MATERIALE PLASTICE
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https://doi.org/10.37358/Mat.Plast.1964

Surface-modified Polyurethane Structures Used as a Carrier for Simvastatin for the Possible Treatment of Atherosclerosis in Patients with Hepatic Arterial Variations

LAURA ANDREEA GHENCIU (BOLINTINEANU)[1], SORIN LUCIAN BOLINTINEANU[1*], FLORIN BORCAN[2], DELIA ELENA ZAHOI[1]

[1] "Victor Babes" University of Medicine and Pharmacy, Faculty of Medicine, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[2] "Victor Babes" University of Medicine and Pharmacy, Faculty of Pharmacy, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

Abstract: *Simvastatin, a lipid-lowering drug from the statins group, is used in various dyslipidemias. It appears like a very useful medication to decrease the level of total cholesterol, of the low-density-lipids and triglycerides from blood, but it is well-known that the administration of statins have serious side effects, like muscle pain and weakness that can lead to kidney damage, rash on the skin and inflammation of the joints and of the blood vessels, shortness of breath, inflammation of the liver, dark urine, anemia, memory and sleep disorders, and problems in performing the sexual act. The main aims of this research were to obtain and to characterize a polymer carrier used for the transmembrane transfer of simvastatin. Hepatic arterial variations are common knowledge and it has been studied that these types of arteries have a narrower endoluminal diameter, therefore the patients may be prone to develop atherosclerosis more rapid that the ones with standard hepatic arterial patterns. The samples based on polyurethane structures with and respectively without the active agent were synthesized and characterized by measurements of pH, encapsulation efficacy, cumulative drug release in a simulated body fluid, scanning electron microscopy, Zetasizer measurements, thermal stability, cytotoxicity assay, and non-invasive skin irritation assessment. The results indicate the obtaining of heterogenous polyurethane structures with mean sizes between 80-440 nm and neutral pH, that have a good stability against the agglomeration tendency and a prolonged release rate. The results on structures cytotoxicity and their non-irritative potential are important clues that can be used in further clinical investigations.*

Keywords*: drug delivery, pH, release rate, SEM, skin tests, statins, Zetasizer*

1. Introduction

It is considered that mortality from cardiovascular diseases ranks first worldwide and represents an important public health issue. In the last decades we can observe a clear evidence of direct proportional correlation between the value of the total cholesterol (especially of its fraction with low density, LDLc) and the coronary risk. Examples in this sense are the Framingham and MRFIT studies (Multiple Risk Factor Intervention Trial) [1, 2]. The recommendations of the current guidelines indicate that the effective therapeutic intervention on hyperlipidemia and to maintain low LDLc values in the blood is very important in preventing cardiovascular events. It was found that statin therapy decreases the total cholesterol and LDLc levels and improve the cardiovascular morbidity and mortality both in primary and secondary prevention [3].

Statins are a category of drugs that reduce circulating cholesterol levels by inhibiting its synthesis. The hydrophilic statins include pravastatin and rosuvastatin, while the group of lipophilic statins consist of lovastatin, fluvastatin, cerivastatin, atorvastatin, simvastatin, and pitavastatin [4]. Simvastatin ($C_{25}H_{38}O_5$, M = 418.6, Figure 1) is a semi-synthetic compound derived from a fermentation product of the fungus *Aspergillus terreus*; it was patented in 1980s and nowadays is on The WHO List of Essential Medicines (aka Essential Medicines List or EML) [5].

