BIOPOLYMER BASED MICRO/NANOPARTICLES AS DRUG CARRIERS FOR THE TREATMENT OF SKIN DISEASES

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

BIOPOLYMER BASED MICRO/NANOPARTICLES AS DRUG CARRIERS FOR THE TREATMENT OF SKIN DISEASES

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Controlled drug delivery systems are becoming increasingly interesting with the contribution of nanotechnology. In the case of transdermal applications the greatest limitation is the highly impermeable outermost layer of the skin, the stratum corneum. One promising method of controlled transdermal drug delivery of the skin therapeutics is the use of nanoparticles as carriers. Encapsulation of the drug, as opposed to classical topical application of creams or emulsions, allows the drug to diffuse into hair follicles where drug release can occur in the deeper layers of the skin.

The aim of this study was to develop micro and nano sized carriers as drug delivery systems to achieve treatment for skin conditions like psoriasis, aging or UV damage, caused by radiation or health problems. Two different types of bioactive agents, retinyl palmitate (RP) and Dead Sea Water (DSW), were used by encapsulating in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) carriers.

In some tests $MgCl_2$ was used as a substitute for DSW when quantification was needed. Bioactive agent loaded nanospheres and nanocapsules were prepared with o/w and w/o/w methods in low micron (1.9 µm), mid nano (426 nm) and nano (166 nm) sizes. Loading, encapsulation efficiency and release kinetics were studied. The encapsulation efficiency and loading values are low especially for the water soluble agents, DSW and $MgCl_2$. It was observed that the capsules loaded with hydrophilic agents released their content in the first 24 h in aqueous media. The encapsulation efficiency and loading values for RP were higher because of the insolubility of the agent in water.

In the *in vitro* studies carried out with L929 mouse fibroblast cells, the nano sized PHBV capsules were detected in the cytoplasm of the cells. Cell viability assay (MTT) for L929 cells showed a growth trend indicating that the particles were not cytotoxic and the values were close to the controls.

Hemolytic activity was examined using human erythrocytes and micro/nanoparticles of PHBV were found to be non hemolytic.

In vivo testing with BALB/c mice, nanocapsule penetration revealed that a small amount of nano sized particles penetrated the mice skin, despite the highly impermeable outer skin layer.

As a result, PHBV micro/nanoparticles have a significant potential for use as topical drug delivery systems in the treatment of skin diseases.

Keywords: Drug delivery systems, controlled release, biopolymer, microparticles, nanoparticles, skin diseases.

ÖZ

DERİ HASTALIKLARI TEDAVİSİNDE İLAÇ TAŞIYICILAR OLARAK BİYOPOLİMERİK MİKRO/NANOTANECİKLERİN GELİŞTİRİLMESİ

Eke, Gözde

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Kontrollü ilaç salım sistemleri, nanoteknolojinin katkısı ile devamlı artan bir şekilde ilgi çekici hale gelmektedir. Transdermal uygulamalardaki en büyük sınırlama, derinin en dış ve geçirimsiz tabakası olan stratum korneumdur. Deri hastalıklarının tedavisinde ilacın transdermal olarak kontrollü bir şekilde salınması konusunda umut vaat eden yöntemlerden biri nanoparçacıkların taşıyıcı sistemler olarak kullanılmasıdır. Kapsülün içine yüklenen ilaç, kremler ve emülsiyonların topikal olarak kullanılması şeklindeki klasik yöntemlerden farklı olarak, ilacın derinin daha alt tabakalarında salınabilmesi için kıl köklerine nüfuz etmesine izin vermektedir.

Bu çalışmanın amacı, radyasyon ya da sağlık problemleri nedeniyle ortaya çıkan sedef hastalığı, derinin yaşlanması veya UV hasarı gibi deri sorunlarının tedavisi için mikro ve nano boyutlu ilaç taşıyıcıların geliştirilmesidir.

İki farklı biyoaktif ajan, retinil palmitat (RP) ve Lut Gölü Suyu (Dead Sea Water, DSW), poli(3-hidroksibütirat-ko-3-hidroksivalerat) (PHBV) taşıyıcılara yüklenerek kullanıldı.

Bazı testlerde, miktar ölçümü gerektiğinde DSW yerine $MgCl_2$ kullanıldı. Biyoaktif ajan yüklü nanoküreler ve nanokapsüller, düşük mikron (1.9 µm), orta nano (426 nm) ve nano (166 nm) boyutlarda o/w ve w/o/w yöntemleri kullanılarak hazırlandı. Yükleme, kapsülleme verimi ve salım kinetiği incelendi. Kapsülleme verimleri ve yükleme değerleri, özellikle suda çözünen ajanlardan DSW ve $MgCl_2$ için düşüktü. Hidrofilik ilaç yüklenen kapsüllerin içeriklerini sulu ortamda ilk 24 saat içinde saldığı gözlemlendi. RP için bulunan kapsülleme verimleri ve yükleme değerleri, molekülün su içinde çözünmemesinden ötürü daha yüksek çıkmıştır.

L929 fare fibroblast hücreleri kullanılarak yapılan *in vitro* çalışmalarda, nano boyuttaki PHBV kapsülleri hücre sitoplazmasında gözlemlendi. L929 hücreleri ile yapılan hücre canlılık deneyi (MTT), taneciklerin toksik olmadığını ve değerlerin kontrollere yakın olduğunu göstermiştir.

Hemolitik aktivite, insan eritrositleri kullanarak incelenmiş ve PHBV mikro/nanoparçacıklarının hemolitik olmadığı saptanmıştır.

BALB/c fareler ile yapılan *in vivo* çalışmaları, yüksek derecede geçirimsiz olan dış deriye rağmen, nano boyutlu kapsüllerin düşük miktarlarda da olsa farelerin derisine girdiğini göstermiştir.

Sonuç olarak, PHBV mikro/nanoparçacıklarının, deri hastalıklarının tedavisinde topikal ilaç salım sistemleri olarak kullanılabilme potansiyeline sahip olduğu görülmüştür.

Anahtar Kelimeler: İlaç salım sistemleri, kontrollü salım, biyopolimer, mikroparçacıklar, nanoparçacıklar, deri hastalıkları.

Dedicated to Özge EKE …
For breathing safely for 23 years

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BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscope
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSW	Dead Sea Water
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
MW	Molecular Weight
NC	Nanocapsule
NS	Nanosphere
PBS	Phosphate Buffered Saline
PEG	Poly(ethylene glycol)
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PVA	Polyvinylalcohol
RP	Retinyl Palmitate
SEM	Scanning Electron Microscope
TCPS	Tissue Culture Polystyrene
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Skin

The skin is the largest organ in the body, covers the area of 1.7 m^2 and constitutes 16% of the body weight. The main function of the skin is to protect body from the external environment such as microorganisms, UV radiation, toxic agents and shearing forces (Ro and Dawson 2005). It prevents the loss of water and electrolytes and helps control the temperature of the body. The thickness and color of skin varies in different parts of the body. Several studies show that the pH on the surface of healthy, undamaged skin of adults is slightly acidic (about 5). Lactic acid, butyric acid, amino acids, and free fatty acids are secreted onto the skin through sweat and decrease the surface pH (Parra and Paye, 2003).

1.1.1. Structure and Organization of Skin

The skin is basically a three layered tissue: epidermis, dermis and subcutaneous tissue. The epidermis is the outer layer and serves as a barrier between the body and the environment. The dermis, which gives the skin its mechanical strength, is the thickest structure of the skin and consists of collagen fibers and glycoprotein filaments embedded in amorphous connective tissue (Figure 1.1). Capillaries in the dermis constitute a vascular surface of 1- 2 cm^2 of skin surface and serve the exchange of substances between blood and skin.

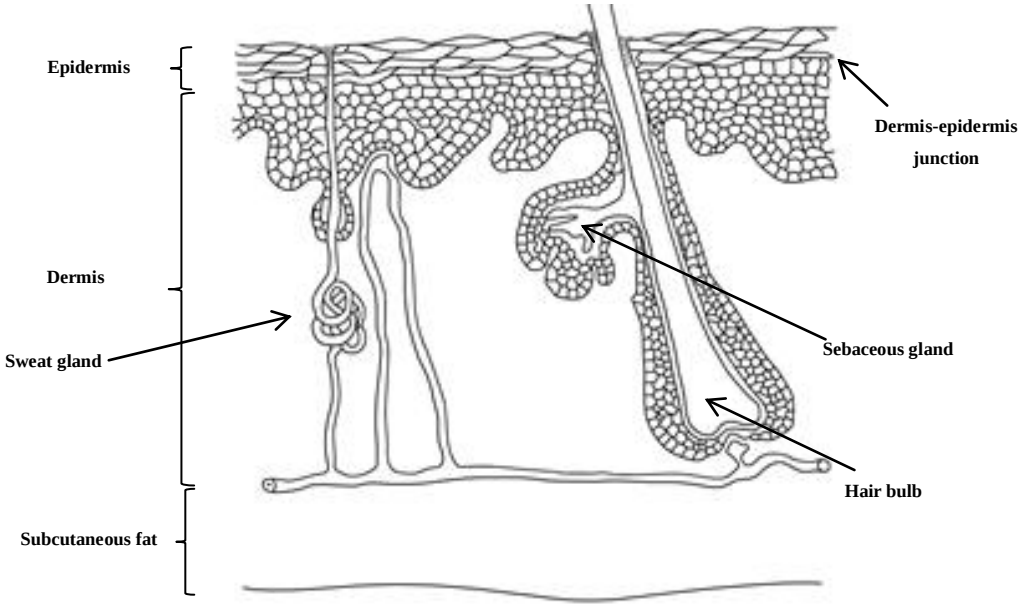

Figure 1.1. Cross section of skin.

1.1.1.1. Subcutaneous Tissue

Beneath the dermis there is a layer of loose connective tissue and fat called subcutaneous fat layer. It contains blood vessels that provide nutrition and control the body temperature, and nerves that provide sensations. This layer also contains fat cells. Subcutaneous fat protects the body from cold, reserves nutrition, and cushions the body from physical trauma (Gawkroder et. al., 2002).

1.1.1.2. Dermis

Dermis is the tough, fibrous layer of the skin. It consists of collagen fibers, elastic fibers, ground substance (glucosaminoglycans), fibroblasts, dermal dendrocytes (skin dendritic cells known as Langerhan cells), mast cells, histiocytes, blood vessels, nerves, and lymphatics (Sneddon and Church, 1971). The thickness of dermis is 1–2 mm (Prausnitz and Langer, 2008).

Collagen fibers (collagen types 1 and 3) provide the mechanical support to the skin. Ground substance holds water, and helps nutrient, hormon, and fluid

transfer through the dermis. This layer is tightly connected to the epidermis, the most upper layer of the skin through the basement membrane (Wolf et. al, 2008).

1.1.1.3. Epidermis

This layer does not contain any blood vessels, obtains nutrient from the blood vessels in dermis through the dermis-epidermis junction. Eighty percent of the cells in the epidermis are derived from keratinocytes, the cells that carry intracellular keratin. This fibrous protein forms a cytoskeleton that through a supracellular network create cell-to-cell contact between the keratinocytes (Buraczewska, 2008). Keratinocytes are continuously generated at the innermost layer of the epidermis. The other prominent cells are melanocytes,

The cells that originate from keratinocytes are flattened cells in the stratum spinosum and corneocytes in the stratum corneum. The corneocytes are arranged into 15 to 25 cell layers in a regular pattern, and are surrounded by a lipid matrix of primarily neutral lipids that form stacked bilayers of membranes (Wolff, 2008).

1.1.1.4. Stratum Corneum

The stratum corneum is 10–20 μm thick and it is the most essential layer of epidermis as a barrier and protective layer (Wolf et. al., 2008). It forms a relatively impermeable layer at the surface of skin that restricts the evaporation of water and the penetration of molecules across the skin (Gawkrodger, 2002). Stratum corneum consists of the flat, hexagonal shaped keratinized corneocytes and intercellular domains rich in lipid. This lipoid layer forms a continuous structure surrounding the corneocytes. Since transference of substances has to be through these lipoid domains their roles in the barrier function of the skin is very critical. Stratum corneum has a very low water content. This water is found in the corneocytes (Prausnitz and Lagner, 2008).

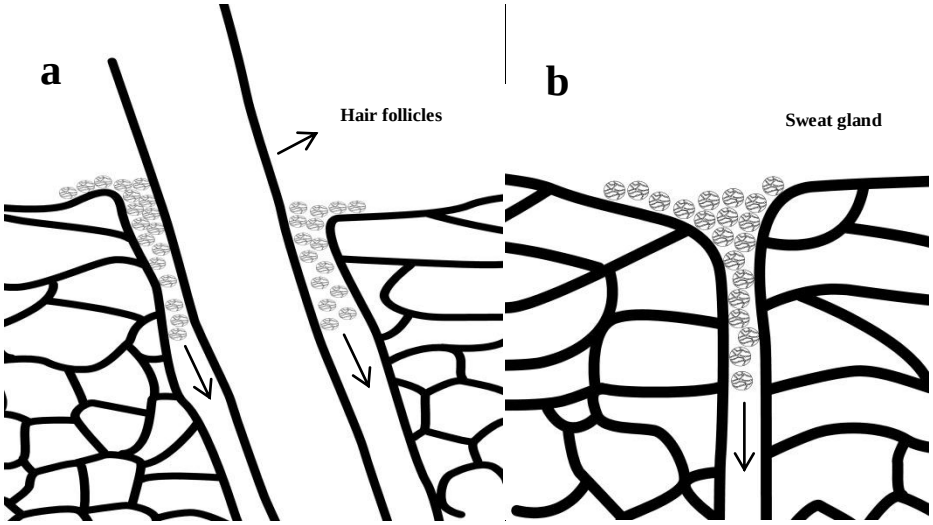

Figure 1.2. Penetration of nanoparticles through stratum corneum. Penetration from a) hair follicles, b) sweat glands

The lipid matrix and the corneocytes, together, create a barrier, which is broken by hair follicles (Figure 1.2a) and sweat glands (Figure 1.2b). These components cover less than 1% of the surface area, are believed to serve as a route of entry for nanoparticles.

1.2. Skin Diseases

Skin disorders may be generalized or localized to one or several sites of abnormality known as lesions that appear on the skin. A common sign of skin diseases is redness or erythema (Sneddon and Church, 1971). Many patients with skin diseases believe that because this sign is on the surface it should be easy to cure. However, this approach reduces the rate of correct diagnosis and treatment, and leads to a social isolation and depression of patients.

Skin is a mirror for the internal organs. Therefore, scars, inflammations and discoloration on the skin may also indicate systemic disorders (Lebwohl, 1988). There is a broad spectrum of skin diseases, and only aging, cancer, psoriasis, eczema and UV damages will be discussed in this section.

1.2.1. Aging

There is very little information on why skin ages. However, there are two components of aging: intrinsic and extrinsic. Intrinsic aging is caused by hormones, genetic make up of the patient and passage of time. Extrinsic aging is caused by the environmental factors, especially solar radiation which especially is damaging in the form of UV-A and UV-B.

With aging, both the epidermis and the dermis become thinner. The degree of thinning varies with the location in the body such as scalp, near the eyes, and genitalia. As the thickness of the epidermis is decreased, the keratinocytes shrink and form clumps and the number of blood vessels decrease. Epidermal turnover rate decreases by 50% (Balin et. al., 1989). In the dermal layer, connective tissue loses much of its proteoglycan and the amount of mast cells, collagen and vascular network decreases. Cell size increases, thin and long cells become flattened and irregular. Membrane rigidity increases as seen in the Figure 1.3a. Also some biochemical changes occur in aging skin. The number of damaged, abnormal proteins and level of macromolecular crosslinking increases (Stroikin et. al., 2005). The loss of skin elasticity, dermal thickening, and elastic degradation results with wrinkling. The amount and the depth of the wrinkles increase with the increasing age (Figure 1.3b).

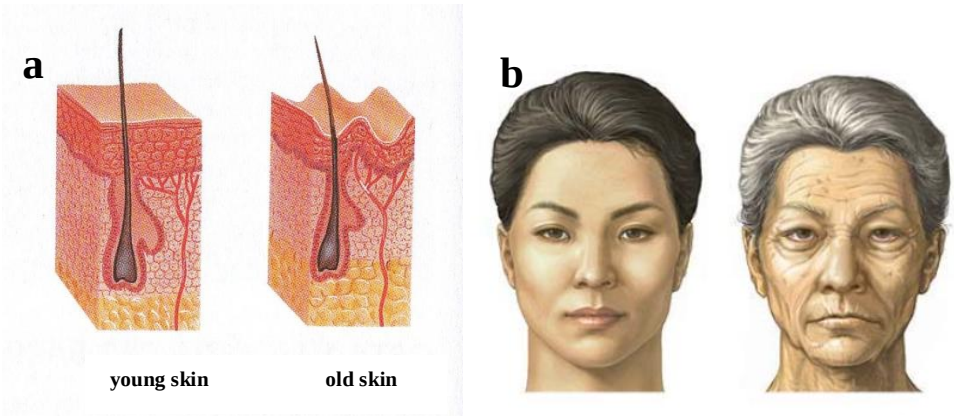

Figure 1.3. Aging of the skin. a) Scheme of skin aging showing structural changes (Ostrov, 1999). Decreased number of blood vessels, dermal and epidermal thinning, changed cell shape and wrinkle formation is seen. b) Texture changes caused by skin (https://bohone09.wikispaces.com/Group)

Environmental conditions such as exposure to sun, smoking, and stress can trigger early skin aging. In order to resist wrinkles, generally non-surgical ways such as botox, filler substances, and nonablative lasers are used. Sometimes more aggressive methods such as chemical peeling, dermabrasion, and ablative lasers or more traditional, surgical procedures such as, liposuction, face and neck lifting can be used.

1.2.2. Cancer

There are three types of commonly known skin cancers; basal cell cancer (BCC), squamous cell carcinoma (SCC), and melanoma. Each is named after skin cell from which they arise. BCC is a locally invasive malignant epithelial tumor of basaloid cells. SCC is a pink or grey, usually 2–5 mm diameter tumor. Melanoma is an invasive, neoplastic disorder of melanocytes which has a tendency to metastasize the epidermis (Figure 1.4a). All types of skin cancers generally develop in the epidermis, therefore, a tumor can usually be visible from the outside This means that it is often possible to detect skin cancers at an early stage (Marks, 1995). In Figure 1.4b melanoma on the dorsal region of the patient can be seen with the naked eye.

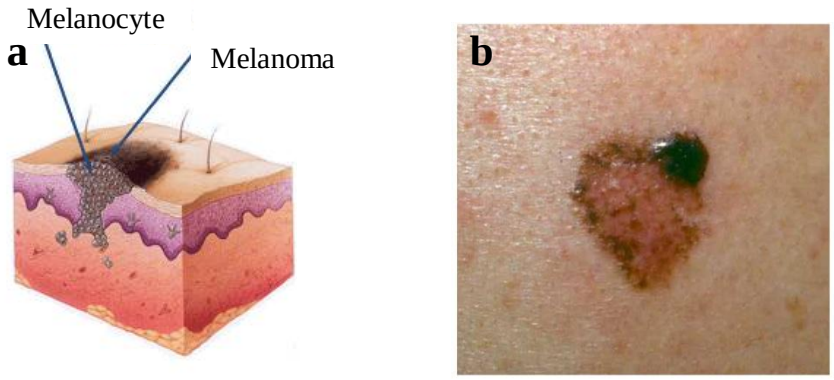

Figure 1.4. Melanoma as a skin cancer type. a) The growth of malignant melanoma on skin, b) visible melanoma on the dorsal region of the patient (http://www. articlesbase.com/cancer-articles/the-increasing-incidence-of-cutaneous-malignant-melanoma-cmm-in-ireland-2871081.html)

Melanoma has a high mortality rate. At advanced stages, conventional therapies such as chemotherapy and radiation are ineffective. The primary cause of skin cancer is the sun and exposure to UV. These cancers are also related with pigmentary features. For example, people with red hair, freckles and who never tan are at a high risk of skin cancer (melanoma, SCC and BCC) (Zaidi and Lanigan, 2010).

Smoking and non-healing wounds also trigger the skin cancer. Treatment is dependent on type and location of the cancer, the age of the patient, and whether the cancer is primary or a recurrence. Along with chemotherapy, surgery, radiation, immunotherapy or combinations of these are used for aggressive cancer types (Roth, 2010).

1.2.3. Eczema

The term eczema covers several disorders. These are atopic dermatitis, seborrhoeic dermatitis, discoid eczema, venous eczema, allergic contact dermatitis, and primary irritant contact dermatitis in which an inflammation occurs on the epidermis and epidermal cells accumulate edema fluid (Figure 1.5). Most of these

types of eczema affect children, adolescents and young adults. Inflammatory cells and vasodilatation are associated with the edema of the dermis of the affected area.

