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Manufacturing of substitutes for spongy bone with increased absorbability

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Composite scaffolds with increased hydrophilicity were prepared for cancellous bone regeneration by the freeze-extraction method. As a construction material, a poly–L–lactide (PLLA) was applied. As a hydrophilic, modifying agent a methacrylic acid copolymer, trade name Eudragit®, was used. A preliminary investigation and optimization of the process were performed. For the obtained scaffolds, regression equations determining the effect of: Eudragit®E100/PLLA weight ratio; volume ratio of methanol (porophore)/PLLA solution in dioxane on interconnected porosity and mass absorbability of obtained implants were calculated.

Keywords: porous implant, polylactide, Eudragit®, freeze-extraction method

1. INTRODUCTION

A commonly used treatment for large bone defects is a transplant, which involves taking a part of the tissue and prefixing it in the place of the defect. Autogenic grafts, i.e. those from the treated patient, most often from the iliac crest, are considered to be the standard treatment of bone implantology (Ficek et al., 2015). Because of many problems such as risk of infection of the site of transplantation, hemorrhage or nerve damage, other synthetic substitutes are wanted. The perfect synthetic bone substitute has not yet been designed (Błaszczyk et al., 2018). Among synthetic materials polymers quickly gained wide acceptance of surgeons, because of low mass and elastic and frictional properties that bring them closer to the properties of the synovial joint tissue. Resorbable polymers gain the advantage because they are gradually replaced by the host bone and there is no need for a second operation. Due to their biocompatibility and ability to control degradation time, polyesters (for example polylactide, polycaprolactone, copolymers of lactic acid and glycolic acid) occupy an important position among polymers used in bone regeneration (Blanco et al., 2005; Ip and Gogolewski, 2007; Pretzl et al., 2008; Slomkowski, 2007).

Polylactide (PLA) is a polyester widely used in medicine. PLA was used in controlled release systems of the active substance, in hydrogels, for the production of orthopedic screws, as well as in tissue engineering. Polylactide has already demonstrated its osteogenic properties and capabilities of healing bone defects (Gogolewski et al., 2000). However, polylactide is a hydrophobic polymer and has insufficient osteoconductive and osteoinductive properties in terms of large bone defects (Błaszczyk et al., 2018). Consequently, synthetic PLA scaffold should be applied together with osteoinductive/osteogenic materials, such as platelet-rich plasma (PRP) (Szpalski et al., 2010). To overcome the problem of poor impregnability of PLA with hydrophilic substances, modification of polylactide surface may be applied to make PLA more wettable and therefore to obtain better biological activity of PLA.

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Methacrylic acid copolymers are used in the drug form technology (Sonje and Chandra, 2013; Wyszomierski and Sawicki, 2010) and are known under the trade name of Eudragit®. They are auxiliary substances in tablets which facilitate swallowing, mask an unpleasant taste, release the active substance with a delay in a specific section of the gastrointestinal tract. The presence of many lateral ester groups imparts methacrylic acid copolymers greater hydrophilic properties than in the case of polylactide. In addition, depending on the substituent, hydrophilic polymethacrylates with anionic (carboxylate group) or cationic (trimethylammonium group) properties may also be available. The presence of suitable moieties such as hydroxyl, carboxyl, amino groups have proven an effect on adhesion and differentiation of osteoblasts. Therefore, these polymers were considered by our group as an additive to PLA implants.

