Preliminary testing of silicone-urethane elastomers as substrates in human cell culture

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Abstract: Silicone-urethanes, polymers combining the characteristics of two widely used biomaterials, i.e. polyurethanes and silicones, are highly valued in many applications, including medical implants. To assess properties of these materials in contact with living cells, a set of different silicone-urethane materials, candidates for tissue engineering scaffolds, was synthesized and characterized. Two different oligomeric siloxane diols: Tegomer-2111 (Teg) and KF-6001 (KF), and two different types of diisocyanate, MDI and IPDI, were used in synthesis. Blood platelets adhesion to surfaces of selected materials showed a higher thrombogenicity of material based on Teg. Human fibroblasts were used in in vitro biocompatibility tests. The viability of cells cultured on silicone-urethanes was tested by XTT assay. Teg-based silicone–urethanes showed a significantly higher biocompatibility than those based on KF. Materials based on MDI compared to IPDI were found to be significantly more favoured by cells, not necessarily due to the type of diisocyanate but maybe also because of the necessity of using potentially toxic catalyst which accompanies the use of IPDI. Our studies indicate that silicone-urethanes are potent materials for tissue engineering products development. On the basis of the observations performed in cell culture, Tegomer-2111 as oligomeric siloxane diol and MDI as diisocyanate are recommended as starting materials for silicone-urethane scaffolds synthesis.

Introduction

The commercial use of silicone-urethane elastomers (i.e. polyurethane elastomers containing polysiloxane segments in the main polymer chain) in medical applications has been reported [1]. However, only little is known of the interaction of silicone-urethanes with living cells in culture. Taking into account the good biocompatibility of both polyurethanes and silicones [2-7], it can be expected that silicone-urethanes would be well tolerated by live cells and no adverse effects would be observed in tissue culture. In order to confirm this assumption, a series of silicone-urethane...
elastomers was synthesized from two different oligomeric siloxane diols and two different diisocyanates. The resulting films were used as substrates in human cell culture. In order to perform a preliminary evaluation of the samples the viability of human fibroblasts was determined. The selected samples of the materials were subjected to standard simple preliminary biocompatibility tests (including blood contact test). The mechanical properties of the selected samples were also evaluated.

**Results**

Results of water extract investigations and mechanical properties of selected samples of silicone-urethanes investigated in this study are presented in Table 1.

**Tab. 1.** Results of water extract testing and mechanical properties of selected silicone-urethane samples.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Properties of water extracts (70°C, 24 hrs)</th>
<th>Mechanical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV absorption at λ equal to nm</td>
<td>Mass change εᵣ δᵣ δ₁₀₀</td>
</tr>
<tr>
<td></td>
<td>228.2 248.9 260.2</td>
<td>%</td>
</tr>
<tr>
<td>Teg/MDI 2</td>
<td>0.155 0.082 0.064</td>
<td>0.17</td>
</tr>
<tr>
<td>KF/IPDI 2</td>
<td>0.025 nt 0.011 nt</td>
<td>nt</td>
</tr>
<tr>
<td>Teg/IPDI 2</td>
<td>0.050 0.043 0.015</td>
<td>1.66</td>
</tr>
<tr>
<td>KF/IPDI 3.5</td>
<td>0.114 0.106 0.077</td>
<td>-0.18</td>
</tr>
<tr>
<td>Teg/IPDI 3.5</td>
<td>0.086 0.075 0.034</td>
<td>1.32</td>
</tr>
<tr>
<td>Teg/MDI 3.5</td>
<td>0.233 0.095 0.093</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*nt = not tested

The results of testing the traces of organic substances and traces of heavy metals in oligomeric siloxane diols are presented below. (1) absorption spectrometry: No presence of organic groups showing significant UV absorption in the range of 190 to 400 nm was detected in either of the oligomeric siloxane diol. (2) GC-MS (gas chromatography combined with mass spectrometry): Traces of the following organic compounds were detected in KF and not detected in Teg: ethylene glycol, ethyl dioxolane, acetic acid, ethanol, isopropanol. Presence of ethyl dioxolane can be explained by the cyclization of 2-allyloxyethanol (presumably one of the substrates for KF synthesis) (3) EM-spectrometry: Traces of platinum were detected in Teg and not detected in KF. The presence of rhutene and iridium in KF was excluded while the presence of osmium could not be excluded.

