et al.*, 2010).

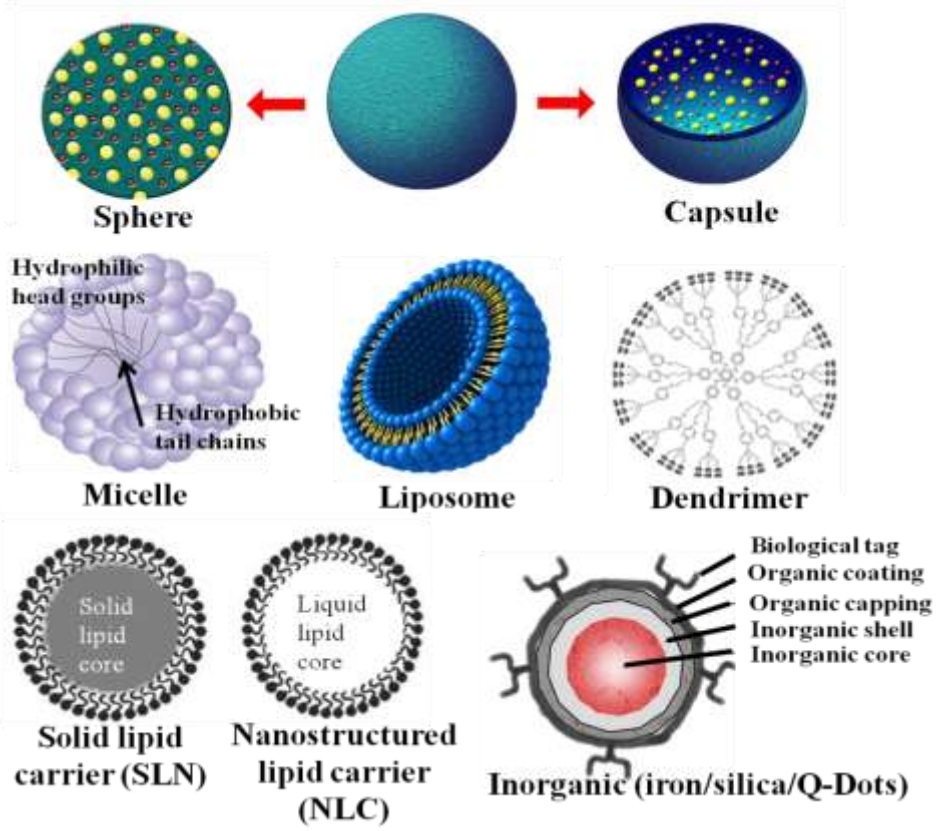


Figure 1. 10 Commonly used nanoparticulates for drug delivery.

1.5.1. Lipoid Systems

Phospholipids, triglycerides, and cholesterol are the main ingredients of liposomes and lipid nanoparticles. Phospholipids spontaneously form bilayer membrane vesicles (liposomes) or may act as surfactants in forming micro or nanoemulsions or solid–lipid nanoparticles upon hydration. Since they are natural components of biological membranes and lipoproteins, therefore are highly biocompatible (Wu *et al.*, 2007).

1.5.1.1. Liposomes

Liposomes are colloidal particles made up of phospholipids and organized in bilayers. Depending on the number of layers (lamellarity) and diameter, liposomes are classified into multilamellar vesicles (MLVs, diameter > 200 nm),

large unilamellar vesicles (diameter 100 – 400 nm), and small unilamellar vesicles (diameter < 100 nm). According to the surface charge (zeta potential), they are classified into cationic, neutral, and anionic liposomes. They can incorporate different kind of substances, independent of MW, solubility, or electric charge within the aqueous core or the lipoid membrane or both.

Studies have shown that liposomes adsorb and fuse with the skin surface and form a favorable environment for the partitioning of lipophilic drugs (Ramon *et al.*, 2005, El Maghraby *et al.*, 2006, Kitagawa and Kasamaki, 2006).

1.5.1.2. Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles (SLN) are 50 - 1000 nm in diameter and were introduced in 1991 as an alternative carrier system to traditional colloidal carriers. These colloidal systems are made from highly purified triglycerides, complex glyceride mixtures, or waxes and stabilized by surfactant(s). Thus, organic solvents are not needed for their production (Müller *et al.*, 2000, Cruz *et al.*, 2009).

The SLNs offer many advantages over other lipid carriers for skin. The lipid nanoparticles form an occluding film when applied to the skin (Wissing *et al.*, 2001), and this leads to increased skin hydration, which, in turn, reduces the corneocyte packing and increases skin penetration. Furthermore, the lipids can interact with the skin lipids and act as penetration enhancers (Souto and Müller, 2007).

1.5.1.3. Nanostructured Lipid Carriers (NLC)

The main difference between SLNs and NLCs is that, in the case of SLNs, the core consists of a solid lipid, while NLCs have a liquid lipid core. NLCs consist of a mixture of solid and liquid lipids (Souto and Müller, 2007, Jain and Ram, 2011). The drug is incorporated between the voids in the crystal lattice of SLNs, on the other hand, the NLC matrix has more imperfections than SLNs and hence, higher drug encapsulation is possible (Müller *et al.*, 2000, Müller, 2007).

1.5.2. Polymeric Systems

Materials used to augment or substitute the functions of organs or tissues for short or long periods are called biomaterials and can be metals, ceramics, biological or synthetic polymers and composites. Their uses range from traditional products such as catheters, syringes, blood contacting extra corporeal devices, drug delivery, to systems for cell encapsulation and tissue regeneration (Rokkanen, 2002).

Polymers have significant potential in the production of biomedical devices because their chemistry allows materials to be tailored to provide versatility of the desired physical and mechanical properties (Ulery *et al.*, 2011).

Polymers can be classified in different ways; the most common classification is based on the origin of the polymer, such as, natural or synthetic. Other classifications are based on the polymer structure, polymerization mechanism, preparative techniques, or thermal behavior (Ebewele, 2000, Hasirci and Yucel, 2008). Meanwhile, stability of the polymers is a very important factor especially in drug delivery.

1.5.2.1. Nondegradable (Stable) Polymers

Stability of polymers is determined by the nature of the chemical bonds of the polymer backbone, which is a function of the chemistry of the monomers and the linkages between them. In general a carbon-carbon bond is chemically and biological quite inert and therefore stable. The inert nature of the C-C bond can be modulated by oxidation. Thus, stability of the polymer could be changed by introduction of chemical linkages such as anhydride, ester, or amide bonds into the backbone (Shastri, 2003). Most commonly used non-degradable polymers and their applications in the biomedical field and especially in drug delivery are presented in Table 1.5.

Table 1. 5 Some key properties and applications of most commonly used nondegradable polymers in the biomedical field (adopted from Shastri, 2003).

Chemical	Trade Name	Key Property	Application area
Poly(ethylene) (PE) (HDPE, UHMWPE)		Strength and lubricity	Orthopaedic implants and catheters
Poly(propylene) (PP)		Chemical inertness and rigidity	Drug delivery, meshes and sutures
Poly(tetrafluroethylene) (PTFE)	Teflon	Chemical and biological inertness and lubricity	Hollow fibers for enzyme immobilization, vascular graft, guided tissue regeneration, barrier membrane in the prevention of tissue adhesion
Poly(methymethacrylate)	Palacos®	Hardness, excellent optical transparency	Bone cement, intraocular lens
Ethylene-co-vinylacetate (EVA)	Elvax®	Elasticity, film forming property	Implantable drug delivery devices
Poly(dimethylsiloxane) (PDMS) (silicone rubber)	Silastic®	Ease of processing, chemical and biological inertness, excellent oxygen permeability, excellent optical transparency	Implantable drug delivery devices, device coatings, gas exchange membranes, intraocular lens, orbital implants
Low MW Poly(dimethylsiloxane) (Silicone oil)		Gel-like characteristics	Filler in silicone breast implants
Poly(ether-urethanes) (PU)	Tecoflex® Tecothane® BioSpan®	Blood compatibility, rubber-like elasticity	Vascular grafts, heart valves, blood contacting devices, coatings
Poly(ethylene terphthalate) (PET)	Dacron®	Fiber forming property, slow in vivo degradation	Knitted Dacron vascular grafts, coatings on degradable sutures, meshes in abdominal surgery

Table 1. 5 Cont'd Some key properties and applications of most commonly used non-degradable polymers in the biomedical field (adopted from Shastri, 2003).

Chemical	Trade Name	Key Property	Application area
Polysulphone (PS)		Chemical inertness, creep resistance	Hollow fibers, membranes for immobilization of biomolecules in extra-corporeal devices
Poly(ethylene oxide) (PEO, PEG)		Negligible protein adsorption, hydrogel forming characteristics	Passsivation of devices toward protein adsorption, cell encapsulation

1.5.2.2. Biodegradable Polymers

Biodegradable polymers degrade *in vitro* and *in vivo* either into products some of which are normally metabolized by the body or into products that can be completely eliminated from the body with or without further metabolic transformations. A biodegradable polymer for use as a biomaterial has to have nontoxic degradation products and the degradation rate and mechanical properties of the material should match the intended application (Nair and Laurencin, 2006).

Biodegradable polymers can be of either natural or synthetic origin. In general, synthetic polymers offer greater advantages over natural ones as they can be tailored to have a wider range of properties. Some of the natural polymers have functional groups appropriate for applications such as tissue engineering and tend to show less toxic effects (Jain *et al.*, 2011). A representative list of natural and synthetic biodegradable polymers used in drug delivery applications are given in Table 1.6.

Table 1. 6 Biodegradable polymers used in drug delivery (adopted from Pillai and Panchagnula, 2001, Nair and Laurencin, 2006).

Polymers		
Natural	**Protein-based polymers**	Collagen, albumin, gelatin
	Polysaccharides	Cellulose, starch, alginic acid, dextran, hyaluronic acid, chitin, chitosan, agarose
Synthetic	**Polyesters**	Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactid acid-co-glycolic acid) (PLGA), poly(hydroxybutyrate) (PHB), poly(ϵ-caprolactone) (PCL), poly(β-malic acid), poly(dioxanes)
	Polyanhydrides	Poly(sebacic acid), poly(adipic acid), poly(terephthalic acid)
	Polyamides	Poly(imino carbonates), polyamino acids
	Phosphorous-based polymers	Polyphosphates, polyphosphonates, polyphosphazenes

1.5.2.2.1. Natural Polymers

Natural polymers such as gelatin, chitosan, proteins and starch are all interesting materials for medical applications since they are biodegradable and bioabsorbable where the degradation products do not have any toxic effect (Hasirci, 2007).

Polysaccharides are polymers of simple sugars and their derivatives can be branched or linear, and chitosan is most commonly used polymer in this class. Taveira et al. (2009) investigated percutaneous absorption of cancer drug doxorubicin and its retention in the skin following iontophoresis. They reported that chitosan appears to interact with the negative charged groups in the skin, and improved drug diffusion into the deeper layers of the skin. Other studies were also

reported about investigations on the mechanisms enhancing transdermal penetration by chitosan and its derivatives (He *et al.*, 2009, Kählig *et al.*, 2009).

Protein based polymers have high molecular weights where the amino acids are bound together by characteristic peptide linkages (Nair and Laurencin, 2006). Collagen is the most abundant protein in the human body and is the major component of bone, skin, ligament, cartilage, and tendon, and also forms the structural network of other tissues such as blood vessels. Collagen is used in dermocosmetic applications such as dermal filler or in injections into the mid to deep dermis for the correction of moderate to severe wrinkles and folds, leading to restoring a smoother and younger appearance. Collagen was the first material to be approved by the U.S. Food and Drug Administration (FDA) for injection into facial scars, furrows, and lines in the form of dermal filler injections (Khan *et al.*, 2011, Ulery *et al.*, 2011).

Gelatin is prepared by the thermal denaturation of collagen, isolated from animal skin and bones in the presence of dilute acid and extensively used in food and medical products. Because of the nontoxic, biodegradable, bioactive and the ease of processability properties make gelatin attractive for the use in controlled release applications. The high biocompatibility of gelatin makes it a suitable candidate, particularly as delivery matrices for growth factors (Nair and Laurencin, 2006, Kumari *et al*., 2010, Totre *et al.*, 2011).

Albumin is another water soluble protein, and has the highest concentration in blood plasma. The primary function of albumin is to carry hydrophobic fatty acid molecules around the bloodstream, and also carefully maintain blood pH. Albumin is essentially found everywhere in the body, nearly all tissues have enzymes that can degrade it which makes it a promising polymer for biomedical applications (Prinsen and Velden, 2004, Ulery *et al.*, 2011). Like gelatin, albumin can be easily processed into membranes, nano and microspheres due to its solubility, besides the presence of reactive functional groups along the polymer chain (Nair and Laurencin, 2006). Due to its high blood compatibility property, albumin has been extensively investigated as a matrix for intravascular drug delivery systems (Merodio *et al.*, 2002).

Although, natural polymers exhibit a lower occurrence of toxicity and inflammation as compared to synthetic materials, than production and isolation of them be often expensive that make synthetic polymers preferable in such applications (Kuhn, 2005).

1.5.2.2.2. Synthetic Polymers

Synthetic polymers and natural macromolecules have been widely studied, specifically for use as nanoparticles in drug delivery. Synthetic polymers have the superiority of high purity and reproducibility of properties compared to natural polymers. Among the broad class of synthetic polymers, the polyester family (e.g. PLA, PCL, PGA and PLGA) are of interest in the biomedical area because of their biocompatibility and biodegradability that could be controlled through composition and form (Astete and Sabliov, 2006).

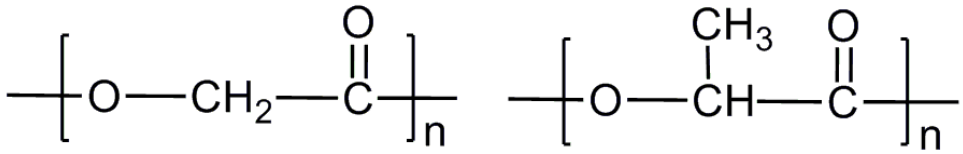
1.5.2.2.2.1. Aliphatic Polyesters

Aliphatic polyesters can be considered as a representative class of synthetic, biodegradable polymers that contain an aliphatic ester bond in their backbone (Okada, 2002). Although a number of polyesters are commercially available and all of them are degradable in theory, only polyesters have adequately short aliphatic side chains that can be used as degradable polymers in biomedical applications. These polymers are often hydrophobic but instability of ester bond causes them to undergo bulk erosion. Poly(α-esters) have been the widely researched biomaterials to date because of the relative ease of their synthesis (via ring-opening and condensation polymerization), commercial availability, biodegradability, bioresorbability, and biocompatibility (Ulery *et al.*, 2011, Tian *et al.*, 2012).

PGA was one of the initially investigated biodegradable polyesters for biomedical use. Due to its high crystallinity, PGA shows high tensile strength and modulus but very low solubility in most organic solvents (Figure 1.11 A). The

initial applications of PGA were directed toward developing biodegradable sutures and the first biodegradable synthetic suture DEXON was developed in 1970. PGA is often fabricated into a mesh network and has been used as a scaffold for bone, cartilage, tendon, tooth, vaginal, intestinal, lymphatic, and spinal regeneration (Chu, 2000, Nair and Laurencin, 2006, Ulery *et al.*, 2011).

A) **B)**



PGA **PLA**

Figure 1. 11 Chemical formula of poly(α-esters) A) PGA and B) PLA, n can be in the range of several thousands.