Table 1. Comparison of scaffold preparation

<table>
<tr><th colspan="2">Method</th><th colspan="2">Advantages</th><th colspan="2">Disadvantages</th></tr>
<tr><td rowspan="2">Phase inversion</td><td>TIPS</td><td rowspan="2">• the ability to change many factors affecting the structure
• interconnected porosity > 80%
• macro-, microporosity</td><td>–</td><td rowspan="2">sensitivity to changing environmental conditions</td><td>thermally sensitive polymers excluded</td></tr>
<tr><td>Freeze extraction</td><td>sample flexibility</td><td>solvent limited choice</td></tr>
<tr><td colspan="2">Salt leaching</td><td colspan="2">• pore size and porosity control</td><td colspan="2">• porophore residues
• flat scaffold limitation
• closed pore structure risk
• fragility</td></tr>
<tr><td colspan="2">Lyophilization</td><td colspan="2">• high porosity > 90%</td><td colspan="2">• limited pore size < 200 μm
• closed pore structure risk</td></tr>
<tr><td colspan="2">Gas foaming</td><td colspan="2">• preparation facility
• solvent free
• high porosity > 90%</td><td colspan="2">• closed pore structure
• nonporous surface
• limited pore size < 100 μm
• pore structure collapse risk</td></tr>
<tr><td colspan="2">Electrospinning</td><td colspan="2">• preparation facility
• high porosity</td><td colspan="2">• limited pore size < 20 μm
• flat structure</td></tr>
<tr><td rowspan="4">Rapid prototyping techniques</td><td>3D printing</td><td colspan="2">• material assortment
• short duration
• possible cell employment
• unlimited shape</td><td colspan="2">• substrate residues risk
• low resolution
• expensive apparatus</td></tr>
<tr><td>SLA</td><td colspan="2">• high resolution
• short duration</td><td colspan="2">• limited materials choice
• expensive apparatus
• cytotoxicity risk</td></tr>
<tr><td>SLS</td><td colspan="2">• material assortment
• high resolution
• short duration</td><td colspan="2">• thermal degradation risk
• expensive apparatus</td></tr>
<tr><td>FDM</td><td colspan="2">• mechanical strength
• solvent free</td><td colspan="2">• thermal degradation risk
• limited materials choice
• expensive apparatus</td></tr>
</table>

A proper bone substitute must be biocompatible and degrade in controlled time into non-toxic products excreted from the body. Mechanical properties and the internal structure should be proper for the tissue to be regenerated. Literature data show that a bone substitute must have interlinked pores of > 100 μm (optimum pore size: 200–350 μm) and interconnected porosity of > 80% (Bose et al., 2012; Olszta et al., 2007).

The requirements set for a given tissue can be also achieved by choosing the right method of production. The methods of manufacturing polymer bone implants include phase inversion (including thermally induced phase separation (TIPS) (Buzarovska et al., 2015), freeze extraction variant (Budyanto et al., 2009), salt leaching (Xie et al., 2018), emulsion method with lyophilization (Bai et al., 2015), gas foaming (Mi et al., 2014), electrospinning (Kołbuk et al., 2019) and rapid prototyping techniques (as 3D printing (Bose et al., 2013), stereolithography (SLA) (Skoog et al., 2014), selective laser sintering (SLS) (Mazzoli, 2013), fused deposition modeling (FDM)) (Popescu et al., 2018). The methods are compared in Table 1.

Freeze extraction method allows to obtain scaffolds of good flexibility compared to rapid prototyping techniques and salt leaching (Kruk et al., 2017; Kruk, 2018). This feature enables the surgeon to adapt the implant size to the defect site in the operating room. Moreover, the adaptation of process conditions can lead to a proper internal structure of a scaffold.

In this work, a novel implant was obtained, consisting of two materials: polylactide and methacrylic acid copolymer. The influence of the addition of three methacrylic acid copolymers on implant mass absorbability and interconnected porosity was examined. Subsequently, using the design of experiments (DOE) (Hajmowicz et al., 2011; Politis et al., 2017; Davim, 2016), preparation of polylactide cancellous bone implants modified with methacrylic acid copolymer was optimized to obtain a scaffold with maximum mass absorbability at the interconnected porosity of more than 90%. We suggest using the implant as a platelet reach-plasma carrier. The maximization of mass absorbability is important as it makes it possible to insert a greater volume of platelet-rich plasma into the scaffold.

2. MATERIALS AND METHODS

2.1. Materials

Poly–L–lactide (PLLA) of M_n 86 000 g/mol NW 2003D was distributed by Nature Works. Eudragit®E100 (methyl methacrylate, dimethylaminoethyl methacrylate, *n*–butyl methacrylate copolymer 1:2:1) of M_w 47 000 g/mol was kindly donated by EVONIK Industries. Eudragit®S100 (methacrylic acid, methyl methacrylate copolymer 1:2) of M_w 125 000 g/mol, Eudragit®L100 (methacrylic acid, methyl methacrylate copolymer 1:1) of 125 000 g/mol was distributed by EVONIK Industries. 1,4–Dioxane, methanol were distributed by POCh S.A. 2–propanol was distributed by Chempur. Ultrapure water with 18.2 MΩ cm conductivity was obtained using a Milli-Q device.

