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Fate of Poly-3-Hydroxybutyrate-co-3-Hydroxyvalerate on Skin

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Transdermal drug delivery systems have become increasingly sophisticated over time. However, the greatest limitation for developing an effective drug delivery system is the highly impermeable outermost layer of the skin called the stratum corneum. Therefore, materials penetrating the skin must be of low molecular weight, and lipophilic. There are many techniques to safely pass the stratum corneum and one of the promising method of transdermal drug delivery is the use of micro and nano sized particles. The aim of this study was to develop three different micro and nano sized carriers to study their skin penetration and to judge their effectiveness within the skin. Polymeric micro/nanocapsules carrying a fluorescent dye Nile Red, were prepared using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (5 %mol. hydroxyvalerate). The in vivo transdermal permeation of PHBV micro/nanoparticles were studied using a mouse model. According to the particle size analysis with Master Sizer and Zeta Potential Measurement System, the PHBV micro/nanocapsules were 1.9 μm, 426 nm and 166 nm in diameter. The particles were applied to healthy skin of the dorsal region of

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BALB/c mice. Penetration of the particles was determined by GC-MS analysis of the skin for PHBV. Scanning electron microscope (SEM) imaging was used to study their morphology. GC-MS results showed that the capsules penetrated into the skin in relation with their particle size, despite the highly impermeable outer skin layer. However, histology cross-section revealed that uncompromised skin could not penetrate; the transport of the polymeric particle was clearly impeded by the stratum corneum. It was thus shown in this study that control of penetration depth, and therefore, the target size within into the skin is possible by varying the size of the drug carrying nanocapsules.

Keywords: transdermal drug delivery, micro/nanocapsules, polymeric carriers, in vivo drug permeation.

Introduction

Transdermal drug applications are frequently used because of the effectiveness of the localized treatment, low cost, relatively low side effects, maximum drug availability at the target site, and avoids systemic circulation. Many bioactive agents, however, do not have the necessary physicochemical properties for satisfactory efficacy when applied topically (Sloan et al., 2006).

Transdermal skin treatment requires the absorption of drug through the skin into the body. One of the biggest challenges in developing an effective system is to transfer the drug through the tightly structured stratum corneum when the skin is not compromised (Yow et al., 2009). There are basically three layers of skin that needs to be passed: 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. 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 (Gawkrodger et al., 2002). The outermost layer of skin (stratum corneum) consists of keratinized corneocytes and intercellular lipid rich domains. The lipid matrix and the corneocytes, together, create a barrier, which is broken by hair follicles and sweat glands (Prausnitz, Lagner, 2008). These components covered <1 % of the surface area are believed to serve as a route of entry for nanoparticles. 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).

For the drugs to be able to cross the skin, in other words, to have skin permeability they need to be lipophilic and of low molecular weight. When the bioactive agent is prone to enzymatic attack or simple hydrolysis, it must be protected until it reaches the target site through complexation being attached to or entrapped by a carrier, preferably small size. Such labile molecules include proteins, DNA and other genetic materials, and vaccines. An important approach in this direction is the use of nanoscale drug delivery systems. Nanoparticles were developed as an important strategy to deliver conventional drugs like antibiotics (Gursel et al., 2001), recombinant proteins (Jahanshahi et al., 2008), vaccines (Rieuxet al., 2006), nucleotides (Xia et al., 2009) and growth factors (Yilgor et al., 2010). The special advantage of nanoparticles is their ability to reach tissues that other controlled release depots cannot and to release their contents there rather than staying in the circulation and releasing their

content systemically. With the developments in nanotechnology and their introduction to the biomaterials field, various types of nanosized drug delivery systems such as nanocapsules, nanospheres, complexes, liposomes, dendrimers and emulsions were developed. Among these are the uses of nanosized drug carriers constructed from biodegradable polymers are also increasingly used because even if they penetrate untargeted tissues they do not stay long and disintegrate (Yin, Feng 2005; Hasirci et al.; 2007, Yilgor et al., 2010).