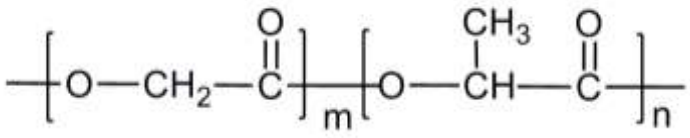
D, L-PLA is preferred over L-PLA because it provides a more homogenous dispersion of a drug in the polymer matrix due to its chain structure. Moreover, additional methyl group in PLA causes the polymer to be much more hydrophobic and stable against hydrolysis than PGA (Jain, 2000, Ulery *et al.*, 2011). General molecular structure of PLA is given in Figure 1.11 B.

Copolymer of PLA and PGA, known as poly(lactic acid-*co*-glycolic acid) (PLGA) is the polymer of main concern for this study and given in the following section 1.5.2.2.2.2.

1.5.2.2.2.2. Poly(lactic acid-*co*-glycolic acid) (PLGA)

Poly(lactic acid-*co*-glycolic acid) (PLGA) is a copolymer synthesized with direct polycondensation of two different monomers, PLA (L-lactide and D, L-lactide) and PGA, specifically in most of the cases by ring opening copolymerization of these monomers to produce high MW copolymers (Figure 1.12). PLGA is one of the most successfully used biodegradable polymers in the

development of the nanosystems for biomedical applications because it undergoes hydrolysis in the body and degrades into nontoxic by-products that are salt forms of lactic acid (LA) and glycolic acid (GA) which are eliminated from the body easily (Avgoustakis, 2008, Kumari *et al*., 2010) by normal excretion.



GA **LA**

Figure 1. 12 Chemical formula of PLGA copolymer of glycolic acid and lactic acid monomers, m and n can be in the range of several thousands.

Ability to produce polymers with different physico-chemical properties is an advantage of PLGA which can be achieved by altering monomer composition, molecular weight, crystallinity and so on (Fredenberg *et al.*, 2011). Crystallinity of PLGA directly affects its mechanical strength, swelling behavior, capacity to undergo hydrolysis, and subsequently the biodegradation rate. The final crystallinity of the PLGA copolymer is dependent on the type and the molar ratio of the monomer components (LA and GA) in its structure (Jain, 2000, Domb *et al*., 2002, Avgoustakis, 2008). PLGA with 50:50, 75:25, 85:15 (LA:GA) ratios are the most commonly used PLGA types biomedical applications. Because of the LA is more hydrophobic than the GA, LA rich PLGA copolymers (such as 75:25, 85:15) are more hydrophobic, absorb less water and degrade more slowly. Degradation times of 50:50, 75:25, and 85:15 PLGA were determined as 1-2 months, 4-5 months, and 5-6 months, respectively, and PLGA 50:50 is hydrolyzed much faster than the other types (Miller *et al.*, 1977, Middleton and Tripton, 1998, Jain, 2000, Ulery *et al.*, 2011). Depending on these differences between the degradation times, the duration of the bioactive agents release can be varied from hours to several months from the PLGA nano-microsystems (Fredenberg *et al.*, 2011).

PLGA undergoes biodegradation both *in vitro* and *in viv*o through hydrolysis of the ester linkages in the backbone by homogeneous degradation or by bulk erosion of the PLGA nano-microsystems (Pitt and Gu, 1987). Relatively large sized PLGA devices degrade through a heterogeneous process with the core degrading faster than the surface (Grizzi *et al.*, 1995). Lactate and glycolate (salts of the LA and GA) are the nontoxic, final degradation products of PLGA and are eliminated easily.

PLGA is frequently converted into nano and micro particles or nanofibers for the sustained delivery of encapsulated or entrapped bioactive agents (Mukerjee and Vishwanatha, 2009, Yilgor *et al.*, 2010, Liu *et al.*, 2011). Number of PLGA based biomedical systems are found in the market as commercial products, and PLGA based drug delivery systems in the market are given in Table 1.7.

Table 1. 7 Commercial drug delivery products of PLGA (adopted from Avgoustakis, 2008).

Drug delivery systems	Drug	Illness
Zoladex	Goserelin acetate	Prostate cancer
Nutropic Depot	Human growth hormone (hGF)	Growth deficiencies
Trelstar Depot	Triptorelin pamoate	Prostate cancer
Sandostatin LAR	Octreotide	Acromegaly

1.5.2.3. Preparation Techniques for Polymeric Nanoparticles

The term "polymeric nanoparticle" includes irregular particulates, nanospheres and nanocapsules. A nanosphere is defined as a polymeric matrix with the drug dispersed in its matrix, and nanocapsules are described as a polymeric coat surrounding the drug in the core (Anton *et al.*, 2008) (Figure 1.13).

A) Nanosphere **B)** Nanocapsule

Figure 1. 13 Polymeric nanoparticles. A) Nanospheres, B) nanocapsules.

The properties of polymeric nanoparticles have an important role on their release behavior and therefore, preparation techniques are tools used to obtain polymeric nanoparticles with the desired properties for a particular application (Rao and Geckeler, 2011).

PLGA nanoparticles have been mostly prepared by emulsion-solvent evaporation, nanoprecipitation (solvent displacement), salting out, and emulsion-solvent diffusion methods (Kumari et al., 2010).

1.5.2.3.1. Emulsion-Solvent Evaporation

Emulsion-solvent evaporation method was developed as one first methods of nanoparticle preparation from polymers (Vanderhoff et al., 1979). There are two important sub-groups of this process oil-in-water (o/w) and water-in-oil-in-water (w/o/w). First an organic polymer solution is emulsified in an aqueous phase (o/w) containing the surfactant and then nanoparticles are formed upon evaporation of the organic solvent. The polymer and the bioactive drug have to be in the volatile organic solvent such as chloroform or dichloromethane (DCM). Emulsification is carried out under high shear stress (high energy homogenization) to reduce the size of the droplets. This method is used to entrap hydrophobic agents. In the water-in-oil-in-water (w/o/w) double emulsion approach, which encapsulation of hydrophilic compounds is achieved in hydrophobic particles. In this approach the hydrophilic drug is dissolved in an aqueous phase which is then emulsified in a polymer solution (the “oil” phase). This emulsion is then added to a larger aqueous phase forming capsules with

aqueous cores and organic walls. The preparation approaches are given in Figure 1.14. The size of the nanoparticles can be controlled by adjusting the stir rate and agitation power, type and amount of dispersing agents (surfactants), viscosity of organic and aqueous phases, and temperature. Emulsion-solvent evaporation method is widely used for preparation of nanoparticles of PLA, PLGA and PCL. Use of water as non-solvent in the process makes this method is economic; however, it is not easy to scale up because the need of high energy requirements in homogenization (Tice and Gilley, 1985, Jain, 2000, Pinto Reis et al., 2006, Vauthier and Bouchemal, 2009).

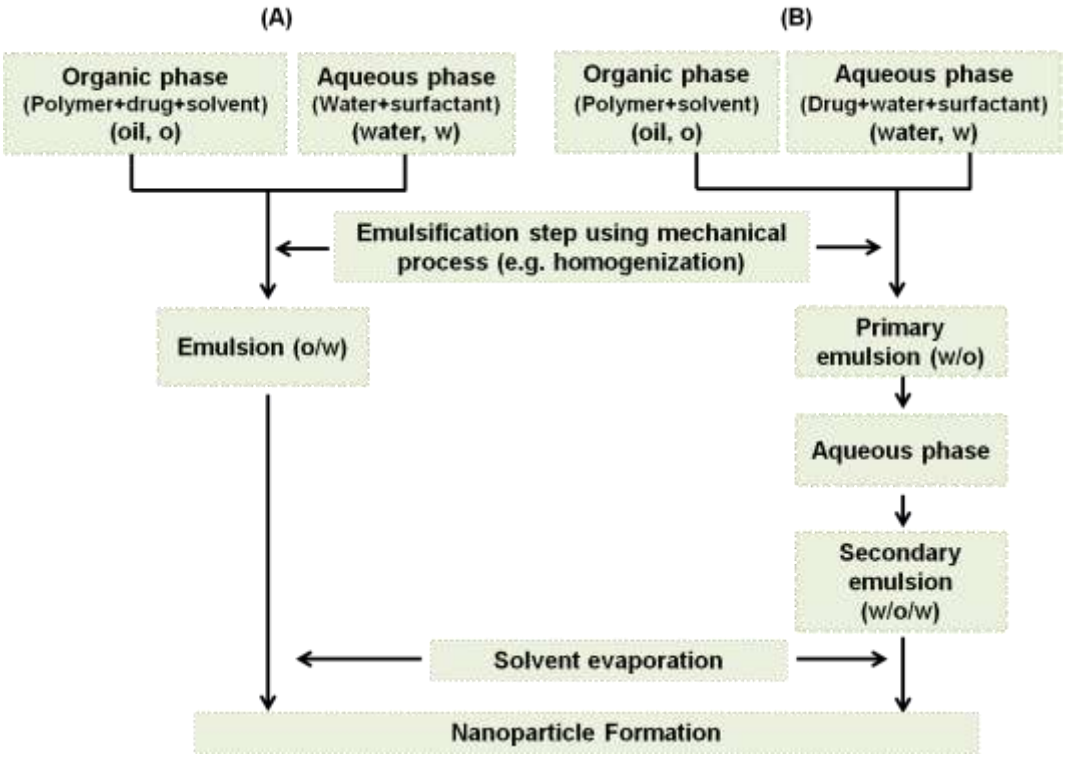


Figure 1. 14 Nanosphere and nanocapsule preparation by emulsion-solvent evaporation method. A) Oil-in-water (o/w) emulsion, B) water-in-oil-in-water (w/o/w) double emulsion (adopted from Vauthier and Bouchemal, 2009).

1.5.2.3.2. Nanoprecipitation

Nanoprecipitation, is also known as solvent diffusion or solvent displacement method, is typically used for entrapment of hydrophobic drug; however, it is adapted for hydrophilic drugs, as well. In this method, polymer and drug must dissolve in the solvent, but not in the non-solvent. Nanoprecipitation occurs by a rapid desolvation of the polymer when the polymer solution is added to the non-solvent, and then solvent is removed under reduced pressure. This rapid nanoparticle formation occurs due to the Marangoni effect, which is caused of interfacial turbulences that take place at the interface of the solvent and the non-solvent (Bilati et al., 2005, Blouza et al., 2006). This method is one of the easiest nanoparticle preparation procedures which is reproducible, fast and economic, and additionally can be applied to a wide range of polymers including peptides, drugs. Large amounts of toxic solvents and external energy sources are not used in this method and the basic limitation of the method is the solubility of the drug in organic solvents. The main problem with the nanoprecipitation method is the frequent agglomeration of particles due to the lack of a stabilizer (Astete and Sabliov, 2006, Lassalle *et al.*, 2007, Vauthier and Bouchemal, 2009).

1.5.2.3.3. Salting Out

Salting-out is based on the separation of a water miscible solvent from aqueous solution via a salting out effect. Polymer and bioactive agent are dissolved in water miscible organic solvent (e.g. acetone or tetrahydrofuran (THF)) and this solution is emulsified in an aqueous phase under strong mechanical shear stress in this method. The aqueous phase consists of salting out agents such as magnesium chloride, calcium chloride, and magnesium acetate, and a stabilizer. These components retain the water molecules for their own solubilization; hence modify the miscibility properties of water with other solvent in use. This oil/water emulsion is diluted with a sufficient volume of water or aqueous solution to enhance the diffusion of solvent (e.g. acetone) into the aqueous phase, thus inducing the formation of nanoparticles. Finally,

nanoparticles are eliminated from both the solvent and the salting out agent by purification (e.g. cross-flow filtration). This method can be easily scaled up and minimizes the stress on the particles; however, requires extensive purification step for salting out agent elimination (Ibrahim et al., 1992, Quintanar-Guerrero et al., 1998, Pinto Reis et al., 2006).

1.5.2.3.4. Emulsion-Solvent Diffusion

Emulsion-solvent diffusion method was adapted from salting-out technique. Polymer and drug is dissolved in a partially water miscible solvent and before the emulsification of the polymer solution, this solution is saturated with water. Then, this polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, causing to solvent diffusion to the external aqueous phase and nanoparticles are formed. At the end, solvent is eliminated by evaporation or filtration depending on its boiling point. This technique is simple, provides easier scale up, high encapsulation efficiencies, and narrow size distribution, but leakage of water soluble drug into the aqueous phase reducing the encapsulation efficiency. Thus this method is efficient for encapsulating hydrophilic drugs (Pinto Reis et al., 2006, Moinard-Chécot et al., 2008).

1.6. Approach

The aim of this study was to develop nanocarriers for the treatment of skin diseases specifically for psoriasis, atopic dermatitis, aging or UV damage. Nanoparticulate delivery systems used in this study were prepared from PLGA, a polymer which is FDA approved especially for use in drug delivery. Two different bioactive agents, retinyl palmitate (RP) and Dead Sea Water (DSW), were loaded into PLGA nanospheres and nanocapsules, respectively. The PLGA nanocarriers were expected to release RP and DSW into the SC and deeper in a sustained manner. Except a parallel study where PLGA was used to deliver same drugs, this was the first study where RP and DSW were delivered by PLGA nanoparticles. The particulate delivery systems were prepared at mid nano sizes

and then characterized, and the release profiles of the bioactive agents were studied *in situ*.

Then cytotoxicities of the produced nanoparticles were assessed. The effect of bioactive agent loaded nanoparticles on cell proliferation and their uptake by cells were studied *in vitro* using Saos 2 human osteosarcoma cells and L929 mouse fibroblasts, using cell counting techniques and various microscopical approaches including Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microcopy (CLSM).

Novelty of the study: For the first time in the literature DSW and RP loaded PLGA nanoparticles were shown to penetrate into the cells, accumulate around the nucleus, thus showing a potential as an active agent carrier in skin therapy. It can also be considered as a potential gene therapy tool for skin and other diseases. Moreover, producing the particles in nano sizes prevents them to penetrate through the lower level of the dermis that accumulate in the SC which enables them to be more effective in the treatment of psoriasis, atopic dermatitis, and UV damaged skin.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

PLGA (50:50) (inherent viscosity (i.v.) 2.2 – 2.8 dL/g) was bought from For U Sorb Company (PR China). Dead Sea Water (DSW) was a kind gift of AHAVA Dead Sea Laboratories (Israel). $MgCl_2.6H_2O$ was obtained from ATABAY Ltd. (Turkey). Retinyl palmitate (RP, without antioxidants), Bovine serum albumin (BSA), Thiazolyl blue tetrazolium bromide cell proliferation assay kit (MTT) and dichloromethane (DCM) were obtained from Sigma-Aldrich (USA). Polyvinyl alcohol (PVA, MW $1.5x10^4$, degree of hydrolysis 86 - 89 mol %) was obtained from Fluka (USA). Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Germany). Coomassie Blue (Pierce, USA) was bought from Thermo Scientific (USA). Chloride specific electrode and ion meter were from Cole Parmer (USA) and UV-Visible Spectrophotometer (Thermo Scientific, MultiSkan Spectrum Type 1500) was from Thermo Scientific (USA). L929 fibroblast cell line was purchased from the Foot-and-Mouth Disease Institute (Ankara, Turkey). The human osteosarcoma cell line (Saos 2) was purchased from the American Type Culture Collection (ATCC HTB-85). Erythrocytes used in Hemolytic Activity Test were obtained from human donors with consent and used as described in Section 2.2.6. Dulbecco's Modified Eagle Medium (DMEM, high glucose (with phenol red) and high glucose modified without phenol red), RPMI 1640 (Roswell Park Memorial Institute) medium, fetal bovine serum (FBS), and laboratory grade Penicillin/Streptomycin (10,000 units/mL Penicillin, 10 mg/mL Streptomycin) were purchased from HyClone (USA). Vancomycin hydrochloride (10 mg/mL)

was bought from Hospira (UK) and LincoSpectin (50 mg/mL Lincomycin, 100 mg/mL Spectiromycin) was obtained from Pfizer (USA).