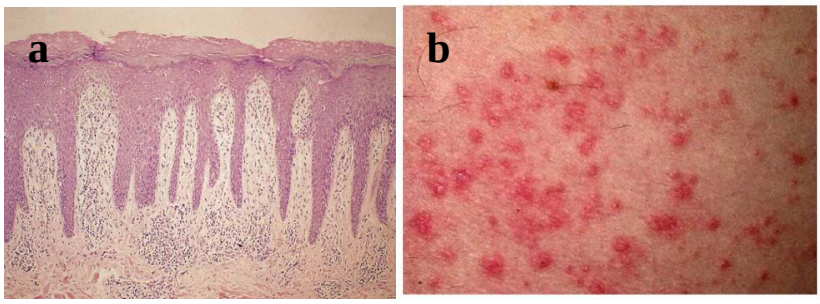

Figure 1.5. Micro and macro images of eczema. a) Micrograph of inflamed, and thickened eczematous area (Marks, 2003), b) atopic eczema on the dorsal region on the patient (http://www.picturesdepot.com/medical/12434/atopic+dermatitis.html)

The cause of eczema may be a microbial infection, low humidity, vigorous washing with detergents, contact with medicaments or scratching the wounds (Marks, 2003). Food allergens, air borne allergens, soaps and detergents, and emotional stress also trigger eczema.

The treatment for eczema must be local, and aim to relieve the inflammation, the itch and infections. Generally topically applied softening skin care products are used to help to hydrate the skin and prevent evaporation and allow repair through reconstruction of new lipids (Leung and Bieber, 2003).

1.2.4. Psoriasis

Psoriasis is a common, chronic inflammatory skin disorder with genetically and immunologically modulated components and may have a profound effect on the quality of life with physical, psychological and social consequences. Psoriasis is not a life threatening disease. Several factors such as infection, medication, physiological stress, local trauma and environmental factors trigger psoriasis and also influence the effectiveness of the treatment. The structural changes such as excess growth of skin cells, formation of plaques and inflammation as seen in Figure 1.6a are common features of psoriasis. Psoriasis is essentially incurable, and both acute and long term treatments are needed. Still,

long term uses of conventional systemic agents are limited due to their toxicity and change efficacy over time (Roth, 2010).

The most popular drugs for psoriasis are corticosteroids because these small molecules can penetrate to the stratum corneum, bind the steroid receptors of the keratinocytes and alter their DNA synthesis (Camisa, 2004). Psoriasis and its treatments may increase the risk of skin cancer, including melanoma (Roth, 2010). Patients with psoriasis perceive a reduction in mental and physical functioning comparable to those with cancer, diabetes, and depression due to the localization of the disease. As seen in Figure 1.6b, the appearance of the psoriatic skin is dramatically different than a normal skin.

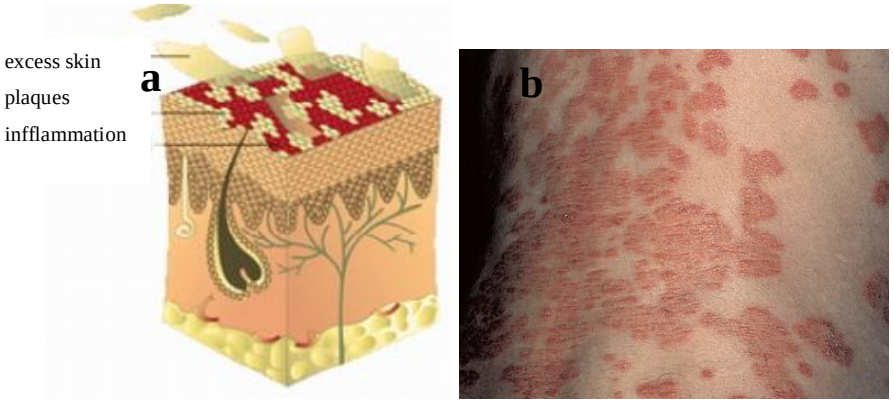

Figure 1.6. Psoriatic skin. a) Cross section where the epidermis gets thicker (http://www.realage.com.tr/v2/Sedef-Hastaligi_3_52.htm), b) Plaques of psoriasis with typical red color (Marks, 2003)

Novel treatments involve fusion proteins, recombinant human interleukins, and monoclonal antibodies that target specific cell types, block T-cell activation and migration into skin (Roth,2010).

1.2.5. Damages due to Exposure to UV Radiation

The sun emits radiation over a broad spectrum, however, the UV radiation (250–400 nm) is the most important factor that damages the skin. There are three ranges of UV radiation: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C

(250–280 nm). UV-C is mostly filtered by the ozone layer and is not biologically important as long as the ozone layer is maintained. UV-A has little short term effects on normal skin and can be mostly tolerated.

UV-B (especially around 290 nm) has the most harmful effect. It is mainly responsible for sun burn, sun tan and skin cancer, although the other two ranges also contribute. It is not possible to have a tanned skin without epidermal damage. Sun burn is recognized by the redness and when severe, by swelling and blistering. The affected area becomes very sore and, if blistered and extensive, makes the human feel sick. UV-B only penetrates as far as the basal layer of the epidermis, however, it causes the death of scattered keratinocytes and damages others so that they release cytokines and mediators. Therefore, it causes superficial burn and the thickness of epidermis may increase two-fold. The thickness of stratum corneum also increases (Figure 1.7a). Cellular proliferation, division rate and the amount of amino acid production decreases (Fisher et. al., 1997, Yin et. al., 2001).

Approximately 2 days after exposure to UV, the rate of melanin synthesis increases. They protect the nuclei of the keratinocytes to prevent the further DNA damage (Zaidi and Lanigan, 2010). However, long exposure to UV-B is absorbed by DNA and cyclobutane pyrimidine dimers form. Also the repair mechanism is unable to restore the damage (Griffiths et al., 1998, Agar et al., 2004). The changes in skin color can be permanent as seen in the Figure 1.7b.

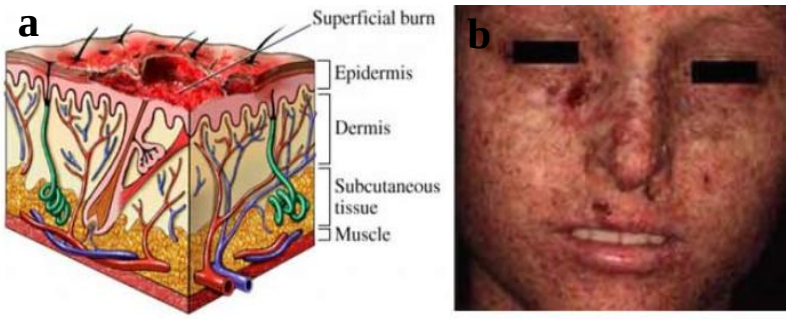

Figure 1.7. UV damaged skin. a) Cross section of long term UV exposure skin (http:/ /www. empowher. com/ media/ reference/ sunburn), b) Damages are visible on UV-exposed skin (http://www.skintreat.eu/index.php?option=com_content&view=article&id=47&Itemid=55)

Not only sunlight but also the commercial tanning lamps cause DNA damage in human cells. Extrinsic aging, known as photoaging is caused by these artificial UV sources. The intrinsic rate of skin aging can also be dramatically influenced by the amount of exposure to ultraviolet light (Puizina et. al., 2008).

1.3. Routes of Administration of Skin Therapeutics

Skin is the most accessible organ to treat. There are many therapeutic approaches to skin disease. These are by topical application of drugs, oral medication, subcutaneous injections, intramuscular and intravenous injections, UV therapy, and surgery. The topical approach is time consuming and large amount of medication is required to produce the desired effect. However, it does not have the side effects of systemic toxicity if used within the required limits.

The physical methods are used in the management of skin diseases such as, phototherapy with psoralens (PUVA) for psoriasis, and eczema, photodynamic therapy for SCC caused by UV damage, surgery for malignant growths.

Systemic applications are still commonly used for skin diseases despite their high doses and broad side effects.

In this section, commercially available administration of drug therapeutics will be discussed.

1.3.1. Oral

Despite many disadvantages, oral route is still a very successful, convenient, and therefore, preferred route of administration for normal drugs. In this application the drug applied has to pass through the highly variable gastrointestinal system. The oral administration has certain limitations because of the special demands of the target site and therefore a case-by-case solution has to be provided.

Methotrexate, cyclosporin and mostly psoralens are used for oral psoriasis treatments. Methotrexate is a folic acid antagonist and an

immunosuppressant especially used in psoriasis. It is given on a one day a week protocol, either as a single dose or the oral triple dose procedure which is the most common method used. The three doses are given at 12 hourly intervals during a 36-h period, once each week. Methotrexate has a toxic effect on the bone marrow and intestinal epithelium. Methotrexate is also hepatotoxic on long term use (Sneddon and Church, 1971). Cyclosporin suppresses cell-mediated immunity. It may also have some direct effect of DNA synthesis and proliferation of keratinocytes. It is used in psoriasis and some cases of eczema treatments and the daily dose is 2-5 mg/kg. Psoralens are very commonly used for psoriasis. They are available as methoxypsoralen and trimethylpsoralen. Psoralens react with DNA on exposure to UV-A, and suppress mast cell degradation. Therefore two hours after oral intake (0.6 mg/kg daily), the patient is exposed to UV-A. This method of treatment is called PUVA and should be given two or three times a week. However, long term applications cause malignant skin cancer (Arndt, 1989).

Azathioprine is a purine analogue that inhibits DNA and RNA synthesis. It is an immunosuppressive drug, and provides steroids. It is used for the treatment of eczema. It is suitable for older patients as a single therapy. Younger patients are not generally treated with azathioprine as a single agent because of the risk of malignancy. 2.5 mg/kg/daily or 150 mg daily doses are required.

In order to treat UV damaged skin, protection from sunlight is important. B-carotene (an active oxygen suppressor) dose is 150 mg/day in most cases of UV damages. Low dose of chloroquine is 125 mg applied twice a week is helpful in a number of cases, too. These drugs are deposited in the liver, form water soluble compounds and are excreted in the urine. Chloroquine is toxic in higher doses and cause irreversible ocular changes. Psoralens are used to increase skin pigment production, and thereby, help in tanning.

In the treatment of UV damaged skin, daily use of vitamin C (2 g), and vitamin E (1000 unit) has been shown to reduce skin reactions and erythema (Arndt, 1989).

1.3.2. Subcutaneous

Immunosuppressive drugs have been used in severe cases of skin diseases. The toxicity of these drugs limits their use via subcutaneous administration.

Efalizumab (a recombinant humanized monoclonal IgG-1 antibody) is commonly used by subcutaneous administration for psoriasis treatment. It inhibits interaction and activation of T cells. It is given as 0.7 mg/kg in the first dose and then in 1 mg/kg weekly doses. However, this type of recombinants must be used in highly needed patients in long term applications due to thrombocyte decreasing (Roth, 2010). Etanercep is a fusion protein that inhibits cytokines and psoriasis is treated with this drug by subcutaneous injection. The drug is given in doses of 25 mg twice a week. It has more side effects than Methotrexate (Zaidi and Lanigan, 2010).

For eczema, injection of corticosteroids under the skin can also be used. However, they have significant side effects: skin becomes thinner and fragile, and therefore becomes very sensitive to further applications of medicine (Atherton, 2003).

In order to treat skin aging, botox injection and application of fillers are mostly subcutaneous. Fillers can be biodegradable (12–18 months), slowly biodegradable (2–5 years), or permanent. Autologous fat, human collagen, bovine collagen, and hyaluronic acid are used as biodegradable fillers. Calcium hydroxylapatite and poly-(L-lactic acid) are for slowly biodegradable applications and polymethylmethacrylate is for permenant fillers (Carruthers et. al.2008).

1.3.3. Intramuscular

In melanoma treatments, drugs are administered intramuscularly. However, tracing studies suggest that this route of injection may significantly affect the immune response (Roth, 2010). Kyte et. al. (2006) investigated the

dendritic cell vaccine delivery for melanoma therapy. Treatment was well tolerated, metastases were prevented, however, the toxicity of vaccines was recorded on the 77 % of the patients. Therefore, this route of vaccine injection is still under investigation.

Alefacept is a fusion protein that binds T cells and causes apoptosis of memory T cells. It is used in the treatment of psoriasis. It is given in a dose of 10–15 mg every week for 12 weeks by intramuscular injection.

In the use of corticosteroids intramuscularly for the treatment of psoriasis and eczema, the age of the patient, disease severity, and type and extent of surface area involvement must be considered. Typical side effects associated with intramuscular injection include mood changes, nervousness, insomnia, gastrointestinal intolerance, muscle weakness, increased risk of infections, acne, glucose intolerance, and slower wound healing (Del Rosso and Friedlander, 2005).

1.3.4. Intravenous

Intravenous (i.v.) administration is mostly used in various types of skin cancers. In the case of cancer that has spread (metastasized), surgical procedures or chemotherapy are used as mentioned in the section 1.2.2. Intravenous administration of these drugs needs to be developed, because the drug is delivered through the veins are efficient and precise. However, targeting is difficult and the concentration of the drug in the diseased site and in the healthy tissue is similar. An aggressive chemotherapy is required for skin cancer types like melanoma. The fluctuation of the drug level, significant side effects of aggressive cancer drugs and the cost of the treatments limit the use of this i.v. route (Roth, 2010). For non-malignant skin cancer types generally alpha-2β is injected twice a week. This drug is suitable only for very large lesions where surgery is not possible (WHO, 2005).

This route was also used in the treatment of psoriasis and inflamed eczema. Infliximab is a monoclonal antibody that triggers complement mediated lysis of T cells. It is used in a number of disorders, especially in psoriasis and eczema. It is given in a dose of 5 mg/kg by an intravenous infusion at 0, 2, and 6 weeks. It can be repeated every 4–6 weeks, but there is an increased risk of serious

infections during therapy. Amphotericin B is also given by intravenous infusion in a dose of 0.4–1 mg/kg/day for inflamed eczema. Side effects of amphotericin B are anorexia, nausea, vomiting, bronchospasm, and hypotension (Lebwohl, 1988).

1.3.5. Transdermal delivery

Transdermal drug applications are always preferred because of the effectiveness of the localized treatment, low cost, relatively low side effects, and maximum drug availability at the target site. Topical delivery, is very promising because of avoiding systemic circulation. Many potential drugs for topical delivery, however, do not have the necessary physicochemical properties for satisfactory efficacy (Sloan et. al., 2006).

Transdermal skin treatment involves the absorption of drug through the skin into the body. Research in these transdermal drug delivery systems has received much attention in the past thirty years. One of the biggest challenges in developing an effective system is to get past the tightly structured stratum corneum if the skin is not compromised (Yow et. al., 2009). Encapsulation of the drug in a carrier allows the drug diffusing into hair follicles where drug release can occur in the deeper layers of the skin (Arora et. al., 2008).

Transdermal delivery has a number of advantages over the other routes. Especially, it is used when there is a significant ‘first-pass effect’ the liver which tends to metabolize drugs. In addition, transdermal systems are non-invasive and easy enough to be self-administered. If properly designed they can provide release for long periods (up to one week) (Prausnitz and Langer, 2008).

Transdermal delivery methods are classified in three ‘generations’ according to their enhancement strategies. The first of these three generations is constituted by the patches that were loaded with drugs. The drugs selected for this application can cross the skin with little or no enhancement. The second generation involves use of increased skin permeability and driving forces for transdermal transport. This generation of delivery has additional advances for small molecules. The third generation enables the transdermal delivery of low molecular

weight drugs and macromolecules (such as proteins and DNA) other vaccines through targeted permeabilization of the stratum corneum.

1.3.5.1. First Generation Transdermal Drug Delivery

The first generation of transdermal delivery systems consists of most of the transdermal patches that have been in clinical use for quite sometime (Morgan et. al., 1998). Drugs for first generation delivery systems must be low molecular weight, lipophilic and have high efficacy. The use of first generation transdermal delivery systems in the clinical studies for delivery of small, lipophilic, lower dosed drugs is increasing. Usually, the transdermal delivery of the drugs is more suitable than oral delivery due to their low oral bioavailability. However, the first generation approach to transdermal delivery is limited by the stratum corneum (Figure 1.2). (Prausnitz and Langer, 2008). A variation of first generation delivery systems not only involves patches, but covers also liquid sprays, gels or other topical formulations that, upon evaporation or absorption, can drive small lipophilic drugs into the stratum corneum, which then becomes a drug reservoir for extended release into epidermis. For example, sex hormone gels and sprays have been in use for several years (Morgan et. al., 1998).

1.3.5.2. Second Generation Transdermal Delivery

The second generation of transdermal delivery systems takes into consideration the need for skin permeability enhancement. The ideal enhancer should increase skin permeability, and avoid injury to deeper, living tissues. All the enhancement methods developed in this context, such as conventional chemical enhancers (Karande et. al., 2005), iontophoresis (Zempsky et. al., 2004) and ultrasound (Park et. al., 2007) must achieve this balance. The ability of iontophoresis to control delivery rates is still in progress.

1.3.5.3. Third Generation Transdermal Delivery

The third generation of transdermal delivery systems targets its effects to the stratum corneum and involves electrically assisted devices. This approach leads to a stronger disruption of the stratum corneum, and thereby, to more effective transdermal delivery, while protecting deeper tissues. This type of transdermal delivery system includes electroporation (Zhao et. al., 2006), ultrasound (Becker et. al., 2005), microneedles (Wermeling et. al., 2008), thermal ablation (Badkar et. al., 2007) and microdermabrasion (Glenn et. al., 2007). This generation of drug delivery is more advantageous than the other two because it is able to deliver macromolecules, including therapeutic proteins, vaccines and hormones, across the skin.

1.4. Transdermal Particulate Drug Delivery Systems

In addition to the approaches mentioned above where generally creams and ointments are used and achieve controlled release to a limited degree, more controlled delivery of drugs can be achieved when the active agents are transported in nanoparticles made using a number of materials. Below they will be presented.

1.4.1. Inorganic Materials

Transdermal studies involving inorganic nanoparticles, such as nanotubes, fullerenes, quantum dots and magnetic nanoparticles are increasingly being used in nanomedicine and transdermal studies (Baroli, 2010). These nanomaterials are mostly used in creams. Silver is very popular for transdermal delivery purposes due to its antimicrobial activity and ease of modification of its surface (Kath et. al., 2011). It was reported that silver containing fibers provide protection against some common bacteria found on burn wounds (Rujitanaroj et.

al., 2008). Since it is commonly used on burn wounds, there are a number of wound dressing products that are available on the market such as Acticoat®. Wound dressings serve as drug release reservoir and provide protection against bacteria and also they have high permeability towards oxygen. This specific wound dressing consists of a polyester core coated with an upper and lower layer of silver particles loaded in a mesh. These layers are designed to be barriers against microbial infection (Holder et. al., 2003).

Generally quantum dots and magnetic particles are used for penetration ability and imaging purposes but not for treatment (Stolyar et. al., 2006, Goope et. al., 2009).

1.4.2. Liposomes

Liposomes are nano and microparticles that are commonly used for transdermal studies. There are number of approved liposome containing creams.

Liposomes when used topically may serve as a solubilization media, as a local store for dermally active compounds for sustained release, and as a chemical enhancer. The interaction mechanism between the liposome and skin were investigated and researchers proposed that a direct contact between the liposome and skin is essential for efficient delivery of its contents (Schreier and Bouwstra, 1993).

At the moment the most promising delivery systems for skin cells are based on liposomes and polymers, by which the improvements with liposomes seems to be more advanced.

1.4.3. Polymer Based Systems

Particles prepared from polymers, consisting of micro and nanospheres or capsules, have been developed for the controlled release of the entrapped drug. These carriers may be administered at all body locations. They can be found as particulate drug carriers in emulsions or creams in matrices, in patches and in wound dressings. Besides all these polymers (such as PEG) may be used as

permeation enhancers. Recently, Puglia et. al. (2010) investigated skin penetration ability of polymeric particles. According to their study, hydrophobicity of the polymer and the particles size have important impact on the penetration.

There are two sources of polymers used in drug delivery studies: synthetic and natural, and these will be provided below.

1.4.3.1. Synthetic Polymers

The advantages of synthetic polymers can be stated as low risk of immunogenicity and disease transmission, easier processibility and higher flexibility (Lee et. al., 2007). The most popular synthetic polymeric materials for transdermal studies are polyethylene glycol (PEG), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers poly(lactic acid-co-glycolic acid) (PLGA).

PLA, PGA and their copolymer PLGA are FDA approved and therefore are the most commonly used synthetic polymers in transdermal studies. Since their degradation products are acidic (lactic acid and glycolic acid), they are not only used in skin diseases but also for responsive release purposes (Lazerri et al., 2005). Rollan et. al. (1993) studied PLGA microspheres in an aqueous gel that was applied to rabbit skin and to human volunteers, and it was found to be safe to be used on skin.

PEG is known as a popular chemical enhancer especially when used in low concentrations (Mitragotri, 2000). Goope et. al. (2009) studied skin penetration of PEG coated CdSe quantum dots in dermabraded (skin abraded) mouse skin. They detected significant increase in the level of cadmium in the lymph nodes and liver of mice pretreated with dermabrasion.

Some synthetic polymers are used for scar treatments. For example, silicone gels are used for some of these scar treatments (Mustoe et. al., 2002). There are two commercially available products named Silastic Gel Sheeting® and Cica-Care® in the treatment of hypertrophic scars. The efficacy of the two gels is similar. Vitamin E can be also added into silicone gels in order to heal the scar faster (Palmieri et. al., 1995).

