APPLICATION OF PHB-LIPOSOME PARTICLES AND NANOFIBERS IN COSMETICS

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Abstract

Liposomes are versatile systems for drug delivery. They are utilized in various industries such as pharmaceutical, food industry and cosmetics. Even though they are capable to encapsulate both hydrophilic and lipophilic compounds, they can be modified using cholesterol, proteins and polymers to enhance their properties.

Presented work is focused on development and preparation of PHB-liposomes particles with different PHB content as well as PHB nanofibers. Characterization in terms of particle size, polydispersity index, colloidal stability and long-term stability were done. Sun protection factor was measured, too. Electron microscopy techniques (SEM, cryoTEM) were used to observe structure of prepared materials. To assess whether prepared particles are suitable for further application in cosmetics, MTT and LDH cytotoxicity assays on HaCaT cell line were carried out.

Our goal was to prepare and characterize PHB containing liposomes and nanofibers for potential application in cosmetic, pharmaceutical and food industry. Prepared particles were stable and had proper properties to be used as vehicles. Cytotoxicity assays showed that all materials were nontoxic to HaCaT cell line and, therefore, they can be ceded for further cytotoxicity as well as genotoxicity testing and in future potentially applicable in cosmetic industry.

Keywords: PBH, liposomes, nanofibers, cyto-toxicity, cosmetics

1. INTRODUCTION

Liposomes are versatile vehicles that are used for encapsulation and drug delivery in pharmaceutical industry as well as in cosmetics. They were firstly observed by Alec Bangham et al in 1960s [1]. Liposomes comprises of particles made of one or more phospholipid bilayer from biodegradable and biocompatible materials and aqueous environment entrapped in the middle part of the vesicle. Besides phospho- and sphingolipids their compositions could be altered by different compounds such as cholesterol, lipids and others [2]. Polyhydroxy alcanoates like poly-3-hydroxy butyrate are polyesters accumulated by numerous prokaryotes in the form of intracellular granules. The primary function of PHAs is the storage of carbon and energy. Recent research has shown that the biological function of PHAs is much more complex and that the capability to accumulate PHA has many biochemical and biophysical consequences.Their UV-protective action might be explained by their efficient UV-radiation scattering properties with high scattering efficiency in the wavelengths close to the DNA absorption maxima [3].

Our goal was to prepare and characterize liposomal particles enriched with poly-3-hydroxy butyrate and its nanofibers. As a new nanomaterial, both particles and fibers need to be evaluated by cytotoxicity assays like MTT and LDH. All these characterizations are important steps for final decision whether prepared materials are suitable for further application in cosmetics and other industries.

2. MATERIALS AND METHODS

2.1. Preparation of PHA/Liposomes

Liposomal particles from phosphatidylcholine (PC, Sigma) and cholesterol (Serva) with different content of PHB (Hydal, Nafigate Co.) were prepared using sonication technique. Briefly, all compounds were dissolved in chloroform and add to water. Resulting mixture was sonicated for 1 min using 13-mm-diameter 20 kHz ultrasound probe (Sonopuls, Bandelin). The organic solvent was evaporated. For further cytotoxicity testing, the particle suspension was centrifuged at 3600×g for 5 min. The supernatant was removed and centrifuged at 12 200×g for 1 h. Finally, the liposomal pellet was resuspended in double amount of sterile distilled water and filtered through 0.22 μm syringe filter to obtain uniform particle size.

2.2. preparation of nanofibers

Nanofibers were prepared using electrospinning technique, where PHB was dissolved in chloroform and 0.5 % TEAB was add to enhanced conductivity. Parameters for electrospinning were 15 kV, 15 cm distance between needle and collector, relative humidity 60 %, temperature 20 °C.

2.3. Particle size and coloidal stability analysis

Evaluation of particle size, polydispersity index (PdI) and colloidal stability in solution was done by standard procedure with Malvern Zetasizer Nano ZS. All samples of prepared particles were diluted 100times and 1 ml od solution was plated into the cuvette. Colloidal stability was measured with same solution but with Dipp Cell.

2.4. Long term stability

Particle size, polydispersity index and colloidal stability were measured for 3 months to determine long-term stability.

2.5. SPF measurment

Prepared extracts were diluted with UV-VIS ethanol to concentration of 200 μg·ml^{-1} and absorption spectra were measured at wavelengths 290–320 nm. UV-VIS ethanol was used as blank. Calculation of SPF was done according to following Mansur equation (1).

$$SPF = CF \cdot \sum_{290}^{320} EE(\lambda) \cdot I(\lambda) \cdot Abs(\lambda) \quad (1)$$

Where CF is correlation factor (10), EE (λ) is erythemogenic effect at certain wavelength and Abs (λ) is absorption of sample according to wavelength. Values of EE·I are constants defined by Sayre et. Al. (1979) and are given in **Table 1**.