2.2. Methods

2.2.1. Purification of PLGA

A PLGA (50:50) solution in acetone was prepared (5%, w/v) and added dropwise into 10 fold higher volume of sterile, cold, ultrapure water with vigorous stirring. The coagulated polymer fibers were collected and washed with excess ultrapure water, and lyophilized (Labconco, FreeZone6P Plus, USA).

2.2.2. Preparation of Bioactive Agent Loaded PLGA Nanoparticles (NP)

Oil-in-water (o/w) and water-in-oil-in water ($w_1/o/w_2$) methods were used to produce the PLGA nanospheres (NS) and nanocapsules (NC), respectively.

2.2.2.1. Preparation of Retinyl Palmitate (RP) Loaded PLGA Nanospheres (NS) Using Oil-in-Water (o/w) Emulsion

PLGA NS were prepared to carry the hydrophobic bioactive agent, retinyl palmitate (RP), by oil-in-water (o/w) emulsion technique. For this purpose, PLGA was dissolved in DCM (10%, w/v, 600 µL) and RP was dissolved in DCM (15 µL) and added into the polymer solution in a 4:1 polymer:drug ratio (w/w). This polymer-drug solution (615 µL) was added into an aqueous PVA solution (2 mL, 4%, w/v) and probe sonication (Cole-Parmer Instruments, Ultrasonic homogenizer, 4710 series, USA) was applied for 15 s with 5 s on and 5 s off sequences, (at an output of 50 W, in an ice bath) to form the o/w emulsion. This nanoemulsion was stabilized by transferring into another, higher volume aqueous PVA solution (50 mL, 0.3%, w/v). Organic solvent (DCM) was evaporated by vigorous stirring overnight. NS were collected by centrifugation (Sigma 3K30, rotor: 15156-H, Germany) (13,000 rpm, 11 min, 20 ^{o}C). The obtained pellet was washed twice with excess ultrapure water after the supernatant was discarded.

Finally, NS were resuspended in 10 mL ultrapure water and lyophilized. The whole process is presented schematically in Figure 2.1.

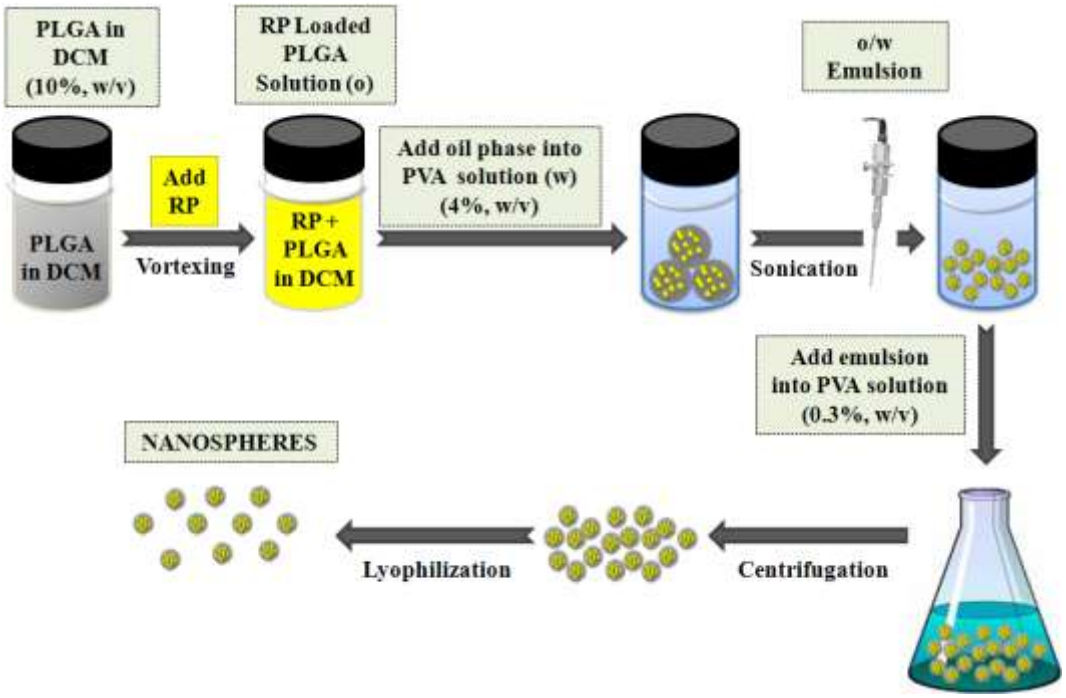


Figure 2. 1 Preparation of RP loaded PLGA NS with oil-in-water (o/w) emulsion approach.

2.2.2.2. Preparation of Dead Sea Water (DSW) and Magnesium Chloride ($MgCl_2$) Loaded PLGA Nanocapsules (NC) Using Water-in-Oil-in-Water (w/o/w) Double Emulsion

Water-in-oil-in-water ($w_1/o/w_2$) double emulsion method was used to produce hydrophilic drug, Dead Sea Water (DSW) and magnesium chloride ($MgCl_2$), loaded PLGA NC. After a PLGA solution (10%, w/v, 600 µL) in DCM was prepared, DSW or $MgCl_2$ solution (1 g/mL) was added into this solution to form the w/o. The solution was then sonicated for 15 s with 5 s on and 5 s off intervals (at an output of 50 W, in an ice bath). This first emulsion (w_1/o) was added into an aqueous phase containing PVA (2 mL, 4%, w/v) and $w_1/o/w_2$ double emulsion was formed. This double emulsion was then added into larger volume of

PVA (50 mL, 0.3%, w/v). Solvent (DCM) was evaporated by vigorous stirring overnight to yield the capsules. Centrifugation was used to collect the NC from the solution as was mentioned in Section 2.2.2.1. The obtained pellet was washed twice with ultrapure water, resuspended in 10 mL ultrapure water and then lyophilized. The whole process is presented schematically in Figure 2.2.

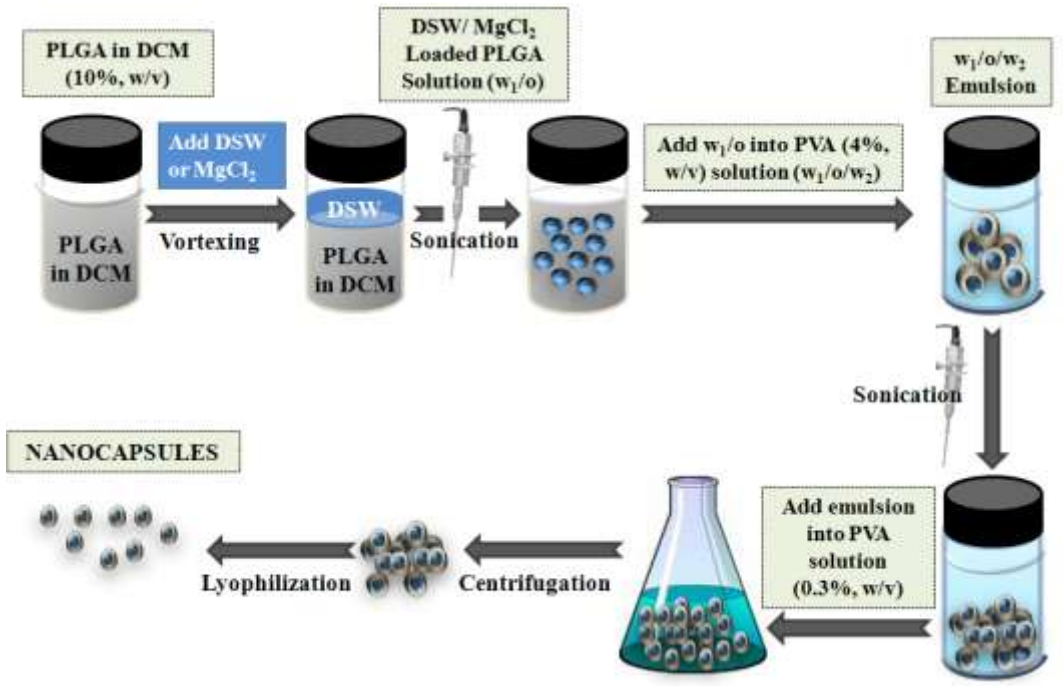


Figure 2. 2 Preparation of DSW or $MgCl_2$ loaded PLGA NC using the water-in-oil-in-water ($w_1/o/w_2$) double emulsion method.

2.2.2.3. Preparation of Bovine Serum Albumin (BSA) Loaded PLGA Nanocapsules (NC)

Bovine serum albumin (BSA) is a high molecular weight protein (~66 kDa), and was used as a model drug to compare the entrapment efficiency (E.E.) and the loading capability of the PLGA nanoparticles. BSA loaded NC were also prepared by $w_1/o/w_2$ double emulsion technique except that an aqueous solution of BSA (2 mg BSA in 0.1 mL ultrapure water) was added into the DCM solution of PLGA in place of using DSW or $MgCl_2$.

2.2.2.4. Preparation of Nile Red Loaded PLGA Nanocapsules (NC)

In order to microscopically trace the nanoparticles *in vitro,* Nile Red was loaded into the PLGA NC by $w_1/o/w_2$ technique (as in Section 2.2.2.2) except that Nile Red (0.1 mL, 0.01%, w/v, in acetone) was added in the PLGA solution (10%, w/v, in DCM) in place of DSW or $MgCl_2$ solution.

2.2.2.5. Preparation of $MgCl_2$ and Bovine Serum Albumin (BSA) Loaded PLGA Nanocapsules (NC) Using Water-in-Oil-in-Water (w/o/w) Double Emulsion

In order to control the encapsulation efficiency (E.E.) of Cl^- ions, $MgCl_2$ was loaded into the PLGA NC mixing with a high molecular weight substance (BSA) by $w_1/o/w_2$ technique (as in Section 2.2.2.2). In this case, solution of BSA (2 mg BSA in 0.1 mL ultrapure water) was mixed with $MgCl_2$ solution (1 g/mL), and was added in the PLGA solution (10%, w/v, in DCM) in place of only $MgCl_2$ solution.

2.2.3. Characterization

The nanospheres and capsules were characterized by using SEM and light scattering for topography and particle size. Stability in excipient solutions, encapsulation efficiency and loading of the active compounds were studied.

2.2.3.1. Scanning Electron Microscopy (SEM)

SEM was used to study the topographic and morphologic properties of the particles. Aqueous suspensions (50 μL) of nanoparticles (Section 2.2.2.1 and Section 2.2.2.2) just before drying with lyophilization were added onto carbon tapes on SEM stubs and were allowed to dry at room temperature, sputter coated with Au-Pd (average coat thickness 10 nm) under vacuum and examined (Nova, NanoSEM, USA and Quanta 400F Field Emission SEM, Netherlands).

Image J software (NIH, USA) was used to determine the particle size distribution and the wall thicknesses of the particles using the SEM micrographs.

2.2.3.2. Particle Size Distribution Analysis

Dynamic Light Scattering (DLS) (Malvern, Mastersizer 2000, UK) was used to determine the particle size distribution of the nanoparticles. Aqueous suspensions (2 mL) of nanoparticles just before drying were tested. The data was provided in the form of particle size distribution and polydispersity index.

2.2.3.3. Stability

Stability of the PLGA nanoparticles in certain vehicles (carrier solutions) was studied at Dead Sea Laboratories (DSL, Israel) within the context of the EU FP7 SkinTreat Project. Stability was the preservation of unprecipitated state in a given vehicle mainly at 37 ^{o}C. Stabilities were tested in the vehicles ST_01, ST_022, and gel ST_03 at different temperatures. The composition of the vehicles is presented in Table 2.1.

Table 2. 1 Ingredients of the vehicles used to study the suspension stability of the nanoparticles.

w/o emulsion (ST_01)		o/w emulsion (ST_022)		Gel (ST_03)	
Ingredients (%)		Ingredients (%)		Ingredients (%)	
Water	65	Water	82.8	Water	85.35
Glycerin USP K	3.5	Glycerin	2	Keltrol T	0.05
Cyclomethicone	8.1	Propanediol	1	Natrosol	1
ABIL EM 90	0.5	Lanette N	1	Solubilizer	2
BENTONE GEL GTCC	2.5	Octyl palmitate	6	Water	11.4
DOW 5225	10	Dimethicone	1	Microcare MT	0.1
DOW CORNING 9045	9.7	Cetyl alcohol C16	2	Glicacyl	0.1
ISOLAN GI 34	0.5	Lanette AO	4	-	-

2.2.3.4. Inclusion of Bioactive Agents into Nanoparticles

E.E. (%) was used to define "entrapment efficiency" when nanospheres were used and "encapsulation efficiency" when nanocapsules were used.

2.2.3.4.1. Entrapment Efficiency and Loading of Retinyl Palmitate (RP)

The amount of RP loaded in PLGA NS was determined by UV-Visible Spectrophotometry (Thermo Scientific, MultiSkan Spectrum, Type 1500, USA), after dissolving the NS (20 mg) in DCM (4 mL) and determining the absorbance at 330 nm and using a calibration curve to convert it into concentration and amount (Appendix A). Entrapment efficiency and loading were calculated according to Equations 1 and 2 given below.

$$\text{E.E. (\%)} = \frac{\text{Amount of RP, DSW, } MgCl_2 \text{ or BSA (mg)}}{\text{Input RP, DSW, } MgCl_2 \text{ or BSA (mg)}} \text{ X } 100 \quad (1)$$

$$\text{Loading (\%)} = \frac{\text{Amount of RP, DSW, } MgCl_2 \text{ or BSA (mg)}}{\text{Tested amount of nanoparticles (mg)}} \text{ X } 100 \quad (2)$$

2.2.3.4.2. Dead Sea Water (DSW) and Magnesium Chloride ($MgCl_2$) Encapsulation Efficiency and Loading

DSW is mainly a mixture of soluble salts obtained from the Dead Sea in Israel. Cl^- ion is the major constituent (350 mg Cl^-/mL DSW) as shown in Table 2.2. In order to calculate the encapsulation efficiency of DSW, the amount of chloride entrapped within the nanoparticles was determined by using a chloride ion specific electrode.

Table 2. 2 Major constituents of DSW.

Major Constituents	Concentration (mg/mL)
Chloride (Cl^-)	320,000 – 370,000
Calcium (Ca^{2+})	36,000 – 40,000
Magnesium (Mg^{2+})	90,000 – 95,000
Potassium (K^+)	1,300 – 1,500
Sodium (Na^+)	1,500 – 2,500
Bromide (Br^-)	11,000 – 12,000
Strontium (Sr^{2+})	750 – 850
Others (Heavy metals, As, Pb, Hg, I)	< 2 ppm

In the determination of the amount of Cl^-, the presence of other ions in the DSW could lead to interference. Therefore, magnesium chloride was chosen as a model compound to represent DSW, a solution of it with chloride ion concentration same as that of DSW was prepared and loaded into the nanocapsules.

NC (60 mg) was dissolved in DCM (3 mL) in a glass tube and Cl^- was extracted several times by adding ultrapure water (3 mL) onto this DCM solution and vortexing. The water phase was removed and the "Chloride ion specific electrode" of the ion meter (Cole-Parmer Instrument, USA) was immersed it to measure the concentration of the Cl^- ions. The measurements were done after calibrating the ion meter.