1.4.3.2. Natural Polymers

Natural polymers are usually biodegradable and have excellent biocompatibility, but their properties differ with the source (Pillai et al., 2001).
The most widely used natural polymer is collagen which is the predominant protein in the body and has excellent biocompatibility (Cascone et al., 1995). Many systems were constructed using collagen for drug delivery (Yannas, 1980). It was used in wound and burn dressings (Marks et al., 1991).

Gelatin is more convenient to use than collagen and is known to have no antigenicity while collagen expresses some under physiological conditions. Also, it is easier to prepare concentrated solutions of gelatin than that of native collagen. Finally its low cost is also a great advantage of gelatin for use in skin treatments. It was reported that gelatin microspheres loaded with epidermal growth factor (EGF) prepared for wound dressing purposes had a positive effect on wound healing process as was shown in *in vivo* studies (Ulubayram et. al., 2001). It is also possible to build up a three dimensional networks using gelatin.

Chitosan is a nontoxic, inexpensive and biodegradable natural polymer and it can also be used in hydrogel formation. By modifying the degree of acetylation and molecular weight, the hydrophilicity and mechanical properties of chitosan can be adjusted to fit the requirements (Valenta and Auner, 2004). For example, it was reported that chitosan-EDTA composition showed a high anti-microbial activity against skin irritation when compared to different polymeric gels (Valente et. al., 1998).

Hyaluronic acid is a polyanionic, natural polymer that is mostly present in connective tissues. It is non-immunogenic, biodegradable and has viscoeleastic properties, and therefore, it has been considered to be ideal for topical applications. Moreover, chitosan and hyaluronic acid mixtures are also used in wound healing (Weindl et. al., 2004, Xu et. al., 2007). Chondroitin sulfate is also a polyanion and used in wound dressings. Kirker et al. (2002) applied crosslinked chondroitin

sulfate hydrogels on injured mice skin. They observed a significant re-epithelialization on their skin after 10 days.

Another material used for skin therapies is silk fibroin. Min et al. (2004) fabricated these nanofibers by electrospinning and prepared a scaffold for growing human keratinocytes and fibroblasts for use as a wound dressing and tissue engineering scaffold.

Today large amounts of such natural compounds are available in standard quality due to the improvement in analytical and purification methods, and therefore, they are expected to be used more in the future (Valenta and Auner, 2004).

1.4.3.3. PHAs

Polyhydroxyalkanoates (PHAs) are the most commonly used natural biotechnological polymers, because they are produced by bacteria as well as by other organisms some of which are transgenic (Hasirci and Yucel, 2007). PHAs (Figure 1.8) are linear, thermoplastic, bioresorbable and biocompatible polymers of microbiological origin which have a certain degree of crystallinity and biodegradability (Ammas et al, 1998, Sudech et al., 2000, Martin and Williams, 2003, Volova, 2004, Volova et al, 2003 and 2006, Hasirci et al., 2006) and as such have the advantage over the polylactides of being synthesized from non-petroleum origin such as transgenic plants and by bacteria. The crystallinity and degradability of the biopolymer is determined by the composition. The mechanical properties, crystallinity and solubility of the polyesters are controlled by their composition and the molecular weight and heterogeneity index (the molecular weight distribution in a given batch) (Nair et. al., 2006). Thus, changing PHA composition allows mechanical properties, biocompatibility, and degradation times suitable for the desired application.

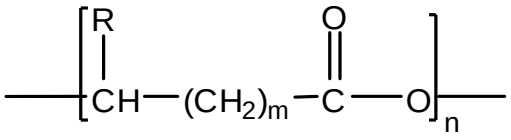

Figure 1.8. General molecular structure of polyhydroxyalkanoates. When m=1, and R=CH_3, the product is poly(3-hydroxybutyrate) (P3HB), and when R=CH_2CH_3 the product is poly(3-hydroxyvalerate) (PHV)

They are rapidly hydrolyzed in the environment but more slowly in the human body and this rate could be regulated by varying the composition, form and the size of the biomedical device. Over the past years, PHA and its composites were used in many forms (sponges, films, fibers, tubes, etc.) in many different devices (Figure 1.9). The most abundantly tested PHA is poly(3-hydroxybutyrate) (P3HB) and its copolymers with 3-hydroxyvalerate, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), with varying proportions of HV. PHBV has been used in the controlled release systems (in the delivery of anticancer agents, pain relievers, antibiotics, growth factors), biodegradable bone plates, and in tissue engineering (of cornea, bone, cartilage) applications (Hasirci and Yucel, 2007).

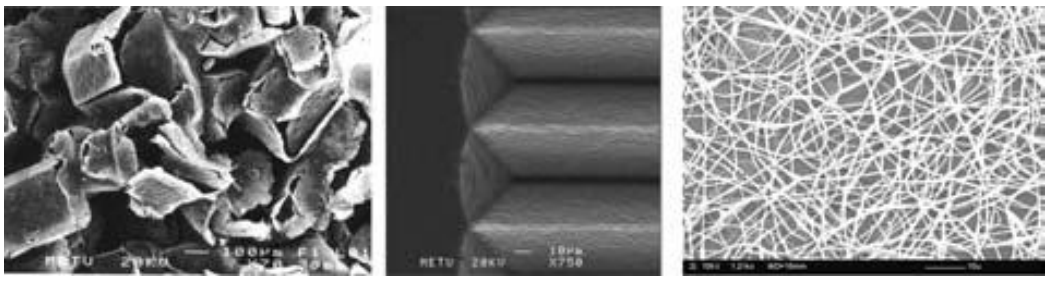

Figure 1.9. SEM of different forms of PHBV used in biomedical field. a) sponge, b) micropatterned film, c) electrospun fibers (Yilgor et. al., 2007).

Poly(3-hydroxybutyrate) (P3HB) is the PHA homopolymer that was investigated most intensively and for various applications (Shishatskaya et. al., 2005). Poly(R-3-hydroxybutyrate-co-R-3-hydroxyhexanoate) (PHBHHx), medium chain length PHA copolymer, has also been investigated extensively for biomedical applications (Qui et. al., 2004).

There have been some studies where PHB and PHBV were used to examine their *in vivo* degradation and biocompatibility (Shishatskaya et. al., 2004). Table 1.1 shows PHA types produced by microorganisms (Gursel, 1995).

Table 1.1. PHAs produced by microorganisms (Gursel, 1995).

Monomer	**R-Group**	**Polymer**
β-hydroxybutyrate (HB)	CH_3 (methyl)	PHB
β -hydroxyvalerate (HV)	CH_2CH_3 (ethyl)	PHV
β -hydroxycaproate (HC)	$CH_2CH_2CH_3$ (n-propyl)	PHC
β -hydroxyheptanoate (HH)	$CH_2(CH_2)_2CH_3$ (n-butyl)	PHH
β -hydroxyoctanoate (HO)	$CH_2(CH_2)_3CH_3$ (n-pentyl)	PHO
β -hydroxynonanoate (HN)	$CH_2(CH_2)_4CH_3$ (n-hexyl)	PHN
β -hdroxydecanoate (HD)	$CH_2(CH_2)_5CH_3$ (n-hepyl)	PHD
β - hydroxyundecanoate (HUD)	$CH_2(CH_2)_6CH_3$ (n-octyl)	PHUD
β -hydroxydodecanoate (HDD)	$CH_2(CH_2)_7CH_3$ (n-nonyl)	PHDD

Among the PHAs used in biomedicine PHBV has a special importance (Figure 1.10). Like the other PHAs, it is degraded in the biological environment to the end products carbon dioxide and water.

Depending on the requirements of different applications, PHBV can be surface modified, turned into a composite or blended with other polymers or inorganic materials, and treated with enzymes to further adjust their properties.

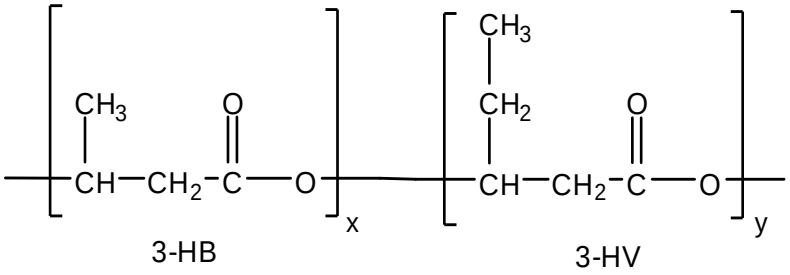

Figure 1.10. Chemical formula of PHBV. x and y can be in the range of several thousands.

1.5. Micro and Nanoparticles for Drug Delivery

Nanotechnology is focused on processes and materials at the atomic and molecular level. Main focus of this area is the scale of 1-100 nm range (Farokhzad and Langer, 2009), but in the biomedical field, structures in the 1-1000 nm range are considered as the nanomaterials. Within the broad category of nanoparticles, "nanospheres" refers to spherical particles with full interior while "nanocapsules" apply to particles which have a core-shell structure. The core may be left empty or loaded with solid or liquid drugs. It is usually assumed that a formulation described as a nanosphere is comprised of a mixture of polymer and active agent, whereas nanocapsules have two discrete domains into which active agents of different chemistries can be loaded.

1.5.1. Contribution of Nanotechnology to Drug Delivery Systems

Nanotechnology has a wide range of applications in a number of fields, such as fiber and textile industry (Dubas et. al. 2006), agriculture (Gonzales et. al., 2008) electronics (Kruis et. al., 1998), forensic science (Choi et. al., 2008), and medical area (Salata et. al., 2004, Rieux et. al., 2006, Davidson et. al., 2009) Application of nanotechnology in engineering sciences and medicine has created opportunities in new interdisciplinary fields. Two important such fields are nanobiotechnology and nanomedicine, which are described as the application of principles of nanotechnology to biological systems and to medicine, respectively

(Roco et. al., 2003). An important application in this direction is the nanoscale drug delivery systems. Nanoparticles were developed as an important strategy to deliver conventional drugs (Gursel et. al., 2000), recombinant proteins (Jahanshahi et. al., 2008), vaccines (Rieux et. al., 2006), nucleotides (Xio et. al., 2009) and growth factors (Yilgor et. al., 2009). Nanoparticles and other colloidal drug delivery systems modify the release kinetics, body distribution (Wolbank et. al., 2006) and release kinetics of an encapsulated drug (Yilgor et. al., 2009). Other important applications are tissue or cell specific targeting of drugs (Rensen et. al., 2001), the reduction of unwanted side effects through controlled release (Yousefpour et. al., 2011, Gardikis et. al., 2011) and the large therapeutic index (Kayser 2005). The above cited advantages are found in most controlled release systems. The special advantage of nanoparticles is their ability to reach tissues that other controlled release systems cannot and to release their contents there rather than staying in the circulation and releasing their content in the blood stream. Another property of nanoparticles is their high surface-to-volume ratio allowing faster release of drugs into the medium. These are especially important in anticancer agent delivery because solid tumors are known to have leaky blood vessels (enhances extravasation) and poor lymphatic removal (prolonging the bioavailability of the drug at the release site).

With the developments in nanotechnology and their introduction to the biomaterials field, various types of nanosized drug delivery systems were developed. Among these are the uses of nanosized drug carriers constructed from biodegradable polymers (Feng, 2006, Hasirci et al., 2008, Yilgor et. al., 2009).

Nanoparticles were used in the delivery of DNA (Kumar et al., 2004), antisense RNA (siRNA) and antitumor drugs (Park et al., 2005a), and in the delivery of substances across the blood brain barrier (BBB), a property which is not found in many other drug carriers (Olivier, 2005). Due to their small size they can easily penetrate the capillary walls, and the tight territorial/extracellular matrix. Because of this high penetrating ability, local delivery of nanoparticles can be realized in the necessary locus.

The “nanoness” of the systems impart three important properties to the designed drug delivery systems:

- A high rate of release due to increased surface-to-volume ratio,
- Extravasation leading to release of the drugs in the regions with compromised vasculature.
- Ease of transportation across biological membranes including the blood brain barrier (BBB)

1.5.2. Preparation Methods for Polymeric Nanoparticles

The most widely used polymers in drug delivery applications are biodegradable, biocompatible and FDA approved ones. Poly(D,L-lactide) (PLA), poly(ε-caprolactone) (PCL), poly(L-glycolide) (PLG) and poly(lactide-co-gycolide) (PLGA), alginate and chitosan are among these. Another polymer which will be focused on in this study is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), which is considered to be a promising biopolymer for use in drug delivery systems (Volova et. al., 2004, Yilgor et al., 2009).

In the preparation of nanoparticles, the use of a surfactant, typically 0.2-2 % (w/v) (Poletto et. al., 2008, Legrand et. al., 1999) is recommended because of the tendency of nanoparticles to aggregate (Poletto et. al., 2008). One of the most widely used polymeric surfactants is polyvinyl alcohol, which is an amphiphilic material that prevents agglomeration. However, it is not suitable for intravenous usage and this limits its applications. Tween and Span are alternatives surfactants that are used.

The main preparation methods of polymeric nanoparticulate are; nanoprecipitation, solvent evaporation, salting out, emulsification-solvent diffusion, gelling processes and supercritical fluids. The method of choice depends on the final characteristics of the nanoparticles desired (e.g. size and polydispersity, type of the polymer, and properties of the drug).

When both the drug and the polymer are soluble in water miscible organic solvents, nanoprecipitation is generally the method of choice (Barichello et. al., 1999). It is an easy, reproducible and low cost method, therefore, it has been widely used especially with PLA and PLGA based nanoparticles. It is based on

interfacial deposition of polymers in a low polarity solvent in the aqueous phase. The polymer is dissolved in this solvent and then the solution is injected into a stirred aqueous phase to achieve emulsification. The polymer spontaneously deposits on the interface of solvent and aqueous phase. By this method, without using extra energy, large amounts of nanoparticles with a narrow size distribution can be obtained (Lassalle and Ferreira, 2007)

Another commonly used method is the solvent evaporation method in which a water insoluble solvent is used as the organic phase (Baran et. al., 2002, Tong et. al., 2008). Among the main solvents used in this technique are two chlorinated compounds, chloroform and dichloromethane. This method involves the addition of the polymer and drug dissolved in the organic phase to an aqueous phase containing a surfactant. An oil-in-water emulsion is then achieved using emulsification methods such as sonication or mechanical stirring. A modification of this method, the double emulsion water-in-oil-in-water (w/o/w), is generally used to encapsulate hydrophilic compounds within hydrophobic particles (Porjazoska et. al., 2004). In this method, the polymer is dissolved in an organic solvent with a surfactant. An aqueous phase containing the hydrophilic drug is emulsified in the organic solvent, and this emulsion is added to a continuous phase and the emulsification process is completed. Then nanoparticle formation is carried out with mechanical stirring. Nanoparticle size can be controlled by changing the rate of stirring, sonication time, concentration of surfactant or viscosity of the organic solvent. It is a popular method for producing nanoparticles, because water is generally used as the nonsolvent, and therefore, is low cost. However it is difficult to scale up because of excess amount of energy requirement.

The salting out method is a variation of emulsification-solvent evaporation and nanoprecipitation. In this method, drug and polymer are dissolved in a solvent that is miscible with water, such as acetone, which is then emulsified in an aqueous phase containing the salting out agent. Commonly used salts for this purpose are the electrolytes magnesium chloride, calcium chloride, and magnesium acetate or non electrolytes like sucrose (Farago et. al., 2008). This ornanic phase in water emulsion is diluted with water or aqueous solution in order to increase acetone diffusion into the aqueous phase. This method is easy to scale up

(Quintanar-Guerrero et. al., 1998), but requires extensive washing (Couvreur, 1995).

The emulsification-solvent diffusion (ESD) method is amodification of the salting out procedure. A partially water miscible solvent such as ethyl acetate is used. Before preparing an emulsion, saturation of solvent and water is obtained by first mixing them and separating the phases. The polymer and drug are dissolved in the organic solvent saturated with water, while surfactant is dissolved in the aqueous phase. These phases are then emulsified allowing solvent diffusion to the external aqueous phase and the formation of nanoparticles. The solvent is then removed by evaporation or filtration, depending on its boiling point (Reis et. al., 2006). This technique provides narrow size distribution, however, leakage of water soluble drug into the aqueous phase during emulsification can occur. It is efficient in encapsulating lipophilic drugs (Saxena et. al., 2004, Lu et. al., 2005).

Another method of nanoparticle preparation is the gelling process which involves the addition of a gelling agent (sometimes low concentration of calcium). Most polymers used in this method are soluble in water, such as alginate or chitosan. The preparation of alginate nanoparticles is obtained starting with an aqueous sodium alginate solution. Gelation is achieved using calcium ion addition. This triggers to the formation of transparent clusters of calcium alginate gels. Alginate particles are produced by dropwise extrusion of sodium alginate solution into calcium chloride solution. The particle size usually depends on the size of the initial droplet of the solution (Reis et. al., 2005). It is a method suitable for the encapsulation of hydrophilic compounds and fragile molecules.

There are some novel nanopreparation methods based on supercritical fluids. In these techniques, polymers are dissolved in a supercritical fluid, and the solution is extruded through a nozzle. The supercritical fluid is evaporated during the spraying process, and the particles are recovered (Reis et. al., 2006). This technique yields products free of solvent. These methods can be used especially in the protein drug delivery systems (Elvassore et. al., 2001, Meziani et. al., 2004).

1.5.3. Characterization for Micro and Nano Sized Polymeric Particles

Nanoparticle systems are generally characterized by studying their morphology, size and size distribution, stability, zeta potential, degradability and drug release kinetics (Beck et. al., 2011).

Controlling the particle size and polydispersity is necessary to ensure the properties of the nanoparticles. Laser light scattering is widely used for size determination since nanocapsule diameters are near the visible spectrum of light (Prasad et. al., 2007).

Certain properties can be determined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The treatment of nanocapsules for SEM analysis involves sample drying and coating with a thin layer of gold or platinum. The sample surface is then scanned by a high energy beam of electrons. These electrons interact with atoms of the sample producing signals which can provide information about the surface topography, composition, and shape. This method can be useful in qualitatively or quantitively evaluating the formulations.

Smaller nanoparticles (<100 nm), are difficult to observe with SEM because of the resolution limitation. The most popular characterization technique for smaller particles is TEM that consist of a beam of electrons transmitted through the sample, which was previously dried and stained with contrast agents. TEM is more useful to provide information about the wall and the core morphology of the polymeric nanocapsules (Beck et. al., 2011).

Another technique applied to investigate the nanocapsule structure is atomic force microscopy (AFM), where three dimensional images of the samples can be achieved. Electron microscopy techniques are useful in analyzing individual particles, however, it has to be remembered that all microscopic techniques require image treatment of a large number of particles to determine a reliable mean particle size. Therefore, a combination of at least two methods (such as light scattering and microscopy) is recommended to determine nanocapsule size and distribution (Beck et. al, 2011).

1.6. Scope, Aim and Novelty of the Study

The aim of this study was to develop a novel generation of nanocarrier based drug delivery system for the treatment of skin diseases such as psoriasis, aging or UV damage and improving the treatment efficacy for skin. The nanoparticulate delivery systems loaded with bioactive agents against skin conditions prepared from the PHBV were expected to offer a remedy for several skin diseases. Two different bioactive agents, retinyl palmitate (RP) and dead sea water (DSW), were used to load into PHBV nanospheres and nanocapsules. The goal was to illustrate the effectiveness of drug release *in situ* and *in vitro*. Initially, these nanoparticulate delivery systems were prepared and characterized, *in situ* release was studied and then the effect of bioactive agent loaded nanoparticles on cell proliferation and their uptake by cells were studied *in vitro* using L929 mouse fibroblasts.

An important concern with nanoparticles is their toxicity and this is becoming an extremely important research area. The dependence of cytotoxic effect of nanoparticulate drug delivery systems on their sizes is under active investigation. In this research, it was studied *in vitro* by using particulate material with different sizes. The toxic effects of micron, submicron and nano sized particles was studied *in vitro* using mouse fibroblast cell line L929. Penetration of each size group particles into L929 cells was observed.

Moreover, the *in vivo* transdermal permeation behavior of PHBV micro/nanoparticles were studied using a mouse model. Transdermal transfer of polymeric particles was studied by using Nile Red as a fluorescent agent to visualize the particles. A reproducible method to prepare 3 different sized PHBV micro/nanocarriers was developed and the influence of particle size on the penetration through the skin and cells was studied.

For *in vivo* penetration studies mouse skin was used and penetration of the particles was determined by GC-MS analysis.

Novelty of the study: In this study PHBV nanoparticles were shown to penetrate into cells, accumulate near the nucleus, thus potential as drug and bioactive agent carriers into the cytoplasm without further modification. It was also shown in this study that control of penetration depth into the skin is possible by varying the size of the nanoparticles.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

PHBV (HV content 5% molar), Retinyl palmitate (RP, MW 524.9) and Thiazolyl blue tetrazolium bromide cell proliferation assay (*MTT*) were purchased from Sigma-Aldrich (USA). Polyvinyl alcohol (PVA, MW $1.5.10^4$) was purchased from Fluka (USA). Dead Sea Water (DSW) was obtained from AHAVA Dead Sea Laboratories (Israel). Chloride specific electrode and ionmeter (for the determination of DSW encapsulation) were purchased from Cole Parmer (USA). Erythrocytes were obtained from human donors as described in Section 2.2.5. L929 cell line was obtained from the Foot-and-Mouth Disease Institute (Ankara, Turkey). Human osteosarcoma cells (Saos 2) was purchased from the American Type Culture Collection (No: CCL-1, ATCC). Dulbecco's Modified Eagle Medium (DMEM, high glucose), RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were all purchased from HyClone® (USA).