Polyhydroxyalkanoates (PHAs) are among the preferred natural polymers, because they are produced in variety compositions with different properties including degradation rate by bacteria as well as by transgenic organisms (Hasirci, Yucel, 2007). The versatility of the products is due to ease of changing of the carbon source, bacteria type and the growth conditions in the reactor. PHAs are linear, semicrystalline, thermoplastic, bioresorbable and biocompatible polymers of microbiological origin which have a certain degree of biodegradability in the body (Amass et al, 1998; Sudesh et al., 2000; Martin, Williams, 2003; Volova et al., 2003; Volova 2004; Volova et al., 2006; Hasirci et al., 2006) and as such have the advantage over the other polyester group, polylactides, which are petroleum based. 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 with various HV contents has been used in controlled release systems (anticancer agents, pain relievers, antibiotics, growth factors), biodegradable bone plates, and in tissue engineering (cornea, bone, cartilage) applications (Hasirci, Yucel, 2007; Goreva et al., 2012).

In this study, *in vivo* transdermal permeation of PHBV micro/nanoparticles was studied by using Nile Red as a fluorescent agent to follow transdermal mobility of polymeric particles. The delivery system was constructed using PHBV micro/nanocapsules with different diameters, and whether the size of the particles influences the penetration depth was studied. The size distribution, morphology and skin penetration of the capsules were studied. Mouse skin was used for *in vivo* penetration studies and by histology examinations penetration depth of the particles were determined.

Materials and Methods

Nile Red loaded PHBV capsule preparation

A solution of PHBV in dichloromethane (1.2 mL, 10 %, w/v) was prepared and then Nile Red (0.1 mL, 0.01 % in acetone) was added to this solution. An emulsion was formed by sonication for 15 s in an ice bath, added into a solution of PVA in distilled water (4 mL, 4 %, w/v) and sonicated again for 15 s. This emulsion (water/oil/water) was further diluted with aqueous polyvinyl alcohol solution (PVA) (10 mL, 0.3 %, w/v), mixed with a magnetic stirrer overnight at room temperature for solvent evaporation. The capsules were precipitated by centrifugation and lyophilized.

Preparation of micro/nanocapsules with different sizes

In order to obtain different sized particles, different centrifuge speeds and durations were employed. To obtain low micron sized (largest) fraction, the suspension was centrifuged at 12000 rpm (15455 g) for 10 min, in the centrifugation step of the preparation to recover the particles. The supernatant was further centrifuged at 13500 rpm (18138 g) for 10 min to obtain high nano-sized particles. Finally, the remaining supernatant was centrifuged at 14500 rpm (22566 g) for 40 min to obtain the lowest diameter nanoparticles.

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 (Netherlands). The diameters of the nanoparticles were measured from the SEM images using the Image J software (NIH).

Particle size distribution analysis

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

In vivo studies: preparation of PVA suspension containing PHBV micro/nanocapsules to enhance skin penetration

Two types of amphiphilic molecules, with low and high molecular weights, were tested as skin penetration enhancers, polyethyleneglycol (PEG) (MW 10^3) and PVA (MW $1.5x10^4$). PEG and PVA were dissolved in distilled water at a concentration of 15 % (w/v) and 8 % (w/v), respectively. Solutions were heated to 50°C, filtered, and then 50 mg and 5 mg of Nile Red loaded PHBV capsules were suspended in these solutions to form 1 % (w/v) and 0.1 % (w/v) nanoparticle suspensions, respectively. Then, these suspensions were applied to the dorsal skin of the mice that was shaved one day earlier.

In vivo studies: animal model

Experiments were conducted on 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 of Russian Federation (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 Siberian Federal University, Krasnoyarsk, Russia.

In vivo studies: treatment of mice and application of polymeric particles

Dorsal sections of the mice were shaved before application of the formulations prepared as above desribed. After removal of the hair, skin was swabbed with pure ethanol to sterilize and dry the skin (Goope et al., 2009).

On the dorsal left side of the mice (12 individuals), low concentration (0.5 mg/mL), and on the right side, high concentration (5 mg/mL) particle suspension (ca. 1 mL/day) was applied daily 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 micro and nanocapsule suspension were applied to the shaved skin followed by a 2 min massage using a cosmetic applicator. This application was repeated everyday for 10 days. The control was the intact skin of the mice. Controls were treated exactly same way, except the application of the formulation.

After 10 days, the animals were sacrificed with an overdose of formalin vapor. Skin patches (approximately 1x1 cm^2) were removed and used in the determination of the polymer content, and thus, the amount of particles penetrated.

Determination of the polymer of the nanocapsules in the skin with GC-MS

The skin samples were dried at 60°C overnight and weighed. Then approximately

10 mg of sample was weighed and the polymer amount was determined with GC-MS.