2.2.3.4.3. Encapsulation Efficiency and Loading of BSA

The amount of BSA encapsulated was determined by extraction as in Section 2.2.3.4.2. Briefly, BSA loaded NC (5 mg) was dissolved in DCM (2 mL) and the encapsulated BSA was extracted several times with ultrapure water (2 mL) and vortexing. When the phases separated, water phase was removed and the amount of BSA was determined with Coomassie Plus Bradford Assay (Pierce, USA). For this assay, Bradford reagent (150 μL) was added into 150 μL of extract in a 96 well plate. The protein in the sample binds to the Coomassie dye which results in a spectral shift in its color from the reddish/brown (λ_{max} 465 nm) to blue (λ_{max} 600 nm). The absorbance at 600 nm is measured after storage for 10 min at room temperature using a plate reader (Thermo Scientific, MultiSkan Spectrum, Type 1500, USA). Protein concentration was determined from a calibration curve constructed in the range 0 – 25 μg/mL (Appendix B) and the encapsulation efficiency and loading were calculated according to Equations 1 and 2.

2.2.4. *In situ* Release of DSW and $MgCl_2$ from NC

Release kinetics of DSW and $MgCl_2$ from PLGA NC were studied *in situ*. DSW (or $MgCl_2$) loaded NC (30 mg) were put in Eppendorf tubes, 2 mL ultrapure water was added and incubated at 37 ^{o}C in a shaking incubator (shaking rate 55 rpm, New Brunswick Scientific, Innova 4000, USA). At various time points the tubes were centrifuged, supernatant removed and the amount of released Cl^- ions were determined with the chloride ion specific electrode and ion meter as described in Section 2.2.3.4.2. Then, 2 mL of fresh ultrapure water was added to the Eppendorf tubes, pellets were resuspended and incubation was continued. These experiments were carried out in triplicates.

2.2.5. *In situ* Release of BSA from NC

BSA released from the NC was determined using the Coomassie Plus Bradford Assay (Pierce, USA) as given in Section 2.2.3.4.3 and the BSA release from PLGA NC was carried out as described above (Section 2.2.4). In these experiments, instead of ultrapure water PBS (0.01 M, pH 7.4) was used as the medium and released protein amount was determined from a calibration curve constructed at 600 nm (Appendix C).

2.2.6. Hemolytic Activity of the PLGA Nanoparticles

Hemolytic activity tests were made as a quick test for blood compatibility of the PLGA nanoparticles, according to ASTM F756-08 procedure (Standard Practice for Assessment of Hemolytic Properties of Materials).

In order to conduct the hemolytic activity test, fresh human peripheral blood was collected from volunteers into EDTA containing tubes at the METU Health Center and immediately centrifuged (Sigma 3K30, rotor: 15154-H, Germany) (1250 g, 15 min, 20 ºC). The supernatant was discarded and the pellet was resuspended in PBS (pellet:PBS 1:6, v/v; 0.01 M, pH 7.4) to prepare Red Blood Cell (RBC) solution.

DSW loaded and unloaded (DSW free) NC were suspended in PBS (2 mg/mL, pH 7.4) and diluted to yield 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL NC in PBS in Eppendorfs by diluting with RBC solution. These nanoparticle - RBC suspensions were incubated at 37 ºC in a water bath (Julabo, F34 Grant LTD 6, UK) for 1 h, and centrifuged (1500 rpm, 20 min, 20 ºC). The absorbance of the hemoglobin in the supernatants were determined spectrophotometrically at 540 nm.

The control groups used were: a) Complete hemolysis (100% hemolysis) (positive control), achieved by diluting the RBC pellet 10-fold with ultrapure water (RBC:Ultrapure water, 1:10, v/v), b) Negative Control (or Blank) was just the RBC suspension without any nanoparticles was diluted 1:1 with PBS. These controls were also incubated at 37 ºC for 1 h and then centrifuged as before.

Hemolytic activity (HA) were calculated as:

$$\text{Hemolytic activity (HA) (\%)} = \frac{A_{sample} - A_{blank}}{A_{100\%} - A_{blank}} \times 100 \quad (3)$$

where A is absorbance at 540 nm.

2.2.7. *In vitro* Studies

2.2.7.1. Cell Types and Components of Culture Media

Osteosarcoma cells (human osteosarcoma cell line, Saos 2, passage numbers between 17 and 21) and L929 cells (fibroblasts, passage numbers between 14 and 19) were used in the *in vitro* studies. Effect of different bioactive agents loaded in PLGA nanoparticles on cells was studied with L929 cells, and uptake of PLGA nanoparticles by cells was studied with both cell types (Saos 2 and L929).

Saos 2 cells were grown in RPMI 1640 medium (HyClone, USA) containing 10% of fetal bovine serum (FBS) in addition to 100 U/mL penicillin - 100 µg/mL streptomycin (1.0%), 100 µg/mL vancomycin (1.0%), and 200 µg/mL lincospectin (0.2%) against bacterial contamination. L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (with phenol red, HyClone, USA) supplemented with 10% fetal bovine serum in addition to antibiotics.

2.2.7.2. Effect of Bioactive Agent (RP, DSW, $MgCl_2$ or BSA) Loaded in Nanoparticles on the Proliferation of L929

MTT assay was used on the determination of the effect of bioactive agents loaded in PLGA nanoparticles on L929 proliferation. This is a colorimetric assay that measures the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide) (MTT) and conversion to water-insoluble, purple colored formazan product by the mitochondria of viable cells (Figure 2.3).

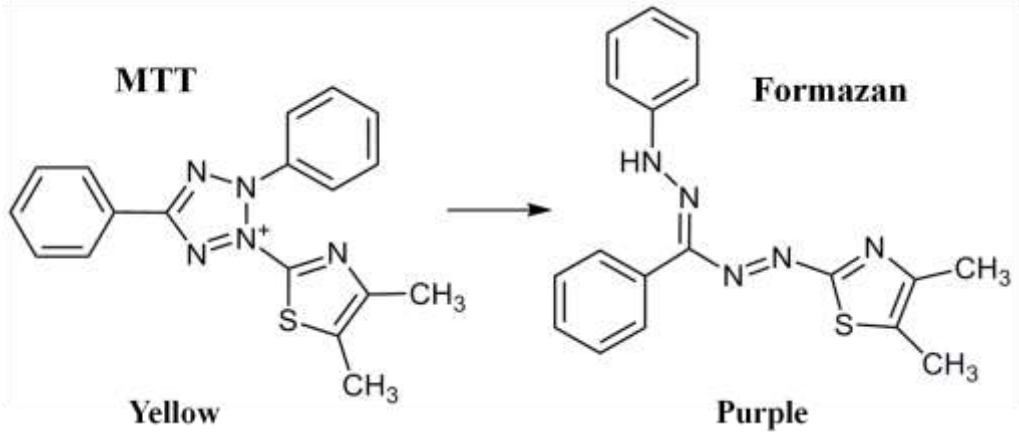


Figure 2. 3 Reduction of MTT (Yellow) into formazan crystals (Purple)

L929 stored at -80 ^{o}C were thawed, incubated in DMEM High Glucose Medium (with phenol red, HyClone, USA) supplemented with 10% FBS in addition to antibiotics, under standard culture conditions (37 °C, 5% CO_2) in a CO_2 incubator until confluency. Cells were detached from the flask surface using Trypsin-EDTA solution (0.05% in PBS) (5 min at 37 °C). After detachment, culture medium was added into the flask, cells were collected by centrifugation (3000 rpm, 5 min) and pellet was resuspended in DMEM High Glucose Medium (supplemented with 10% FBS in addition to antibiotics) prior to count. Cells were counted with a hemocytometer (Blau Brand, Germany) and seeded in 24 well plates ($3x10^4$ cells/well) in triplicates and incubated for 4 h for cell attachment.

Meanwhile, bioactive agent (RP, DSW, $MgCl_2$, or BSA) loaded nanoparticles and their unloaded counterparts were sterilized with UV (1.5 h with 15 min on-15 min off intervals). After 4 h of cell seeding, the growth medium was discarded, 0.2 mg of nanoparticles resuspended in 1 mL fresh DMEM High Glucose Medium (supplemented with 10% FBS in addition to antibiotics) was added into the wells containing the cells, and incubation continued (37 °C, 5% CO_2) in a CO_2 incubator. Cell seeded tissue culture polystyrene (TCPS) containing only the growth medium but no nanoparticles served as the control.

MTT test was performed at predetermined time points (Days 1, 3 and 7). The medium in the wells was removed; the wells containing the cells were washed twice with colorless DMEM High Modified Medium (without phenol red, high glucose, 1 mL/well). Then MTT solution (1 mL, 1 mg/mL, in colorless DMEM High Modified Medium, without phenol red) was added to each well and the plates were incubated at 37 °C, 5% CO_2 for 3 h. The MTT solution was gently withdrawn to avoid disturbing the formed formazan crystals. Acidified isopropanol (1 mL, 4%, v/v, 1 N HCl in 2-propanol) was added to dissolve the formazan crystals, 200 μL aliquots were put in 96 well plates, and the absorbances of the solutions were measured at 550 nm using a UV-Visible Spectrophotometer (Thermo Scientific, MultiSkan Spectrum, Type 1500, USA). The absorbances were converted to number of viable cells using a calibration curve (Appendix D). Pure acidified isopropanol was used as blank.

2.2.7.3. Uptake of Nile Red Loaded Nanocapsules by Saos 2 and L929 Cells

Uptake of nanoparticles was studied both with Saos 2 and L929 cells. Saos 2 cells were incubated in RPMI-1640 medium (HyClone, USA) supplemented with 10% FBS while L929 cells were incubated in DMEM High Glucose Medium (HyClone, USA) with 10% FBS in addition to antibiotics. The cells were detached from the flask surfaces after confluency and seeded after counting as in Section 2.2.6.2.

Saos 2 cells were seeded ($2.5x10^4$ cells/well) onto steam sterilized cover slips placed in each well of 6 well plate in triplicate with their controls, and incubated in RPMI-1640 medium (supplemented with 10% FBS in addition to antibiotics) under standard culture conditions (37 °C, 5% CO_2) in a CO_2 incubator. Growth medium was removed after 1 day of seeding, and Nile Red (Sigma, USA) loaded NC (Section 2.2.2.4) suspended in the growth medium (0.4 mg/mL) were added on cell seeded cover slips. The incubation was continued (37 °C) in a CO_2 incubator. Growth medium without nanoparticles was added into the cell seeded TCPS to serve as control. Cells on the cover slips were washed with PBS (1 mL,

0.01 M, pH 7.4) twice prior to fixing with paraformaldehyde (1 mL, 4%, 15 min) at predetermined time points (4, 24, and 48 h).

Similar to Saos 2 cells as was described above, L929 cells were incubated in DMEM High Glucose Medium (supplemented with 10% FBS in addition to antibiotics) under standard culture conditions (37 °C, 5% CO_2). The samples were fixed at the same time points (4, 24, and 48 h) as described for Saos 2 cells.

Cell nuclei and cytoskeletons were stained after fixation with 4',6-diamidino-2-phenylindole (DAPI) and FITC-labeled Phalloidin, respectively. The dyes, FITC-labeled Phalloidin and DAPI, were used after 100 and 3000 fold dilution, respectively, in PBS solution containing 0.1% BSA. After fixation, cells were washed once with 1 mL PBS and then treated with Triton X in PBS (1 mL, 1%, v/v) at room temperature for 5 min. Afterwards, cells were washed twice with 1 mL PBS. BSA in PBS (1 mL, 1%, w/v) was added on the cells and incubated for 30 min at 37 ^{o}C. FITC-labeled Phalloidin (1 mL) was then added into each well, incubated at 37 ^{o}C for 1 h and then the cells were washed with PBS again. After this step, the cells were stained with DAPI (1 mL) for 5 min, and then each well was washed with 1 mL PBS.

Uptake of nanoparticles by cells was examined microscopically with fluorescence microscopy (Olympus, IX-70, Japan) and Confocal Laser Scanning Microscopy (CLSM) (Leica DM2500, Germany). Nile Red, FITC-labeled Phalloidin and DAPI labels were examined by using green, blue and UV filters of the microscope, respectively.

Nile Red was traced by excitation at 533 nm and emission at 525 - 605 nm. FITC-labeled Phalloidin was detected after excitation at 488 nm and emission at 525 - 600 nm in CLSM. To follow the relation of the particles and the cells cross-section (z-stacks) and 3D composite images were obtained.

2.2.8. Statistical analysis

All the characterization and in vitro studies were performed in duplicates or triplicates. Arithmetic means and standard deviations were calculated and afterwards, significant differences between mean values in control and test groups were determined using 1-tail Student's t-test that is standard software of Microsoft Excel. Means were considered to be significantly different for $p \leq 0.05$ values.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Characterization of PLGA Nanoparticles

3.1.1. Microscopical Evaluation and Size Measurements

PLGA nanoparticles were produced by using o/w solvent evaporation and w/o/w double emulsion-solvent evaporation methods, for nanospheres (NS) and nanocapsules (NC), respectively, using 10% (w/v) polymer concentration. According to Bivas-Benita et al. (2004) and Yilgor et al. (2009), uniformly distributed, smooth surfaces, round and spherical particles with nano and low micron diameters were expected with 10% (w/v) PLGA polymer solutions.

Figure 3.1 shows SEM micrographs of both unloaded (empty) and bioactive agent (RP, DSW, or $MgCl_2$) loaded PLGA nanoparticles. Sizes and the size distribution of the particles were determined using dynamic light scattering (DLS) system and also SEM micrographs with NIH Image J program. Size distribution was detected by DLS system and was found in the range of 5 – 21 μm for all, unloaded and bioactive agent loaded particles. In contrast SEM micrographs showed that the particles were distributed in the nano range. The reason of the difference in between these two separate measurements was probably caused of agglomeration of the particles through the lyophilization. In DLS measurements, particles were resuspended in distilled water when they were in lyophilized powder; however, for SEM analysis, very small volume (50 μL) of nanoemulsion was air dried on the carbon tape before examination (See Section 2.2.3.1). It is expected that, lyophilization may cause particle agglomeration especially if the particles are in the nano range as given in literature (Sommerfeld *et al.*, 1997). Thus, in order to determine the size distribution and wall thickness

of the particles, SEM micrographs with NIH Image J program was used afterwards in this study. Particle sizes were found to be 300 ± 110 nm for unloaded spheres, and addition of RP caused an increase in size leading to 522 nm. For $MgCl_2$ and DSW loaded capsules, the sizes were in the range of 280 – 311 nm. Wall thicknesses of $MgCl_2$ and DSW loaded NC were found to be 85 ± 29 nm and 53 ± 12 nm wall thicknesses, respectively (Table 3.1).