2.2. Preparation of implant-forming solutions

A PLLA solution was prepared in 1,4–dioxane at a concentration of 3 wt. %. PLLA was dissolved in dioxane for 24 h with constant agitation using a magnetic stirrer without heating. Then a suitable methacrylic acid copolymer (Eudragit®E100 or Eudragit®S100 or Eudragit®L100) was added in a 4:60 or a 4:6 weight ratio relative to PLLA. After complete dissolution of Eudragit®, the porophore (methanol or 2–propanol) was added in a 1:10 volume ratio relative to PLLA/dioxane solution. The mixture was stirred for the next 24 h at 25 °C, with a speed of 200 rpm.

2.3. Preparation of implants

PLLA solutions in dioxane with additives were poured into polyethylene forms at 25 °C. Then the forms were cooled to a temperature of −18 °C for 24 h. The frozen solutions were removed from the forms and extracted in a 300 mL of a gelling bath at −18 °C (methanol/water in a 4:6 volume ratio) or 4 °C (water) for 5 days, without stirring. Then samples were vacuum dried at 45 °C, 10 mbar for 24 h (Gadomska-Gajadhur et al., 2018; Gadomska-Gajadhur et al., 2018).

2.4. Analytical methods

Implant interconnected porosity and mass absorbability

Implants were weighed using Mettler Toledo XS 104 scales. Dry scaffolds were weighed in air (m_s). Then scaffolds were impregnated in the isopropanol/water solution in a 4:6 volume ratio (*vac*, 30 min). Impregnated scaffolds were weighted in isopropanol/water solution (m_{ww}) and finally the scaffolds impregnated in isopropanol/water solution were weighted in air (m_w).

Interconnected porosity (P_o) was defined according to Eq. (1)

$$P_o = \frac{V_3}{V_1 + V_2 + V_3} \cdot 100\% \tag{1}$$

where V_1 is the volume of material without pores, V_2 is the volume of all closed pores in the material, V_3 is the volume of all interconnected pores in the material. On the basis of Archimedes' law, Eq. (2) holds.

$$(m_s - m_{ww}) \cdot g = (V_1 + V_2) \cdot d_0 \cdot g \tag{2}$$

where g is the standard gravity, d_0 is the density of the liquid at the measurement temperature. The volume of open pores was defined as (3).

$$V_3 = \frac{(m_w - m_s)}{d_0} \tag{3}$$

After substitution of Eq. (2) and (3) into Eq. (1), implant interconnected porosity was calculated according to Eq. (4).

$$P_o = \frac{m_w - m_s}{m_w - m_{ww}} \cdot 100\% \tag{4}$$

Mass absorbability (N_m) was determined according to Eq. (5).

$$N_m = \frac{m_w - m_s}{m_s} \cdot 100\% \tag{5}$$

3. RESULTS AND DISCUSSION

Poly–L–lactide bone implants with the addition of a suitable methacrylic acid copolymer were obtained. Implants were in the form of a cylinder with a volume of about 10 cm^3 (Fig. 1).

Three types of methacrylic acid copolymer were used: methyl methacrylate, dimethylaminoethyl methacrylate, n-butyl methacrylate copolymer (group ratio of 1:2:1 – Eudragit®E100), methacrylic acid, methyl methacrylate copolymer (group ratio of 1:2 – Eudragit®S100, group ratio of 1:1 – Eudragit®L100). As a preparation method phase inversion with freeze extraction variant was applied. Two types of porophore were used: methanol and 2–propanol.