To determine the polymer amount of the particles, 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, v/v) for 140 min at 100°C in a thermostatically regulated bath. This method is called methanolysis, and degrades the polymer to its constituent, fatty 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 (GCD Plus, Hewlett Packard, USA), equipped with a 30 m x 0.25 mm HP-5 capillary column (Sevastianov et al., 2003).

Histological preparation studies

The tissue samples were fixed in 10 % formalin and paraffin-embedded. The microtome sections (5µm) were obtained using microtome and stained with hematoxylin and eosin and examined under a light microscope (on fluorescence) (Leica DM6000B, Germany). This analysis was qualitative and analyzed either the presence or absence of polymer penetration. The general tissue reaction to micro and nanocapsules was investigated using conventional histological techniques.

Statistical analysis

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

Results and Discussion

Micro/nanocapsule characterization

Production of PHBV particles were carried out with 10 % (w/v) polymer solution in dichloromethane. The choice of this concentration was based on previous studies conducted with similar polymers (Chen et al., 2009; Yilgor et al., 2010). SEM micrographs show that round, spherical capsules with smooth surfaces and were obtained with 10 % (w/v) polymer solutions (Fig. 1). 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 (Crowley et al., 2000; Ericco et al., 2009; Yilgor et al., 2010). In this study fractionation using successive centrifugations was employed.

Three different capsule populations with different sizes were obtained. The SEM micrographs revealed that by changing the parameters of time and speed of centrifugation, it was possible to fractionate the particles into groups with different sizes (Table 1, Fig.1). The largest (1900 nm) particles were obtained in the first fractionation step (10 min, 12000 rpm). The medium sized particles (426 nm) were obtained after the second step (10 min, 13500 rpm) as seen in the Fig. 1a and 1b. The smallest PHBV particles (186 nm) were obtained after the third centrifugation (40 min, 14500 rpm) (Fig. 1c). SEM micrographs also show that all particles were round and spherical with smooth surfaces.

The samples are referred to as micro, nano and low nano, based on their average particle size, size range and polydispersity. Size range of the particles was determined by the Master Sizer for the microcapsules and Zeta Sizer for the nano and low nanocapsules. Polydispersity indices (PI) of these micro, submicro and nanoparticles were found to be 0.8, 0.3 and 0.1, respectively, indicating that the smallest fraction had the most

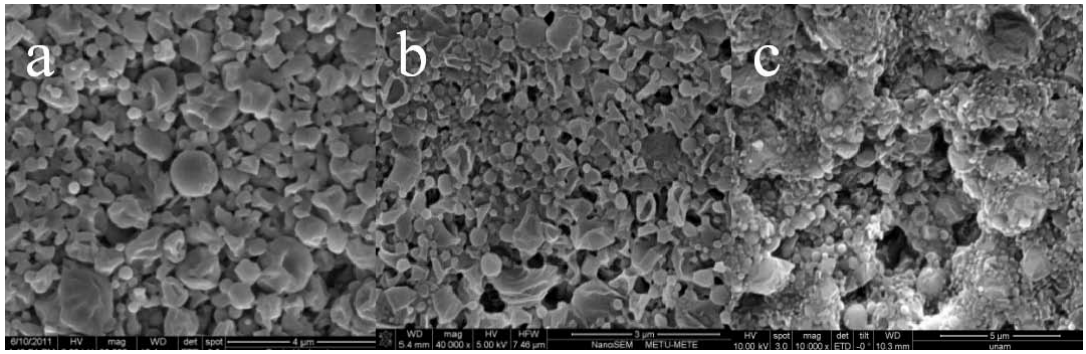

Fig. 1. SEM micrographs of PHBV particles prepared with different duration and speed of centrifugation: a) 10 min at 12000 rpm. Bar 4 μm, b) 10 min at 13500 rpm. Bar 3 μm, c) 40 min at 14500 rpm. Bar 5 μm

Table 1. Sizes and polydispersity indices of PHBV micro/nanocapsules

Property	Sample		
	Micro	Nano	Low Nano
Mean Diameter, nm	1900	426	166
PI	0.8	0.3	0.1
Size Range, nm	400-12000	190-712	80-542

narrow particle size distribution as confirmed by the sharpness of the peak in Fig. 2c and the size range in Table 1.

