1 INTRODUCTION

1.1. Different approaches to heart tissue regeneration

Since heart diseases have become the number one cause of death worldwide\(^1\), there is a strong interest in searching of efficient methods of heart tissue regeneration. The possible way of therapy depends on a degree of tissue damage. In case of i.e. nonischemic cardiomyopathy there is the opportunity of applying the left ventricular assist device
(LVAD) therapy. LVAD-induced mechanical unlock of a heart would lead to a complete myocardium recovery and a patient may not need any additional treatment. However, in case of more serious heart diseases, it is obligatory for a patient to undergo a replacement procedure of a whole heart or its parts.

In order to avoid such an extensive and dangerous surgery, for more than fifty years attention has been paid to tissue engineering methods of heart tissue regeneration. These include an injured heart support by introduction of cell-seeded or cell-free materials into the heart. The function of cell-free materials is to replace a dilated, soft myocardium and thus improve ventricular function and then can be repopulated with endogenous cells. Another approach comprises the use of pre-formed three-dimensional scaffolds of synthetic and/or natural polymers that would aid in the introduction of the cells to the heart. In vivo tissue formation method living tissue implants are generated outside the human body. In situ tissue engineering includes applying materials presenting active biomolecules. These allow controlling and guiding endogenous cell repopulation and subsequent tissue formation inside the human body.

1.2 Artificial heart as an alternative approach

The main problem concerning approaches mentioned above are weak mechanical properties of fabricated implants. Hence, replace the damaged heart with an artificial heart made of synthetic material seems to be an interesting point of view. However, there are many expectations that have to be met to minimize harmful effects that could arise.

Because of its destination, an artificial prosthesis would directly and constantly contact blood for a long time – if possible the lifetime of the patient. An implant surface becomes a border between living body and artificial live-rescuing device. Although implantable materials are carefully chosen, they still do not interact with blood components as natural vessel tissue does. That results with a number of unwanted phenomena occurring on a prosthesis surface. These effects form a complex biochemical process that has a great influence on a patient’s health, even long time after implantation. Hence, artificial heart has to be made from a material that should cooperate with blood without any side-effects. The only way to achieve this goal is to cover artificial prosthesis’s material with endothelial cells that form a monolayer in the inner part of blood vessels and heart. This approach considers in vitro cell pre-seeding and cultivation before implantation. That is the subject of the present work.

Below, we describe the advantages of PU in terms of artificial heart construction, the necessity of covering the surface of a PU implant with endothelial cells and the method of in vitro introducing endothelial cells onto PU.

1.3 Physiological effects caused by artificial heart prosthesis

The very first event that happens on the surface of artificial material is the adsorption of water and small ions together with small proteins, such as kininogen or fibrinogen. These are components that can trigger the blood coagulation cascade. This is a rapid process that requires just traces of initializing factors and results with formation of blood clots. In case of artificial heart, vessels or any other artificial implant it is strongly unwanted, because clots are likely to detach from the surface and cause thrombosis that can lead to a stroke. Moreover, the presence of proteins increase hydrophilicity of implanted material surface and so blood cells start to attach. Monocytes adhesion can cause activation of extensive inflammation response: activation of neutrophils and leukocytes and later also
macrophages\textsuperscript{15}. Porosity of the surface increases as well. That stimulates adhesion of platelets and other factors that eventually leads to formation of blood clots\textsuperscript{16}.

Since artificial heart should serve well for the whole patient’s life, it is crucial that a heart prosthesis’s material would not trigger any of these harmful effects.

1.4 Artificial heart prosthesis made from PU

Nowadays PUs are the most promising polymers used in fabrication of artificial heart prosthesis. Therefore, PUs were chosen to be the focus of the present work.

PUs are segmented copolymers that constitute a diverse group of chemical compounds. The selection of monomers used in the PU synthesis can produce materials with different mechanical characteristics, which makes PU an attractive biomaterial. Also, among all polymers PUs are characterized by the best hemocompatibility (compatibility with blood)\textsuperscript{17}.

Hemocompatibility of PUs is satisfying but not impeccable. Also, \textit{in vivo} studies indicated degradation of unprotected PU by surface oxidation\textsuperscript{18,19}, hydrolysis\textsuperscript{20} and radical processes caused by blood cells metabolism\textsuperscript{21}. Usually, a polymer such as PU undergoes further chemical or physical modification. A number of methods have been applied: hydrophilization\textsuperscript{22} or hydrophobization\textsuperscript{23,24} of the polymer surface and immobilization of biomolecules such as phospholipids\textsuperscript{25} or heparin\textsuperscript{20}. None of them was completely successful. The most advanced method is to cultivate endothelial cells on previously prepared polymer surface, which is described below.