The results of testing selected samples of silicone-urethanes in contact with blood are presented in Fig 1. Adhesion of a number of blood platelets, either individually or in aggregates (Fig. 1A, 1B) was found on the surface of the sample synthesized from Teg (Teg/IPDI 2). On the surface of the sample synthesized from KF (KF/IPDI 2) only some deformed cell structures (12-25 μm in size) were observed (Fig. 1C, 1D), only
few of which could be possibly identified as blood platelets. The image of blood
platelets in contact with Ti (Fig. 1E) shows its morphology on the thrombogenic
surface of metal.

![Fig. 1. SEM observation of the adhesion of blood platelets on the surfaces of two
chosen silicone-urethane materials differed in the type of siloxane diol used for their
synthesis; Teg/IPDI 2 sample - pictures A, B, KF/IPDI 2 sample -C, D, titanium
sample - E. The bars showed under the left (A, C, E) and right (B, D) columns are
proportional to the size of 10 and 50 μm of the visualised structures, respectively.

The results of testing the viability of fibroblasts by XTT assay are gathered in Table 2.
The same values are shown in the form of diagrams in order to facilitate a
comparison of results obtained on the samples differing in the type of siloxane diols
(Fig. 2) and the type of diisocyanate (Fig. 3).

Figure 2 shows the comparison of the viability of cells for pairs of silicone-urethanes
differing only in oligomeric siloxane diols used for their synthesis. In all cases the
viability of fibroblasts is significantly higher on materials synthesized from Teg than
from KF.
Tab. 2. Results of viability of fibroblasts tested using XTT assay.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Viability of fibroblasts (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teg/MDI 2</td>
<td>74.19 ± 4.89</td>
</tr>
<tr>
<td>KF/MDI 2</td>
<td>29.84 ± 23.28</td>
</tr>
<tr>
<td>Teg/MDI 3.5</td>
<td>112.90 ± 20.23</td>
</tr>
<tr>
<td>KF/MDI 3.5</td>
<td>71.77 ± 11.22</td>
</tr>
<tr>
<td>Teg/IPDI 2</td>
<td>34.68 ± 6.59</td>
</tr>
<tr>
<td>KF/IPDI 2</td>
<td>0.00 ± 11.20</td>
</tr>
<tr>
<td>Teg/IPDI 3.5</td>
<td>30.91 ± 4.84</td>
</tr>
<tr>
<td>KF/IPDI 3.5</td>
<td>4.57 ± 4.06</td>
</tr>
</tbody>
</table>

Fig. 2. Influence of the type of oligosiloxane diol monomer used in the synthesis of silicone-urethane elastomers on the viability of human fibroblasts – results of XTT assay (percentage of absorbance in positive control).

When the materials synthesized with different diisocyanate were compared (Fig. 3), higher viability of fibroblasts was observed on samples synthesized with aromatic diisocyanate (MDI) in 3 from 4 pairs.
Discussion

The main finding of our study is that silicone-urethane materials based on Tegomer 2111 are significantly better tolerated by cells than materials based on KF-6001. This was found in human fibroblast culture, where cell viability determined in XTT assay was significantly lower on silicone-urethanes based on KF-6001 siloxane diol as compared to those based on Teg. Poor adhesion of cells to KF-based support was also observed in blood platelets adhesion tests. Platelets did not spread on the surface of KF-based materials while they adhered very well to the surface of Teg-based support. On the one hand, non-thrombogenic surface may be wanted if the investigated material is designated for biomedical devices that remain in contact with blood. However, on the other hand, the lack of platelet adhesion analysed alongside with the diminished fibroblast viability may confirm the cytotoxicity of KF-based silicone-urethanes in vitro. Thus, these polymers could not be considered as substrates for tissue culture designed for scaffold in tissue engineering.