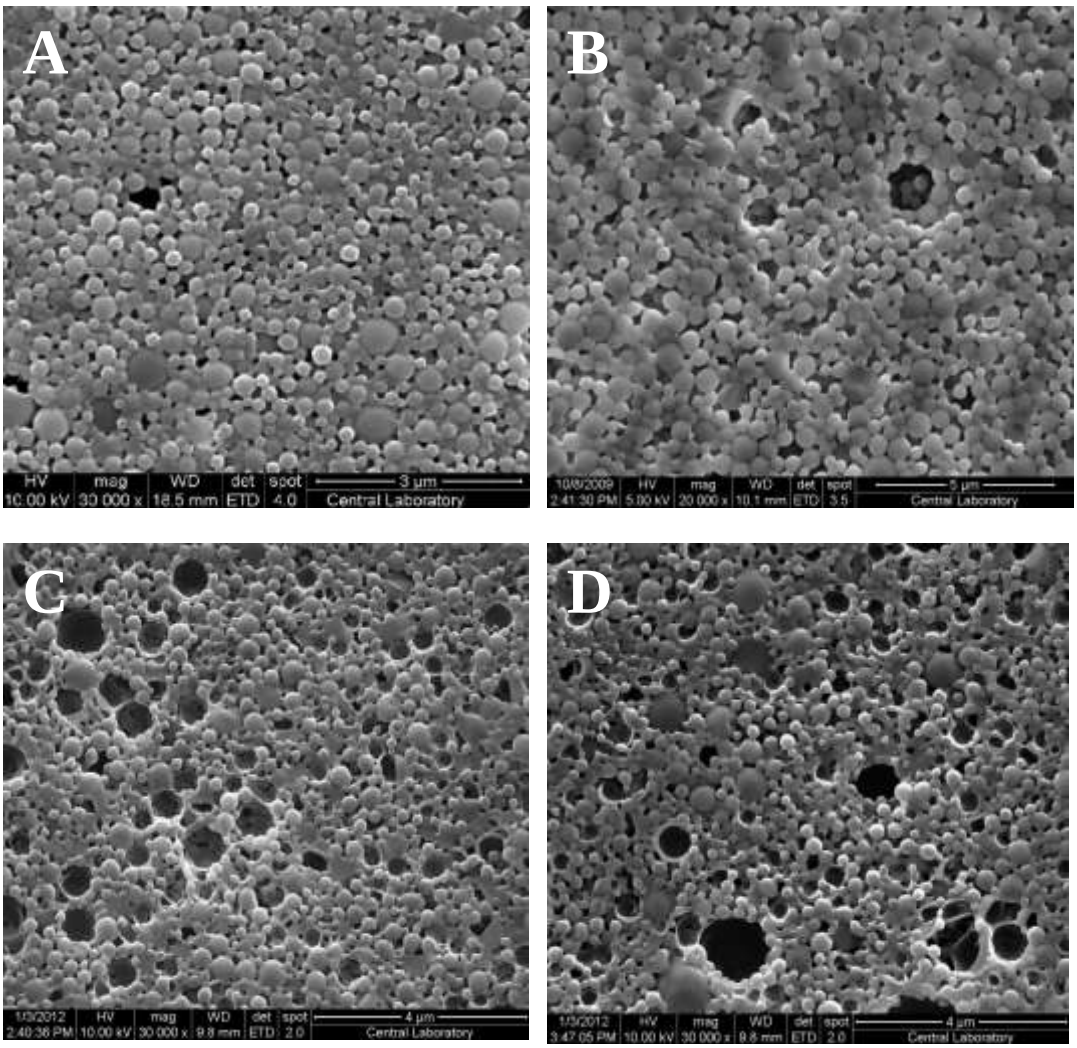


Figure 3. 1 SEM micrographs of PLGA nanoparticles. A) Unloaded NS (x30,000), B) RP loaded NS (x20,000), C) $MgCl_2$ Loaded NC (x30,000), D) DSW Loaded NC (x30,000).

Table 3. 1 Size distribution and the wall thicknesses of the bioactive agent loaded particles.

Bioactive agent and type of particles	Sizes of particles (nm)	Wall thickness of particles
Unloaded spheres	300 ± 110	N/A
RP loaded spheres	522 ± 167	N/A
$MgCl_2$ loaded capsules	280 ± 105	85 ± 29
DSW loaded capsules	280 ± 133	53 ± 12
BSA loaded capsules	300 ± 85	100 ± 26
Capsules carrying $MgCl_2$ co-encapsulated with BSA	311 ± 136	84 ± 33
(n=75 for particle size measurement and n=35 for wall thickness measurement) N/A: Not applicable		

In addition to bioactive agents, bovine serum albumin (BSA) was loaded into the NC as a model drug, as was mentioned in Section 2.2.2.3 with the same polymer concentration. Figure 3.2 shows the SEM micrographs of BSA loaded NC. Size measurements with SEM and Image J showed that the average diameter of the particles were 300 ± 85 nm with 100 ± 26 nm wall thickness (Table 3.1).

Similarly, a test was carried out to study the effect of co-encapsulation of BSA on that of $MgCl_2$, they were loaded together in PLGA capsules as was mentioned in Section 2.2.2.5. Size measurements showed that the average diameter of the particles were 311 ± 136 nm with 84 ± 33 nm wall thickness (Table 3.1, Figure 3.3).

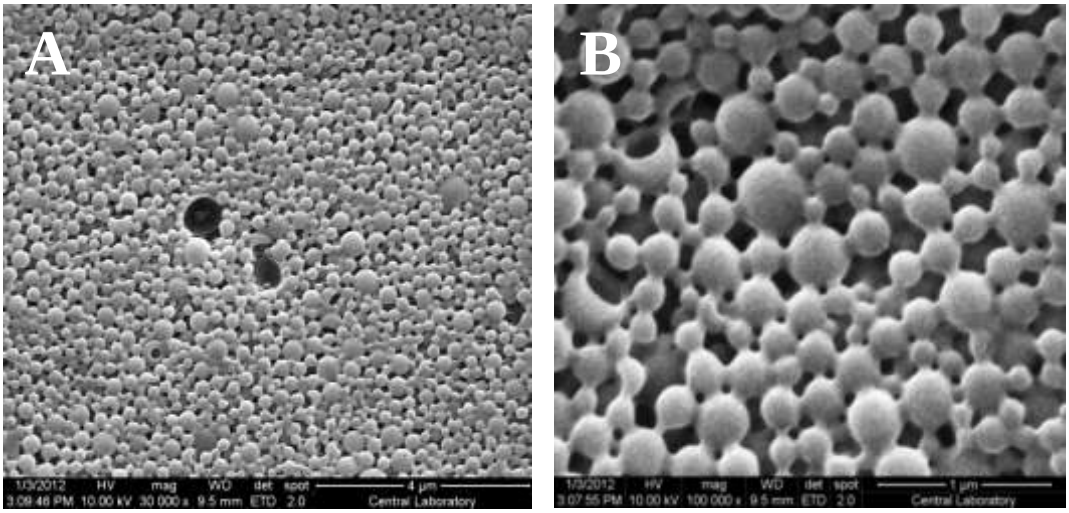


Figure 3. 2 SEM micrographs of BSA loaded NC at two different magnifications. A) x30,000, B) x100,000.

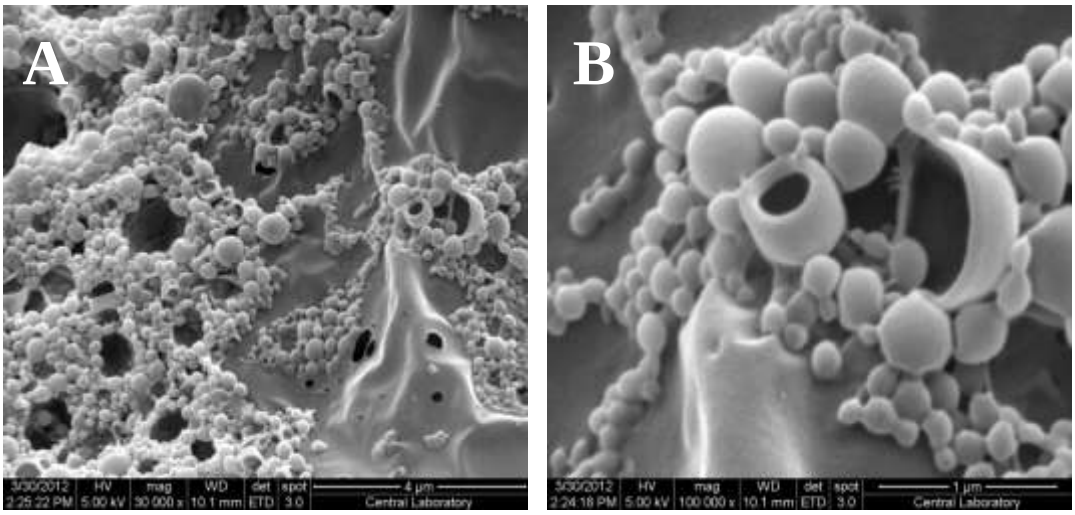


Figure 3. 3 SEM micrographs NC loaded with $MgCl_2$ and BSA. Magnifications: A) x30,000, B) x100,000.

Although there is a difference between the wall thicknesses of BSA loaded and $MgCl_2$ loaded NC (BSA loaded capsules: 100 ± 26 nm, $MgCl_2$ loaded capsules: 85 ± 29 nm), it is not statistically significant. The wall thicknesses of NC after co-encapsulation of $MgCl_2$ with BSA were 84 ± 33 nm.

In brief, SEM analyses revealed that, uniformly distributed, smooth surfaces and spherical particles with several hundred nano meter diameters were obtained. Drug loading did not affect the smoothness and roundness of the particles. Size differences were observed with SEM analyses only for the RP loaded NS; loading of hydrophobic drug was found to increase the particle size. Errico et al. (2009) reported that vitamin A derivatives loaded in nanoparticles slightly increased the size of particles similar to our observations.

3.1.2. Stability

Stability of the nanoparticle suspensions or emulsions (not settling of the nanoparticles) is important especially for the shelf life and storage conditions of the products. Unloaded and bioactive agent (RP and DSW) loaded PLGA nanoparticles were dispersed in the vehicles (carrier solutions) as mentioned in Section 2.2.3.3 and their stabilities were tested at different temperatures. These stability tests of the particles were carried out at Dead Sea Laboratories (DSL) (Israel) within the context of the EU FP7 SkinTreat Project.

Unloaded and RP loaded spheres were prepared in BIOMATEN and sent to DSL as lyophilized powder. Two separate runs were done to determine the stability of the particles in ST_01, ST_022 and ST_03 vehicles. In the first run, both types of PLGA spheres (unloaded and RP loaded) were tested and were found to be stable for 14 days at room temperature (RT) in ST_01 vehicle, unloaded NS and RP loaded NS were found to be stable in both ST_01 and ST_022 vehicles. Afterwards, in order to increase the stability, RP loaded NS were lyophilized in sucrose solution. In this way it would be easy to resuspend the spheres after drying and agglomeration would be prevented. Second run was repeated only with these NS and they were found to be stable in ST_03 carrier for 3 days at 45 ^{o}C (Table 3.2).

Table 3. 2 Stability test results of PLGA nanospheres.

Sample Type	First Run			Second Run		
	T= Room Temperature (RT)			T= 45 ^{o}C		
	ST_01	ST_022	ST_03	ST_01	ST_022	ST_03
Unloaded NS	Stable for 14 days	Not stable	Not stable	-	-	-
RP loaded NS	Stable for 14 days	Stable for 14 days	NT	-	-	-
RP loaded NS (Lyophilized in sucrose solution)	NT	NT	NT	NT	Not stable	Stable for 3 days
NT: Not tested ST_01: w/o emulsion, ST_022: o/w emulsion, ST_03: aqueous gel						

Teixeira et al. (2005) used the same polymer, PLGA, in their study and both Xanthone or 3-methoxyxanthone loaded nanocapsules were found to be stable at 4 ^{o}C for 4 months without any change in particle size, zeta potential and sedimentation or without any phase alteration in the vehicles. In another study Lutein loaded solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) were prepared and all the batches remained stable at both 4 ^{o}C and RT for 30 days. However, significant changes were reported from the samples that were stored at 40 ^{o}C after a month; especially aggregation and increase in the particle sizes were observed (Mitri *et al.*, 2011). Kim et al. (2005) reported that polyethylenimine (PEI) containing PLGA (50:50) nanoparticles were stored at RT for 12 days without any aggregation and precipitation. It was stated that, stability of the nanoparticles could be improved by the use of sufficient quantity of a stabilizer in the formulation to prevent the agglomeration and increase the hydrophilic moiety around the nanoparticles (Kim *et al.*, 2005, Mitra *et al.*, 2011).

The main reasons of stability differences between two runs were the changes in the temperature and the vehicle types. Since the unloaded and RP loaded PLGA nanospheres were found to be stable in their carriers for 14 days at RT, they were found to be suitable for further testing.

3.1.3. Inclusion of Bioactive Agents into Nanoparticles

3.1.3.1. Entrapment Efficiency and Loading of Retinyl Palmitate (RP)

RP is a hydrophobic compound and the most stable form of Vitamin A which has an important role in cellular differentiation and in the prevention of carcinogenesis. Use of RP is also applicable in the field of anti-ageing pharmaceutical preparations (Teixeria *et al.*, 2010). RP encapsulation efficiency by the PLGA nanoparticles was determined as was reported in Section 2.2.3.4.1.

In general, HPLC is accepted as one of the frequently used and highly sensitive technique for detection and quantification of retinoids and carotenoids. The use of HPLC involves chromatography coupled with detection of compounds with UV and visible light absorbance detectors and provides rapid qualitative and quantitative analysis results from small volumes of extracts of the sample in a short time.

RP loaded PLGA nanospheres were dissolved in dichloromethane (DCM), and the RP content was detected with simple UV-VIS detectors at 330 nm. The absorbances were converted to concentrations using the calibration curve presented in Appendix A, and the calculations were done using Equations 1 and 2 (Section 2.2.3.4.1). The percentages of RP encapsulation efficiency (E.E.) and loading were found as $0.8 \pm 0.3\%$ and $0.3 \pm 0.1\%$, respectively. The E.E. (%) and loading (%) values were quite low than expected. Errico et al. (2009) reported the loading of retinoic acid (another form of Vitamin A) was 1% (w/w) for PLGA particles. In another study, it was also stated that, through the loading process of hydrophobic drugs into the particles hydrophobic interactions between the drug and polymer might be involved (Jeong *et al.*, 2001).

3.1.3.2. Encapsulation Efficiency and Loading of Bovine Serum Albumin (BSA)

In addition to small sized and/or ionic bioactive agents (RP, DSW and $MgCl_2$), a larger molecule, bovine serum albumin (BSA) was loaded into the NC as a model drug with the hope of achieving much higher loading efficiency. BSA was loaded in the NC as reported in Section 2.2.2.3.

Determination of BSA was done by Micro Bradford Assay at 600 nm using a calibration curve (Appendix B). E.E. (%) and loading (%) of BSA was calculated by using Equations 1 and 2 (Section 2.2.3.4.1) and the values were found to be much higher than the bioactive agents used: 41.0 ± 9.9 and 2.2 ± 0.5, respectively.

These results were quite similar to the results obtained in the study of Blanco and Alonso (1997). In their study, BSA was loaded in the nanocapsules prepared from the same polymer, PLGA, and E.E. (%) was found as 46.80 ± 1.52 using an extraction procedure and spectrophotometry. Same set of particles were also examined by hydrolysis method, and E.E. (%) was found to be 63.89 ± 4.71. Same basic preparation and extraction procedure were used for BSA loaded PLGA nanoparticles, and the E.E. (%) was found to be 84.75 ± 1.47 (Yilgor *et al.*, 2009). On the whole, the loading efficiencies are quite high. The differences between the results reported in literature can be explained as the effects of MW of the polymer, viscosity of the polymer solution, the type and amount of surfactants and the determination method that was used as was done by Blanco and Alonso (1997).