**email: s.bolintineanu@umft.ro*

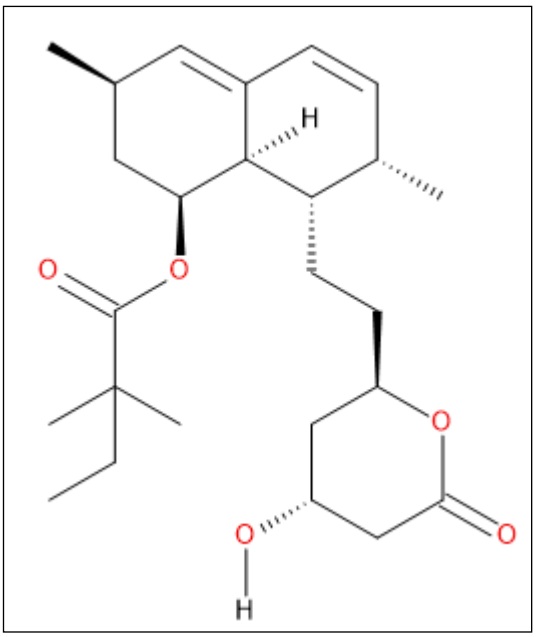

Figure 1. 2D chemical structure of simvastatin

Although lowering cholesterol is a beneficial effect for the body, the long administration of statins must be avoided due to a series of side-effects such as memory loss, nausea, constipation, the symptomatic increase of transaminases in the blood, myalgia and rhabdomyolysis (rare, 1/100,000), and toxic effects on the kidneys, with a risk of proteinuria and hematuria [6]. These are the main reasons why a series of new studies are targeted on the controlled release of statins; the new drug delivery systems based on the development of the nanotechnology represent a good alternative to deliver statins with a decreased risk to develop side effects [7].

The polyurethane (PU) nanostructures were not synthesized at all as a drug delivery system for statins, even though their potential as carriers have been proven in previous researches, when they were used for the transmembranar transport of anti-cancer drugs [8], natural extracts [9, 10], organic liquids [11], etc.

Arterial variations are serendipitous findings during surgeries and paraclinical investigations. Such anatomical anomalies are frequently encountered in the supramesocolic region. Knowledge of the anatomical variations of the hepatic arteries is crucial because a few studies have shown that the endoluminal diameter at the origin is narrower in ambiguous anatomy versus standard anatomy [12, 13]. The only variant that appears to have an endoluminal diameter within the normal range described in the specialized literature is a replaced common hepatic artery arising from the superior mesenteric artery [14, 15]**.**

The main aims of the present research were to develop and to characterize a PU carrier that can be used as a drug delivery system for simvastatin in the possible treatment of patients with arterial variations and atherosclerosis. The novelty of this study is represented by the use of polyurethane in simvastatin delivery and by the modification of the surface of structures to enhance the controlled release.

2. Materials and methods

2.1. Materials and animals

The following chemicals were used in this research: simvastatin **(**≥97% HPLC) and polyethyleneglycol (PEG, M≈200) were supplied by Sigma-Aldrich (Schnelldorf, Germany), while Tween® 20 and isophorone-diisocyanate (IPDI) from Merck (Darmstadt, Germany); mono-ethylene glycol (MEG) was achieved from Lach-Ner s.r.o. (Neratovice, Czech Rep.) and 1,4-butanediol (BD) from Carl Roth GmbH (Karlsruhe, Germany) and acetone from Honeywell | Riedel-de Haen (Seelze, Germany); inorganic salts

($NaCl$, $NaHCO_3$, Na_2HPO_4, KCl, K_2HPO_4, KH_2PO_4 and $MgCl_2$) were acquired from Sigma-Aldrich (St. Louis, USA). All reagents were used without any previous purification. Double distilled water prepared at in-house facility (JP Selecta Dest-4 distiller) was used throughout.

A culture of Human Dermal Fibroblast (HDFa) was obtained from Invitrogen (Waltham, USA), while the specific reagents (media, fetal bovine serum, antibiotic, phosphate buffered saline) and various supplies (flasks, pipettes and pipette tips, tubes and microplates) were supplied by Thermo Fischer Scientific (Waltham, USA).

Ten healthy female, 10-12-week-old Nu/Nu, Balb-c mice were achieved from Charles River (Sulzfeld, Germany); they were kept under standard conditions of temperature (23±2°C) and humidity (50%), 12h light/dark cycle.

2.2. The obtaining of PU carrier

The passive transport of active agents across the cell membranes is influenced by its solubility, ionization, molecular size, etc. Thus, the following procedure was repeated four times to obtain two heterogeneous samples based on particles with different sizes, respectively to have a reference sample - PU_0 (empty PU structures without simvastatin) and another sample whose raw materials containing 10 mg simvastatin - PU_1.

The drug delivery system was synthesized by mixing of two main phases:

(A) a hydroxylic component containing different volumes of MEG and BD (Table 1), 0.020 mmols PEG, 0.002 mmols Tween®20, and 1.940 mmols distilled water;

(B) an organic component based on 0.031 mmols IPDI in 0.473 mmols acetone.

Table 1. The ratio of the raw materials.