2.2. Methods

2.2.1. Nanoparticle Preparation

Oil-in-water (o/w) and water-in-oil-in-water (w/o/w) methods were utilized for the production of nanospheres and nanocapsules, respectively. These methods are based on emulsification process using dichloromethane as the hydrophobic solvent (for PHBV) distilled water as the continuous medium and PVA (4%, w/v) as the emulsifier.

2.2.1.1. Preparation of RP loaded PHBV Nano and Microspheres

The RP loaded PHBV spheres (PHBV:RP; 4:1, w/w) were prepared with the o/w technique. Briefly, in the first step, an oil-in-water emulsion was prepared. A solution of PHBV (10% w/v) was prepared in dichloromethane (DCM, 0.6 mL). RP was added to this solution (PHBV:RP, 4:1 w/w) and this was added into an aqueous solution of PVA (2 mL, 4% w/v) and sonicated (Ultrasonic Homogenizer, 4710 series, Cole-Parmer Instruments, USA) in an ice bath for 15 s. This emulsion (o/w) was diluted in more aqueous PVA solution (50 mL, 0.3 %, w/v), and mixed with a magnetic stirrer overnight at room temperature to achieve solvent evaporation. The emulsion was centrifuged (12000 rpm, 10 min) (rotor: 15156-H Sigma, 3K30), the pellet was washed twice with distilled water or sucrose suspension (1%, w/v), then resuspended in distilled water or sucrose suspension (1%, w/v) and lyophilized after freezing at -80^{o}C as shown in Figure 2.1.

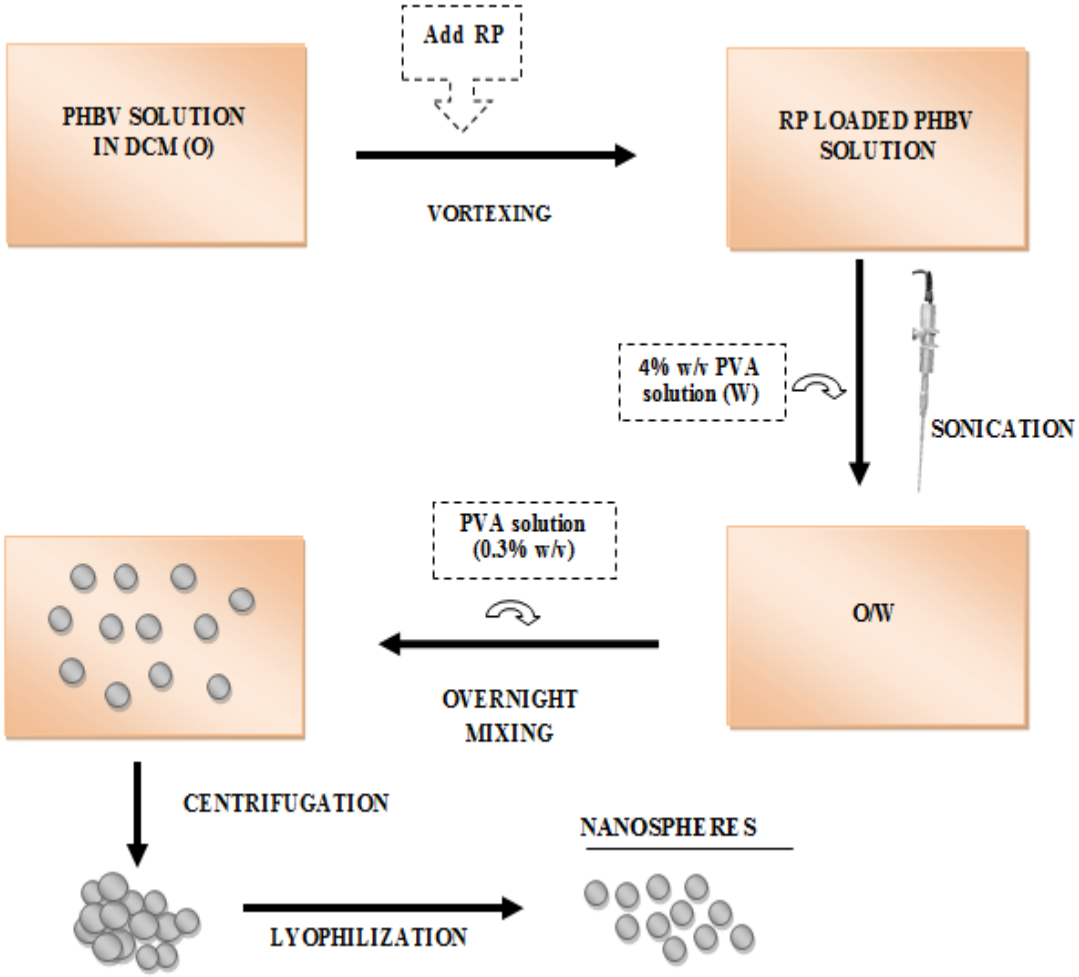

Figure 2.1. Preparation of RP loaded PHBV micro/nanospheres with o/w emulsion method

2.2.1.2. Preparation of DSW loaded PHBV Nano and Microcapsules

DSW loaded PHBV capsules were prepared with the $w_1/o/w_2$ solvent evaporation technique based on the two step emulsification process. Initially, a water-in-oil (w/o) emulsion was prepared. For this, a solution of PHBV in dichloromethane (0.6 mL, 10%, w/v) was prepared and then DSW (100 µL) was added. Emulsion was formed by sonication for 15 s in an ice bath. This emulsion was added into an aqueous solution of PVA (w_2) (2 mL, 4%, w/v) and sonicated for 15 s. This emulsion ($w_1/o/w_2$) was further diluted in aqueous PVA solution (50 mL, 0.3 % w/v), mixed with a magnetic stirrer overnight at room temperature for solvent (DCM) evaporation. The capsules were precipitated by centrifugation (12000 rpm, 10 min) (rotor: 15156-H, Sigma 3K30), washed twice with distilled water or sucrose suspension (1%, w/v), then resuspended in distilled water or sucrose suspension (1%, w/v) and lyophilized at -80^{o}C. A schematic presentation of the process is given in Figure 2.2.

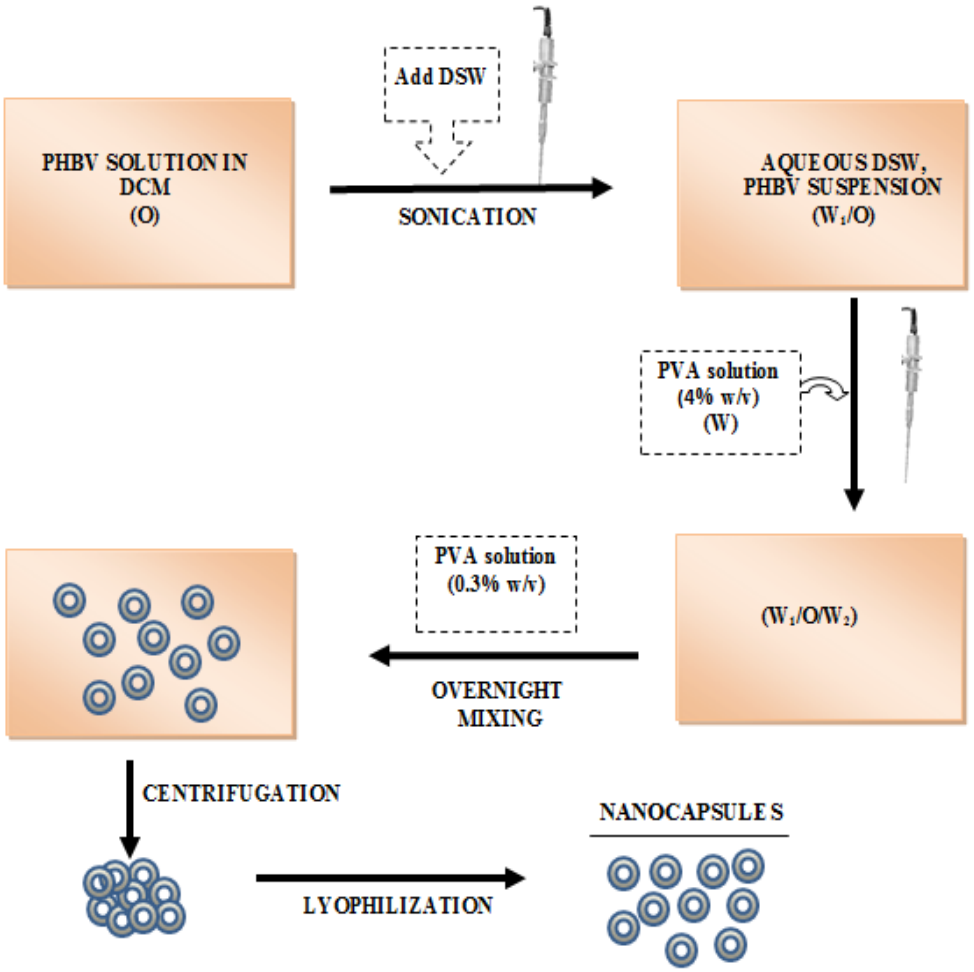

Figure 2.2. Preparation of DSW loaded PHBV micro/nanocapsules with $w_1/o/w_2$ double emulsion method.

2.2.1.3. Loading of Nile Red into the Nanocapsule Shell

PHBV nanocapsules loaded with Nile Red were prepared by w/o/w technique in order to be able to trace the particles *in vitro* and *in vivo*. The procedure was the same as in Section 2.2.1.2 except that Nile Red (0.1 mL, 0.01% in acetone) was added into the DCM solution of PHBV.

2.2.1.4. Preparation of Micro/Nanocapsules with Different Sizes

The particle suspensions were subjected to two different types of treatments in order to measure the diameter of the particles. In order to obtain different sized particles, different centrifuge speeds and durations were employed. To obtain micron sized (largest) particles the particle suspension was centrifuged at 12,000 rpm for 10 min, in the centrifugation step to recover the particles. The supernatant was separated after centrifugation at 13,500 rpm for 10 min to obtain mid-size particles. Finally, the remaining supernatant was centrifuged at 14,500 rpm for 40 min to get the smallest particles.

2.2.2. Characterization

2.2.2.1. Nanoparticle Topography with Scanning Electron Microscopy

An aqueous suspension of PHBV nanoparticles (100 µL, 1.2 %) was added onto carbon tapes (Electron Microscopy Sciences, USA) attached to SEM stubs, and Au-Pd sputter coating (2 nm) was performed under vacuum before the SEM study with QUANTA 400F Field Emission SEM (Netherland). The diameters of the nanoparticles were measured from the SEM images using the Image J software (NIH).

2.2.2.2. Chemical Composition of PHBV5 with Gas Chromatography-Mass Spectrometry

The chemical compositions of commercial PHBV5 (Sigma Aldrich Co.) and the nanoparticles produced from this PHBV were determined by GC-MS. To determine the 3-HV/3-HB ratio in the polymer structure, approximately 4 mg of PHBV powder or its nanoparticles was refluxed in a solution of chloroform, methanol and sulfuric acid (1:0.85:0.15) for 140 min at 100^{o}C in a thermostatically regulated bath. This method is called methanolysis, and degrades the polymer to its constituent β-hydroxycarboxylic acid methyl esters (FAME). After the digestion, distilled water (0.5 mL) was added and the tube was shaken for 1 min. After phase separation, the organic phase was transferred into a vial and analyzed in a gas chromatograph-mass spectrometer (GC-MS, GCD Plus, Hewlett Packard, USA), equipped with a 30 m x 0.25 mm HP-5 (polysiloxane composed of 5 % diphenyl and 95 % dimethyl monomers) fused silica capillary column (Sevastianov *et. al.*, 2003).

2.2.2.3. Particle Size Distribution Analysis

The size distribution of the micron sized PHBV particles were determined by Mastersizer (Malvern Instruments 2000, UK) and the nano sized PHBV particles were studied with the Zeta Potential and Mobility Measurement System (Malvern Nano ZS90, UK).

2.2.2.4. RP Loading and Entrapment Efficiency

The concentration of RP in PHBV particles was determined with UV spectrophotometer. After dissolution of RP containing PHBV (10 mg) in DCM (3 mL), the absorbance values at 325 nm were recorded and the amount was calculated according to the calibration curve (Appendix B).

The amount of retinyl palmitate contained within PHBV micro/nanospheres was also quantified by using HPLC. Analysis was conducted on

an ODS 3 column with methylene chloride in methanol (60% v/v) as the mobile phase with the flow rate of 1 mL/min. Retinyl palmitate was quantified using a fluorescence detector. The concentration of RP in standards and samples were calculated using the average peak areas at 325 and 470 nm for excitation and emission wavelengths after duplicate injections. Calibration curve is given in Appendix C.

2.2.2.5. DSW and $MgCl_2$ Loadings and Entrapment Efficiencies

DSW has a significant amount of Cl^- ions in its composition (350 mg/mL) in addition to many other ions (such as Ca^{+2}, Mg^{+2}, Na^+, Br^-, Sr^{+2}). These ions interfere with each other during any analysis. In order to determine the encapsulation efficiency, magnesium chloride was chosen as the molecule to load inside the nanoparticles instead of the DSW itself. The encapsulation efficiency in the particles was determined with a chloride specific electrode and an ionmeter (Cole-Parmer Instrument Company, USA). Briefly, nanocapsules loaded with magnesium chloride (100 µL) were disrupted in DCM (6 mL). Then ultrapure (ion free) water (2 mL) was added to the solution and vortexed. After phase separation, the upper phase containing the Cl^- ions was removed by pipetting. Extraction step was repeated two more times. The chloride ion contents were determined by inserting the chloride-specific electrode into the extract. Each test was run in triplicate. The loading and encapsulation efficiency (E.E.) values of $MgCl_2$were calculated using the following equations:

$$\text{Loading (\%)} = \frac{encapsulated\ MgCl_2\ (mg)}{total\ weight\ of\ nanocapsules\ (mg)} \times 100$$

$$\text{E.E. (\%)} = \frac{amount\ of\ MgCl_2}{input\ amount\ of\ MgCl_2} \times 100$$

2.2.3. *In Situ* DSW and $MgCl_2$ Release From Micro/nanocapsules

Briefly, particles were suspended in ultrapure water (5 mL, 10 mg/mL) and placed in the dialysis tubing (Snake skin, MW 10,000 cut off, pleated, Thermo Scientific, USA). The tubing was then introduced to ultrapure water (30 mL). This medium was kept shaking at 37°C for a week. At various time points (1 h, 3 h, 6 h, 12 h, 2 days and 7 days) chloride specific electrode was dipped into the release medium and concentration was recorded. The data was plotted as released $MgCl_2$ and DSW concentration versus time and treated according to Higuchi Equation (M_t/M_∞ vs $t^{1/2}$).

2.2.4. Determination of Hemolytic Activity of the Micro/nanoparticles

Human peripheral blood was collected at the Health Center in METU from volunteers into EDTA containing tubes to prevent coagulation. Blood samples were centrifuged (20ºC, 3400 rpm, 15 min) in order to collect the red blood cells (RBC). Then the RBCs were resuspended in PBS (3 mL) which was then further diluted to 20 mL with PBS.

Bioactive agent (DSW and RP) loaded dry nanoparticles were suspended in PBS (2 mg/mL). Serial dilutions were made to obtain suspension with (0.1, 0.2, 0.3, 0.4 and 1 mg/mL particles in RBC solution. These nanoparticle-RBC samples were incubated at 37ºC for 1 h. Then, the samples were centrifuged (1500 rpm, 20 min) and 200 μL of the supernatants were transferred to 96-well plate in triplicate. Absorbances at 540 nm were determined with a UV spectrophotometer (Thermo Scientific Multiscan Spectrum, Type 1500, USA).

Complete hemolysis (control) was achieved by diluting the RBC suspension with ultrapure water (1:10) and then incubating at 37ºC for 1 h centrifuged as before.

RBC solution itself was also treated similarly but without nanoparticles and was used as negative control.

These tests were made for assessment of blood compatibility of samples in accordance with a modification of an ASTM standards procedure [Standard Practice for Assesment of Hemolytic Properties of Materials, F756-08].

All hemolysis data points were presented as the percentage of the complete hemolysis.

2.2.5. *in vitro* Studies

2.2.5.1. Determination of Cell Proliferation with MTT Assay

L929 cells (passage no 14) were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit) whereas the human osteosarcoma cell line Saos 2 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit). They were maintained in an incubator with 5% CO_2 at 37°C (Sanyo MCO-17AIC, Japan) until confluency. Before seeding, the cells were detached from the flask by treatment with trypsin-EDTA solution (3 mL, 0.05% in PBS) for 5 min. Then culture medium (6 mL, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin 100 unit) was added to the flask to inhibit trypsin activity. The cell suspension was centrifuged (3000 rpm, 5 min) and the pellet was resuspended in the medium (2 mL, 1% penicillin/streptomycin 100 unit, 10% FBS). The cells were counted with a hemocytometer (Blau Brand, Germany) and 20,000 cells were seeded onto 24 well plates TCPS.

Cell numbers were determined with the MTT assay. The 24 well plates seeded with 20,000 L929 cells were incubated (5% CO_2, 37°C) for 3 h in order to achieve cell attachment on TCPS. The medium was removed and bioactive agent loaded nanoparticles which were suspended in the culture medium (0.5 mg particles/mL) were added to the wells containing the cells which were then incubated (37°C, 5% CO_2) for 24 h. The medium was removed, the wells were washed twice with sterile PBS and then MTT solution (1 mL) was put into each

well and incubated for 3 h at 37°C in a humidified 5% CO_2 incubator for formazan crystal formation. The MTT solution was gently removed in order to prevent cell destruction, acidified isopropanol (1 mL) was put in the wells and formazan crystals were dissolved. Aliquots of formazan solution (200 µL) were put in a 96-well plate in triplicates. Absorbances were measured at 550 nm against the blank (acidified isopropanol) using a UV spectrophotometer (Thermo Scientific Multiscan Spectrum, Type 1500, USA) and the OD values were converted to number of cells by a calibration curve (Appendix D). This was repeated on days 3 and 7. The results were evaluated by comparing the experimental group with the control group at the same time points.

All the experiments were conducted in triplicate and cell-free medium was used as the blank.

2.2.5.2. Micro/nanoparticle Uptake by Saos 2 and L929 Cells

Saos 2 and L929 cells were used to determine penetration of nanoparticles into the cells. Culture medium of L929 was DMEM high glucose supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit) whereas RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 units) was used for Saos 2 cells as described in Section 2.2.5.1.

After 24 h of incubation in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit), Saos 2 cells were seeded at a density of 20,000 into each well of 6-well plate. After 2 h, Nile Red loaded nanocapsules (0.2 mg/mL) suspended in the growth medium were added to the wells containing the cells. After 4 h and 24 h of incubation, cells were fixed with paraformaldehyde (4%, 1 mL), stained with 4',6-diamidino-2-phenylindole (DAPI) and FITC-phalloidin for nucleus and cytoskeleton, respectively.

Fluorescent stains, FITC phalloidin and DAPI were used after 100 and 500 fold dilution, respectively, of the stock solution in BSA-PBS solution (0.1 %, w/v).

After incubation, medium was removed and Triton X in PBS (1 % v/v, 1 mL) was added onto the cells. Cells were kept at room temperature for 5 min to achieve permeation and then cells were washed with PBS. Solution of BSA in PBS (1% w/v, 1 mL) was added to the cells and incubated for 30 min at 37^{o}C. FITC-Phalloidin (1 mL) was then added in each well and incubated at 37^{o}C for 1 h. Then cells were washed with PBS again. After this step, DAPI (1 mL) was added to each well for 5 min, and then each well was washed with PBS. After staining process, nanocapsules and cells were observed with fluorescence microscopy (Olympus IX-FLA, Japan) and CLSM (Leica DM2500, Germany). FITC-phalloidin was detected after excitation at 532 nm, emission at 605-635 nm and DAPI was observed after axcitation at 532 nm, emission at 605-635 nm. In order to study particle penetration into the cells, z-stacks were obtained in the CLSM.

2.2.6. *In vivo* Studies

2.2.6.1. Animal Model

Experiments were conducted at Siberian Federal University on sexually mature male, 14 week old BALB/c mice (20-25 mg each). They were kept in an animal house in cages, two animals per cage, fed a standard laboratory diet and water in accordance with the Directives on Maintenance of Animals and Experimentation (Genin et. al., 2001). They were kept under standard environmental conditions. The *in vivo* experimental protocol was approved by the Institutional Animal Ethical Committee on Biomedical Ethics of Sibfu and the protocol is given in the Appendix E.

2.2.6.2. Treatment of Mice and Application of Polymeric Particles

Dorsal section of the mice was shaved before application of the formulations. After removal of the hair, skin was swabbed with pure ethanol to dry the skin (Goope et. al., 2009).

On the left side, low concentration (0.5 mg/mL), and on the right side, high concentration (5 mg/ mL) of the particle suspension was applied for 10 days.