Table 1 Values of constants to calculate SPF by Mansur equation

λ [nm]	290	295	300	305	310	315	320
EE·I	0.015 0	0.081 7	0.287 4	0.327 8	0.186 4	0.083 7	0.018 0

2.6. Imaging of nanomaterials

Prepared particles were imaged by cryogenic transmission electron microscopy (cryo-TEM). Images were recorded at 200 kV on FEI Tecnai F20 transmission electron microscope with 4 k CCD camera FEI Eagle. For cryo-sample preparation, 4 μl was deposited onto Quantifoil R2/1, mesh 200, and copper holey carbon grids.

Vitrification in liquid ethane cooled by liquid nitrogen was conducted using a Vitrobot™ plunge freezing device (FEI) with a blot time of 2.0 s, blot force − 2 and 10.0 s wait time. Samples on grids were stored in liquid nitrogen until transferred to the cryo-holder Gatan 626 that provides cryo-conditions during imaging. Data were collected using automatic software packages EPU and Tomo. Surface structure and diameter of PHB nanofibers was analysed with JEOL JSM-7600F (SEM) electron microscope.

2.7. Keratinocytes cultivation

Cultivation of HaCaT keratinocytes was done in DMEM with High Glucose, with 0,4 mM L-Glutamine, without Sodium Pyruvate, with 10 % FBS and 1 % ATB in Cell cultivation box with atmosphere of 5 %CO_2 at 37 °C, fed every 2-3 days and passaged after reaching 80 % of confluence.

2.8. Cytotoxicity assay

MTT assay [4], [5] was used to assess the cytotoxicity of prepared nanoparticles. Tested particles were treated as follows. Particles were diluted 1:1 with deionized water, pipetted through 200 nm syringe filter and diluted with DMEM till concentration range of 2-14 %. First, 100 µl of cell culture was added into 96-well plate and for nanofiber testing cell culture was added to 6-well plate and let in the cultivator After 24 h medium was replaced by prepared samples in DMEM and placed back to the cultivator. Control sample was the medium alone, and ethanol served as negative control. Subsequently after another 24 h 20 µl of MTT dissolved in PHB (2,5 mg ml^{-1}) was added to each sample and incubated for 3 h in cultivator and then 100 µl of 10% SDS in PBS was added to each well. Plates were stored in darkness and evaluated next day by ELISA Reader at 543 nm.

2.9. LDH Assay

LDH is a stable enzyme occurring in cytosol. When cells undergo some reaction that lead to the damage of plasma membrane, LDH is rapidly released to cell culture media and therefore can be determined 6]. LDH assay was performed according to Brown et al [7] with modifications. For this assay supernatants form exposure were used. If they were not used for the assay immediately, they were kept frozen at −80 °C. A calibration curve was prepared using sodium pyruvate/NADH solution. This solution consists of 1 mg·ml^{-1} NADH in 0.75 mM sodium pyruvate that were equivalent for LDH activity 0-2 000 U ml^{-1}. For determination of LDH activity 10 µl of exposed supernatant, blank and 60 µl calibration solution was pipetted in 96-well plate in triplicates. For samples and blank 50 µl of pyruvate/NADH solution was added and incubated at 37 °C for 30 min. After incubation 50 µl of 4 M sodium hydroxide was added to all wells and incubated at room temperature for 5 min in dark. Last step was measuring the absorbance at 540 nm. Data are measured in absolute LDH activity units.

3. RESULTS AND DISCUSSION

In this study, nanomaterials comprising liposomal particles enriched with PHB and nanofibers from PHB tailored by electrospinning technique were prepared and characterized to see whether they are suitable for further application in cosmetic industry. Characterization of prepared particles were done in terms of particle size, polydispersity index, ζ- potential, Sun Protection Factor (SPF), amount of phospholipids, cytotoxicity and cryo-TEM imaging. Nanofibers were characterized by SEM imaging and cytotoxicity testing.

3.1. Particle characterization

Prepared particles were characterized using Zeta sizer Nano ZS. Results are displayed in **Table 2**.