![Figure 1](image-url)  \textit{Transverse section through the wall of a blood vessel showing its different constituents including the inner endothelial monolayer}\textsuperscript{26}.

1.5. Role of endothelial cells

Endothelial cells form a monolayer that covers the inner part of natural blood vessels and heart. This tight monolayer forms a barrier between blood and smooth muscle cells (Figure 1). Their strategic localization places endothelial cells in very first line of contact with cells and substances migrating from blood to tissue. The endothelium tissue constantly controls blood cells activity since it produces a large variety of both activating and inhibiting
Endothelium, via a large number of mechanisms not yet known in-depth, plays crucial role in regulation of vasomotorics, hemostasis and angiogenesis.

1.6 Adhesion of endothelial cells

Adhesion of endothelial cells on a vessel surface leads to formation of tight monolayer of cells. The process is based on interactions between endothelial transmembrane receptors and ligands that are specific molecules bonded by receptors. Ligands are found in the extracellular matrix (ECM) that in vivo composes a base for endothelial cells. ECM includes peptides, proteins, peptidoglycans and glycoproteins. All of these play a role in regulating endothelial cells metabolism. Receptor-ligand binding starts a cascade of biochemical signals inside a cell that finally results with cell attachment to a surface on which a ligand is present.

**Figure 2.** A picture showing the structure of integrins: α and β subunit, each including a large extracellular domain, a single membrane-spanning region and a short cytoplasmic domain.

**Figure 3**

a) Structure of RGD tripeptide included in adhesion receptors ligands, b) Structure of a cyclic peptide with RGD sequence.
1.6.1 Adhesion receptors - integrins and their ligands. Adhesion receptors have already been widely described in literature\(^35\). Generally, they are divided into four groups: integrin, cadherin, Ig-CAM, and selectin family\(^36\). Endothelial cell adhesion is mostly dependent on integrins. Each integrin is a heterodimer that contains an \(\alpha\) and a \(\beta\) subunit with each subunit having a large extracellular domain, a single membrane-spanning region, and in most cases a short cytoplasmic domain\(^34\) (Figure 2).

Typically, integrins mediate binding to large ECM proteins, such as collagen, laminin, vitronectin and fibronectin\(^33, 37\). Thanks to a development of analytic methods it was discovered that all ligands dedicated to cell adhesion receptors contain specific amino acid sequence that determines a proper interaction with a receptor. The sequence that is most commonly mentioned in literature is a RGD\(^33, 38-40\) tripeptide (Figure 3 a). It has been found e.g. in fibronectin and vitronectin\(^38\).

It has also been proven that stereochemistry of an adhesion sequence affects cell attachment activity. Cyclopeptides containing RGD sequence (Figure 3 b) are more affine to integrins than corresponding linear ones\(^38, 41-43\). Apart from RGD, there are also many different adhesion sequence known, such as LDV\(^44\), REDV\(^45, 46\), YIGSR\(^47\) and PDSGR\(^33, 38\).

1.6.2 Effects of integrin-ligand interaction. Figure 4 illustrates the idea of endothelial cells attachment on the surface rich with adhesion ligands. Binding to integrin receptors activates various signaling paths that, among other functions, mediate cell attachment, proliferation, differentiation and organization of the actin cytoskeleton\(^33, 37, 48\).

Thus, a change of cell shape is a visible symptom of proper attachment and is a result of biochemical processes initiated by ligand binding. Cells become more flattened and able to form a network of focal adhesions to a surface\(^48\). Finally, a cell is strongly anchored to a surface thanks to integrins and other adhesive membrane molecules (e.g. lipids and syndecans) and capable of communicating with other cells next to it\(^44, 49\).

1.6.3 Conditions of proper endothelial cell anchorage to a surface. Receptor-ligand interaction requires free space around the two components so that both of them can take the right conformation\(^39\). Strong covalent bond between a ligand and a surface is very important. Also, any unbounded peptides or proteins should be removed. Endothelial cells that are deprived of anchorage to a ligand die by an apoptotic signal\(^50, 51\).

1.7. Aim of work

The goal of this work was to develop the repeatable and easy to proceed method of fabricating a material that could construct the inner side of an artificial heart. The final material would comprise PU coated with endothelial cells \textit{in vitro}. Thus, the material would present suitable mechanical properties and interact with blood in the way natural tissue does.