In total, 5 mg nanocapsules were applied to the left side and 50 mg to the right side of the mice.

Aliquots (200 μL) of nanocapsule suspension were applied to the mice (Figure 2.3a) and with a 2 min massage was performed using a cosmetic applicator (Figure 2.3.b). This suspension was applied everyday for 10 days. The control was the intact skin of the mice.

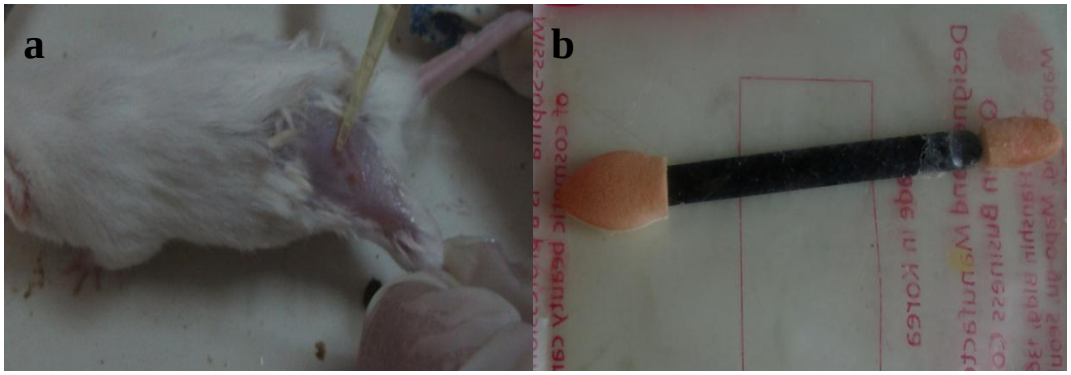

Figure 2.3. Nanoparticle suspension was applied onto shaved mice skin with a cosmetic applicator. a) Application of nanoparticle suspension, b) cosmetic applicator. Different applicators were used for each group in order to prevent cross contamination of the experimental site with the samples of the earlier treatment.

After 10 days, the animals were sacrificed with an overdose of formalin vapor. Skin patches were removed and used to measure the polymer content, and thus, the amount of polymer penetrated (Figure 2.4). After removal, skin sections were dried at 60^{o}C overnight and weighed. Then approximately 10 mg of sections were weighed and the polymer amount was determined with GC-MS (as described in section 2.2.2.2).

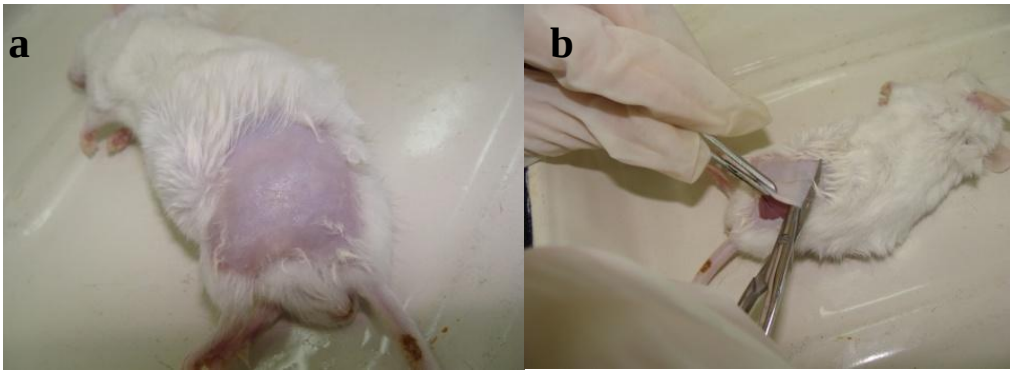

Figure 2.4. Nanoparticle patches areas of the mice skin were removed for analysis. a) Before removal, b) removal of the skin.

2.2.6.3. Determination of the Polymer in the Skin with GC-MS

Skin samples were dried at 60^{o}C overnight, allowed to cool to room temperature and 10.00 ± 0.05 mg skin samples were subjected to methanolysis (section 2.2.2.2). Analysis was through determination of the fatty acid methyl esters with a GCD plus gas chromatograph-mass spectrometer (Hewlett Packard, USA) equipped with a 30 m x 0.25 mm HP-5 (5 % polydiphenylsiloxane and 95 % polydimethylsiloxane) fused silica capillary column (Sevastianov et. al., 2003).

2.2.7. Statistical Analysis

All *in vitro* experiments were carried out in triplicates and the *in vivo* in duplicates. Statistical analysis of the results was made using the standard software of Microsoft Excel. Arithmetic means and standard deviations were calculated. Significant differences between mean values in control and test groups were determined using Student's t-test. Means were considered to be significantly different for $p \leq 0.05$ values.

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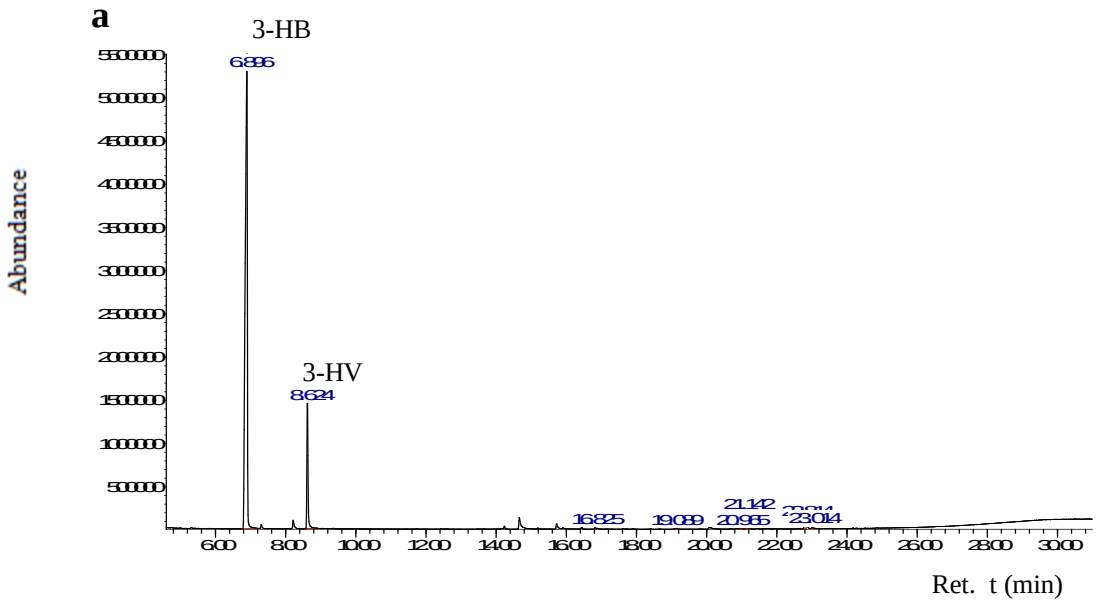
CHAPTER 3

RESULTS AND DISCUSSION

3.1. Quantification of PHBV with GC-MS.

The GC-MS analysis of PHBV particles were made to detect their presence in the *in vivo* testing. The initial analysis was carried out with Nile Red loaded PHBV micro/nanoparticles containing sorbic acid as a preservative (antimicrobial) agent. In Figures 3.1.a and 3.1.b pure PHBV polymer and unloaded PHBV nanoparticles are presented, respectively. It is seen that the spectra are basically the same as expected. Figure 3.1.c shows that the nanoparticles consist of 92% PHBV and 7% of sorbic acid. The peak due to sorbic acid (at 9.151) can be observed.



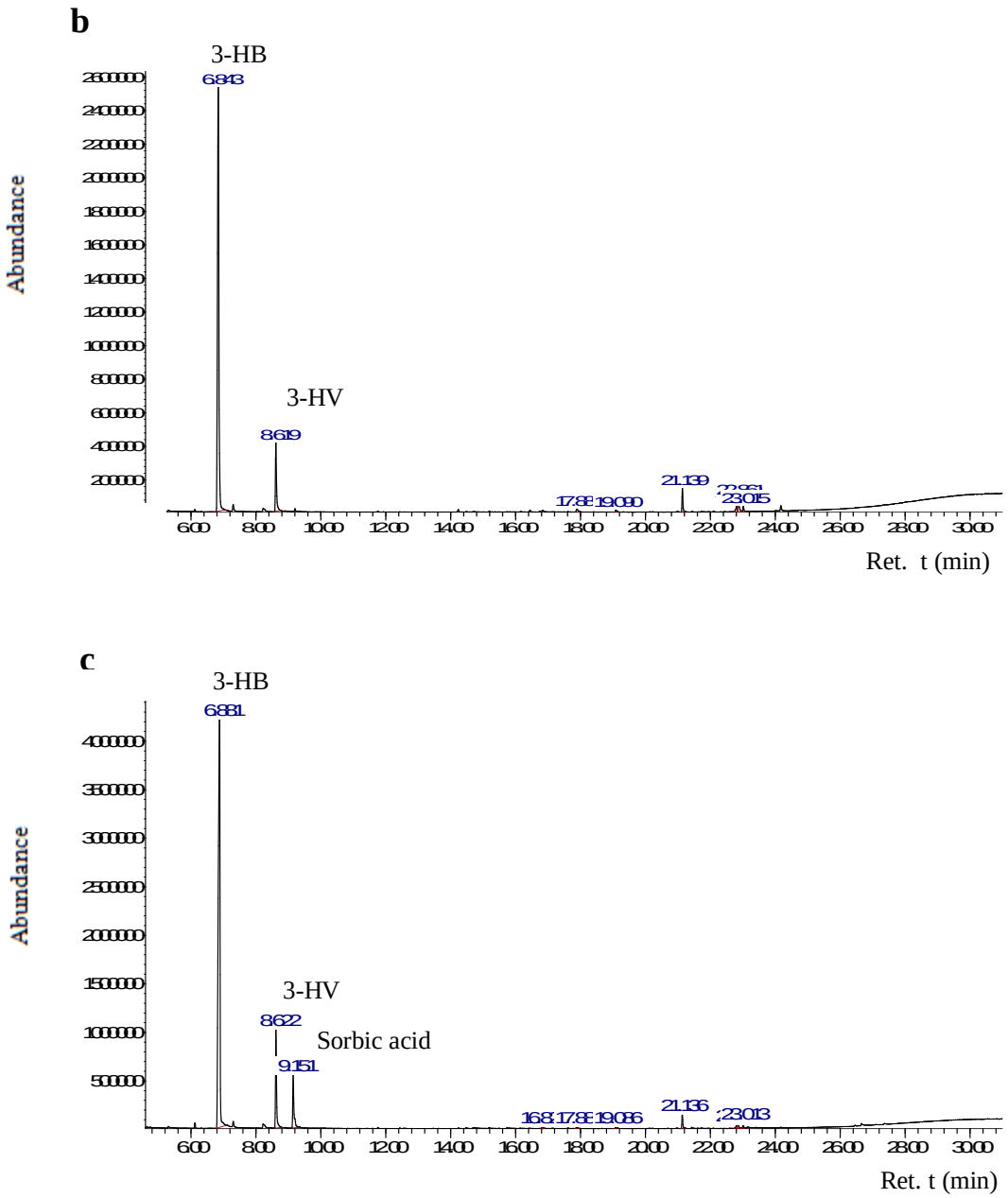


Figure 3.1. GC-MS chromatogram of methyl esters of PHBV and sorbic acid. a) PHBV polymer, b) PHBV nanoparticles, c) Sorbic acid containing PHBV nanoparticle. Sorbic acid presence is seen at 9.151 min.

The HV content of PHBV was calculated from the ratios of the areas of peaks at 6.84 min for 3-HB and at 8.62 min for 3-HV. Area of the peaks for each chromatogram is given in Appendix 1. Amount of fatty acids were also calculated from the peak areas to determine purity of the polymer. Thus, the HV content of

the PHBV was found to be 11 % and there were no other ingredients such as fatty acids, indicating the polymer's purity.

3.2. Preparation of Nanospheres and Nanocapsules for Drug Delivery

3.2.1. Particle Morphology, Size and Size Distribution

Production of PHBV particles were carried out with 10% (w/v) polymer solution in dichloromethane. The choice of this concentration was based on the previous studies conducted with similar polymers (Chen et. al., 2008, Yilgor et. al., 2009). Round, spherical particles with submicron diameters were expected with 10% (w/v) polymer solutions.

Figure 3.2. shows the SEM images of micro/nanocapsules prepared from PHBV. Size measurements showed that average diameter of DSW loaded capsules was 4.3 μm and they had a wall thickness of ~105 nm.

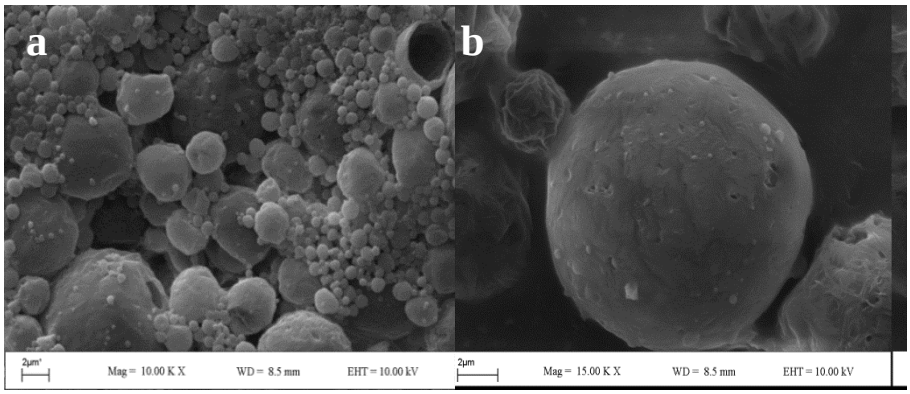


Figure 3.2. Unloaded PHBV capsules. a) Wall thickness of the capsule is 105 nm, x10,000, b) Same samples, higher magnification, x 15,000.

Figure 3.3 shows PHBV micro/nanocapsules and spheres loaded with bioactive agents DSW and RP, respectively. SEM micrographs reveal that drug loading did not affect the smoothness and roundness of the particles. Another observation is that capsule and sphere forms are very similar in terms of average size and spherical form.

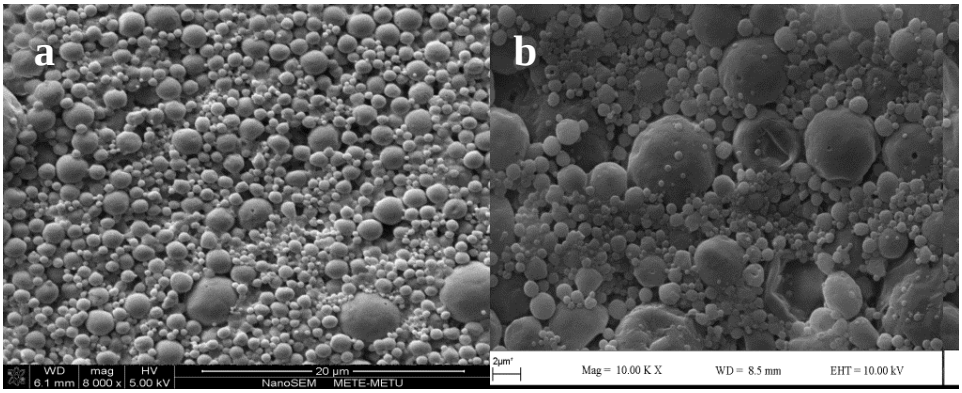


Figure 3.3. Bioactive agent loaded PHBV particles. a) RP loaded spheres (x5,000), b) DSW loaded capsules (x10,000)

The average diameter of unloaded particles was found to be in the range 185 nm-10 µm with average of 4.0 µm, whereas, DSW loaded particles were in the range of 240 nm-10 µm with average of 4.3 µm, and RP loaded particles with the range of 270 nm-14 µm with an average of 6.1 µm. This might mean that if the drug is to be loaded in the central cavity (core) of the particle, the dimension does not change but when it is in the wall, it does. This is logical and similar observations are given in the literature. For example, vitamin A derivatives loaded on the nanoparticles had a slight effect on particle size (Ericco et. al., 2009).

3.2.2. Micro/nanocapsules with Different Sizes for *in vivo* Studies

Unloaded and bioactive agent loaded particles had a broad size distribution. However, the change in the particle size could be controlled by changing the preparation parameters, such as the sonication time and speed (Xiong et. al., 2010), centrifugation speed, surfactant concentration, and polymer concentration (Yokoyama et. al., 1998, Crowley et. al., 2000, Ericco et. al., 2009, Yilgor et. al., 2009).

In order to study the effect of nanocapsule size on skin penetration depth in a topical delivery system, nanoparticles with three different sizes were prepared. Their morphologies were investigated by SEM and their sizes were determined with Mastersizer and Zetasizer. The micrographs revealed that by

changing the parameters of time and speed of centrifugation, it was possible to separate particles with different sizes (Table 3.1 and Figures 3.4 and 3.5). The smallest PHBV particles were obtained with the longest centrifugation duration 40 min (14,500 rpm) and the highest velocity of centrifugation (Figure 3.4.c). SEM micrographs also show that all particles had smooth surfaces.

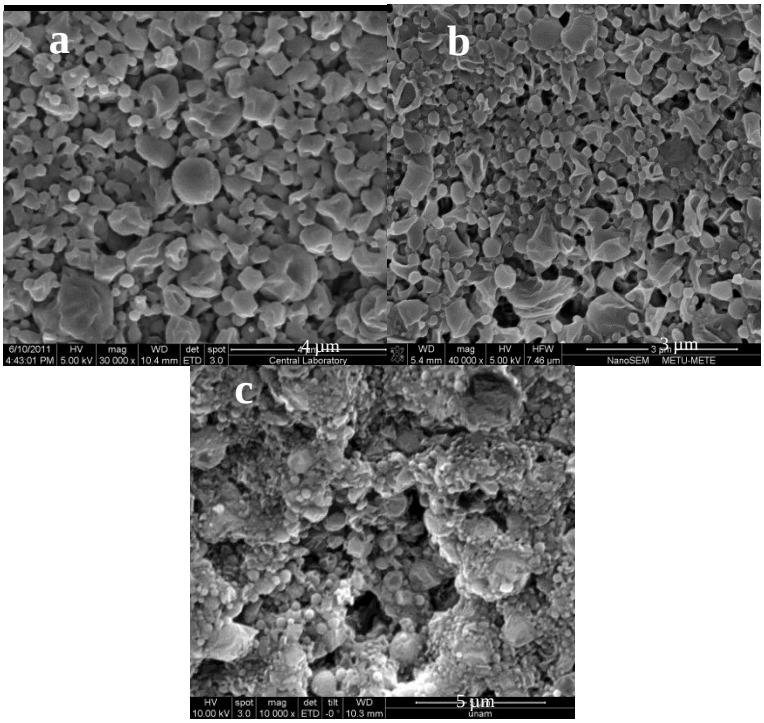


Figure 3.4. SEM micrographs of PHBV particles prepared according to section 2.2.1.3 using different duration and velocity of the centrifugation: a) 10 min at 12,000 rpm (15,455 g). Bar 4 µm, b) 10 min at 13,000 rpm (18,138 g). Bar 3 µm, c) 40 min at 14,500 rpm (22,566 g) Bar 5 µm.

According to the average particle size, size range and polydispersity, the samples were labeled as micro, submicro and nano. Polydispersity indices (PI) of these micro, submicro and nano particles were found to be 0.8, 0.3 and 0.1, respectively, indicating that the smallest had the most narrow particle size distribution as shown by the sharp peak in Figure 3.5c.