In vivo tests: penetration of micro/nanocapsules into mouse skin

Transdermal delivery systems are being increasingly used in the clinic for delivery of small lipophilic drugs for low doses (Wermeling et al., 2008; Baroli, 2010). The question being addressed in this study was whether the penetration depth of different sized particles in healthy skin differs. Therefore, the penetration of Nile Red stained PHBV micro/nanocapsules 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 2.5.2, 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 are shown in Table 2. It is apparent that particles penetrated into the skin at different rates, when different chemical enhancers were used.

GC-MS results indicate that PHBV capsules penetrated into the skin to different extents (percent of the applied amount) depending on the chemical enhancer type used and on size (Table 2). Application of PVA solution (8 %) led to a 3-fold higher penetration of the same particles than PEG (15 %) did. For large particles (micro) penetration was calculated as 0.10 % and was lower than the nano and low nano sized particles, penetration of which were 50 % higher (0.15 % and 0.14 %, respectively), indicating that smaller particles penetrated the skin more effectively. After enhancement with PEG solution, however, the values were too low and very similar. It is probably the sensitivity of the analysis that could

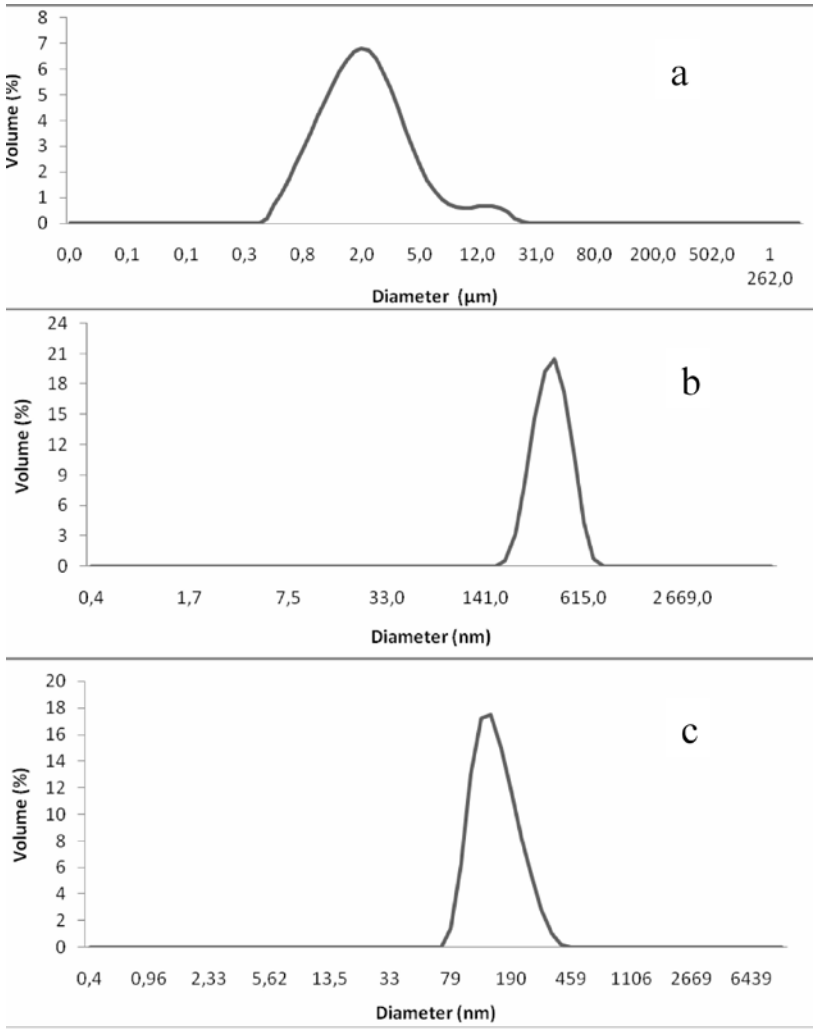

Fig. 2. Particle size distribution of PHBV particles. a) micron, b) submicron, c) nano sized samples

Table 2. PHBV micro/nanocapsule penetration into mice skin. High dose, 50 mg total, 5 mg/mL, application (n=2)

PHBV Capsule Type	Enhancer Type	Polymer Penetrated (mg/test area)	Polymer Penetration Extent Input (%)
Micron	PVA (8 %)	0.030	0.10±0.00
Nano		0.040	0.15±0.01
Low Nano		0.038	0.14±0.01
Micron	PEG (15 %)	0.011	0.04±0.01
Nano		0.013	0.05±0.00
Low Nano		0.011	0.04±0.02

not differentiate the differences if there were any.