PU surface was grafted with collagen that is a ligand for endothelial cells adhesion receptors – integrins. Then human endothelial cells were cultivated on the material. The present work includes a detailed description of the collagen binding to the PU surface and results from the cell seeding. The main challenge was to obtain a stable junction between collagen and PU surface so endothelial cells would properly interact with it. Also, we focused on the elimination of any factors that could interfere a correct cell-material interaction, such as surface roughness or improper collagen conformation.
2 MATERIALS AND METHODS

2.1 Materials

For the PU film preparation we used biomedical PU Estane 5715 P in form of grains (Lubrizol) and THF (Fluka), 30% (v/v), as the solvent. During the PU modification process we used: \( \text{H}_2\text{O}_2 \) (Carlo Erba), 65% (v/v) \( \text{HNO}_3 \) (Lach-Ner s.r.o), \( (\text{NH}_4)_2\text{Ce(SO}_3\text{)}_4 \) (Riedel-de-Haën), \( \text{CH}_2\text{CHCOOH} \) (Fluka), EDC (Sigma Aldrich), NHS (Sigma Aldrich), soluble collagen in citric buffer, average molecular mass 340kDa (Proteina). For –COOH groups analysis TBO (Sigma Aldrich), \( \text{NaOH} \) (Carlo Erba) and were applied. For endothelial cells culture, HUVEC (Lonza), medium was used. Cells were stained with the use of EBM-2 (Lonza), Double Staining Kit (Sigma Aldrich).

![Figure 4](image.png) Model of endothelial cells attachment on a surface presenting adhesion ligands.

![Figure 5](image.png) PU surface after modification shown schematically. Distinct elements that were introduced at each step of the modification process are pointed out.
2.2 Methods

In this work PU underwent superficial modification. The aim was to enrich its surface with collagen molecules since it is a compound of ECM and was reported to interact with integrins. PU grafted with collagen was used in endothelial cells culture to check whether cells would anchor to the modified surface.

Surface modification was accomplished by a three-step chemical procedure. Figure 5 schematically shows PU surface after modification. Our assumption was that only surface undergoes a modification process, therefore mechanical properties provided by PU would remain unaffected. Applied conditions were as mild as possible, because the material must not be toxic or harmful for the cells. All experiments and measurements were carried out once.

2.2.1 Preparation of PU films. A 20% (w/v) solution of PU in THF was prepared. It was stirred for 24h to dissolve the polymer and then left to rest for another 24h. Then the solution was poured on a glass via a film applicator. The PU film was air-dried for 3 days and next in an oven at 40°C for another 3 days. Obtained PU films were 0.3mm in thickness.

2.2.2 Activation of PU surface. PU is chemically inert - it does not contain any reactive superficial chemical groups. Thus, the very first step of modification is to activate PU surface. In the present work a photooxidation method was applied. Figure 6 presents a scheme of this step. Photooxidation is a popular method allowing the introduction of –OOH and –OH groups on the polymer surface. It does not require complex equipment. Also, radicals produced by this method are very reactive, so the reaction yield is high.

Pieces of PU foil were put into flat containers filled with small amount of a 30% (v/v) solution of H₂O₂. Containers were then put inside a UV lamp chamber. Samples were exposed to UV-C radiation for a defined period of time. Different times of exposure were tested – form 1h to 12h. After that samples were washed three times with distilled water and dried in an oven at 40°C. Samples were analyzed with the use of FTIR-ATR spectroscopy. A Thermo Scientific Nicolet™ 6700 spectrometer was used for obtaining the spectra, and OMNIC 8 software was used for spectrum analysis.

![Figure 6. A scheme of the activation of PU surface process. The exposure to UV-C radiation leads to superficial –OH groups appearing.](image-url)
2.2.3 Spacer molecules grafting. As it was mentioned in pp.1.4.4, it is essential that there is some free space provided between a ligand and cell receptor. Thus, it is recommended not to couple ligands directly to superficial reactive groups, but to do it indirectly through long spacer molecules grafting. At this stage, *in situ* radical polymerization of acrylic acid initiated by ceric ions was proposed\textsuperscript{56}. The process is presented in the Figure 7.