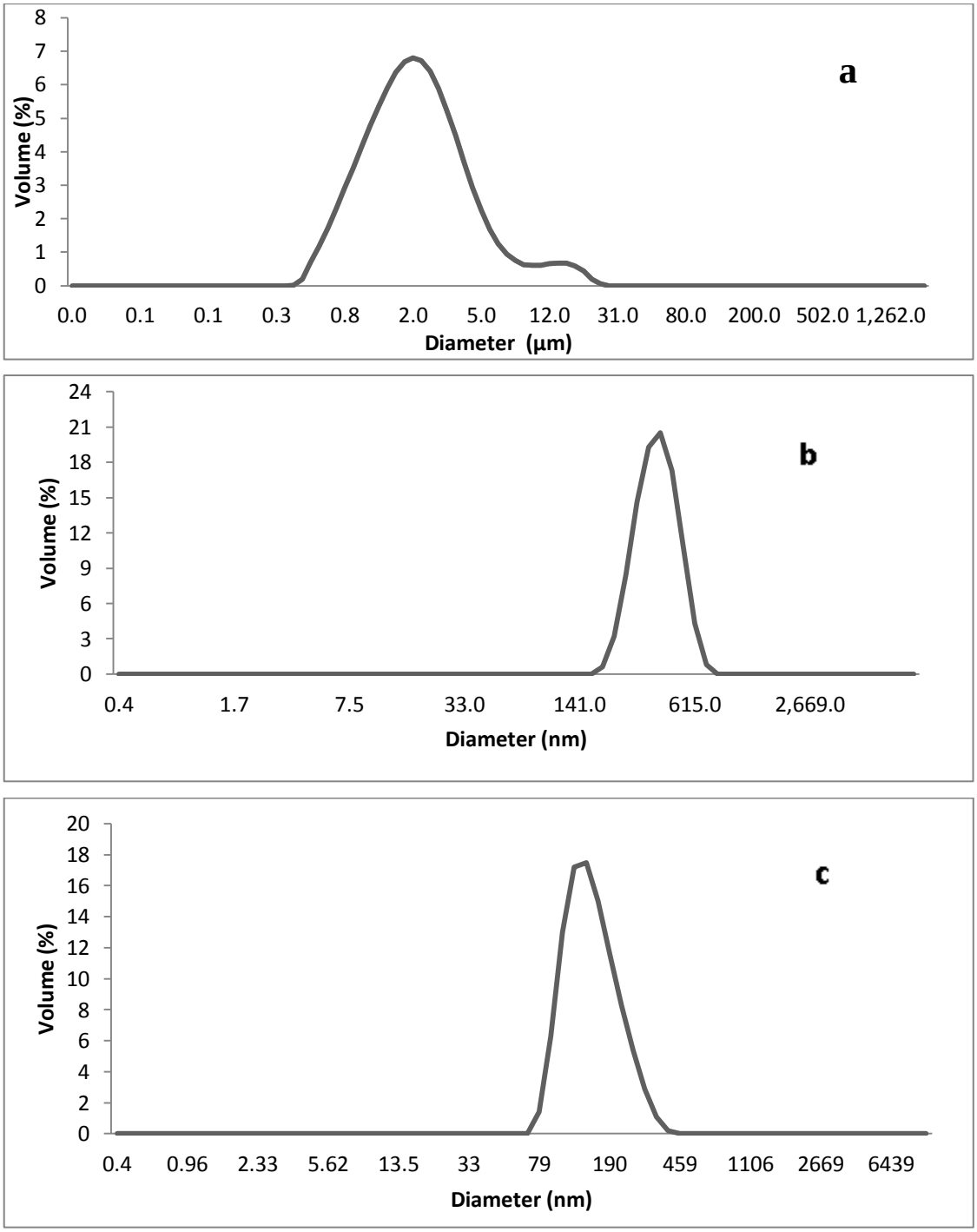


Figure 3.5. Particle size distribution of PHBV particles. a) micron, b) submicron, c) nano sized samples.

Table 3.1. Sizes and polydispersity indices of PHBV micro/nanoparticles

Property	Sample		
	Micro	Submicro	Nano
Mean Diameter (nm)	1900	426	166
PI	0.8	0.3	0.1
Size range (nm)	400-12000	190-712	80-542

3.3. Encapsulation Efficiency and Loading of RP

RP is a hydrophobic compound and an ester of retinol (a derivative of vitamin A) and palmitic acid. RP encapsulation efficiency was determined with both HPLC with a fluorescence detector (Figure 3.6) and with UV-VIS spectrophotometry. With both methods similar encapsulation efficiencies of around 8% were found (Table 3.2).

In general, HPLC is accepted as a more sensitive technique than UV-VIS because the chromatographic step separates the components of a mixture from each other preventing the effects of others and also requires much lower amounts of samples (µL vs mL). Apart from this there is no difference because the detection mechanism is the same. However, in this specific case there were no mixtures, and therefore, HPLC was not specifically advantageous. With HPLC using a fluorescence detector, the concentration of RP in the sample was found to be 8.0% of the input RP in the PHBV sphere preparation medium. A representative HPLC chromatogram is given in Figure 3.6.

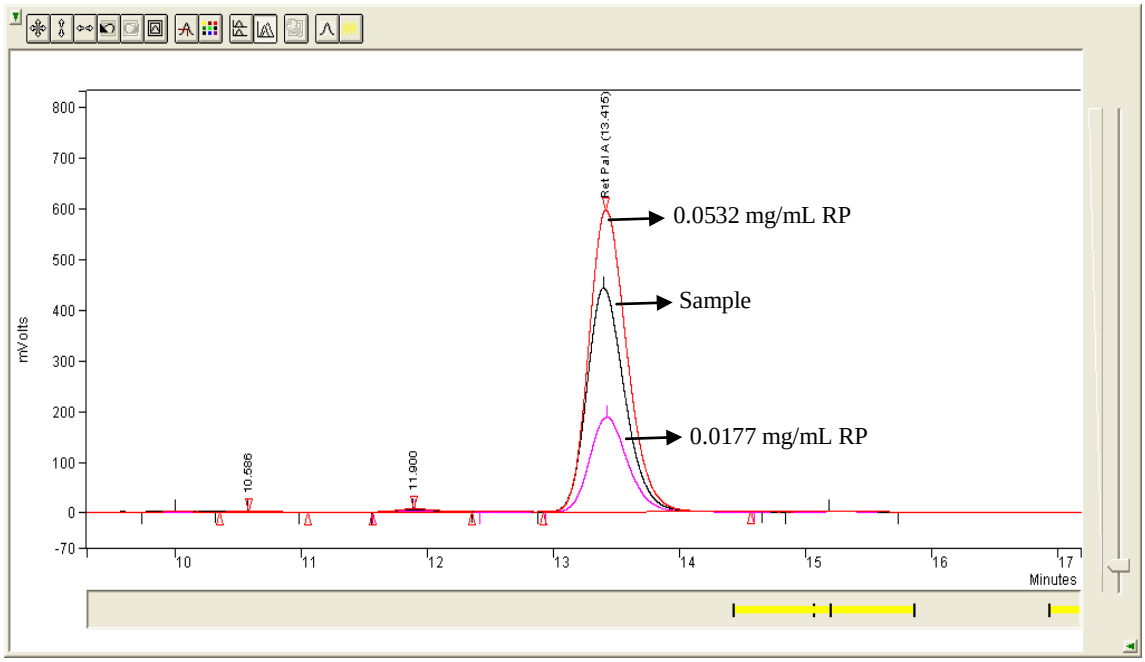


Figure 3.6. HPLC of RP loaded PHBV. Mobile phase: DCM in methanol (60% v/v), Flow rate: 1 mL/min, Column: ODS 3, λ_{ex}: 325 nm and λ_{em}: 470 nm.

Simple UV-VIS spectrophotometry was used as the second (control) method with 540 nm as the absorbance wavelength. The absorbances were converted to concentrations using the calibration curve in Appendix B. Results are presented in the Table 3.2.

Table 3.2. RP Encapsulation Efficiency and Loading of PHBV Spheres

Method	**E.E (%)**	**Loading (%)**
HPLC with fluorescence detector λ_{ex}: 325 nm, λ_{em}: 470 nm	8.0±0.0	1.4±0.1
UV-VIS Spectrophotometry λ_{max} =540 nm	8.5±0.2	1.5±0.1

The loading process of hydrophobic drugs into the particles is considered to involve hydrophobic interactions between the drug and the hydrophobic segment of the polymeric chains (Jeong et. al., 2001). It was reported that retinoic acid (a hydrophilic molecule) loading was 1%, w/w for PLGA and 0.94%, w/w for PHBV particles (Errico et. al., 2009). These values are lower compared to that of the ones obtained in present study because retinoic acid is polar and this hampers the establishment of hydrophobic interactions with the polymer and decreases its encapsulation (Noy, 2000). Sane et. al. (2009) on the other hand reported 6.2% loading of RP in PLLA nanoparticles by using supercritical CO_2 supporting the results obtained in this study.

3.4. Encapsulation Efficiency of $MgCl_2$ and DSW

Encapsulation efficiency and release rate of active agents from PHBV capsules were investigated by using $MgCl_2$ as a molecule representative of DSW because the composition of DSW is very complex (many different salts are dissolved in it with $MgCl_2$ being the most abundant) and determination of encapsulation efficiency or release kinetics is difficult. With both $MgCl_2$ and DSW, it was observed that the maximum encapsulation efficiency is not higher than 2.0% with either method (Table 3.3).

Table 3.3. $MgCl_2$ and DSW encapsulation efficiency and loading of PHBV capsules

Hydrophilic Agents	**E.E. (%)**	**Loading (%)**
$MgCl_2$	1.7±0.2	1.0±0.1
DSW	1.1±0.1	0.9±0.1

Encapsulation efficiency of $MgCl_2$ loaded PHBV capsules appears higher than DSW loaded counterparts (1.7±0.2 vs. 1.1±0.1). However, it must not

be forgotten that the DSW sample is a mixed medium of ions and contains significant amount of bromide ions (11-12 g/L) and these bromide ions interfere with the chloride ions (Rhodes and Buck 1980). The percent encapsulation values of the model molecule with PHBV nanocapsules are in agreement with the literature values: encapsulation efficiency of 1.29% was observed with metformin hydrochloride (MH) in PHBV8 nanocapsules (Farago et. al., 2008) and 1.5% was observed with carboxyfluorescein (CF) in PHBV14 (Gursel, 1995).

Considering that $MgCl_2$ is a water soluble electrolyte with a very low molecular weight (MW 95.2 g/mol) like the other ions of the dead sea water the low encapsulation values obtained for these nanoparticles can be considered acceptable due to substantial leakage of the drug from the first emulsion to the external aqueous phase during the emulsification and solvent evaporation stages.

3.5. Release Kinetics of $MgCl_2$ and DSW

The release kinetics of MgCl2 and DSW were assessed by measuring the released chloride ions with a chloride specific electrode. The obtained data was fitted to the release relation of Higuchi as well as to equations of Zero and First Order Kinetics.

Zero Order release model was assessed by plotting (amount released in time) M_t vs t according to $M_t/M_\infty = k_0 t$. First Order release model requires plotting $\ln M_t$ vs t according to of M_t/M_∞ e^{k1t}. Higuchi release model requires plotting M_t/M_∞ vs $t^{1/2}$ according to $M_t/M_\infty = k_H t^{1/2}$. M_∞ is the amount of drug released at time infinity (h), where t is time (h) and k_0, k_1 and k_H are rate constants for Zero Order and First Order and Higuchi Release relations, respectively.

It is observed that most of the salts were released into the aqueous medium in the first 24 h (Figure 3.7). The kinetics of release was investigated by fitting the data to the rate equations and the best fit was obtained with the Higuchi model, which describes release from a monolithic structure via diffusion (Higuchi, 1961). A very good fit of the release profile to this relation was observed with all hydrophilic agents (compared the r^2 values) (Table 3.4 and Figure 3.8).

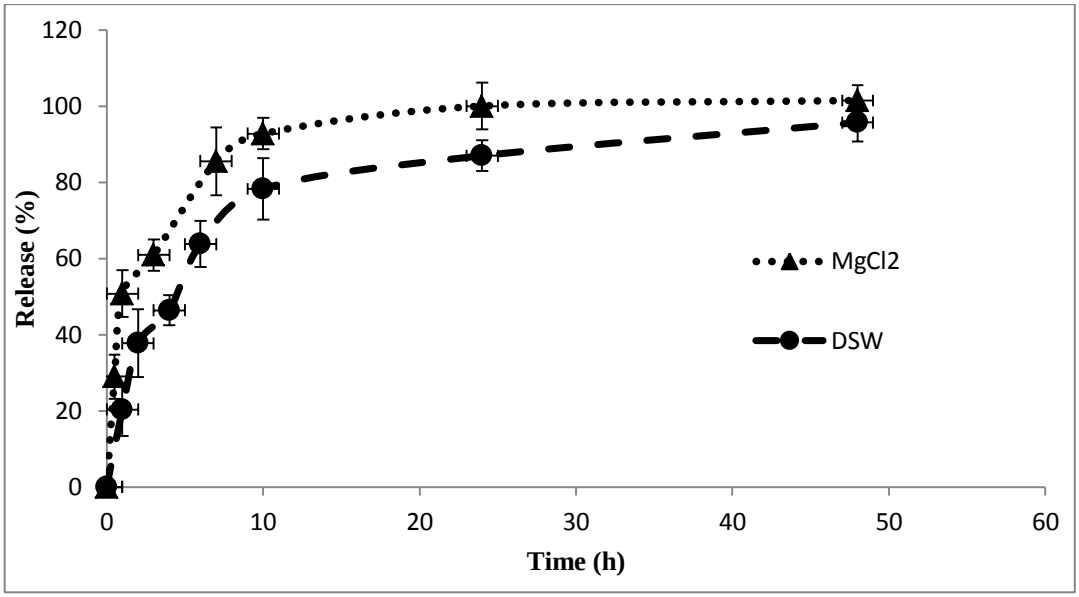


Figure 3.7. $MgCl_2$ and DSW release from PHBV micro/nanocapsules (n=3).

Table 3.4. Kinetic analysis of $MgCl_2$ and DSW release from PHBV micro/nanocapsules.

Samples	**Release Models, Rate Constant (k) and r^2 values**					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$MgCl_2$	0.0053	0.4753	0.0174	0.4242	0.1602	0.9678
DSW	0.0067	0.6105	0.0229	0.5005	0.136	0.9851

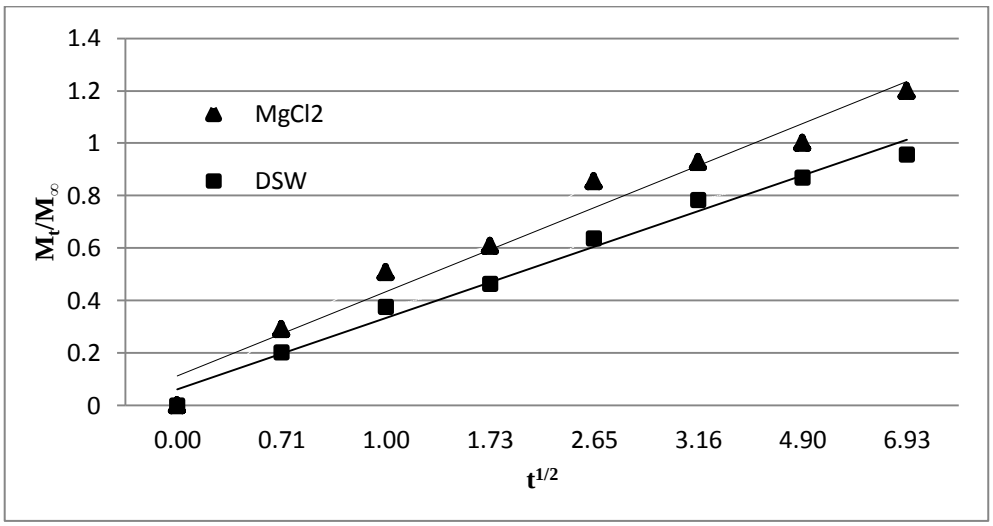


Figure 3.8. The $MgCl_2$ and DSW release data according to Higuchi Model.

3.6. Hemolytic Activity of PHBV Nanoparticles

PHBV micro/nanoparticles were designed for use in skin disease treatment. However, the skin is a multilayer tissue with low permeability for particles. It can, however, be harmed by the nanoparticle. The hemolytic activity of the particles was used as a quick check of toxicity or damage to cells.

Hemolytic activity of PHBV spheres and capsules loaded with different bioactive agents was studied (Table 3.5). The quantitive results of hemolytic activity show that percent hemolysis is less than 0.8% for unloaded nanoparticles. For DSW loaded nanocapsules and RP loaded nanospheres, these values are found to be less than 1.0% and 1.4% respectively. According to the ISO/TR 7405-1 984(f), a sample is considered as hemolytic if the hemolytic activity is above 5%.

It can, therefore, be safely stated that the particles tested are not hemolytic.

Table 3.5. Hemolytic Activity of Bioactive Agent Loaded Nanoparticles (n=3).

Sample	Hemolysis (%)
Unloaded PHBV NC	< 0.8
DSW Loaded PHBV NC	< 1.0
RP Loaded PHBV NS	< 1.4

NC: Nanocapsules

NS: Nanospheres

Microscopically, no significant differences have been found in the morphology of the erythrocytes were detected.

These findings are in agreement with those in the literature, where it is reported that, PHB and PHBV films and spheres were not hemolytic having a hemolytic value less than 5% (Doyle et. al., 1991, Sevastianov et. al., 2003).

3.7. *In vitro* Studies

3.7.1. Influence of Particle Size and Concentration on Adhesion and Cell Proliferation

In biomedical applications, PHA family has been attracting a great deal of interest, especially as carriers for drug delivery. Therefore, the cytotoxicity of this copolymer was of importance and thus was investigated.

After preparation and characterization of the different sized PHBV micro/nanoparticles, they were tested using L929 cells of mouse fibroblast origin in order to assess their suitability for use in transdermal applications.

The MTT test was used to detect the any changes in cell adhesion (Figure 3.9) and proliferation (Figure 3.10) using Nile Red stained particles. In order to study the effect of micro/nanoparticles on cell adhesion, the micro/nanoparticle suspensions were added in the TCPS and then cells were added. All micro/nanoparticles were tested with L929 (passage no 14) cells with the concentration of the NP in the medium being 0.5 mg/mL and 1 mg/mL. NP free

cell culture was used as the control group. Separate experimental and control groups were used for each time point.

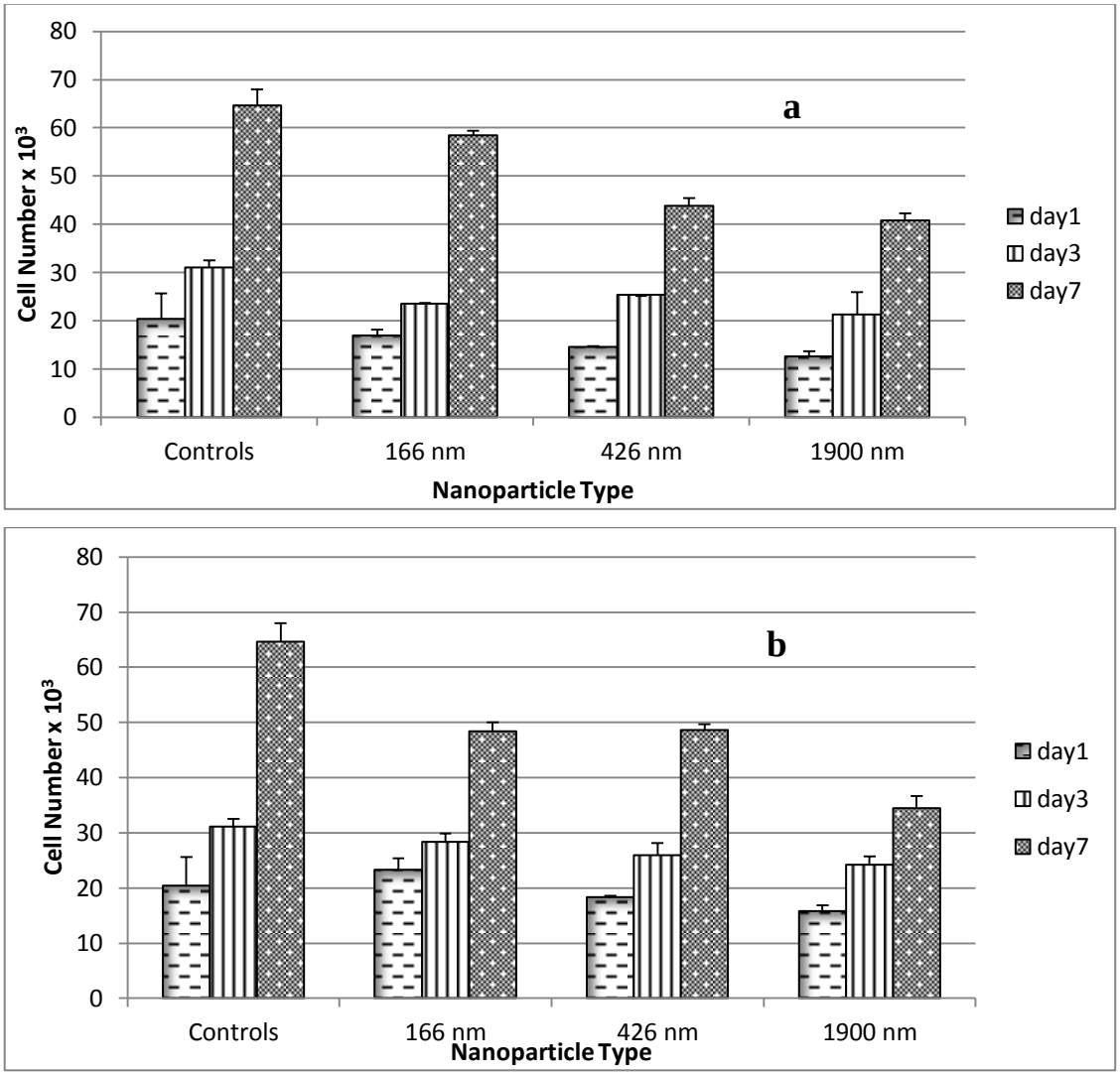


Figure 3.9. Effect of particle size and concentration on L929 cell adhesion (n=3). a) Low dose (0.5 mg/mL), b) High dose (1 mg/mL) of micro/nanocapsules.

On day 1, nanoparticles seem to have some negative effect (Figure 3.9) on cell adhesion onto TCPS. It was observed that adhesion of cells is concentration and size dependent. A slight decrease in the adhesion was observed when the conentration of the particles were increased from 0.5 to 1.0 mg/mL.