Synthesis no	Main differences (amounts)		**Sample label**
	MEG and BD (mmols)	simvastatin (mmols)	
1	0.025 and 0.018	0.0	PU_0
2	0.048 and 0.026	0.0	
3	0.025 and 0.018	0.012	PU_1
4	0.048 and 0.026	0.012	

These two components were separately homogenized for 25 min with 400 rpm at room temperature and then, the organic component was rapidly injected in the hydroxylic one according to the "one-shot method", which means the mixing of the entire amount of all the reagents together. The stirring was continued for 6 h (525 rpm, 40°C) to ensure the complete synthesis of PU chains.

The obtained product was repeatedly washed with a mixture acetone : water (1.4 : 1, v/v) and centrifuged and finally it was dried at 60°C in a PolEko SL115 drying oven for 48h.

The dried product was suspended in a solution obtained from 0.014 mmols IPDI in 0.405 mmols acetone and maintained for 48h (450 rpm, 40°C) to modify the surface of particles and to block all hydroxyl-terminated PU chains. Once again, the purifying process was based on repeated wash-centrifugation processes using the same mixture acetone : water (1.4 : 1, v/v) and it was followed by the drying of sample. On the other hand, the obtaining of particles with two different size was wanted in order to modify the pharmacokinetics of this formulation.

2.3. The characterization of samples

Diluted aqueous solutions (3.5%, w/v) of both samples (PU_0 and PU_1) were used to evaluate the *p*H values of the samples using a Mettler Toledo FiveGo F2 portable pH meter (Schwerzenbach, Switzerland), equipped with an InLab® Expert Go Sensor. The point of zero charge (pH_{zpc}), related to the charge on the surface of particles, that depends on the samples *p*H, was also investigated based on an acid-base titration [15]: aliquots with 0.500 mmols NaOH were prepared in different flasks and their *p*H was adjusted between 2 and 12 by addition of 0.01 M solution of HCl; 0.005 mmols of both samples

(PU_0 and PU_1) were added to every flask and shake for 72 h.

A solution of sample PU_1 in methanol (3.5%, w/v) was investigated using an UVi Line 9400 SI Analytics spectrophotometer (Mainz, Germany) in order to found the encapsulation efficacy (EE) and the cumulative drug release (CDR) based on the following formulas and procedures which were already published in our previous scientific article [16]: EE is the ratio between the amount of the encapsulated drug obtained as difference between the initial weight (Wi) and the free simvastatin (W_f) from the washing mixture *vs*. the initial weight, $EE = [(W_i - W_f)/W_i]\times 100$ %; CDR was found after the maintaining of sample PU_1 in a degradative media, known as T. Kokubo' simulated body fluid [17], which presents similar ions concentrations and *p*H with the human plasma. CDR is equal with the volume of sample withdrawn (mL) / bath volume × $P_{(t-1)}$ + P_t, where P_t is percentage release at time "t" and $P_{(t-1)}$ is percentage release previous to "t" [18]; all UV-Vis analyses were based on the difference between the maximum absorption peak (238 nm for simvastatin and 361 nm for PU structures), a calibration curve (simvastatin absorption vs. its concentration, R^2= 0.9915) and the procedure described in the literature [19].

A combined Zetasizer evaluation instrument from Cordouan Technology (Pessac, France) was used to assess the average diameter and the polydispersity index (PDI) based on the Vasco Particle Size module, respectively the surface charges of structures on the Wallis Zeta potential module; the following parameters have been chosen: evaluation temperature (30±1°C), interval of time (12±3µs), number of channels (420-440), power of laser (80%), acquisition mode (continuous), analysis mode (Pade-Laplace), Wallis resolution (medium), and Smoluchowski model as Henry function.

A Quanta 250 FEI scanning electron microscope (Eindhoven, The Netherlands) was chosen to comparatively evaluate the morphological aspect of the samples. The accelerating voltages value was set at 10 kV via the "electron column" console and the magnifications to 500x.

The thermal behavior of samples was investigated using the differential scanning calorimetry on a Mettler-Toledo DSC1 instrument (Greifensee, Switzerland) between 20 and 350°C in an inert atmosphere (100 mL/min Ar) using aluminum crucibles at a 5 degree/min heating rate.

The cytotoxic activity was tested on fibroblasts that were cultured in Dulbecco's Modified Eagle's Medium containing fetal calf serum and penicillin-streptomycin (PromoCell, Germany). Cells were maintained in an atmosphere of 5 % CO_2 at 37°C. The cells viability was assessed by Alamar Blue test, using a spectrophotometer (570 and 600 nm) according to the procedure presented in the literature [20].