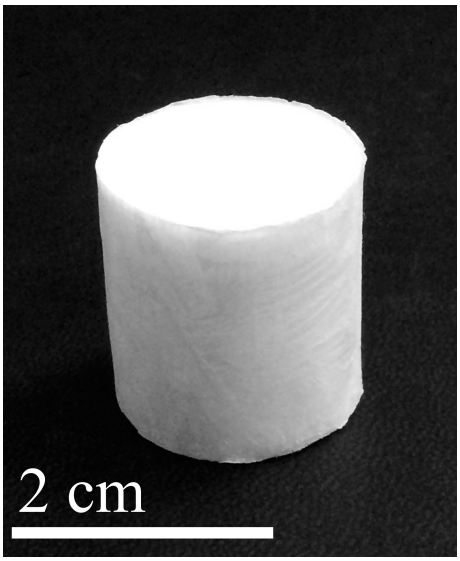

Fig. 1. Poly–L–lactide spongy bone implant modified with Eudragit®E100

3.1. Selection of a porophore

PLLA implants with the addition of three types of Eudragit® in a weight ratio of 4:60 to PLLA were prepared. As a porophore, methanol (MeOH) or 2–propanol (iPrOH) were added. Methanol/water gelling bath was used. Prepared samples were hydrostatically weighed to estimate their mass absorbability and interconnected porosity. Results are presented in Fig. 2.

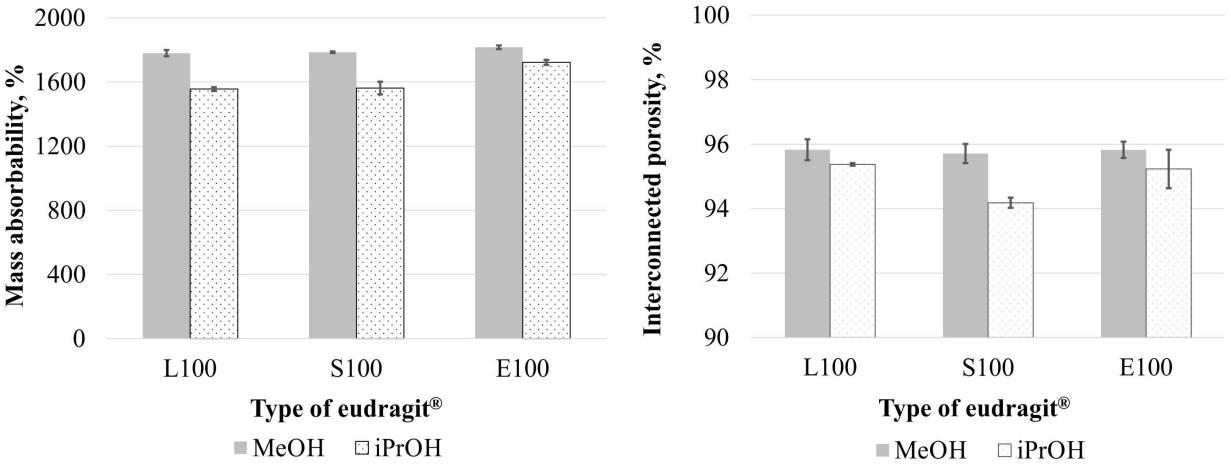

Fig. 2. Dependence of implant mass absorbability (left side) and implant interconnected porosity (right side) on the type of Eudragit® and porophore – methanol (MeOH), 2–propanol (iPrOH). Error bars represent the standard deviation of the mean of three samples

In all cases, mass absorbability was greater when MeOH was used. Obtained mass absorbability values were in the range of 1500−1800 % and corresponded with porosity values in the range of 93−96%. The greater absorbability the greater porosity was observed. Differences between used Eudragits® were not significant. In the next experiments, MeOH was employed as a porophore, as it provided greater absorbability values.

3.2. Eudragit selection

PLLA implants with the addition of three types of Eudragit® in a weight ratio of 4:60 and 4:6 to PLLA were obtained. As a porophore, MeOH was used. Water/methanol gelling bath was applied. Prepared samples were hydrostatically weighed to determine the effect of a type and amount of Eudragit on mass absorbability and interconnected porosity of implants (Fig. 3).