The effect on adhesion of cells is definitely size dependent; higher the particle size the more adverse is its effect on cell adhesion. It was also observed

that the particles have some adverse effect on cell proliferation (Figure 3.10) especially when their concentration was high. When percent of cell proliferation is studied, the low dose (0.5 mg/mL) particles have no negative effect on the rate of cell number increase regardless of their size (Figure 3.10a). It must, however, be remembered that the highest size particles have the lowest number of cells even when their concentration is low (0.5 mg/mL). Cell proliferation further decreased when the dose increased from 0.5 mg/mL to 1 mg/mL indicating that the effect of nanoparticles on cell proliferation is also concentration depended (Figure 3.10b). However, sub-micron and nano sized particles did not cause significant cytotoxicity and cells had a normal shape as observed through light microscope.

Morphological changes of cells were examined with light microscopy, and it was observed that cells spread normally in the nanoparticle containing wells surface (data not shown) which supports the thesis particles are not harmful for the L929 cells.

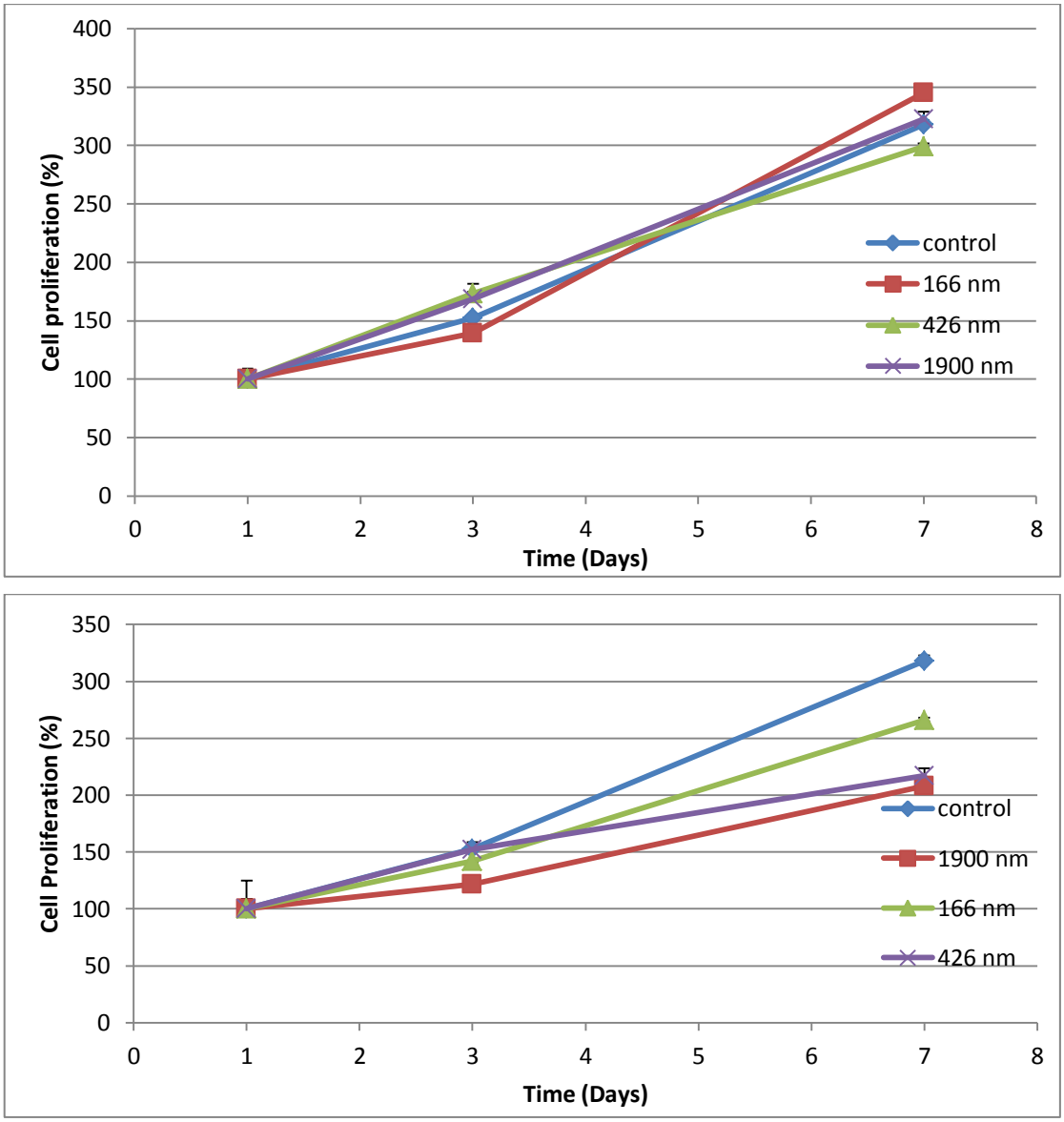


Figure 3.10. Effect of particle size and concentration of nanoparticles on L929 proliferation (n=3). a) Low dose (0.5 mg/mL), b) High dose (1 mg/mL).

These concentration and size dependences of the results are in agreement with those in the literature. It was reported that 1 mg/mL dose of PHB and PLGA nanoparticles led to a slight decrease in L929 cell proliferation and the lesser amounts were safe. (Yin et. al., 2005, Xiong et. al., 2010, Sohaebuddin et. al., 2010). It was also found that the PHB based nanoparticles were safe and

exhibited very good cytocompatibility in low doses when their sizes ranged between 55 and 100 nm (Errico et. al., 2009).

It was reported that increase of size of nanoparticles have a negative influence on cell viability. Wang et. al. (2010) reported that, cells incubated with polymeric nanospheres had a better growth than the ones with microspheres, because microspheres were hardly phagocytized due to their large size.

3.7.2. Influence of Bioactive Agent, DSW and RP, Loaded Particles on Cell Proliferation

In the previous section unloaded PHBV nanoparticles were tested for their effect on cell proliferation. When bioactive agents were loaded, it is expected that the particles should not elicit a toxic response. Therefore, after studying the effect of concentration of unloaded micro/nanoparticles on cell growth, particles loaded with bioactive agents were tested.

Study was conducted by introducing the same amounts of DSW and RP in free form as well as loaded in PHBV micro/nanoparticles (0.5 mg), into the TCPS wells. The amounts of DSW and RP per well were 1.5 ng and 7.5 ng, respectively.

It was observed that the presence of free DSW suppressed cell proliferation and this suppression was more significant with RP (Figure 3.11). Cell proliferation was statistically significant at all time points.

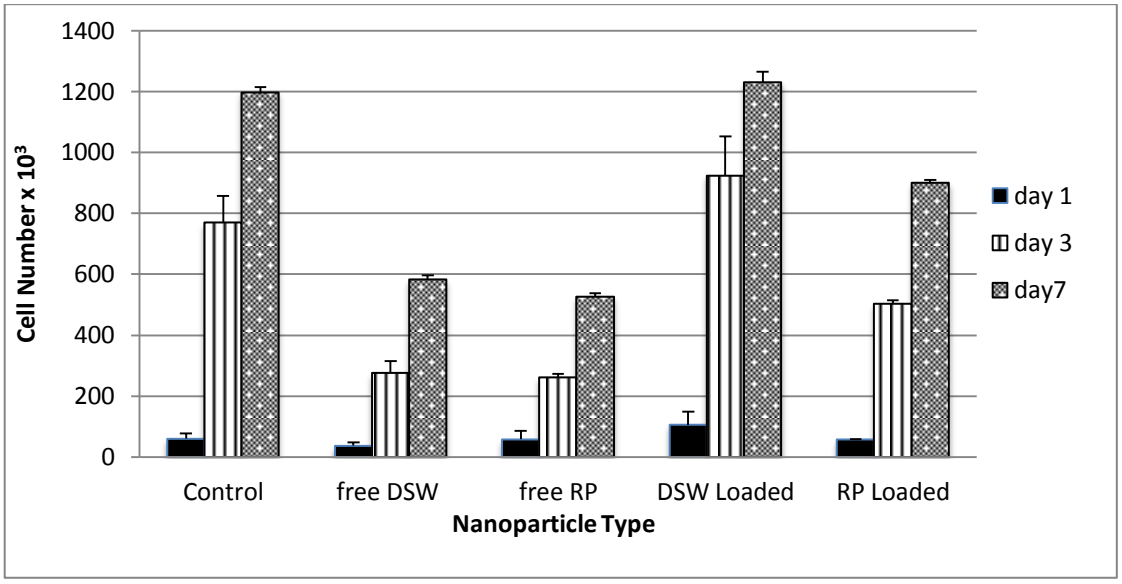


Figure 3.11. Effect of bioactive agent loaded micro/nanoparticles on L929 cell proliferation (n=3). Results of MTT tests.

Upon encapsulation, the RP partially or DSW fully lost their adverse effects. With the introduction of RP in PHBV particles, the cell proliferation increased by 58%. This shows that the toxicity due to DSW and RP is real and entrapment in a carrier helps to mask the toxicity of the bioactive agents.

In the literature, RP has been shown to repair skin damaged by chronological aging or photoaging (Kim et. al., 2008) and it has been reported that photoaging of skin may be decreased by using a cream containing retinoid derivatives (Varani et. al., 2000). It was also known that RP is a skin irritant and has a number of side effects such as skin dryness and wounds, (Kang et. al., 1995, Kim et. al., 2003) and toxicity (Kim et. al., 2008). Thus the damage observed in Figure 3.11 on cell proliferation by free RP was to be expected.

In this study, loading of DSW in PHBV particles significantly prevented cell death (Figure 3.11). DSW loaded capsules demonstrated very good growth of cells; it was quite similar to the control group, where the free DSW strongly suppressed the viability.

3.7.3. Uptake of PHBV Capsules by Different Cells

The interaction of cells with PHBV nanoparticles and the fate of the particles were studied with Saos 2 and L929 Cells. Figure 3.12 shows the location of particles with respect to these cells. The PHBV micro/nanoparticles used were stained with Nile Red (0.1%, v/v) that has a red emission under fluorescence microscopy and were incubated with Saos 2 cells for 4 h and 24 h. Before the microscopic examination, the cells themselves were stained FITC-labeled phalloidin was for the cell cytoskeleton (green) and the DAPI was for cell nuclei (blue). The sizes of most capsules (nano range) were lower than the resolution of the fluorescent microscope, therefore, some of the red (Nile Red stained) regions are probably clusters of nanocapsules. Individual specks observed could be the microparticles. The particle free controls (Figure 3.12a) shows no particles while the samples with the particles show the red specks of the nanoparticles localized in the cytoplasm and some near the cell nuclei after 4 h (Figure 3.12b) and after 24 h (Figure 3.12c) indicating that the nanoparticles can penetrate the cell membrane but not the nuclear membrane.

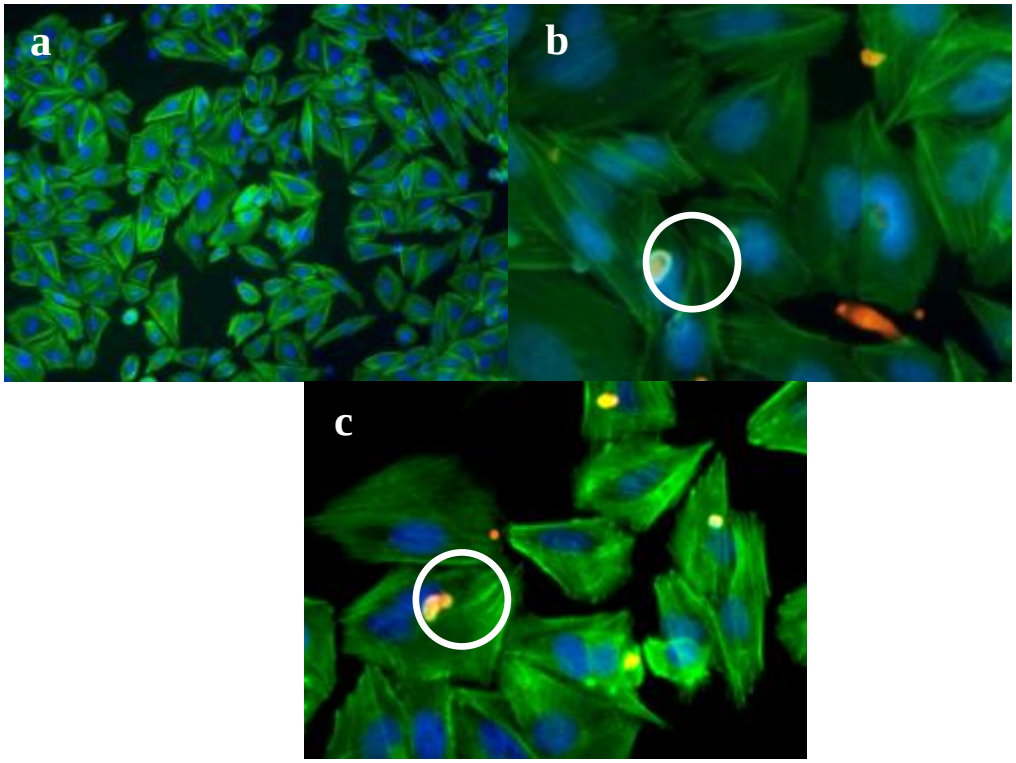


Figure 3.12. Fluorescence microscopy of Saos 2 cells stained with Nile red loaded PHBV nanocapsules. a) Cells without nanocapsules (control) (x20), b) Nanocapsules after 4 h of culture. Capsules are located on the nucleus are indicated by a circle (x40). c) Nanocapsules after 24 h of culture (x40). Cells were stained with FITC-labeled phalloidin and DAPI for the cell cytoskeleton (green) and the cell nuclei (blue), respectively.

After the studying cell penetration into the Saos 2 cancer cells, fibroblastic L929 cells were used to study both the penetration and the effect of size on cell penetration. In order to determine the effect of size on penetration, fibroblasts were used. Micrographs were obtained with CLSM and results are presented in Figures 3.13, 3.14 and 3.15 for nano, sub micron and micron sized particles, respectively.

Initial studies were carried out with nanoparticles with 3 different sizes. There was no significant difference in the uptake of nanocapsules with the average diameter of 166 nm (Figure 3.13) and 426 nm (3.14). An interesting finding is that

the nanocapsules were generally located in the cytoplasm, near the nuclei implying that they could serve as carriers of agents for gene therapy because they seem to be able to avoid the lysosomes and accumulate in the vicinity of the nuclei. However, the larger (micro) particles seem to be unable to penetrate the cells (Figure 3.15).

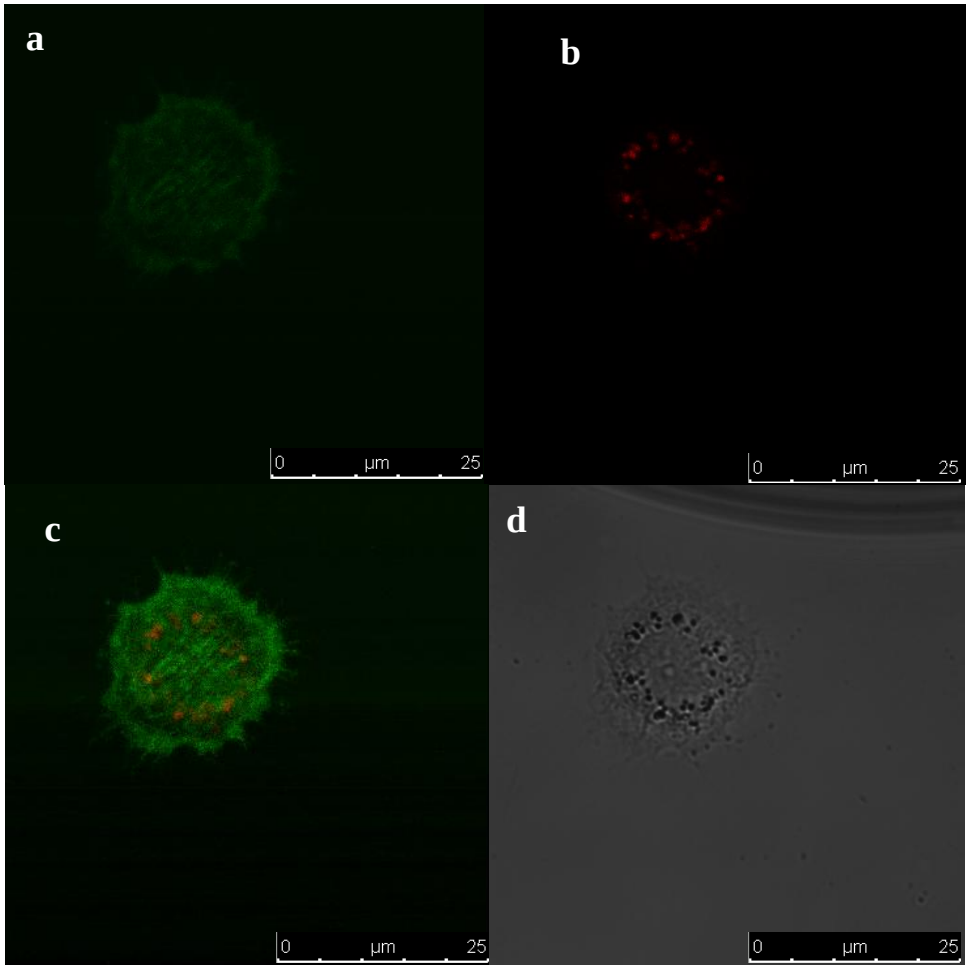


Figure 3.13. Confocal microscopy images of nano (166 nm) sized Nile Red stained PHBV nanocapsules in contact with L929 cells for 24 h (x160). a) Cytoskeleton (green, stained with phalloidin-FITC), b) nanoparticles (stained with Nile Red), c) overlay of (a) and (b), d) transmission image of the cell.

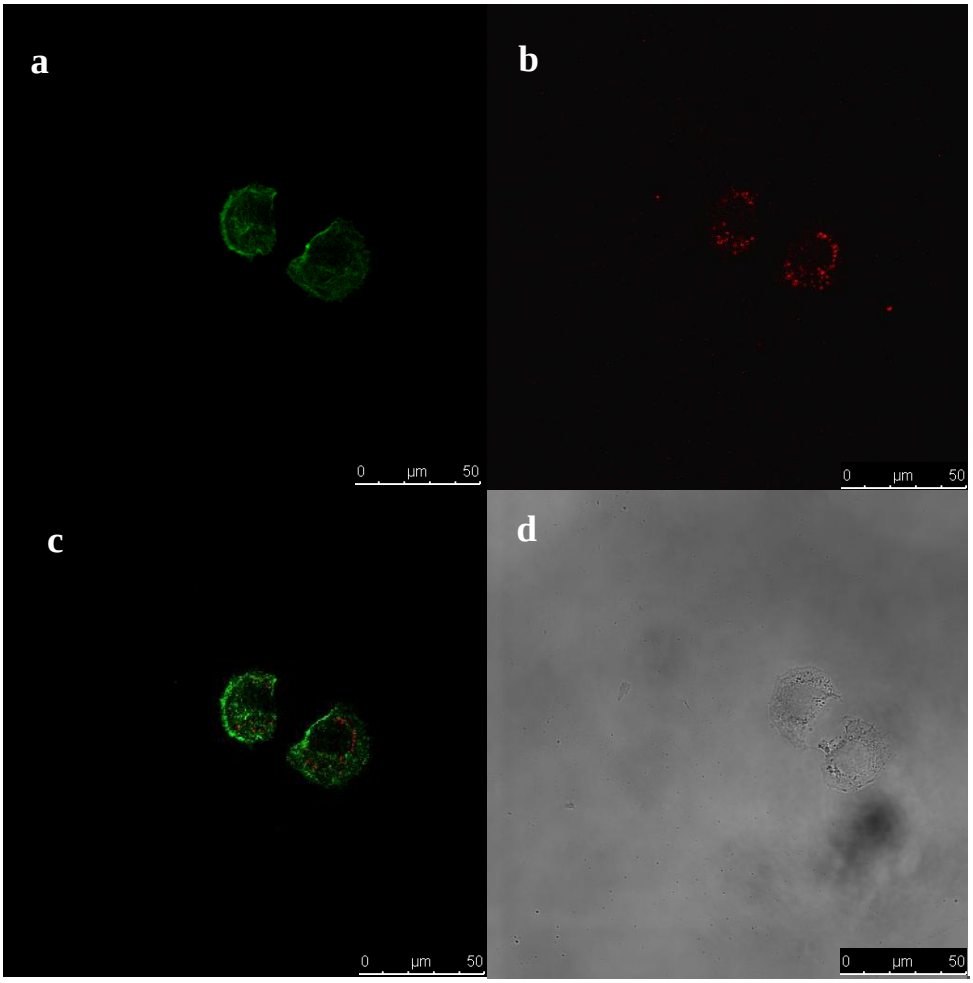


Figure 3.14. Confocal microscopy images of submicron (426 nm) sized Nile Red stained PHBV nanocapsules in contact with L929 cells for 24 h (x40). a) Cytoskeleton (green, stained with phalloidin-FITC), b) nanoparticles (stained with Nile Red), c) overlay of (a) and (b), d) transmission image of the cell.