3.1.3.3. DSW and $MgCl_2$ Encapsulation Efficiency and Loading in PLGA Nanocapsules (NC)

As was stated in Section 2.2.3.4.2, DSW composition is a mixture of a number of soluble salts. Cl^- is the major constituent; therefore, encapsulation efficiency and release rate of bioactive agents from PLGA capsules were investigated by encapsulating $MgCl_2$ as a compound representative of DSW and detecting the chloride ion. Chloride specific electrode of the ion meter was used to

measure the Cl^- ion content in the solutions. In addition to the ion meter and Cl^- specific electrode, titration with $AgNO_3$ was also used as a control method.

In order to study the effect of initial $MgCl_2$ amount on encapsulation efficiency and loading, two different batches of PLGA capsules were prepared for loading of $MgCl_2$ and DSW. The amount of active agent ($MgCl_2$ or DSW) used in loading in SET 2 was approximately one fifth of the amount of the amount used in SET 1. With both $MgCl_2$ and DSW for each set, it can be seen that the encapsulation efficiency is not higher than 1.0% but SET 2 yielded 3-4 fold increases in E.E. even though loading was not significantly affected (Table 3.3).

Table 3. 3 DSW and $MgCl_2$ encapsulation efficiencies and loadings of PLGA NC.

Samples		**Initial Loaded Cl^- Amount (mg)**	**Encapsulation Efficiency (E.E.) (%)**	**Loading (%)**
SET 1	$MgCl_2$	75	0.18 ± 0.12	0.18 ± 0.10
	DSW		0.16 ± 0.10	0.15 ± 0.08
SET 2	$MgCl_2$	16.67	0.86 ± 0.48	0.20 ± 0.11
	DSW		0.50 ± 0.36	0.11 ± 0.07
n=3				

The difference among the E.E. (%) of two sets was thought to be caused by the reduction of the initial amount of electrolyte solution so that drug to polymer ratio was decreased which increased the E.E. as expected. Almost 4 and 3 fold of increase in the E.E. (%) values was obtained after reducing the loaded amount of electrolytes for the second set of $MgCl_2$ and DSW, respectively (Table 3.3).

The drug to polymer ratio in the preparation of the nanoparticles is one of the main factors that affect drug entrapment and loading efficiency. The results obtained in this study are in agreement with the literature that decrease in the drug amount used in loading, increases the entrapment efficiency. In a study, different concentrations of blue dextran (from 2.5% to 12.5%, (w/v)) was loaded into

PLGA in DCM solution, and the highest loading efficiency (41.4%) was obtained for 2.5% concentration of the drug. The other loading efficiencies were varied among 22% - 11% for increasing concentrations of the drug in polymer solution (Ito *et al.*, 2007). Also, high encapsulation efficiency of the drug in PCL microparticles was reported as a result of low drug to polymer ratio (1:9). The main reason for these low E.E. values was stated as insufficient amount of polymer present in the system to cover the drug completely (Jeong *et al.*, 2003).

In addition to the effect of drug to polymer ratio, the low encapsulation efficiencies obtained with highly water soluble molecules with low molecular weights were discussed in a study of Farago et al. (2008). With PHBV microparticles 0.85% E.E. was reported for metformin hydrochloride (MH).

It was also reported in different studies that, low encapsulation values obtained for polymeric nanoparticles loaded with highly hydrophilic drugs with low molecular weights are acceptable due to rapid diffusion of the drug from the first emulsion to the external aqueous phase during the emulsification and solvent evaporation stages (Govender *et al.*, 1999, Hans and Lowman, 2002, Farago *et al.*, 2008). The molecules used in this study, for example $MgCl_2$ and Dead Sea Water are water soluble electrolytes with low molecular weights and thus suffer the consequences.

3.1.3.4. $MgCl_2$ and BSA Co-encapsulation: Encapsulation Efficiency and Loading

As was mentioned in Section 2.2.2.5, $MgCl_2$ was loaded into capsules along with BSA, and E.E. (%) and loading (%) were determined. In Table 3.4 the results of this co-encapsulation along with previous single encapsulations are presented.

E.E. and loading for $MgCl_2$ were found as 0.47% and 0.58%, respectively when encapsulated together with BSA. This is a significant increase over when encapsulated alone (0.47 vs 0.18 E.E. (%) and 0.58 vs 0.18 loading (%)). The increase is about 3-fold in each case. E.E. (%) and loading (%) of BSA was; however, reduced compared to loading alone (Table 3.4).

Table 3. 4 Comparison of the co-encapsulation of BSA and $MgCl_2$ with individual encapsulations.

	Cl^- ions		BSA	
	E.E. (%)	Loading (%)	E.E. (%)	Loading (%)
BSA alone	-	-	41.00 ± 9.90	2.20 ± 0.50
$MgCl_2$ alone	0.18 ± 0.12	0.18 ± 0.10	-	-
$MgCl_2$ with BSA	0.47 ± 0.10	0.58 ± 0.15	9.80 ± 1.12	0.70 ± 0.03

Since the main aim was to improve the efficiency of $MgCl_2$ entrapment, these results are relatively satisfactory. This increase in Cl^- ion E.E. (%) and loading (%) was not caused by possible difference of the wall thicknesses of the particles since there was no significant difference. Probably, the increase in encapsulation and loading efficiencies of $MgCl_2$ was a result of the interactions between BSA and $MgCl_2$. This is to be expected because BSA alone had a quite a high efficiency indicating the inability to leach into the continuous medium and when $MgCl_2$ was co-encapsulated the ions that attached to the protein were also retained in the core.

The interaction between BSA and PLGA chains was reported to result in an increase in encapsulation and loading efficiencies of BSA in nanocapsules (Blanco and Alonso, 1997, Hans and Lowman, 2002).

3.2. *In situ* Release Kinetics of Bioactive Agents

3.2.1. Release Kinetics of BSA

The release kinetics of BSA was assessed with Micro Bradford Assay by determining the amount of the released protein in the medium.

The situation of the release to Zero Order was assessed by plotting amount released (M_t) versus time (t) according to the equation $M_t/M_\infty = k_0t$. A fit to First Order release requires obeying $\ln M_t$ vs t according to $M_t/M_\infty = e^{k1t}$. Higuchi release requires a fit to M_t/M_∞ vs $t^{1/2}$ according to $M_t/M_\infty = k_Ht^{1/2}$. Here M_∞ is the amount of drug released at time infinity (∞), t is time (h) and k_0, k_1 and k_H are rate constants according to Zero Order, First Order and Higuchi Release relations, respectively.

A burst was observed leading to release of 15% of BSA content in the first hour and by the end of the 5 days ca. 30% of BSA was released (Figure 3.4). It was observed that BSA loaded capsules had a triphasic release profile, first started with an initial burst and then reaching a slow release period and then, probably due to the degradation of the system, a higher rate of BSA release. The inset shows the first 40% of the release data fitted to Higuchi relation which should reveal a straight line if the release is by diffusion through monolithic devices as proposed by Higuchi (Figure 3.4).

In a review by Fredenberg et al. (2011) on PLGA based drug delivery systems (DDS), the shape of the release profile was explained by the phases. Initial burst was described as the release of nonencapsulated drug content adsorbed on the surface or drug molecules close to the surface which are easily accessible by hydration. The second, often slow release, a phase is slow drug diffusion either through the relatively dense polymer or through the few existing pores on the carrier surface opened by water diffusion (hydrolysis) or polymer degradation. Finally, phase three is said to be a period of faster release, generally related with the onset of erosion of the system. Thus, it is stated that the release of BSA from PLGA capsules showed triphasic release through 80 days.

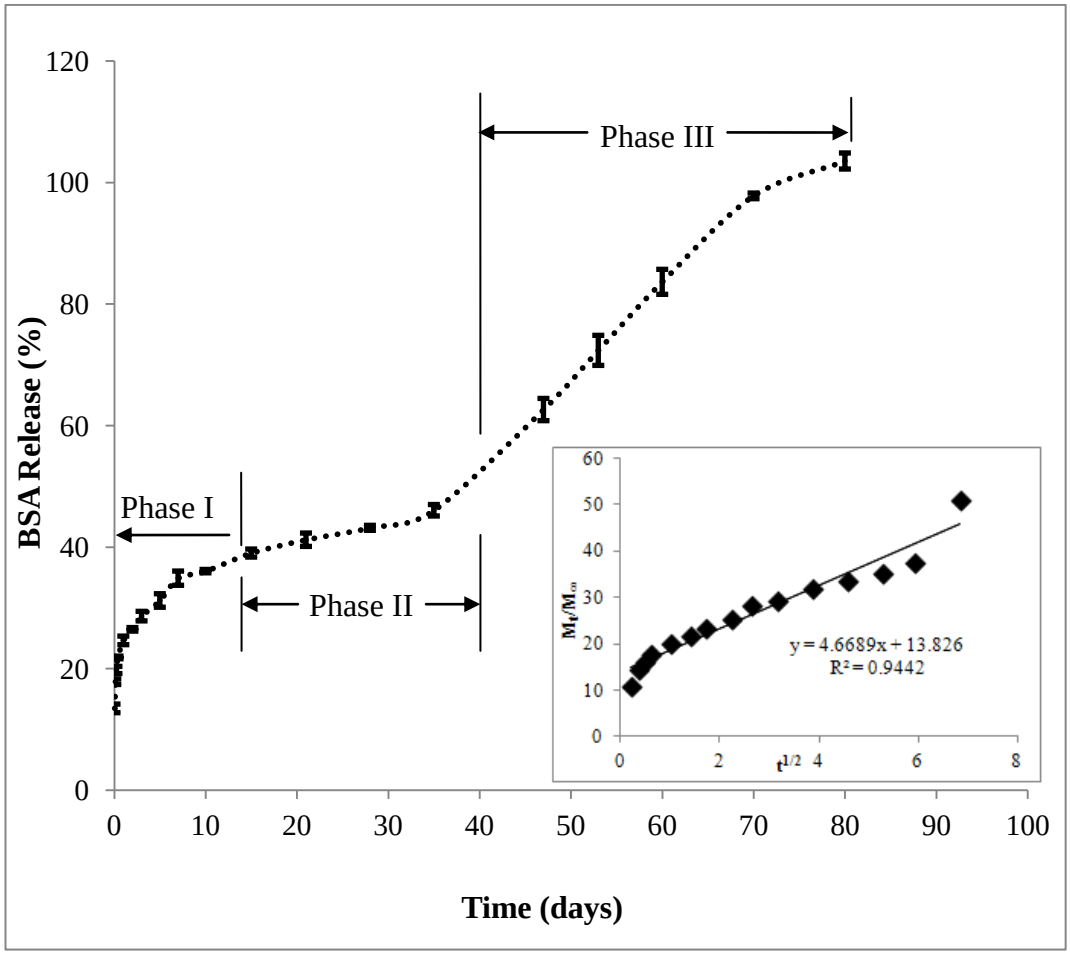


Figure 3. 4 BSA release from PLGA nanocapsules for 80 days (n=3). Inset shows the first 40% of the release data fitted to the Higuchi relation.

3.2.2. Release Kinetics of DSW and $MgCl_2$

The release behavior of $MgCl_2$ and DSW from PLGA nanocapsules were studied by measuring the released chloride ions with a chloride specific electrode and an ion meter. The release behavior was tried to be fitted to Higuchi, Zero and First Order Kinetic relations but was not represented with any of them since it was an initial burst release.

It is observed that almost all of the salt content was released into the aqueous medium in the first 24 h (Figure 3.5) as a burst. Here the chloride content of SET 1 was 75 mg and for SET 2 it was 16.67 mg.

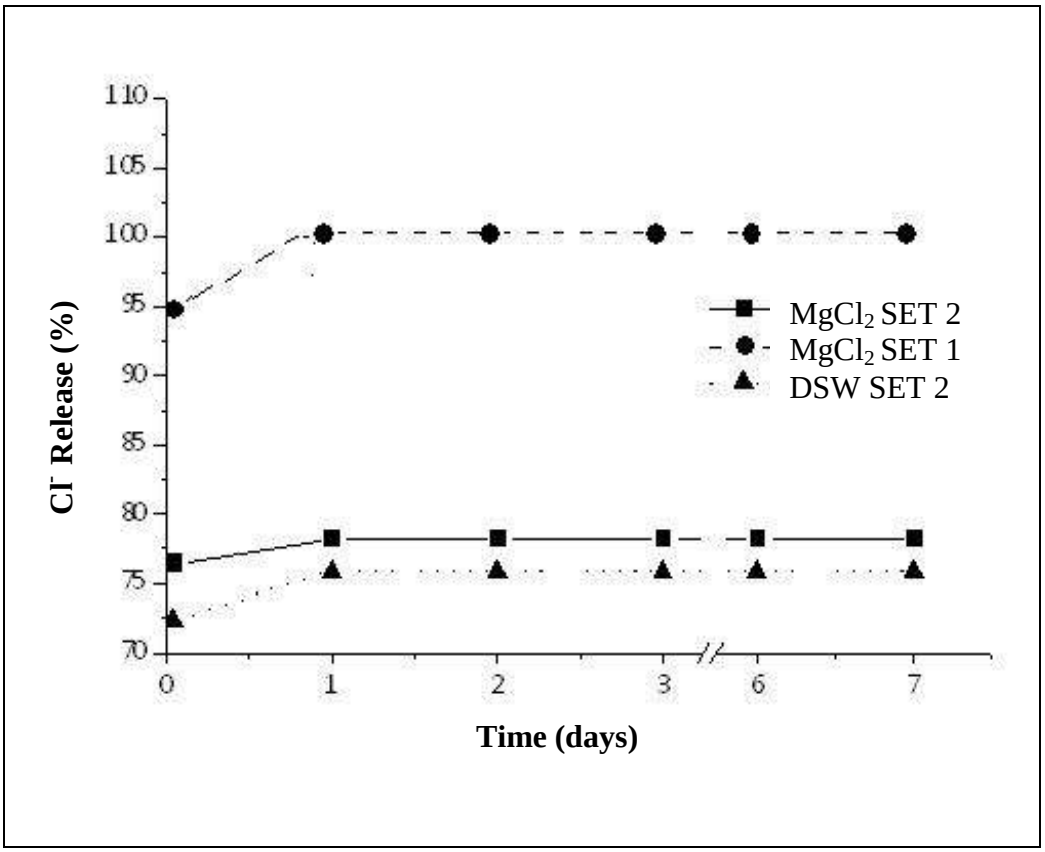


Figure 3. 5 $MgCl_2$ and DSW release from PLGA nanocapsules (NC).

These high burst release rate profiles of the electrolytes were expected because of their small sizes and highly hydrophilic (ionic) nature. In the study of Govender et al. (1999) the drug release of highly water soluble procaine hydrochloride from PLGA nanoparticles was studied and 65% of the drug content was founded to be released within the first 15 min and then slower but exponential release was observed for a further 4 - 6 h. In the literature, the initial burst release of a drug (not necessarily ionic) was thought to be due to poorly entrapped drug, or drug adsorbed on the surface of the particles, or the high surface area to volume ratio leading to faster release from the particles (Hans and Lowman, 2002, Mohanraj and Chen, 2006).

3.2.3. Release Kinetics of $MgCl_2$ Co-encapsulated with BSA

The release kinetics of $MgCl_2$ was earlier assessed by measuring the released chloride ions with a chloride specific electrode and an ion meter, while the release kinetics of BSA was investigated with Micro Bradford Assay.

$MgCl_2$ and BSA were co-encapsulated to improve the loading of $MgCl_2$ and the determinations were achieved as stated before. E.E. and loading of $MgCl_2$ were found to be 0.47% and 0.58%, respectively, and those of BSA were 9.8% and 0.7%, respectively (Section 3.1.3.4). In the Cl^- release from the capsules a decrease in the initial burst was served (Figure 3.6) (from 80% in 1 h to 50% in 1 h).