Table 2 Particle size (d), polydispersity index (PdI) and colloidal stability (ζ-potential)

sample	d (nm)	PdI (-)	ζ- potencial (mV)	SPF (-)
liposomes	148.7 ± 0.4	0.167 ± 0.007	−31.3 ± 2.14	1.70 ± 0.06
10 % PHB	257.2 ± 3.2	0.388 ± 0.023	−40.8 ± 1.2	2.98 ± 0.05
50 %PHB	535.9 ± 28.2	0.567 ± 0.058	−42.0 ± 2.1	15.18 ± 0.30
100 %PHB	308.9 ± 30.6	0.404 ± 0.100	−25.4 ± 1.5	12.74 ± 0.08

From results displayed in **Table 2** we can see that all particles have diameter under 600 nm and their polydispersity is for most cases under 0.4, so, they are monodispersed. Concerning their colloidal stability, we can say they are stable, because their zeta-potential is lower than −25 mV. The same could be told for particles with zeta-potential higher than +25 mV. SPF measuring confirms that with increasing amount of PHB the values of SPF increases as well.

Table 3 Long term stability of prepared particles after 4 months

sample	d (nm)	PdI (-)	ζ- potencial (mV)
liposomes	132.7 ± 0.76	0.177 ± 0.01	−24.2 ± 2.67
10 % PHB	265.8 ± 3.15	0.376 ± 0.06	−35.9 ± 0.95
50 %PHB	415.2 ± 5.11	0.437 ± 0.02	−32.6 ± 5.27
100 %PHB	334.1 ± 38.1	0.398 ± 0.02	−24.2 ± 0.66

After comparison of data from **Tables 2** and **3** we can conclude that all prepared particles are stable for 4 months and, therefore, suitable for further application.

3.2. Imaging of prepared nanomaterials

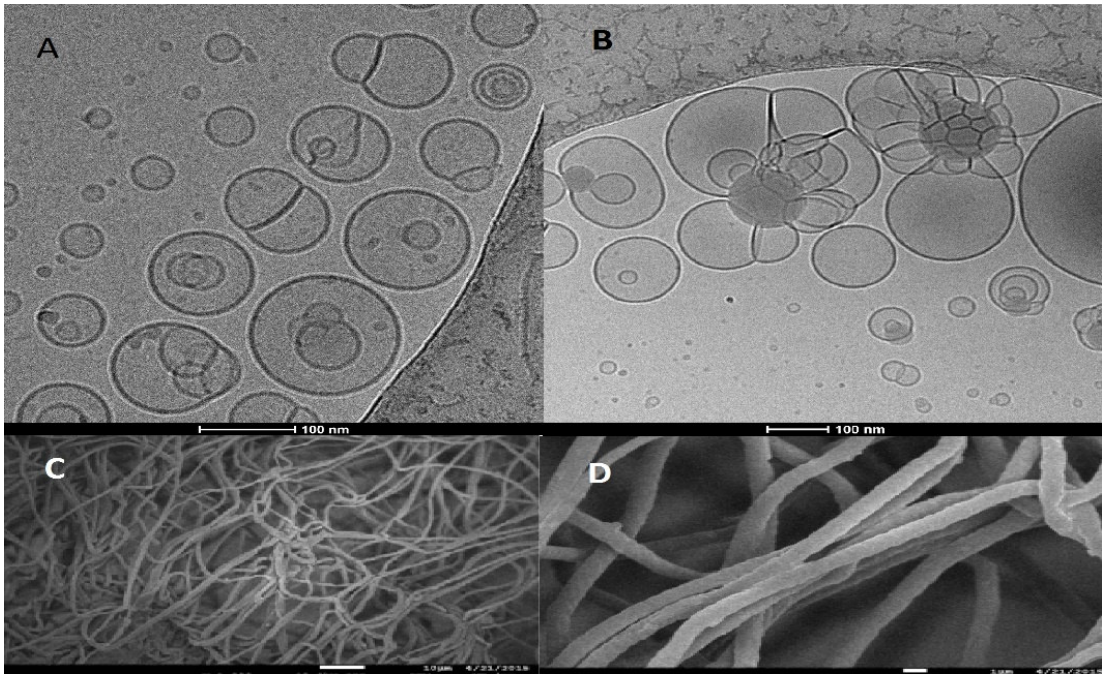

Figure 1 Cry-TEM imaging of A-liposome particles, B-liposome particles with 10 % of PHB, C and D - PHB nanofibers prepared by electrospinning technique

3.3. Cytotoxicity testing

With all prepared materials cytotoxicity testing was done with MTT and LDH assays. On **Figure 2** we can see results from MTT testing on HaCaT cell line for liposomal particles. Liposomes as well as liposomes enriched with 10 % and 50 % of PHB had no cytotoxic effect on cell at all tested concentrations. Particles with 100 % of PHB were not cytotoxic at least at 4 different concentrations in the range of 0 - 8 %. For PHB nanofibers the cell viability was 92.4 % ± 4.2 % so we can say that this material is not cytotoxic to HaCaT cell line.