PEG is a commonly used enhancer, especially effective when it is prepared in high concentrations as was applied in the present study (Heuschkel et al., 2008). But in this study PVA performed much better, and therefore, further tests involving the low dose application were conducted using PVA.

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). The area of the skin was equal in volume for high and low concentration carriers and the amount of penetrated polymers were relatively high with respect to high concentrated polymer suspension. 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 should be considered to be used in further *in vivo* studies.

Histopathological analysis of mouse skin

The depth of penetration of three differently sized Nile Red loaded PHBV micro/nanocapsules was investigated by transdermal application in BALB/c mice using histopathology. Results of the histological examination of the skin of mice from experimental and control groups showed an identical morphological picture of the skin. No signs of necrosis or festering inflammation were observed in any group. Epidermis was of ordinary thickness, pilosebaceous units were fully formed, reaching a hypodermic-fatty layer. Follicles contained hair bars. Slight lymphohistiocytic infiltration was recorded perifollicularly (Fig. 3).

No significant differences were found in tissue response to implantation of three differently sized PHBV micro/nanocapsules used in this study; neither necrosis nor any other adverse morphological changes and tissue transformation in response to the application of the polymeric micro/nanocapsules were recorded. The results of the study suggest that PHBV is a good candidate for fabricating prolonged-action drugs in the form of micro/nanocapsules intended for transdermal application.

Conclusion

The aim of transdermal delivery systems is to deliver low molecular weight agents with the right set of properties without irritating the stratum corneum and deeper tissue layers. Polymeric particles are promising tools for these delivery applications. They can be made in various sizes, from various materials, carry molecules of different chemistries, and be modified for targeting purposes. As such, they are indispensible drug carriers. Polymeric capsules were prepared in micro, submicro and nano sizes. Upon fraction nano sized particles have shown narrower size distribution and lower polydispersity index. *In vivo* testing with BALB/c mice of particle penetration revealed that smaller particles penetrated the skin more effectively. The low concentration of the particles applied to skin was found more effective. These results give us possibility to consider bacterial PHBV, produced in the laboratory HABS and cleaned with the author technique, Bioplastotan®, to develop systems for transdermal transport, based particles smaller than 50 nm, which is necessary for penetration through intact skin, so as use elaborated sizes of particles for the treatment of compromised skin. These results give us ground to consider the use of bacterial PHBV in the development of systems for transdermal transport, taking into account high biocompatibility and feasibility of varying the speed of biodegradation PHA carrier with the composition of monomers and methods for producing the particles.