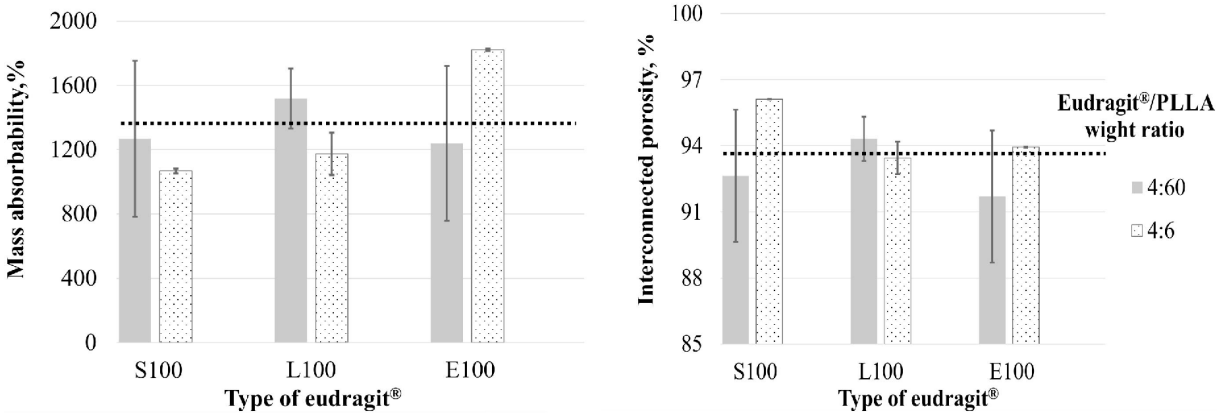

Fig. 3. Dependence of implant mass absorbability (left side) and implant interconnected porosity (right side) on the type and amount of used Eudragit®. Eudragit®/PLLA ratio was 4:60 and 4:6. The dashed line is the outcome for an implant obtained without porophore and Eudragit®. Error bars represent the standard deviation of the mean of three samples

Obtained mass absorbability values were in the range of 1000−1800%, interconnected porosity values were in the range of 92–96%. Absorbability and porosity values were compared to a sample which was prepared without a porophore and Eudragit® (1300%, 94%). In most cases, Eudragit® addition influenced mass absorbability reduction. Influence on porosity is not clear. The greatest absorbability increase was observed in the case of Eudragit®E100 addition in the weight ratio of 4:6. For this reason, Eudragit®E100 was selected for further research. The presence of amino groups in this polymer can increase the differentiation of osteoblasts (Chang and Wang, 2011).

3.3. Influence of mass ratio of Eudragit® E100 to PLLA on implant mass absorbability and interconnected porosity

PLLA implants with a various mass ratio of Eudragit®E100 to PLLA were obtained. Samples were prepared with the addition of porophore (MeOH) in a volume ratio of 1:10 to PLLA solution on dioxane. Water gelling bath was applied to reduce Eudragit® loss during gelation. Prepared samples were hydrostatically weighed to determine the effect of Eudragit®E100 mass ratio to PLLA and the addition of porophore (methanol) on mass absorbability and interconnected porosity of implants (Fig. 4).

The greatest value of absorbability exceeding 2000% was obtained in the case of implant prepared only with a porophore (MeOH). A sample prepared with Eudragit®E100 and porophore addition was characterized by lower absorbability. All implants (with porophore or Eudragit®) were marked by greater absorbability compared to implants prepared without any additive. Because of ambiguous interpretation (no linear dependence), it was decided to optimize the process with the aid of design of experiments. Eudragit®E100 addition was not abandoned because of potential improvement of biological properties of the implant described in detail above.

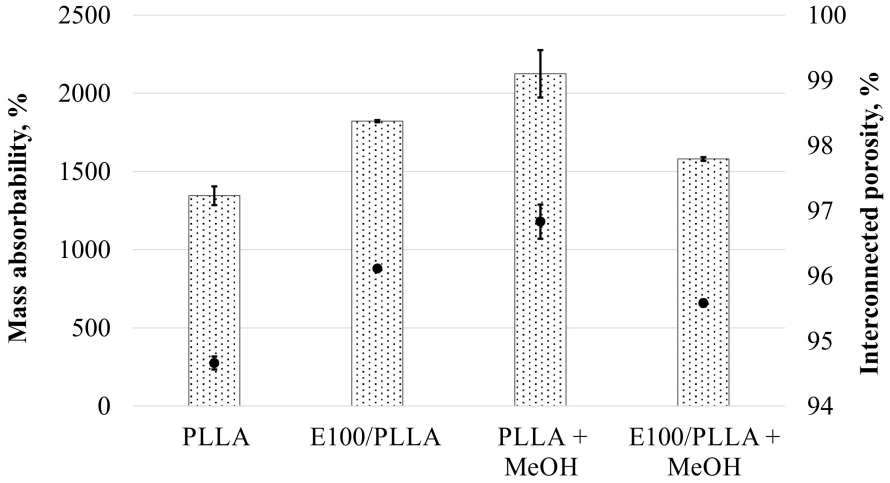

Fig. 4. Dependence of implant mass absorbability (dotted bars) and implant interconnected porosity (black dots) on the amount of used Eudragit®E100. Eudragit®/PLLA mass ratio was 4:60. Porophore/PLLA solution in dioxane volume ratio was 1:10. An implant obtained without porophore and Eudragit® was a control. Error bars represent the standard deviation of the mean of three samples