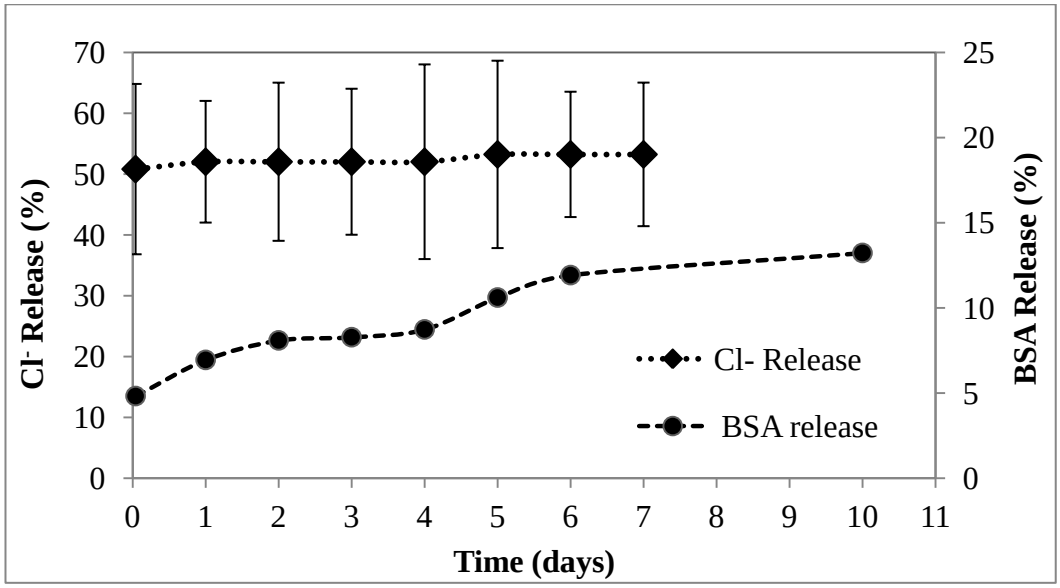


Figure 3. 6 $MgCl_2$ and BSA release from PLGA NC carrying both $MgCl_2$ and BSA.

BSA release was only 14% at the end of the 10 days, and released BSA content was 4% by burst (Figure 3.6).

Co-encapsulation with BSA improved the Cl^- encapsulation and loading efficiencies for $MgCl_2$ but there was a significant decrease in the encapsulation of the BSA. This was thought to be caused by electrolytes changing the viscosity of the BSA and the pH of the inner core therefore effecting PLGA and BSA interactions (Blanco and Alonso, 1997, Hans and Lowman, 2002).

3.3. Hemolytic Activity of PLGA Nanoparticles

Hemolytic activity assessment is a simple biocompatibility test and was applied to PLGA nanoparticles.

Hemolytic activity of PLGA spheres and capsules loaded with different bioactive agents was studied (Table 3.5). Percent hemolysis was found to be less than 1.0% for unloaded and less than 1.5% for DSW loaded NC, and less than 1.3% for RP loaded NS. A sample is considered as hemolytic according to the ISO/TR 7405-1 984(f), if a hemolytic activity above 5% is detected. Thus, it can be safely stated that the PLGA nanoparticles tested were not hemolytic.

Table 3. 5 Hemolytic activity of bioactive agent loaded PLGA nanoparticles

Sample	Hemolysis (%)
Unloaded PLGA NC	< 1.0
DSW loaded PLGA NC	< 1.5
RP loaded PLGA NS	< 1.3
n=3	

The results are in agreement with those reported in the literature, where PLGA nanoparticles were shown to have negligible hemolytic activity (Kim *et al.*, 2005 and Cenni *et al*., 2008).

3.4. *In vitro* Studies

3.4.1. Effect of Bioactive Agent (RP, DSW, $MgCl_2$ or BSA) Loaded PLGA Nanoparticles on Cell Proliferation

PLGA is one of the most commonly used members of poly(α-ester) family, and has been widely used in biomedical applications, especially in drug delivery (Ulery *et al.*, 2011, Tian *et al.*, 2012). Therefore, the cytocompatibility of this copolymer is of importance and was investigated.

Bioactive agent loaded PLGA nanoparticles, were tested using L929 cells (mouse fibroblasts) in order to assess their suitability for use in transdermal drug delivery applications. The MTT test is a cell viability test and all nanoparticle types were tested with L929 cells (passage no 13 - 15) with the concentration of the particles in the medium being 0.2 mg/mL (Figure 3.7). In addition to all types of bioactive agent (RP, DSW, $MgCl_2$ or BSA) loaded particles, DSW was also tested in free form. TCPS (tissue culture polystyrene) containing no nanoparticle served as the control group. Separate experimental and control groups were used for each time point (n=3).

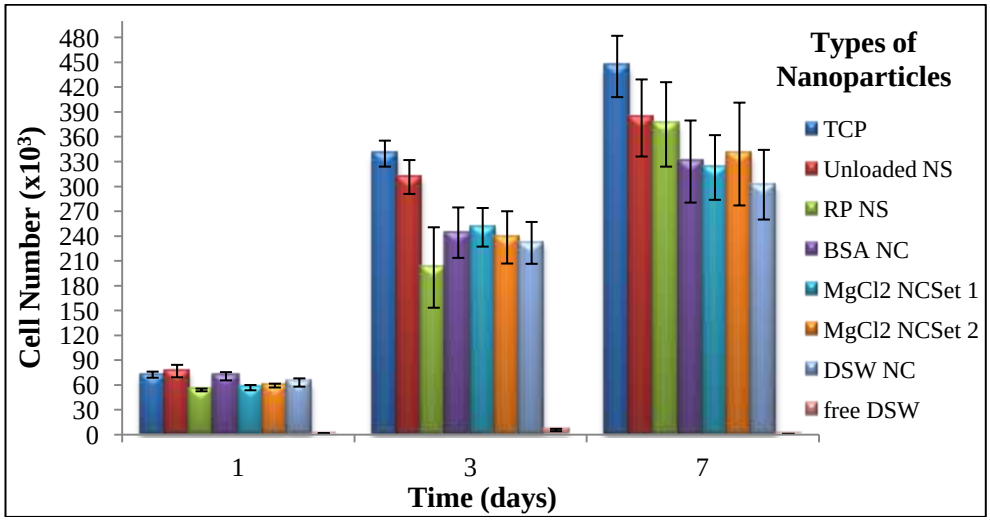


Figure 3. 7 Effect of bioactive agent loaded PLGA nanoparticles on L929 cell viability (n=3). Results of MTT tests.

It was observed that the presence of free DSW substantially suppressed cell proliferation because of its high ionic strength (Figure 3.7).

Upon encapsulation, DSW lost its adverse effect completely. With the introduction of DSW in PLGA particles, the cell proliferation increased significantly. This shows that, the adverse effects due to DSW is real and entrapment in a carrier helps to mask this. On the other hand, $MgCl_2$ and DSW loaded NC did not show a significant difference in their effect on cell proliferation which proved the appropriateness of $MgCl_2$ as a model drug. Reducing the $MgCl_2$ amount loaded in the nanoparticles increased the encapsulation efficiency and loading values of the particles (Section 3.1.3.3), this also did not significantly alter their effect on the proliferation of cells.

NS loaded with RP did not show as significant an adverse effect as the free DSW had on cell proliferation, however, when the cell numbers are compared with these of TCPS control, there was a significant reduction in the viability in the first 3 days, where the viability ratios were 78%, 61% and 86% for the 1st, 3rd and 7th days, respectively. As a result, they were not cytotoxic in accordance with 50% inhibitory concentration of the NS reported by Gaiker Technology Center (Spain).

In addition to the results in Figure 3.7, cytotoxicity of DSW loaded capsules and RP loaded spheres were tested with different cell types at Gaiker Technology Center (Spain) in the context of EU FP7 SkinTreat Project and the results are presented in Table 3.6.

Table 3. 6 Cytotoxicity assesment of bioactive agent loaded nanoparticles on HACAT and 3T3 cells. Results of MTT test.

Nanoparticle Type and Composition	**Cell Type**	
	IC_{50}* Values (µg/mL)	
	HACAT	**3T3**
DSW Loaded PLGA NC	> 3500	1800 ± 8
RP Loaded PLGA NS	1580 ± 5	466 ± 1******

*Samples are not toxic if the toxicity value is larger than the threshold value $IC_{50} \geq 500$ µg/mL

**Sample was very close to toxicity threshold value but it was decided to accept as nontoxic.

At Gaiker Technology Center nanospheres and capsules were tested with two cell types: HACAT (human keratinocyte cell line) and 3T3 (fibroblast cell line). At the end of the tests they were found to be not cytotoxic based on a comparison with their half maximal inhibitory concentration (IC_{50}), which represents the concentration of a drug that is required for 50% inhibition *in vitro*. Neither of the bioactive agent loaded spheres and capsules had any adverse effects on cell proliferation; they had values greater than IC_{50}.

Use of mineral spa water and sea water has been and continues to be a common treatment method for inflammatory skin diseases such as psoriasis, several types of dermatitis (e.g. atopic and irritant contact eczema) (Lee and Maibach, 2005). In the literature, the treating effect of Dead Sea salt baths on psoriatic skin was reported with obtaining a significant decrease in the psoriatic area and in severity index (PASI) scores of patients after treatments (Halevy *et al.*, 1997). At the same time, another study reported that, in an in vitro study of psoriasis, at the end of the 2 h incubation with Dead Sea salts (NaCl, NaBr, KCl, KBr, $MgCl_2$, and MgBr), bromide salts significantly inhibited fibroblast proliferation as compared with chloride salts. Thus, adverse effect of free DSW on cell proliferation was expected and observed in this study (Figure 3.7).

RP is required in many essential biological processes, specifically, epidermal cell growth, normal cell differentiation, and cell maintenance. It has become one of the most commonly used retinoids in cosmetic products because of its higher thermal stability than retinol. As a result its use showed a rapid increase in the market of cosmetic products in the last two decades (Xia *et al.*, 2006). In spite of the frequent use of RP and other retinoids in cosmetic products, they had adverse effects; the most common being erythema, peeling, dryness, tightness and burning sensation through the topical application. Even though these skin responses were stated as temporary, they still were troubling for many patients (Kang and Voorhees, 2008). Thus, the damage observed in the initial 3 days in Figure 3.7 on cell proliferation by RP loaded NS was expected. The good thing is this negative effect did not persist and the response to RP loaded NS became comparable with other test particles and samples.

In this study, loading of DSW in PLGA particles significantly prevented cell death (Figure 3.7). DSW loaded capsules demonstrated very good growth of cells; it was quite comparable to that of the control group, where the free DSW strongly suppressed the viability, which showed the encapsulation of electrolytes in a polymeric carrier is an efficient approach to their delivery.

3.4.2. Uptake of PLGA Nanocapsules by Different Cells

The interaction of cells with PLGA nanoparticles and the fate of the particles were studied with Saos 2 and L929 cells. Figures 3.8 and 3.9 show the particles in these cells. Nile Red (0.01%, w/v, in acetone) is a stain used to dye lipoid structures including membranes and the nanoparticles used in this study. Therefore Nile Red loaded PLGA nanocapsules were incubated with both types of cells up to 48 h. Before the microscopic evaluations, cells were stained with FITC-labeled Phalloidin for the cell cytoskeleton (green) and with DAPI for the cell nuclei (blue). Nile Red has a red emission under fluorescence microscopy which helps to detect the particles.

The sizes of most capsules were in the nano range and were lower than the resolution of the fluorescence microscope preventing their individual visualization; therefore, the Nile Red stained red regions are probably clusters of nanocapsules. In Figures 3.8 A and 3.8 C the particle-free controls showed no particles, while the samples incubated with the particles showed red regions implying that the nanoparticles are generally localized in the cytoplasm and some near the cell nuclei; after 4 h (Figure 3.8 B), and after 24 h (Figure 3.8 D). These results indicate that the nanoparticles produced in this study can penetrate the cell membrane but not the nuclear membrane.

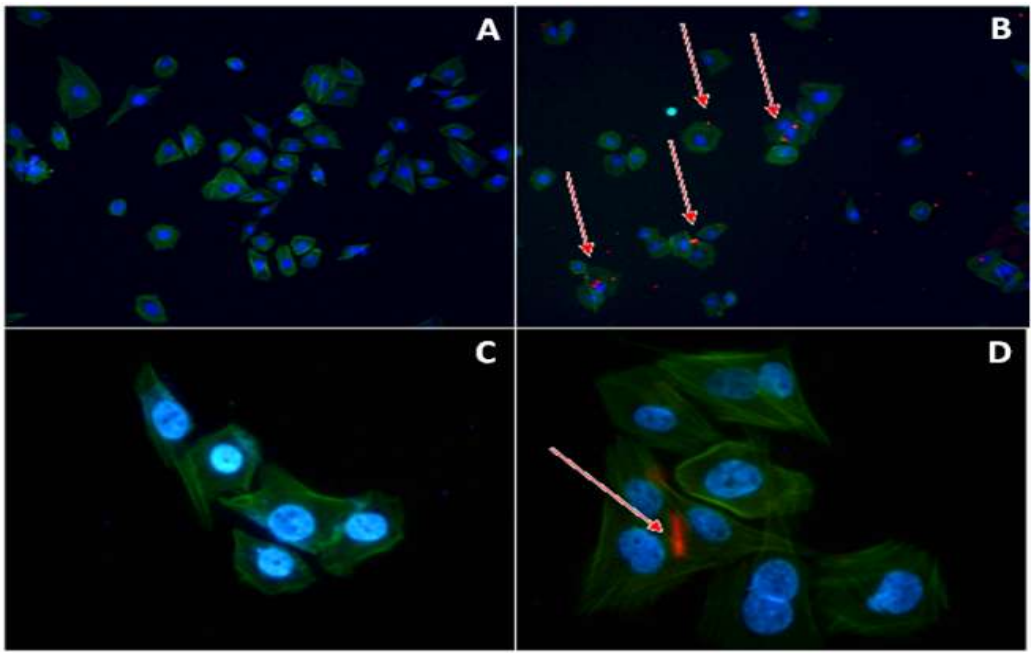


Figure 3. 8 Fluorescence microscopy of uptake of PLGA nanocapsules (NC) by Saos 2 cells. A) NC free control, 4 h, (x10), B) NC containing sample, 4 h, (x10), C) NC free control, 24 h, (x40), D) NC containing sample, 24 h, (x40).

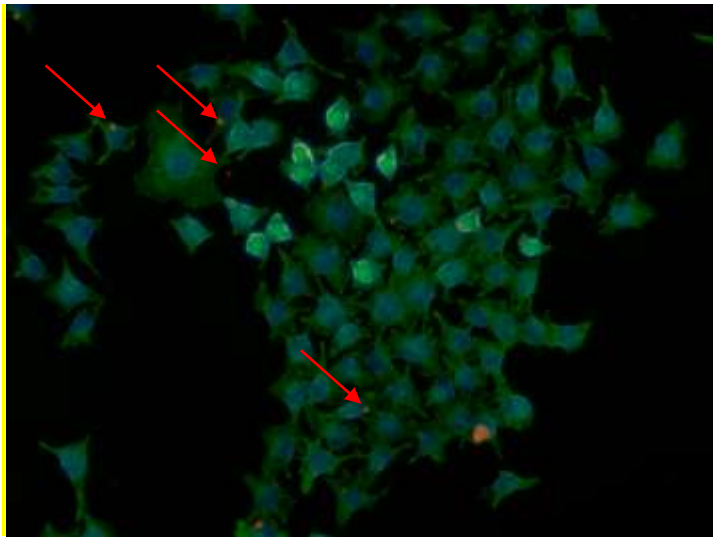


Figure 3. 9 Fluorescence micrograph of uptake of PLGA nanocapsules (NC) by L929 cells, 24 h. Red arrows indicate the Nile Red loaded NC.