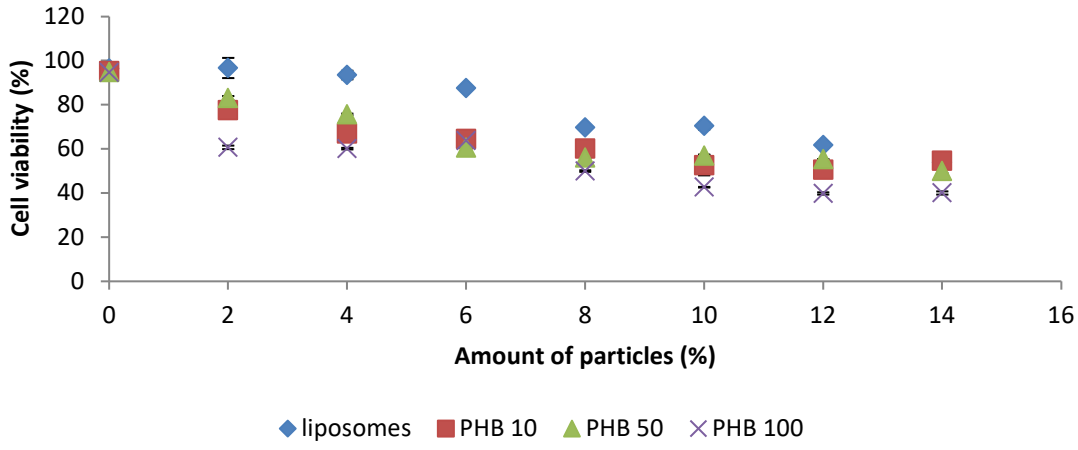

Figure 2 MTT assay of prepared particles

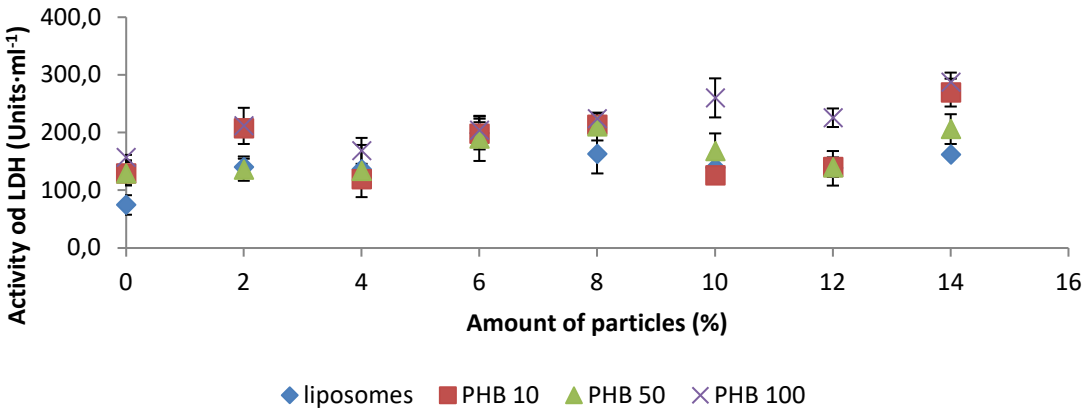

Figure 3 LDH assay of prepared particles

From the data demonstrated in the **Figure 3** we can see that activity of LDH of prepared samples was very similar to cell control. So, no cytotoxic effect was observed on HaCaT cells. The same results came from PHB nanofibers, where the LDH activity was 241.2 ± 16.9 $U \cdot ml^{-1}$ and no cytotoxic activity observed as well.

4. CONCLUSION

We prepared liposome particles enriched with biocompatible polymer PHB. Amount of added PHB altered liposomal size, polydispersity, colloidal stability, and SPF. Cryo-transmission electron microscopy verified that higher concentration of polymer resulted in an increase of particle size. Particles were stable for the period of 3-months with no dramatic change in particle size, polydispersity and zeta-potential. Electro-spun PHB-based nanofibers had diameters ranging 500-800 nm.

MTT and LDH Cytotoxicity assays on HaCaT cell line showed that all types of particles at all tested concentrations as well as nanofibers were non-cytotoxic. Both liposomes and liposomes enriched with PHB are stable with desired properties and if further cytotoxicity and genotoxicity testing will show the same results, they will be suitable for further application in cosmetics.

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