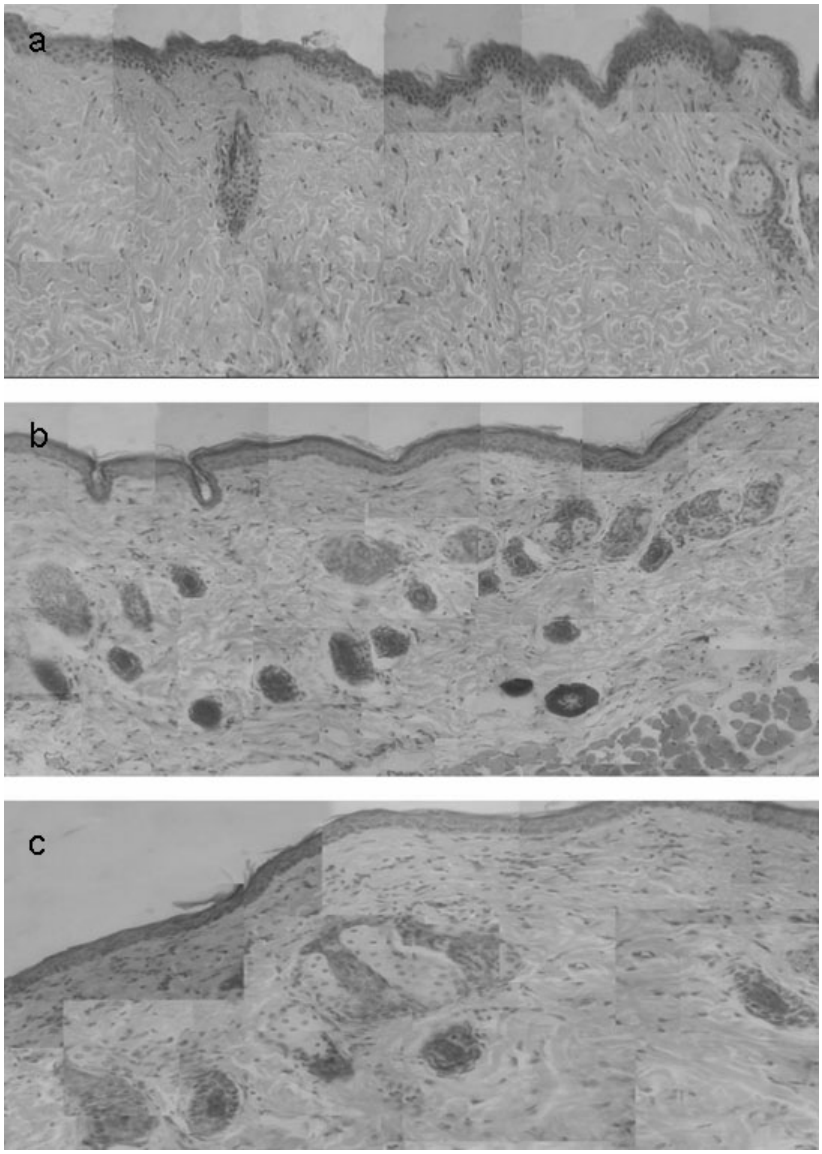

Fig. 3. Histology of the skin sample in BALB/c mice after application PVA (8 %), low dose of Nile Red loaded PHBV nanocapsules. Size of nanocapsules: a – 166 nm, b – 426 nm, c – 1900 nm

Table 3. PHBV micro/nanocapsule penetration into mice skin. Low dose, 5 mg total, 0.5 mg/mL, application (n=2)

PHBV Capsule Type	Enhancer Type	Polymer Penetrated (mg/test area)	Polymer Penetration Extent Input (%)
Micron	PVA (8 %)	0.045	1.21±0.15
Nano		0.060	1.62±0.11
Low Nano		0.067	1.76±0.09

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[ж] БИОМАТЕН, Центр передового опыта в области исследования биоматериалов и биотехнологии тканевой инженерии БВТУ, Анкара, 06800 Турция

Трансдермальные системы доставки лекарственных средств становятся всё более совершенными. Однако самым большим препятствием для разработки систем доставки лекарственных средств является наличие практически непроницаемого верхнего рогового слоя эпидермиса. Материалы, проникающие через кожу, должны иметь низкий молекулярный вес и быть липофильными. Существует много способов преодолеть барьер рогового слоя, и один из перспективных методов – использование микро- и наночастиц. Это исследование было направлено на разработку трёх различных микро- и наноразмерных носителей, изучение их проникновения в кожу и оценку их эффективности в коже. Были сконструированы полимерные микро/нанокапсулы из поли(3-гидроксибутирата/3-гидроксивалерата) (ПГБВ) (ГВ 5 мол. %) с флуоресцентным красителем Nile Red. Трансдермальное проникновение микро/нанокапсул из ПГБВ было изучено in vivo, на мышах. Согласно анализу размера частиц, проведённому с помощью анализатора Master Sizer и системы измерения дзета-потенциала, микро/ нанокапсулы имели диаметр 1,9 мкм, 426 нм и 166 нм. Частицы наносили на здоровую кожу спины мышей линии BALB/c. Чтобы оценить проникновение частиц, определяли присутствие ПГБВ на коже методом хромато-масс-спектрометрии. Морфология исследовалась с помощью растрового электронного микроскопа. Результаты хромато-масс-спектрометрии показали, что проникновение капсул через кожу зависело от размера частиц, несмотря на труднопроницаемый верхний слой кожи. Однако гистологический поперечный разрез показал, что частицы не могли проникнуть через неповреждённую кожу; роговой слой эпидермиса явно

препятствовал переносу полимерных частиц. Данное исследование продемонстрировало, что, изменяя размер наночастиц, несущих лекарственные средства, можно регулировать глубину проникновения частиц и, следовательно, осуществлять целевую доставку лекарственных средств.

Ключевые слова: трансдермальная доставка лекарственных средств, микро/нанокапсулы, полимерные носители, проникновение лекарственных средств in vivo.