L929 cells and micrographs were obtained by both fluorescence microscopy and CLSM (Figure 3.9 and 3.10).

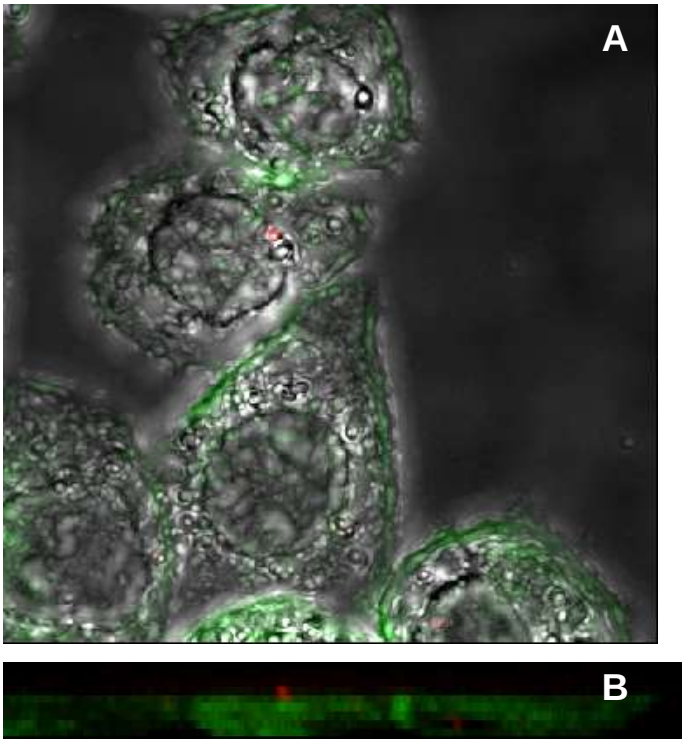


Figure 3. 10 CLSM images of uptake of Nile Red loaded PLGA nanocapsules by L929 cells. A) Overlay of transmission and confocal images, B) cross-sectional image of cells; 24 h. Cytoskeleton and nanoparticles were stained with FITC-labeled phalloidin and Nile Red, respectively.

Both fluorescence microscopy (Figure 3.9) and confocal microscopy 3D images (Figure 3.10) showed that PLGA nanoparticles were taken up by the cells and accumulated in the vicinity of the nucleus probably avoiding the lysosomes. This could indicate that they could serve as carriers of agents developed for gene therapy in addition to other applications.

Hu et al. (2009) reported in their study, that the main factors for controlling the cellular uptake of the colloidal particles were the size, shape, charge and surface chemistry. Nano sized particles were shown to more easily penetrate the cells than the micro sized particles and also spherical shaped particles were found to be taken up faster than the tubular shaped ones. In the study of Cartiera et al. (2009), uptake of PLGA nanoparticles by different epithelial cell lines showed that with appropriate dose and incubation time and cell type, uptake of the particles were successfully achieved. Similar to our

results, polyethylene glycol (PEG)-ylated trimethyl chitosan copolymers loaded with insulin penetrated into epithelial Caco 2 cells and particles were mainly localized around the region of the cell nuclei and suggested endocytic uptake (Mao *et al.*, 2005).

CHAPTER 4

CONCLUSION AND FUTURE STUDIES

In this study, PLGA nanoparticles (NPs) encapsulating bioactive agents namely, RP, DSW, $MgCl_2$, and BSA, were successfully developed as carriers for the treatment of predetermined skin diseases such as psoriasis, atopic dermatitis and UV damaged skin. Morphological analyses, stabilities, entrapment efficiencies and loadings, *in situ* release profiles and kinetics, and *in vitro* cytotoxicities as well as cell penetration of the particles were investigated. PLGA NPs were shown to penetrate into the cells and accumulate around the nucleus by the microscopical evaluations for the first time in literature. Thus, these results show the potential of the system as a gene therapy tool as well as drug carriers.

PLGA spheres and capsules were prepared by oil-in-water (o/w) emulsion solvent evaporation and by water-in-oil-in-water (w/o/w) double emulsion solvent evaporation techniques, respectively. Their microscopical examination showed that, uniformly distributed, smooth surfaced, round and spherical particles within the diameter range of 280 – 550 nm were obtained with selected polymer concentration (10%, w/v). Additionally, stability of the particles were tested in different vehicles and found to be stable in ambient temperature conditions for 14 days.

PLGA is one of the FDA approved polymers especially for use in clinical drug delivery applications. Through this study, the hemolytic activity and *in vitro* cytotoxicity tests showed that the bioactive agent loaded and unloaded PLGA NPs were hemocompatible and had no adverse effect on the cell viability. Furthermore, penetration of the particles through the cell membrane was obtained and shown to be accumulated around the cell nucleus by microscopical examinations. Although PLGA nanoparticles and their use as drug carrier for different compounds has been reported by other scientist in literature, entrapment of RP, DSW and $MgCl_2$

with BSA has been done for the first time. On the other hand, uptake of the particles by the cells and their presence around the nucleus was shown in this study which is also the novel part of the study.

Nevertheless, there are some points that can be further improved. For instance, the encapsulation efficiency (E.E.) of the PLGA particles was found to be low (~1%) for the bioactive agents (RP, DSW, and $MgCl_2$ as the model drug), in addition to high percent of initial burst release profiles. The nano size of the particles with very high surface area to volume ratio and hydrophilic nature of the ionic solutions were the major reasons of this low E.E. and burst release. On the other hand, when macromolecules like bovine serum albumin (BSA) were encapsulated, significantly high E.E. (41%) and triphasic release for 80 days were obtained. The type of the bioactive agents could be altered by applying a different preparation method in order to yield PLGA NPs with higher encapsulation efficiency and with proper zero order release.

Among the important features, a drug delivery system (DDS) has to satisfy a high E.E. and loading to provide administration of high doses of drug into the body via the least amount of material, in order to minimize the possible adverse effects. However, in this study, the aim was the treatment of skin diseases by topical application of the NPs within vehicles (e.g. creams, gels) that hair ducts and follicular route can be used as a depot place for the particles. Therefore, low E.E. of the particles could be overcome by including higher amount of particles into the carrier system to achieve the adequate dose.

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APPENDIX A

RETINYL PALMITATE (RP) CALIBRATION CURVE

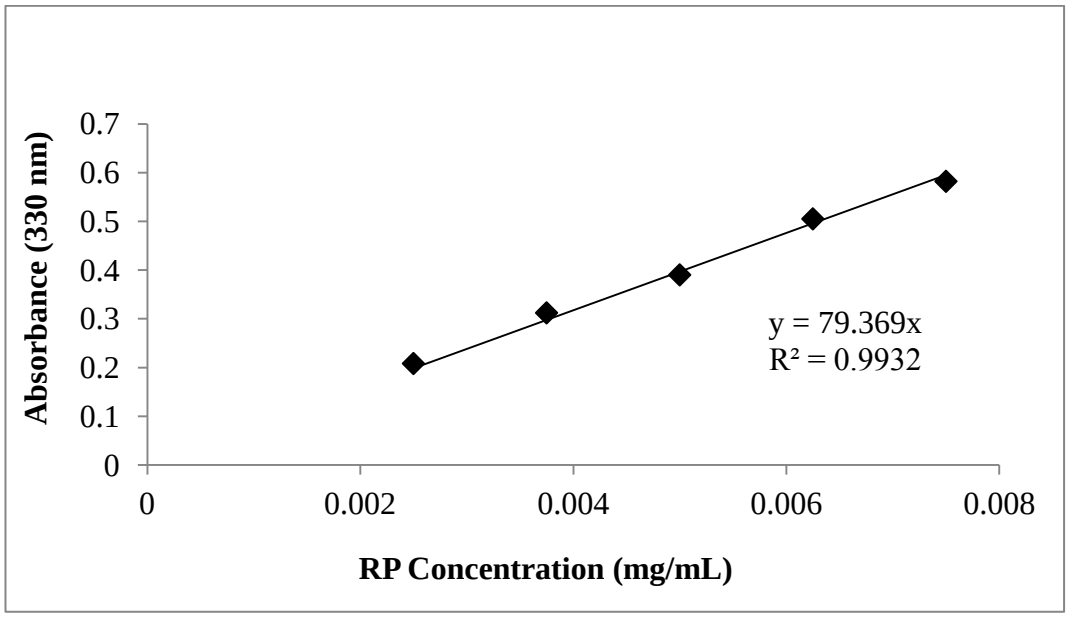

Figure A. 1 Calibration Curve of RP for UV-Vis Spectrophotometer at 330 nm.

APPENDIX B

AMOUNT OF ENCAPSULATED BSA – CALIBRATION CURVE

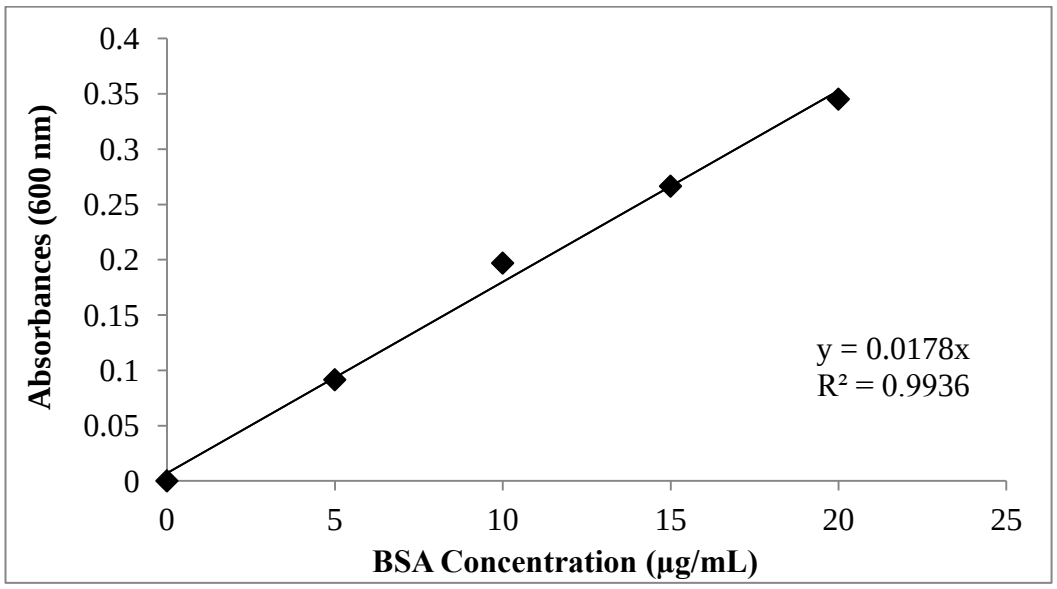

Figure B. 1 Calibration curve of BSA concentration in ultrapure water for Micro-Bradford Assay to determine the encapsulated BSA amount

APPENDIX C

AMOUNT OF RELEASED BSA – CALIBRATION CURVE

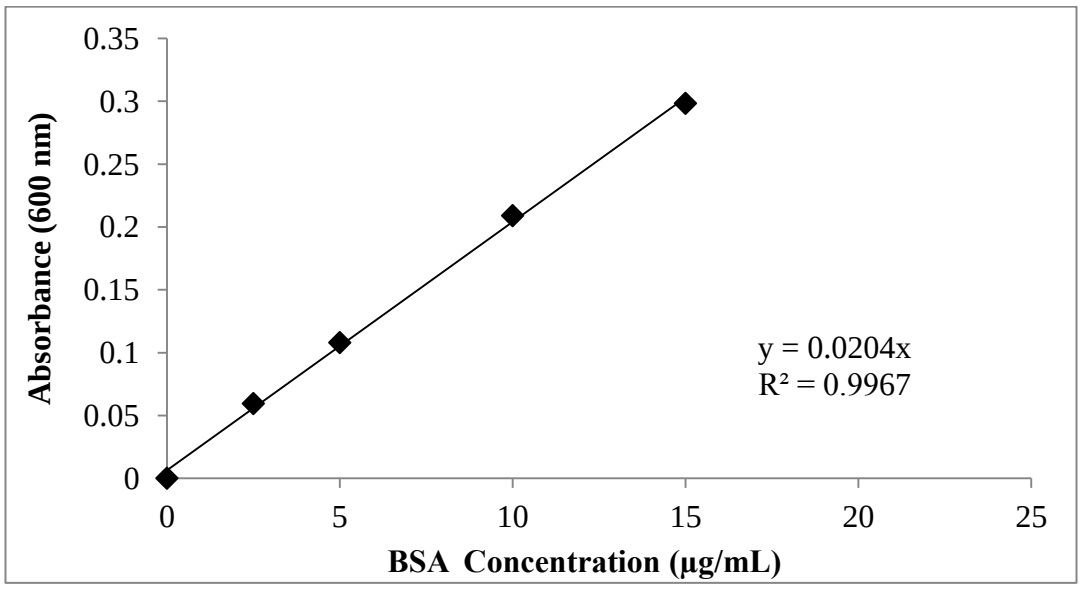

Figure C. 1 Calibration curve of BSA concentration in PBS for Micro-Bradford Assay to determine the released BSA amount

APPENDIX D

MTT CALIBRATION CURVE

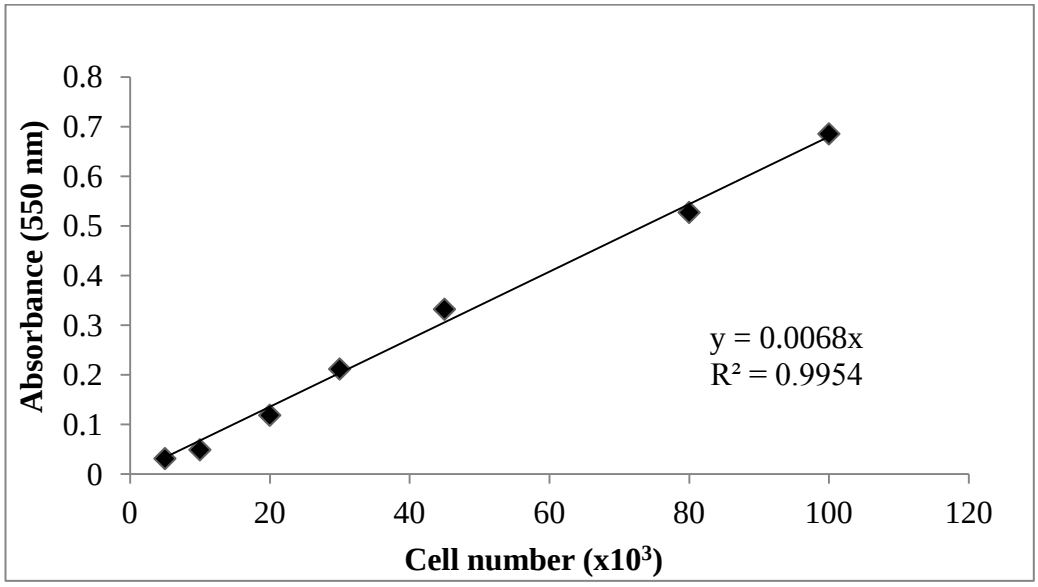

Figure D. 1 MTT calibration curve of L929 cells to determine the cell number