Activated PU samples were placed in a water solution containing 1.5% (v/v) HNO\textsubscript{3}. The solution was heated up to a defined temperature. Then 0.1% (w/v) (NH\textsubscript{4})\textsubscript{4}Ce(SO\textsubscript{3})\textsubscript{4} and a particular amount of CH\textsubscript{2}CHCOOH were added and stirred. Reaction was carried out with constant stirring for particular period of time. Parameters applied were: 25, 35, 40 and 45°C, 0.5, 1.5 and 2.5h of reaction and 5, 10, 15 and 20% (v/v) of CH\textsubscript{2}CHCOOH. After the predefined time of reaction, the samples were taken out from the solution, washed three times with distilled water with stirring and dried in an oven at 40°C. The long chains of polyacrylic acid grafted to the PU surface enrich it with carboxyl groups, thus these groups were used for evaluation. The amount of superficial -COOH groups was evaluated colorimetrically with the use of TBO\textsuperscript{57} (Figure 8), one mole of TBO binding to one mole of –COOH. The absorbance of TBO washing solutions (i.e. acetic acid) was measured at $\lambda=630\text{nm}$ with Helios $\gamma$ spectrophotometer (by Thermo Electron Corporation). Concentration of TBO that correspond to concentration of -COOH was estimated from a calibration curve.

2.2.4 Collagen immobilization. Before this stage was carried out, samples obtained with the use of best parameters were chosen.

For coupling collagen to carboxylated PU surface, a 2-step carbodiimide method\textsuperscript{58} was applied. Figure 9 presents a scheme of this method. Carboxylated PU foils were placed in flat containers filled with a aqueous solution of pH=6 containing 2% (w/v) EDC and 2% (w/v) NHS. After 15minutes samples were taken out and washed with a phosphate buffer solution of pH=6 and carefully dried with paper towel. After that samples were put into an aqueous bovine collagen (mixture of type I and type III) solution. Collagen was used in five-fold molar excess with reference to calculated –COOH groups. Reaction was carried out for 2h. Samples were then washed three times with distilled water and kept in PBS solution.

2.2.5 Endothelial cells culture. Samples of unmodified PU and PU with immobilized collagen were sterilized with UV radiation for 30min. Cells culture was carried out using HUVEC in standard medium for endothelium for 2 days at $37^\circ\text{C}$. In order to asses cells viability, cells were stained with Double Staining Kit\textsuperscript{59}. Samples were
observed every day during cultivation using a transverse optical fluorescence microscope Nikon Eclipse Ti-U.

![Diagram](image)

**Figure 8** A scheme of the colorimetric method of indicating –COOH groups. TBO binds to carboxyl groups that are present on PU surface after spacer grafting. After decoupling the absorbance of washing solution is measured. The amount of –COOH groups are estimated from a calibration curve.

![Diagram](image)

**Figure 9** An illustration of the collagen immobilizing. Amine groups from collagen react with –COOH groups from PU surface. As a result, a peptide bond is formed that provides a strong junction between PU surface and collagen.

### 3 RESULTS AND DISCUSSION

#### 3.1 Activation of PU surface

Figure 10 shows a part of FTIR-ATR spectra obtained for unmodified PU and PU samples activated for 1 to 5h. Samples exposed to UV-C radiation for 12h were thermically damaged because of the heat emitted by the UV lamp, thus they were not analyzed.

There is a characteristic signal appearing at 3600-3000 cm⁻¹ range that comes from –O-H bonds. The peak is clearly increasing and becomes the strongest for a sample
activated for 5h. Hence, 5 hours of exposure was accepted as the most efficient. As for PU surface hydroxylation via UV radiation, other authors propose similar exposure times, e.g. 4h\textsuperscript{56}.

3.2 Spacer molecules grafting

Figure 11a-d shows charts presenting amount of –COOH groups per 1cm\textsuperscript{2} of sample in function of time for different CH\textsubscript{2}CHCOOH concentrations and different reaction times.

![Figure 10](image)

**Figure 10** A part of FTIR-ATR spectra of unmodified PU and PU activated for 1 to 5 hours. A characteristic signal at 3600-3000 cm\textsuperscript{-1} range that comes from –OH groups can be observed.
Figure 11  Charts presenting amount of –COOH groups per 1cm² of sample in function of time for different CH₂CHCOOH concentrations and different reaction times (TBO method).
Charts show that carrying out acrylic acid grafting at higher temperatures (40 and 45°C) does not allow to achieve higher amounts of superficial carboxyl groups. Also, –COOH concentration is roughly constant during the whole process. It is probably caused by a side reaction – homopolymerization of acrylic acid in solution – which rate increases at higher temperatures. Homopolymerization plays a main role also for higher concentrations of acrylic acid. That results in low yields of grafting (Figure 11 d). Homopolymerization as a result of high concentration of acrylic acid and high temperature during the process was also reported by other authors 60. For 5, 10 and 15% of acrylic acid and temperatures of 25 and 35°C results were similar. However, only samples prepared with the use of 5% of acrylic acid presented acceptably smooth surface, without any roughness and clots. The largest amount of superficial -COOH groups (3,28 mM -COOH/cm²) was obtained for 5% (v/v) of acrylic acid, 35°C and 1.5h (Figure 11 a). Therefore, those parameters were chosen as the most suitable.