The mice were divided in two groups in order to comparatively assess the irritation effect of the obtained products based on a protocol that was already described by our research team [21]. The measurements were done using a professional MPA System from Courage&Khazaka (Koln, Germany) equipped with a Tewameter®TM300 probe to evaluate the transepidermal water loss (TWL) and a Mexameter®MX18 probe to find the level of erythema.

All the procedures were done according to the declaration of Helsinki principles. The use of animals (mice) in this biomedical research was first analyzed and approved by the Ethical Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania.

Statistical data were obtained by using SPSS Statistics for Windows, Version 27.0 (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). All measurements were done in triplicate and the results of continuous variables are expressed as average values ± standard error. Non-parametric tests were preferred to determine statistical differences due to the small numbers of data in every dataset; all measurements were done by the same operator in triplicate and the values were the subject of a statistics evaluation; * for $P \leq 0.05$, ** for $P \leq 0.01$, and *** for $P \leq 0.001$.

3. Results and discussions

The following *p*H values were recorded for the synthesized samples, as diluted aqueous solutions: 6.91±0.09 (sample PU_0) and respectively 6.96±0.12 (sample PU_1); it should be noted that the *p*H values for 0.01 M simvastatin solution is 7.68 and for distilled water is 7.00. The drug delivery systems that are developed to be used for various systemic issues such as atherosclerosis must present a neutral

acido-basic character, closed to the *p*H value of blood (7.35-7.45 according to [22]). The *p*H values of the synthesized samples and the small amounts that are used in therapies indicate that this PU carrier can be included in further clinical trials without any risk of metabolic acidosis.

Figure 2 presents the pH_{zpc} of samples.

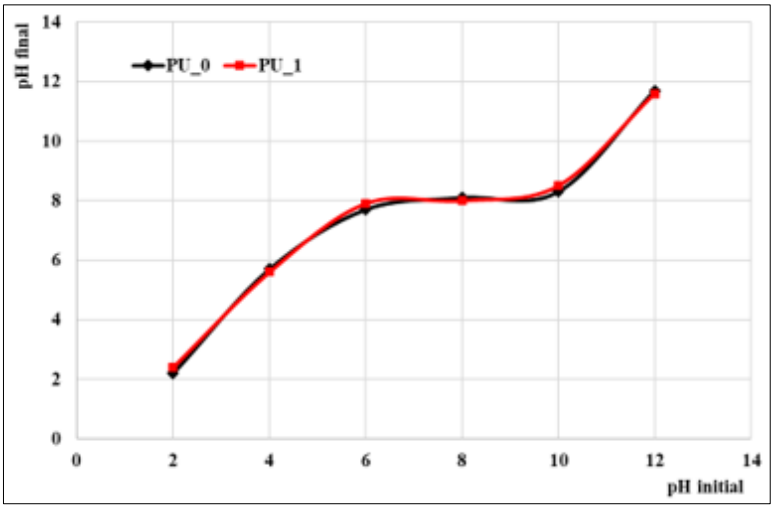

Figure 2. The point of zero charge of samples; temperature 25°C

At *p*H lower than 8.00-8.05, the surface of all particles is positively charged, whereas, at higher *p*H values, the surface became negatively charged. Our results indicated that the pH_{zpc} of samples were quite similar due to the existence of the same polymer chains that were not influenced by the encapsulation of simvastatin; PU_0 presents a pH_{zpc} just a little higher (8.05) than PU_1 (8.00).

The encapsulation efficacy was found at a good level (61.42±0.04 %), based on the simvastatin calibration curve that was drawn between 3 and 50 µg / mL, the total amount used in the synthesis (10.0 mg) and the amount of the free reagent from the water-acetone mixture used to the wash the sample and using the Bouguer-Beer-Lambert law about the relation between the attenuation of light and the concentration of a sample. Based on the same principle, Figure 3 presents the cumulative simvastatin release (CDR) from the PU carrier.