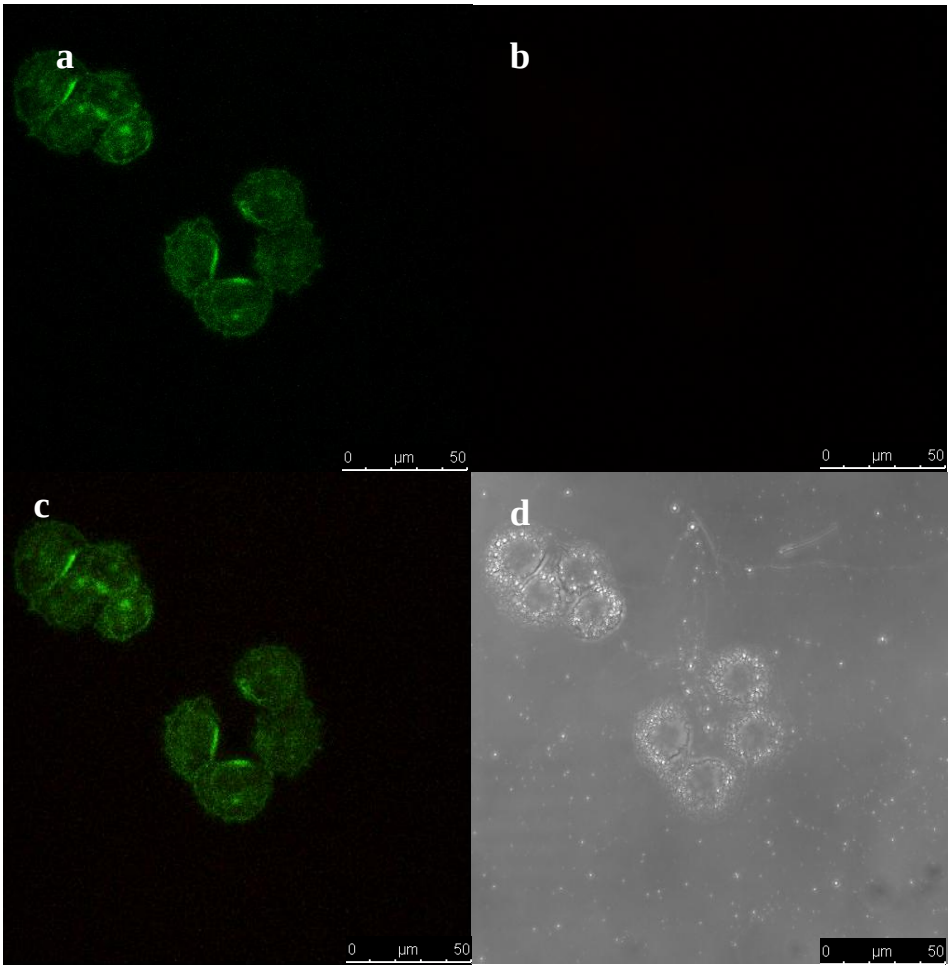


Figure 3.15. Confocal microscopy images of micron (1.9 µm) sized Nile Red stained PHBV nanocapsules in contact with L929 cells for 24 h (x40). a) Cytoskeleton (green, stained with phalloidin-FITC), b) nanoparticles (stained with Nile Red), c) overlay of (a) and (b), d) transmission image of the cell.

Desai et. al., (1997) reported that intracellular uptake of nanoparticles depends on the size and hydrophobicity. In their study the uptake decreased with increasing size and hydrophilicity. Their report stating that it is difficult to internalize the micron sized particles is supported by our not observing the 1.9 µm sized particles inside the cells. Distinction has to be made in terms of the mode of intracellular uptake. Intracellular particulate uptake is proposed to be either by phagocytosis or by endocytosis (Sahoo et. al., 2002). It is reported that microparticles around 0.5 µm are taken up by macrophages by phagocytosis

(Foster et. al., 2001). However, for the smaller nanoparticles, the main route of cellular entry is through fluid phase endocytosis.

Thus, the PHBV nanoparticles prepared in this study were screened by L929 according to their size and penetration into Saos 2 by unfractioned (broad size distribution) particles was probably observed only in the case of the smallest particles.

3.8. *In vivo* Studies

3.8.1. Penetration of Micro/nanoparticles into Mouse Skin

First generation transdermal delivery systems are being increasingly used in the clinic for delivery of small, lipophilic, low dose drugs (Prausnitz et. al., 2008). The question being addressed in this was whether the penetration depth of different sized particles on healthy skin differs. Therefore, the penetration of Nile Red stained PHBV micro/nanoparticles present as emulsions containing simple penetration enhancers (PEG and PVA) was studied on mouse skin.

Upon sacrifice of mice, skin patches were removed, treated according to the section 2.2.6.2, and the amount of micro/nanoparticles in the skin was determined using GC-MS spectroscopy with 0.05 mg of benzoic acid serving as a standard. The results of the analysis of the skins are shown in Table 3.6. It is apparent that particles penetrated into the skin at different rates, when different chemical enhancers were used.

Table 3.6. PHBV micro/nanocapsule penetration into mice skin. High concentration, 5 mg/mL, application (n=2).

PHBV Capsule Size	Enhancer	Polymer penetrated (mg/test area)	Penetration extent of input polymer (%)
Micron	PVA (8%)	0.030	0.10±0.00
Submicron		0.040	0.15±0.01
Nano		0.038	0.14±0.01
Micron	PEG (15%)	0.011	0.04±0.01
Submicron		0.013	0.05±0.00
Nano		0.011	0.04±0.02

PHBV capsules penetrated into the skin to different extents, depending on the chemical enhancer type used and on size (Table 3.6). PVA solution (8 %) led to a 3-fold higher penetration than PEG (15 %). For large particles (micro) penetration was calculated as 0.10%. This amount was less than the submicron and nano sized particles which were 50% higher as 0.15% and 0.14%, respectively, proving that smaller particles penetrated the skin more effectively. The content of polymers in the skin after enhancement with PEG solution was calculated as 0.04%, 0.05% and 0.04% for micron, submicron and nanosized particles, respectively.

PEG is a commonly used enhancer, especially effective when it is prepared in high concentrations (Heuschkel et al, 2007) but in this study PVA was much better as an enhancer. Therefore, further analysis were done using PVA. PEG solution also led to some inflammation whenmacroscopically observed (data not shown).

Table 3.7. PHBV micro/nanocapsule penetration into mice skin. Low concentration, 0.5 mg/mL, application (n=2).

PHBV Capsule Size	Enhancer	Polymer penetrated (mg/test area)	Penetration extent of input polymer (%)
Micron	PVA (8%)	0.045	1.21±0.15
Submicron		0.060	1.62±0.11
Nano		0.067	1.76±0.09

The low concentration of the particles applied to skin was found to be more effective; a higher fraction of the particles penetrated the skin and the size effect was much more distinct (Table 3.7). It is not clear why low concentration got transferred more effectively except may be the higher concentration particles clogged the channels at the hair follicles and decreased the permeants. In brief, low concentration carrier and PVA as enhancer will be used in further in vivo studies.

CHAPTER 4

CONCLUSION

In this study nano and microparticles of PHBV were developed for use in the treatment of various skin diseases. The bioactive agents selected, retinyl palmitate and Deas Sea Water, are only model drugs. For specific diseases special drugs are need to be encapsulated in these particles to achieve the optimum healing if not complete cure. In this context, the systems developed become personalized treatment tools.

It was observed that these particles penetrate the skin and also the cell membrane. This shows that the effectiveness of the application would be high without the potential damage due to the nanoparticle accumulation because of the biodegradability of the particles.

4.1. Ongoing and Future Work

The preliminary results of skin penetration of all sized nanoparticles showed that they penetrated into mice skin indicating that bioactive agents can be transported to the inner parts of the skin if loaded particles were introduced. However, detailed penetration depth should also be studied with histology. Healthy skin penetration tests were partially studied, and compromised skin studies have to be part of the future work. After determination of penetration depth of PHBV particles, the effect of RP and DSW on the healing process of skin has to be studied.

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APPENDIX A

AREA PERCENT REPORT OF GC-MS

Table A.1. Area Percent Report of PHBV Polymer

Peak #	R.T. min	First Scan	Max Scan	Last Scan	PK TY	Peak Height	Corr. Area	Corr. % max.	% of Total
1	6.896	216	230	265	BB	5453127	224051763	100.00%	84.564
2	8.624	393	402	426	BB	1484090	33470467	14.94%	12.633
3	16.825	1215	1219	1222	M	27845	578312	0.26%	0.218
4	19.089	1439	1444	1446	M	19288	429306	0.19%	0.162
5	20.965	1627	1631	1636	M4	11662	251587	0.11%	0.095
6	21.142	1641	1648	1654	BV	177295	3323852	1.48%	1.255
7	22.814	1806	1815	1817	M2	114855	2360614	1.05%	0.891
8	23.014	1832	1835	1837	M	29838	482825	0.22%	0.182

Sum of corrected areas
264948726

Table A.2. Area Percent Report of PHBV Particles with Sorbic Acid.

Peak #	R.T. min	First Scan	Max Scan	Last Scan	PK TY	Peak Height	Corr. Area	Corr. % max.	% of Total
1	6.881	218	228	250	BB 2	4323917	161006216	100.00%	78.737
2	8.622	397	402	420	BV	990052	22722124	14.11%	11.112
3	9.151	449	454	472	BB 2	521409	13935430	8.66%	6.815
4	16.824	1214	1218	1223	M	20975	445917	0.28%	0.218
5	19.086	1439	1444	1445	M	21548	428841	0.27%	0.210
6	21.136	1645	1648	1651	M	150161	2790822	1.73%	1.365
7	22.810	1811	1814	1817	M3	49881	1225841	0.76%	0.599
8	22.855	1818	1819	1828	M2	39178	974131	0.61%	0.476
9	23.013	1831	1835	1839	M	50614	957607	0.59%	0.468

Sum of corrected areas
204486930

Table A.3. Area Percent Report of PHBV Particles without Sorbic Acid.

Peak #	R.T. min	First Scan	Max Scan	Last Scan	PK TY	Peak Height	Corr. Area	Corr. % max.	% of Total
1	6.843	214	225	244	BB 2	2587073	76667483	100.00%	82.020
2	8.619	394	401	416	BB	387555	9399220	12.26%	10.055
3	16.830	1213	1219	1226	M	11510	342337	0.45%	0.366
4	19.090	1438	1444	1446	M	16205	349490	0.46%	0.374
5	21.139	1642	1648	1657	M	144291	2888251	3.77%	3.090
6	22.814	1812	1815	1816	M	65550	1250717	1.63%	1.338
7	22.861	1818	1820	1829	M2	81498	2110657	2.75%	2.258
8	23.015	1833	1835	1839	M	29781	466318	0.61%	0.499

Sum of corrected areas
93474473

APPENDIX B

CALIBRATION CURVE FOR RP CONCENTRATION WITH UV-VIS SPECTROSCOPY

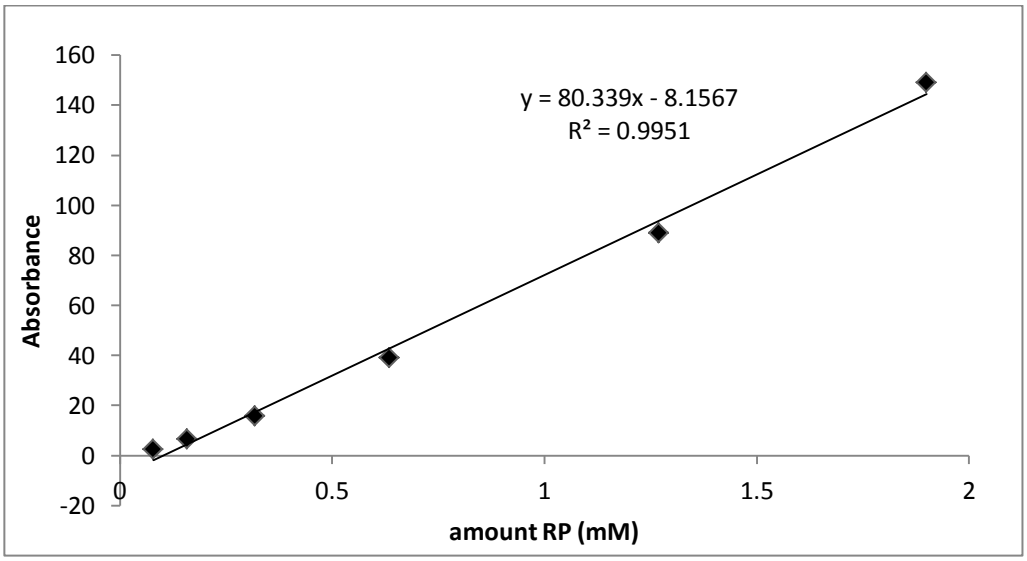

Figure B.1. Calibration curve of RP concentration for UV-VIS Spectrometer.

APPENDIX C

CALIBRATION CURVE FOR RP CONCENTRATION WITH HPLC

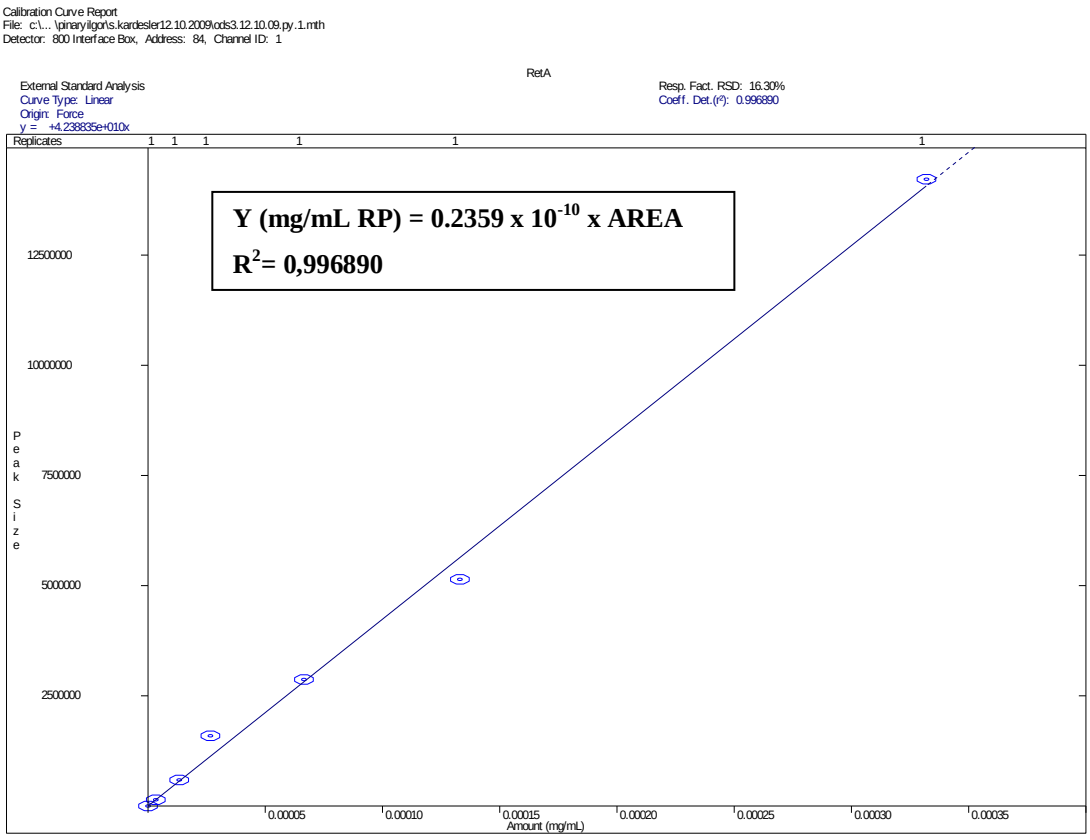

Figure C.1. Calibration curve of RP concentration for HPLC.

APPENDIX D

CALIBRATION CURVE FOR CELL NUMBER DETERMINATION

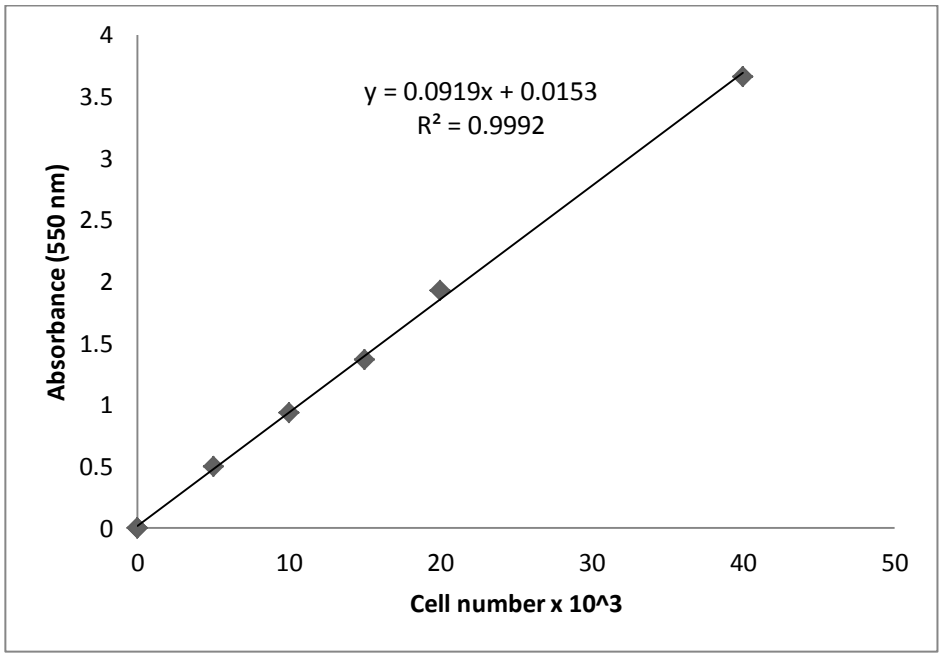

Figure D.1. Calibration Curve of MTT with mouse fibroblast NIH-3T3 Cell Line

APPENDIX E

ETHICAL COMMITTEE REPORT

СИБИРСКИЙ ФЕДЕРАЛЬНЫЙ УНИВЕРСИТЕТ
SIBERIAN FEDERAL UNIVERSITY

Institute of Fundamental Biology and Biotechnology of Siberian Federal University Krasnoyarsk

The protocol of experimental research of "BIOPLASTOTAN" transdermal delivery system based on biopolymers - polyhydroxyalcanoates, PHA, when applied to the undamaged skin of the vertebrata.

Time period of the research – July 1-10, 2011.

Venue - SibFU, the Chair of Medical Biology, rooms 44-02, 44-03.

During the experiment follow the regulations as set in GOST R ISO 10 993.99 "Evaluation of the irritant effect"

1. Shave non-traumatically the skin of the animals on both hips, 24 hours before the application of polymers suspensions.
2. Apply the prepared suspensions of polymer particles on the undamaged skin of mice in the amount of 200 µL every day during 10 days in aseptic conditions using the dosing pipette. Afterwards rub the suspension in using the soft sterile applicator during 2 minutes.
3. Estimate the condition of the animals' skin being under the effect daily during the experiment before and after application of suspensions, and after killing the animals.
4. The animals should be taken out of the experiment by the overdose of the ether anesthesia.
5. Sampling of the skin should be done right after killing of the animals, from both hips, cutting the sections of the skin 2cm x 2cm in size using surgery scissors after disinfecting these sections with the spirit. Then the section should be cut into two pieces, each 2cm x 1cm in size, placing one of them into 10% neutral buffered formaldehyde solution, and another one into the dry Petri dish.
6. Dead bodies of the animals should be disposed of in accordance with the General Regulations (Appendix 1).
7. After finishing the experiment do the microscopic analysis of the skin condition, using light microscopy with coloration by Hematoxylin/ Eosin according to traditional methods. The presence of particles in the animals' skin should be analyzed by the fluorescent microscopy method and HPLC method for chloroform extracts of the skin samples. In all cases the skin of intact animals that were not affected should be used as the control sample.
8. Animals in the experiment: white mice balb/c, males, 12 weeks old, average weight- 20.6 g ±1,2g

Ethic documents

ISO 10993-1:2003
GOSR R ISO 10 993-1.2009
Guide for the Care and Use of Laboratory Animals, Washington, D.C., 1996;
Animal Welfare Act Regulations, Title 9 Code of Federal Regulations, Parts 1, 2 and 3.
USA;
Legislation. Official Journal of European Communities. 1986. Vol.29;
Law Concerning the Protection and Control of Animals (Law #105), October 1, 1973, Japan;
Standards Relating to the Care and Management. Etc. of Experimental Animals
(Notification #6), March 27, 1980, Japan; Use of animals in space biology and medicine. Russian national committee on bioethics of the Russian Academy of Sciences

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Responsible person for observation of the experiment protocol
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The research program was developed by Prof. Ekaterina I. Shishatskaya, M.D., D.Sc., Chief of the Chair of Medical Biology. The program coincides with the SibFU standards for experiments with the vertebrata based on the Appendix No. 2.

The research program was approved by the Committee on Biomedical Ethics of SibFU:

Prof. V.A. Sapozhnikov, Director of the Institute of Fundamental Biology and Biotechnology — 20 June 2011

Prof. T.S. Volova, D.Sc., Head of basic chair of biotechnology

Prof. V.A. Kratasyuk, D.Sc., Head of chair of biophysics

Figure E.1. Ethical Committee Report