It was previously reported that the increase of reaction temperature leads to high acrylic acid grafting yield60. However, unlike in the present work, it has been shown that the grafting yield grows with the temperature increase up to 40°C (cellulose fiber) or 50°C (PET fiber)60, 61. Nevertheless, the roughness of samples after grafting was not mentioned, probably because it was not significant in those research. In the present work, the smoothness of surface is crucial.

A further increase in temperature decreased the graft yield. This was also indicated in other research61. At at higher temperature, the collision between monomer free radicals also increased, wherein the homopolymer formation becomes predominant.

As it was shown in the figure 11, reaction time plays an important role in acrylic acid grafting. The maximum amount of –COOH appears at 1.5h, and when the reaction proceeds further, a decrease can be observed. In the literature, authors mention different times when the largest –COOH amount is achieved (from 1h60, 61 to 2-3h62), yet the decrease is reported in all these works. The explanation may be that the TBO method applied to estimate the amount of –COOH groups has its limitations. The complexation of TBO may be hindered by the presence of the grafted chains when the graft yield is high61.

### 3.3 Endothelial cells culture

Table 1 shows parameters applied to prepare samples used for endothelial cells culture. Figure 12 present pictures from optical microscope (magnification 100x) of HUVEC cultivated on unmodified PU and PU with immobilized collagen. Obtained pictures indicated that for HUVEC deprived of anchorage on unmodified PU surface, after one day of cultivation only red cells were observed. Hence there were no living cells on unmodified PU after one day of cultivation. However, on the surface of PU enriched with collagen the cells survived (Figure 12 b)). Furthermore, after 2 days of cultivation cells significantly
changed their morphology and seemed to be strongly attached to the surface (Figure 12 c). This is a proof of integrin-mediated cells adhesion via ligand (collagen) binding. Besides, there are characteristic twin cells visible (Figure 12 c, circled) that most likely were formed after cell division. This also indicates the proper anchorage via integrins.

The change in the cell shape was expected as the positive premise of the cells anchorage to the surface via integrin-mediated mechanism\textsuperscript{63-66}. However, the density of HUVEC should be higher. It was shown that it is possible to obtain a tight monolayer of HUVEC on a PU grafted with gelatin\textsuperscript{64} and on modified titanium surface\textsuperscript{67}. Besides, obtaining a tight cell coating is crucial because of thrombogenicity of collagen. Collagen molecules exposed on the surface of an implant would cause a strong inflammatory response starting with platelet adhesion\textsuperscript{65}.

We observed cells proliferation 2 days after seeding which has been also previously noted. Sgarioto et al. reported that HUVEC proliferate most intensively 2 days after seeding on materials coated with collagen and other ECM proteins\textsuperscript{68}. Nevertheless, in the present research we did not succeed in longer HUVEC cultivation, whereas other authors managed to obtain viable cells after 7 days of cultivation\textsuperscript{65}. This was most likely due to collagen degradation by the cells enzymes.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure12.png}
\caption{Pictures from optical microscope (magnification 100x) showing HUVEC on a) unmodified PU after 1 day of cultivation, b) PU with immobilized collagen after 1 day of cultivation and c) PU with immobilized collagen after 2 days of cultivation. Cell pairs formed after cell division are circled.}
\end{figure}
4 CONCLUSIONS AND FUTURE PROSPECTS

The aim of the present work was to propose a method to fabricate a material that is composed of PU coated with HUVEC. In considered approach, the patient’s endogenous cells would be pre-seeded and pre-cultivated *in vitro* outside the body until the cells form a tight, healthy monolayer.

It was demonstrated that the process of three-step chemical modification was successful. Samples analysis showed effectiveness of each step. Final materials were not toxic for endothelial cells and clearly promoted cells adhesion, proliferation and change of cell shape. The advantage of the method is that it does not require any complex equipment and thus can be applied in any laboratory and be utilized on a large scale. Besides, it is free from organic solvents, so there is no need of special preparation before cell seeding. Proposed method of endothelialization is promising and can be applied to other base polymers and proteins or peptides.

Since the research was promising, we are going to apply this method to graft short peptides such as RGD or YIGSR onto PU. That may lead to obtain a monolayer of endothelial cells that would completely separate the PU surface from blood. After the tight cell coating would be achieved, the material should be tested by platelet-rich plasma (PRP) incubation to examine platelets activation.

References

59. [www.sigmaaldrich.com](http://www.sigmaaldrich.com).