4. OPTIMIZATION

The effect of the Eudragit®E100 to PLLA weight ratio (x_1), the volume ratio of methanol (porophore) to PLLA solution in dioxane (x_2) on mass absorbability (y_1) and interconnected porosity (y_2) of obtained implants was investigated. Output variables were dependent on each other. However, we focused on mass absorbability and interconnected porosity as controls. The volume of PLLA solution in 1,4–dioxane was constant. The optimization criterion was mass absorbability maximization with interconnected porosity of not less than 90%. Optimization was based on the 2^2 rotatable design (Budnicka et al., 2019; Sebai et al., 2018; Trachtenberg et al., 2016). Input and output variables are shown in Table 2.

Table 2. Identification of input and output variables with a codification of input variables

	Variable	Natural variable	Unit	Coded variables				
				−1.414	−1	0	+1	+1.414
Input	x_1	weight ratio of Eudragit®E100 to PLLA	–	0.10	0.16	0.32	0.48	0.54
	x_2	volume ratio of porophore to PLLA solution in 1,4–dioxane	–	0.03	0.05	0.10	0.15	0.17
Output	y_1	mass absorbability	%			max.		
	y_2	interconnected porosity	%			> 90%		

A 13-run design was used, which consisted of a 2^2 factorial design (4 runs with two input variables in all combinations of +1 and −1 levels), four star points (4 runs with input variables at combinations of −1.414 or +1.414 levels with 0) and five replicates for the center of the design (5 runs with all two variables at 0). All other variables were kept constant (standard conditions). Experiments were performed in a random order, and both of the response variables, y_i, were measured for each experiment. Table 3 shows the design matrix along with the results.

Table 3. The 2^2 rotatable design: experimental matrix[a] and results[b]

Trial no.	x_0	x_1	x_2	$x_1 \cdot x_2$	$(x_1)^2$	$(x_2)^2$	Mass absorbability [%]		Interconnected porosity [%]	
							y_1	$\hat{y}_1$	y_2	$\hat{y}_2$
1	1	−1	−1	1	1	1	1790	1740	95.2	94.8
2	1	1	−1	−1	1	1	1050	1020	93.6	93.5
3	1	−1	1	−1	1	1	1320	1290	93.3	93.7
4	1	1	1	1	1	1	1710	1690	94.4	94.9
5	1	−1.414	0	0	2	0	1530	1570	93.4	93.4
6	1	1.414	0	0	2	0	1310	1340	93.5	93.3
7	1	0	−1.414	0	0	2	1300	1340	94.6	95.0
8	1	0	1.414	0	0	2	1460	1490	95.9	95.3
9	1	0	0	0	0	0	1750	1730	95.6	95.4
10	1	0	0	0	0	0	1840	1730	95.7	95.4
11	1	0	0	0	0	0	1560	1730	94.6	95.4
12	1	0	0	0	0	0	1800	1730	95.7	95.4
13	1	0	0	0	0	0	1700	1730	95.2	95.4

[a]Standard conditions: all experiments were performed using the same raw materials and volume of PLLA solution in 1,4–dioxane. [b]All $\hat{y}_i$ were calculated using a linear model.

In order to shorten the discussion, the details of statistical analysis are not presented in this paper. More detailed information can be seen in (Rodrigues and Iemma, 2014). Here, we present the linear models (without insignificant coefficients) and the most important diagrams only.

4.1. Absorbability, $\hat{y}_1$, %

$$\hat{y}_1 = 1732 \cdot x_0 + 281 \cdot x_1 x_2 - 138 x_1^2 - 158 x_2^2 \tag{6}$$

The diagram of the relation between mass absorbability, $\hat{y}_1$, and weight ratio of Eudragit®E100 to PLLA, x_1, and the volume ratio of porophore to PLLA solution in 1,4–dioxane, x_2 is shown in Fig. 5.