The reasons for the enhanced toxicity of KF-based silicone-urethanes remain unclear. Only traces of ethylene glycol, ethyl oxolane, acetic acid, ethanol, isopropanol were found in KF-based materials by means of gas chromatography combined with mass spectrometry (GC-MS). As regards ethanol, its toxicity in concentrations up to 0.1% in fibroblast culture was excluded, as verified in our laboratory (unpublished data). Not much data about the toxicity of the other substances in cell culture is available. Acetic acid was found to be toxic for Chinese hamster ovary K1 cells at the concentration of 12-16 mM. The effect appeared due to change in pH, as it was cancelled when buffered medium was used [12]. Lethal concentration LC75 for C6 rat glioma cells for isopropanol is about 0.5 M [13]. These
substances were detected in oligomeric siloxane diol KF at a trace level so their potentially negative influence on cell viability seems to be unlikely.

It should be mentioned that synthesis of oligosiloxanes terminated with organic hydroxyl groups is usually conducted by hydrosilylation of relevant unsaturated alcohols with corresponding H-siloxanes and that the catalysts that might be used for hydrosilylation are usually organic complexes of platinum, iridium, rhutenium or osmium. Since all other possible heavy metals (Pt, Rh and Ir) whose complexes could have been applied by the manufacturer in a synthesis of KF were excluded, the presence of osmium traces – which could not be excluded using emission spectroscopy – in KF was very probable and could be the reason for the enhanced cytotoxicity of silicone-urethanes obtained from KF. However, the presence of osmium in KF remains unproved and it was not possible to obtain a manufacturer’s confirmation of the kind of catalyst used in KF synthesis.

Also, examination of water extract did not reveal significant contaminations that could severely affect the interaction of those polymers with biological material.

Summarising, we cannot show any mechanism in which the above discussed substances might influence cell behaviour in contact with the investigated materials. Anyway, their role in KF-based silicone-urethanes toxicity cannot be definitely excluded, even if they were found only at a trace level.

Surface properties of silicone-urethane samples of various compositions were studied earlier by the authors of this paper [14, 15]. The results of dynamic contact angle determinations made using Kruss Tensiometer [14] did not reveal any significant differences between samples prepared using Teg and KF at the same level of NCO/OH. However, more detailed studies of surface behaviour by XPS [15] showed that the concentration of polysiloxane chains on the surface of samples made from KF was much higher than on the surface of samples made of Teg at the same level of NCO/OH. A lower concentration of polysiloxane chains on the surface may, therefore, be one of the factors deciding about a better behaviour of samples synthesized from Teg in cell culture. It is noteworthy that lower concentration of siloxane chains means lower hydrophobicity of the surface. Thus it seems that Teg-based silicone-urethanes are slightly more hydrophilic than those based on KF and, as such, have better abilities as a support for cell spreading.

Another element i.e. mechanical characteristic of the materials may also be taken into account as it is known that cell response to the support may be dependent on surface stiffness [16, 17, 18]. Results presented in Table 1 indicate that the type of starting materials used for synthesis of silicone-urethanes influence their mechanical properties; however, looking for the correlation between material stiffness and cell response would require a series of other experiments.

The obtained results of cell viability indicate that the type of diisocyanate used for synthesis of silicone-urethanes influences the behaviour of the cultured cells. MDI induces more efficient cell growth than IPDI. However, IPDI needs the usage of a catalyst, while MDI does not. The tin-containing catalyst (dibutyltindilaurate – DBTL) applied in experiments may cause loss in cell viability. Therefore, the problem of the influence of the diisocyanate type on biocompatibility of silicone-urethanes needs further investigation.

To sum up, the main conclusion of the present work is that silicone-urethanes based on oligomeric siloxane diol Tegomer 2111 are significantly better tolerated by cells
than those based on KF-6001 siloxane diol. Thus, for practical purposes, Tegomer 2111, and not KF-6001, can be recommended as a starting material for the synthesis of silicone-urethanes which might be applied as scaffolds in tissue engineering. An additional finding concerns MDI diisocyanate, which on the base of obtained results would be recommended as a better starting material as compared to IPDI. However further investigations are required to fully assess this suggestion.