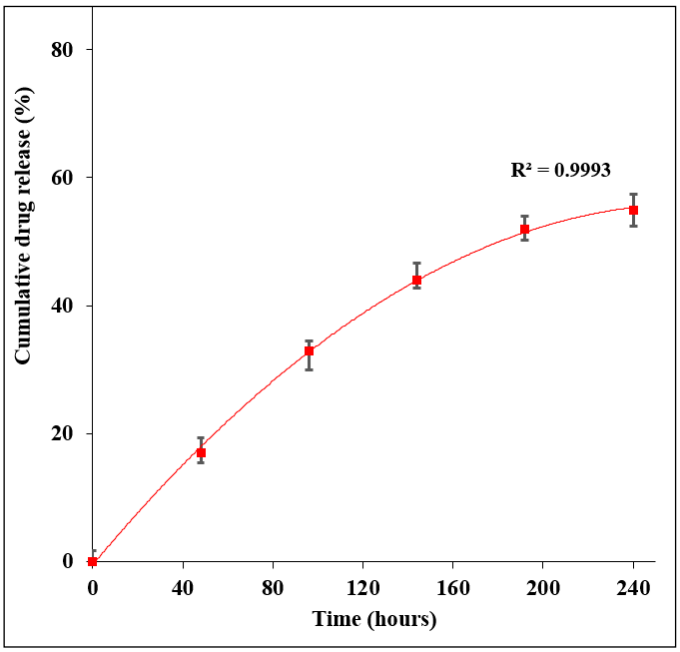

Figure 3. The release profile of sample PU_1 in T. Kokubo medium

The cumulative drug release is related to the degradation of PU structures; it can be enhanced by using mixtures of esteric and etheric polyols on one hand or by using mixtures of structures with different sizes and molecular weights. The release profile is a very important parameter of the drug delivery systems and it can be described by various mathematical models. In the case of simvastatin-loaded PU structures (sample PU_1), the first half seems to fitted to a zero order release model ($CDR = Q_0+K_0*t$, $R^2= 0.9190$), where Q_0 is the initial amount, K_0 is zero order release constant and *t* is time in hours, while the second half of the assessed range correspond to Higuchi square root time model ($M_t / M_\infty = kt^{1/2}$, $R^2= 0.9997$), where M_t is the quantity of simvastatin release at time *t* and M_∞ is the final maximum cumulant, while k is the corresponding release rate constant. The observed profile is a proof that this PU carrier ensures a suitable bioavailability.

Table 2 shows the Zetasizer data for the samples PU_0 and PU_1. The values of the polydispersity index (PDI) indicate the synthesis of two heterogeneous samples with minimum two populations, while the Zeta potential values show a low tendency to form particles clusters according to other published information [23].

Table 2. The obtained Zetasizer information on samples.

Sample	Size of structures (nm)		Zeta potential (mV)
	Mean ± SD	PDI	
PU_0	94 ± 9 427 ± 16	0.9	+27.82
PU_1	82 ± 11 440 ± 13	0.8	+28.19

SEM images is used to describe the aspect of the carrier surface. It is well-known that novel polymer drug delivery systems are developed with different crystallinity degree and this is an important parameter that is corelated to their solubility. On the other hand, any change of carrier surface can be attributed to covalent bonds between the loaded active agents and their delivery system. The modification of the HO-terminated chains leads to a decrease of aqueous solubility and it can simulate the lipophilicity of simvastatin. Figure 4 presents the aspect of the carrier without and with simvastatin: both samples contains very different particles similar to the results obtained by Zetasizer characterization.