4.2. Interconnected porosity, $\hat{y}_2$, %

$$\hat{y}_2 = 95.4 \cdot x_0 - 1.02 \cdot x_1^2 \tag{7}$$

The diagram of the relation between implant interconnected porosity, $\hat{y}_2$, and Eudragit®E100 to PLLA weight ratio, x_1 is shown in Fig. 6.

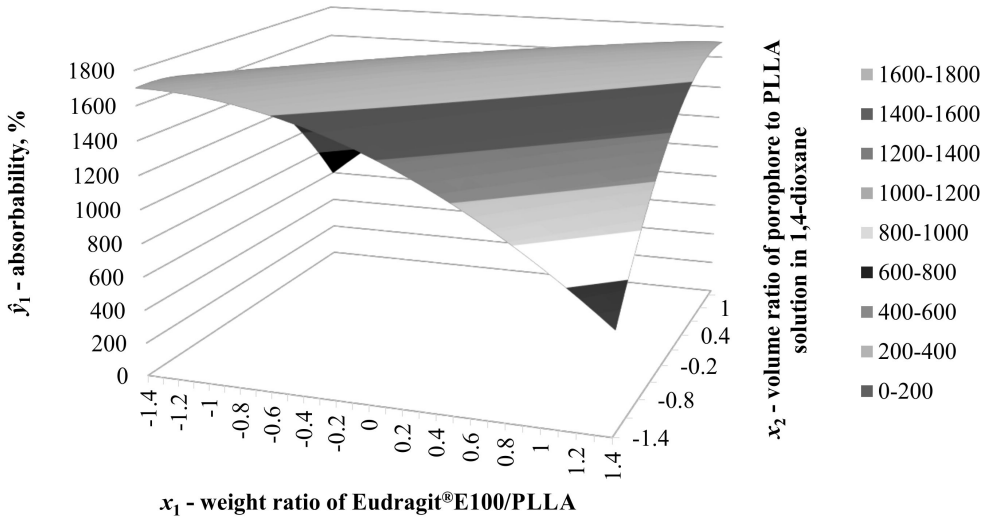

Fig. 5. The relation between mass absorbability ($\hat{y}_1$) and weight ratio of Eudragit®E100 to PLLA (x_1) and volume ratio of porophore to PLLA solution in 1,4–dioxane (x_2)

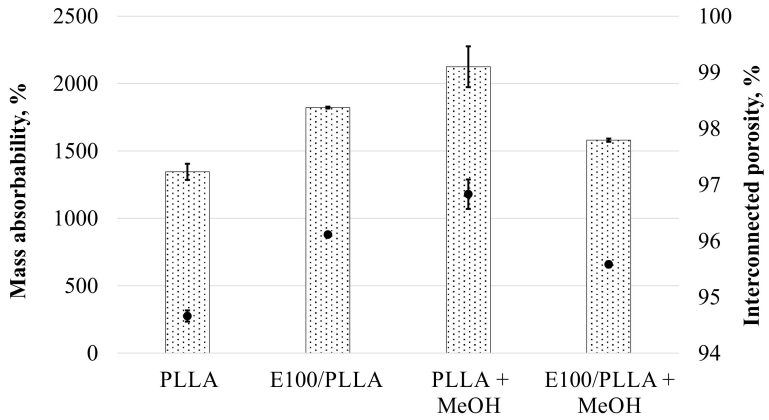

Fig. 6. Interconnected porosity ($\hat{y}_2$) as a function of Eudragit®E100 to PLLA weight ratio (x_1)

5. OPTIMIZATION SUMMARY

The effect of Eudragit®E100 to PLLA weight ratio (x_1), volume ratio of methanol (porophore) to PLLA solution in dioxane (x_2) on mass absorbability (y_1) and interconnected (y_2) porosity of implants was investigated. Using the estimated function (6), it was found that, within the design limits, it is the interaction between Eudragit®E100/PLLA weight ratio and the volume ratio of porophore/PLLA solution in dioxane (x_1x_2) that had the strongest influence on implant mass absorbability. The x_1 and x_2 variables do affect implant mass absorbability only when squared. Because of the positive value of x_1x_2 variable coefficient, extreme variable values decrease mass absorbability. Negative coefficient values of x_1 and x_2 squared variables result in correcting mass absorbability by reducing it. Therefore, the greatest values of mass absorbability correspond to the values of variables approaching the center of the design. A graph in the form of a saddle was obtained on the basis of the regression equation (Fig. 5).