**Experimental**

**Synthesis of silicone-urethanes**

Synthesis of silicone-urethanes was carried out following the procedure described by Kozakiewicz [8], using two oligomeric siloxane diols of structures (I) and (II) obtained from two different producers (KF-6001 from Shin-Etsu, Japan and Tegomer-2111 from Goldschmidt, Germany) and two different diisocyanates: cycloaliphatic diisocyanate – isophoronediisocyanate (IPDI) obtained from Huels and aromatic diisocyanate – diphenylmethanediisocyanate (MDI) obtained from Borsodchem Ltd.

(I)  
\[
\text{Me} \quad \text{Si} \quad \text{(CH}_2\text{)}_2 \text{-O-(CH}_2\text{)}_3 \text{O} \quad \text{Si} \quad \text{Me} \\
\text{(Si-O)}_{20} \quad \text{Me} \\
\text{Me} \quad \text{Si} \quad \text{(CH}_2\text{)}_3 \text{-O-(CH}_2\text{)}_2 \text{-OH} \\
\text{Me} \\
\text{Me}
\]

Oligomeric siloxane diol from Shin-Etsu (Japan). Commercial name: KF-6001. In this paper the abbreviated name KF will be used for this material.

(II)  
\[
\text{Me} \quad \text{Si} \quad \text{(CH}_2\text{)}_6 \text{-O} \\
\text{(Si-O)}_{10} \quad \text{Si} \quad \text{(CH}_2\text{)}_6 \text{-OH} \\
\text{Me} \\
\text{Me}
\]

Oligomeric siloxane diol from Goldschmidt (Germany). Commercial name: Tegomer-2111. In this paper the abbreviated name Teg will be used for this material.

In the first step, a selected oligomeric siloxane diol was reacted with diisocyanate to yield liquid NCO-terminated prepolymer of structure (III) that, in turn, was moisture-cured at 25 °C and 50% relative humidity to form an elastomeric film of silicone-urethane of structure (IV).

(III)  
\[
\text{OCN} \quad \text{U} \quad \text{-O} \quad \text{U} \quad \text{X} \quad \text{NCO}
\]

NCO-terminated silicone-urethane prepolymer

(IV)  
\[
\text{UR} \quad \text{U} \quad \text{-O} \quad \text{U} \quad \text{X} \quad \text{z}
\]

Silicone-urethane elastomer

- urethane moiety (-NHCOO-)
- urea moiety (-NHCONH-)
- diisocyanate segment
- poly siloxane chain

It should be noted here that silicone-urethane is only a common name for that polymer. As it contains not only urethane moieties, but also urea moieties, the correct
full name should read: poly(siloxaneurethaneurea). However, the common name silicone-urethane will be used in this paper. It should also be mentioned that a catalyst (dibutyltinilaurate - DBTL) was used in synthesis of silicone-urethane prepolymer when IPDI was one of the substrates.

Four types of silicone-urethanes were tested in the experiments. They differed in the substrate used for synthesis, the type of siloxane diol and the type of diisocyanate. The set was repeated for two different NCO/OH molar ratios. The following denotation was used in the presented results: Teg means Tegomer 2111, KF means KF-6001, 2 means 2/1 NCO/OH molar ratio and 3.5 means 3.5/1 NCO/OH molar ratio (i.e. Teg/MDI 2 means that material was synthesized based on Tegomer 2111, using MDI diisocyanate and with 2/1 NCO/OH molar ratio).

**Mechanical properties testing**

Mechanical properties of the samples were tested using the Instron 4500 Series tensile machine at a speed of 2.5 cm/min. Elongation at break ($\varepsilon_r$), tensile strength ($\delta_r$) and stress at 100% elongation ($\delta_{100}$) were determined.

Testing of traces of organic substances and traces of heavy metals in both oligomeric siloxane diols used as starting materials for the synthesis of silicone-urethanes were investigated in this study.

**Investigation of starting materials**

Two methods of testing of traces of organic substances (Traces of organic substances above 0.001 ppm could be detected by GC-MS) were used: UV absorption spectrometry PU 8740 Philips UV/Vis apparatus) in the range of 190 to 400 nm and gas chromatography combined with mass spectrometry (Hewlett-Packard GC 5890 Spectrometer, 5989 MBS Engine). Testing of traces of heavy metals was done using emission spectrometry.