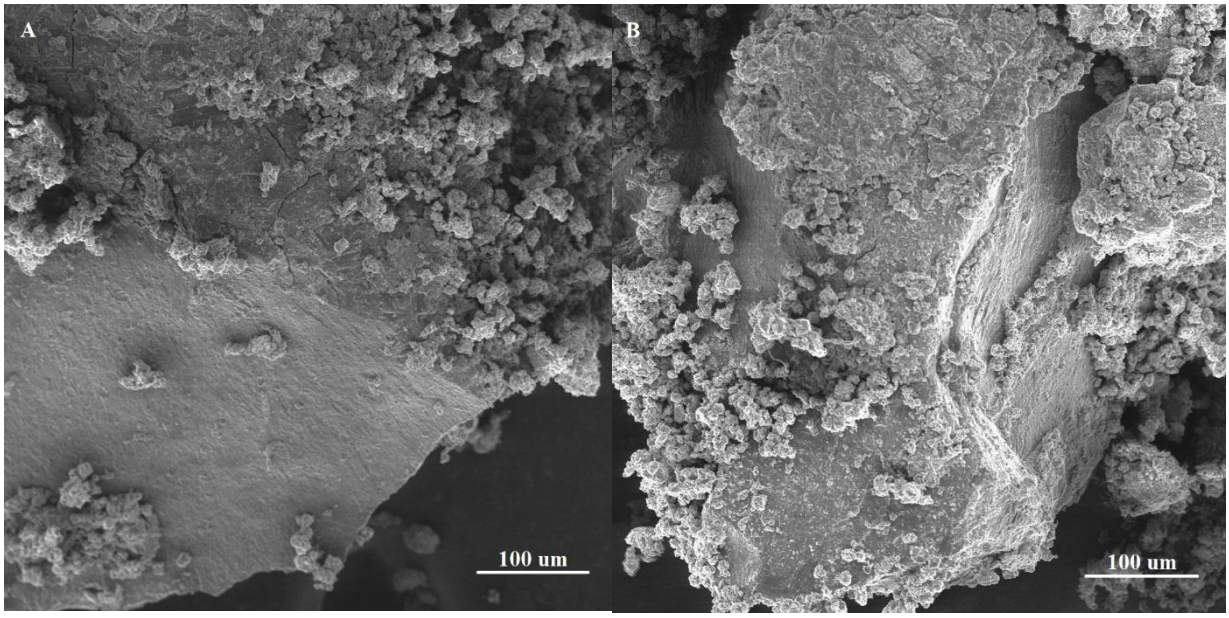

Figure 4. The aspect of the samples: A (PU_0) and B (PU_1)

The thermal stability of the PU drug delivery with and without simvastatin was evaluate by differential scanning calorimetry; the DSC curves (Figure 5) show very similar one to another and are similar to other

thermograms of IPDI-based PU materials: the literature presents polyurethane as a very stable material with a glass transition below 20°C and who pyrolyze at a much higher temperature than 300°C [24]. As can be seen in these curves, there are large endothermic peaks centered at 70.35 and respectively at 53.15°C, that can be attributed to the evaporation of mixture of the solvents; the melting point of free simvastatin was found at 138.10°C in the case of sample PU_1.