In the interconnected porosity equation (7), x_1 variable when squared is the only relevant factor. It indicates that only Eudragit®E100 to PLLA weight ratio affects implant interconnected porosity. A negative coefficient value of x_1^2 variable decreases implant interconnected porosity and increases the x_1^2 variable module. Therefore, the greatest values of interconnected porosity correspond to the x_1^2 variable values equal to 0. A graph in the form of a parabola was obtained on the basis of the regression equation (Fig. 6).

This optimization is appropriate only within the previously established range of variables and it should not be extrapolated beyond that range.

Maximization of mass absorbability of implants with an interconnected porosity of not less than 90% leads to the following optimal conditions:

- Eudragit®E100 to PLLA weight ratio of 1:3 ($x_1 = 0$),
- volume ratio of porophore to PLLA solution in 1,4–dioxane of 1:10 ($x_2 = 0$).

The results are as follows: $\hat{y}_1 = 1732\%$, $\hat{y}_2 = 95.4\%$. Experiments 9–13, which were performed under the same conditions, were accepted as confirmatory experiments. The average results were: $y_1 = 1732\%$, $y_2 = 95.4\%$.

6. CONCLUSIONS

A novel method for the preparation of composite poly–L–lactide/Eudragit®E100 bone implants with increased absorbability was developed. In our group, a spatial implant with a volume of 10 cm^3, interconnected porosity above 90%, microporosity (pore size of ten micrometers) and macroporosity (pore size of 200–250 μm) was obtained (Gadomska-Gajadhur et al., 2018; Gadomska-Gajadhur et al., 2018). These features make the implant suitable for spongy bone regeneration. What is more, the method does not require any special equipment and the cost of used solvents is low.

Among methacrylic acid copolymers, Eudragit®E100 gave the best results of implant mass absorbability. However, the greatest mass absorbability value of implant was obtained using only methanol (porophore) as an additive during the manufacturing process. Still, it is believed the addition of Eudragit®E100 is essential to improve osteogenic properties of the implant, because of favourable groups in its structure. Therefore, it was decided to use Eudragit®E100 as an additive despite slightly lower mass absorbability values recorded during observation. Further research is planned to investigate the influence of Eudragit®E100 addition on cytotoxicity and proliferation of osteoblasts. Also, methanol was used owing to its beneficial influence on implant absorbability. Because of the unclear influence of the addition of methanol and Eudragit®E100 on implant mass absorbability, it was decided to optimize the process using a mathematical model.

The process was successfully optimized with the aid of DOE. After carrying out a 2^2 rotatable design, mathematical models of the influence of input variables: weight ratio of Eudragit®E100 to PLLA; volume ratio of porophore to PLLA solution in 1,4–dioxane, on mass absorbability and interconnected porosity of obtained implants were calculated. Both regression equations are adequate, which is confirmed by the fact that the differences between measured and calculated values are small. The first model enables control over implant mass absorbability, the maximization of which ensures the insertion of the largest possible volume of platelet-rich plasma into the scaffold. The second model makes it possible to control composite implant interconnected porosity. The suitable interconnected porosity (over 90%) enables nutrients to migrate into the scaffold and cell metabolites to migrate out of it. The scaffold design with optimum properties including interconnected porosity and mass absorbability provides an implant which meets the requirements set for cancellous bone regeneration.

Under optimal conditions: Eudragit®E100 to PLLA weight ratio of 1:3, volume ratio of porophore to PLLA solution in 1,4–dioxane of 1:10, the poly–L–lactide bone implant modified with Eudragit®E100 having the interconnected porosity of 95.4% and mass absorbability of 1732% was obtained confirming that the calculated equations describe the entire process very well.

SYMBOLS

V_1 volume of material without pores, cm^3
V_2 volume of all closed pores in the material, cm^3
V_3 volume of all interconnected pores in the material, cm^3
g standard gravity, m/s^2
d_0 density of the liquid at the measurement temperature, g/cm^3

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