**Investigation of water extracts of the final products**

Water extracts of all investigated materials were tested. They were obtained after soaking the samples in water at 70 °C over 24 hours (according to the procedure described in EN ISO 10993-11:1995). 25 g of sample per 500 ml of water was used. The investigation was carried out for the presence of organic compounds - UV spectrum was taken using the UV/VIS apparatus described above in the range of 200 to 300 nm, and the absorption was noted at three fixed points. According to the requirements specified by the Polish Institute of Medicines, the UV absorption should not exceed 0.3 at any of the fixed points. For the change of the mass of the extracted sample, mass change provides some information on the possible water absorption (positive value of mass change) or at the release of some matter in to water (negative value of mass change). There is no fixed approvable limit of mass change, though values not exceeding 1-2% can be considered as a good result.

**Blood contact test - Estimation of blood platelet adhesion to surfaces of chosen materials**

Two types of tested materials which differed in the type of siloxane diol (Teg/IPDI 2 and KF/IPDI 2) were observed in direct contact with blood platelets. For comparison titanium sample was used; some reports indicate that titanium is a thrombogenic
The blood was collected from healthy volunteers who had abstained from any medication for at least 2 weeks prior to the study. The informed consent was collected from all the donors, and the study was performed according to the Helsinki Declaration. The blood was collected into plastic tubes containing 3.8% sodium citrate as an anticoagulant (1:9 ratio v:v). Platelet-poor plasma (PPP) was obtained by centrifugation of citrated whole blood for 10 minutes at 1000xg at RT.

Before the experiment, the samples were washed twice in a supersonic washer, with distilled water, and after that they were placed overnight in PPP at 4 °C. Subsequently, the samples were washed twice with 10 mM phosphate buffered saline (PBS), pH 7.4.

Such pre-treated samples were put into contact with citrated whole blood for one hour with continuous end to end mixing. After that the tested surfaces were carefully washed with PBS and immersed for fixation in a 2.5% solution of glutaraldehyde made in PBS. The samples were dehydrated in ethanol and dried with air. The dry surfaces were coated with 20-30 nm of gold film, in a sputter-coater JEOL JEE-4X, and were observed in a HITACHI 3000N scanning electron microscope at 5 kV.

**Fibroblasts behaviour in direct contact with the studied surfaces**

-**Cells**

Human fibroblasts were isolated from connective tissue explants, harvested during surgery, which would otherwise be discarded. Tissue fragments were cut into small pieces washed with PBS and placed in the collagenase solution on a magnetic stirrer in the temperature of 37 °C for 24 h. The supernatant was decanted and the cells were counted. The cells were cultured in Dulbeco’s Modified Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (all from Gibco BRL, Paisley, UK) hereafter referred to as culture medium. The cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C.

-**XTT assay**

Silicone-urethane samples (irradiation-sterilized at the dosage of 25 kGy) were placed in 96 well tissue culture microplates (Corning Inc., Corning, NY, USA). Then, cells were seeded in each well directly on the surfaces of investigated materials at the density of 15x10³ per well. Cells were cultured on unmodified surfaces of tissue culture dish; polystyrene served as a control. The plates were incubated under humidified 5% CO₂ atmosphere at temperature 37 °C for 7 days. The medium was changed twice a week.

After that time cell survival was assessed by the XTT assay. This test is used in toxicology in vitro and is based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the XTT substrate (2,3-bis(2methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carboxyl]-2H-tetrazolium hydroxide) into a water-soluble formazan product. The final product of the reaction is measured in the ELISA reader at 450 nm. Although the result of this test is not equal to the number of cells, there are reports which confirm that the production of formazans is proportional to the number of living cells [11], which makes this method useful in studies based on the comparison of the number of living cells among groups.
-Statistical analysis

Experiments in which fibroblasts were observed in direct contact with the tested materials were performed twice. Four samples were tested in each repetition. The XTT assay results were expressed as percent of control ±SD. Statistical significance was determined using unpaired t test with Welch’s correction. Values of p<0.05 were considered as statistically significant.

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