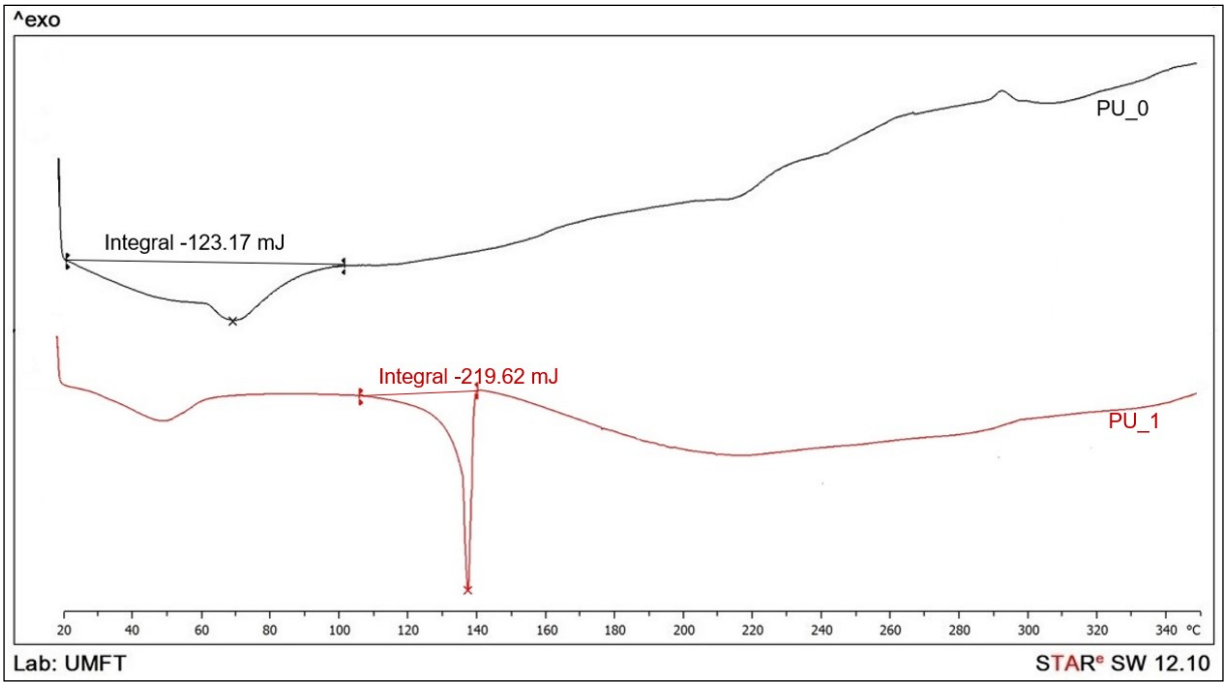

Figure 5. The DSC thermograms

Cell cultures are often used in many toxicological assays. Nowadays, a huge number of cell cultures can be used to mimic the *in vivo* condition. Figure 6 comparatively presents the viability of Human Dermal Fibroblasts-adult (HDFa) at 24 and 48 h exposure to the synthesized samples that were previously solubilized in DMSO. Very good cell viability values (around 90% at 24 and 48 h) were found and these results confirm the good biocompatibility of PU samples [25] and the fact that these samples are safe to be used in further evaluations.

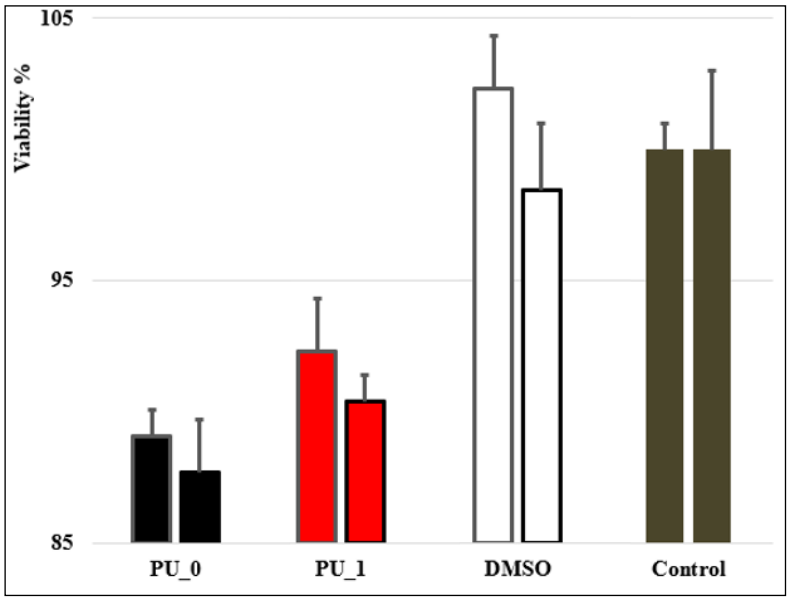

Figure 6. The cells viability, 24 and 48 h exposure on synthesized compounds

The skin represents an alternative procedure to test the toxicity of new synthesized pharmaceutical formulations. The literature describes that European Commission has approved a series of guides based on the 3R principle of William Russell and Rex Burch (reduction, refinement, and replacement) [26]; thus, human and/or animal skin can be used to predict the toxicity. Figure 7 presents the modification of the erythema and of trans-epidermal water loss in a 48 h experiment on mice.

Another study on the modifications of TWL and erythema to mice exposed to pharmaceutical products also revealed very slight and similar increases of these two skin parameters [27]. These increases are specific to an irritant effect due to an unpleasant process, but the amplitude of these changes make the difference between a safe and an unsafe chemical reagent. Courage-Khazaka, as the manufacturer of the professional skin probes, did not revealed the maximum allowed levels for the healthy skin or for the apparition of irritative processes due to the variability of these parameters from one subject to another. However, comparing to the results which were obtained in the previously mentioned research [27], the toxicity of these samples (PU_0 and PU_1) is lower: around 30 arbitr. units in 48 h for erythema level and for TWL just 3.3 g/h/m^2 in the same period. Thus, this assessment indicates that the obtained simvastatin carrier can be used for its controlled delivery.

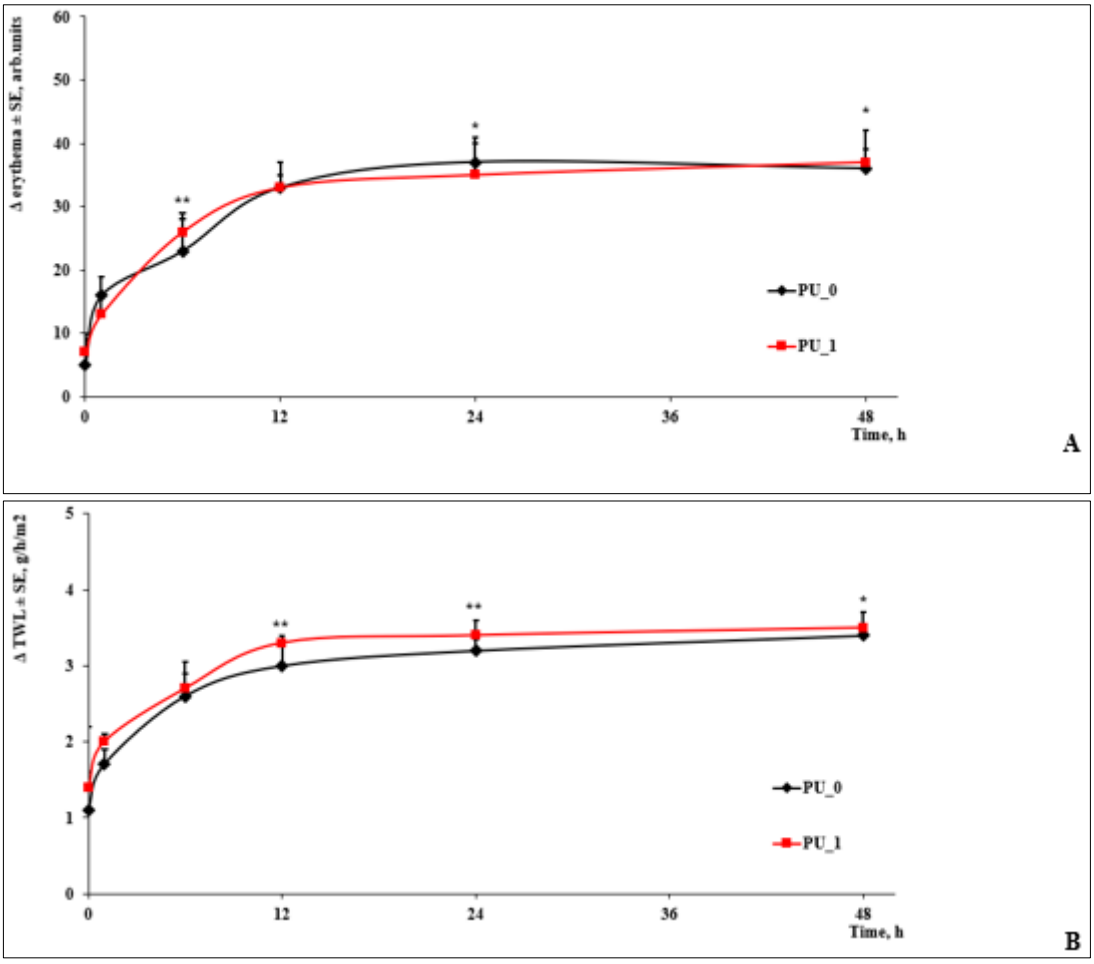

Figure 7. The evolution of (A) erythema and (B) of the trans-epidermal water loss

The encapsulation of biological active agents in polyurethane delivery systems has begun at the beginning of this century [28]: the process based on a simultaneous emulsification and polyaddition reaction is suitable to obtain hollow particles that can exhibit high loading efficiencies. The results of the current research reveal small differences between the two samples and thus may indicate that physical entrapment of simvastatin has been achieved. The increased reactivity of the -NCO functional groups from the raw materials is often responsible for the covalent binding of the loaded

drugs [29]; the existence of these bonds can be demonstrated by the presence of particles with a wide distribution of sizes - the drug can act as a crosslinking agent, extender or interrupter of polymer chains depending on its functionality. In the current study, each sample contains two different populations with narrow distribution of sizes and this is very important because physically encapsulated drugs can be easily released from its carrier [30].

4. Conclusions

This paper is about a study on the encapsulation of simvastatin inside polyurethane structures which are used as a drug delivery system. To the best of our knowledge, polyurethane drug delivery systems are not very well known and used despite the many advantages they present. Hence, various structures with sizes between 80 and 440 nm were synthesized to assure a prolonged release of simvastatin.

The results of the current research reveal the obtaining of heterogenous macromolecular products with an almost neutral *p*H, a very good thermal stability between 20 and 350^{o}C, that are non-cytotoxic and they are free of irritating effects that damage the mucous membranes. Nevertheless, a few clinical trials are necessary in the next step of these products' evaluation.

Acknowledgments: This article was supported by the grant 5EXP/1244/30.01.2020 from Victor Babes University of Medicine and Pharmacy, Timisoara, Romania.

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Manuscript received: 3.05.2023