Structuring of bioactive glass surfaces at the micrometer scale by direct casting intended to influence cell response

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DOI 10.1515/bglass-2016-0008
Received Aug 12, 2016; revised Oct 29, 2016; accepted Oct 31, 2016

Abstract: Defect-free bioactive glass surfaces with a grooved microstructure at the low micrometer scale were achieved by a mold casting process. The process was applied to the well-known glass compositions 45S5 and 13–93. Such microstructured surfaces may exhibit especially favorable conditions for bone cell orientation and growth. The aim of the study was to assess the parameter range for a successful casting process and thus to produce samples suitable to investigate the interaction between structured surfaces and relevant cells. Viscous flow in its temperature dependence and thermal analysis were analyzed to identify a suitable process window and to design a manageable time-temperature process scheme. Countering effects such as formation of chill ripples, mold sticking and build-up of permanent thermal stress in the glass had to be overcome. A platinum gold alloy was chosen as mold material with the mold surface bearing the mother shape of the microstructure to be imprinted on the glass surface. First experiments studying the behavior of osteoblast-like cells, seeded on these microstructured glass surfaces revealed excellent viability and an orientation of the cells along the microgrooves. The presented results show that direct casting is a suitable process to produce defined microstructures on bioactive glass surfaces.

Keywords: casting; surface topography; groove structure; cell guidance

1 Introduction

Large bone defects cannot be healed by the body itself. Since the availability of autologous material is limited, synthetic materials offer a promising solution for bone replacement applications [1]. In this respect, bioactive glasses exhibit excellent properties: They are cytocompatible, bind quickly to bone and can stimulate bone regeneration [2].

Cellular behavior is crucially influenced not only by the chemistry of the underlying substrate but by the surface topography and mechanical properties as well. Surface structures can increase the bone–to–implant contact and thus the fixation in the host bone [3]. It has been shown that cells react to surface structures from the nanometer to the micrometer scale [4, 5]. Furthermore it is well known that cell adhesion and even differentiation of cells can be influenced by surface structures in the low micrometer range [6–8]. Parallel groove–ridge structures promote contact guided alignment of different cell types with the groove depth having more impact as the grooves become deeper [7, 9–11]. More than 5 µm in depth are needed to sufficiently guide the cells [7, 10, 12]. In addition, McBeath et al. and Kumar et al. have shown that elongated stem cells rather differentiate into osteoblasts whereas rounded cells become adipogenic cells [13, 14].

Photolithography and etching methods enabled the possibility to manufacture surface topographies at the micrometer scale [15–18]. A wide variety of materials have thus become available to study the phenomenon of contact guidance [19, 20]. Still, due to their strong crystallization tendency, bioactive glasses are mostly ineligible for a
surface finish after manufacturing and therefore they are rarely available for cell culture experiments regarding cell behavior e.g. guidance phenomena on structured surfaces.

Itälä et al. manufactured micro-rough bioglass surfaces by chemical etching and found an improved osteoblast attachment compared to polished control specimens [21]. Following in vivo experiments even showed a higher amount of incorporated new bone in comparison to smooth implants [22]. Porous structures were realized by lithography-based additive manufacturing as well, creating crystallized bioglass samples [23]. Efforts have also been made to structure bioglass, showing alignment of osteoblast-like MG-63 cells. But as the samples were sintered, the tested material became a glass-ceramic [24]. Surface structures on amorphous bioactive glasses have not been realized before.

To choose an appropriate testing pattern we took into consideration the results of above mentioned former studies, where many cell types react to grooved substrates in the low micrometer range, mesenchymal stem cell size, as well as the requirement that a surface structure on bioactive glass has to endure the different states of corrosion to provide the intended biophysical cue throughout the formation of different layers after all. Moreover, when considering the average viscosity of a glass melt, our calculations and experiments indicated that surface topographies at the scale of a few 10 μm suitable for casting. Finally a parallel groove pattern of 30 μm groove, 10 μm ridge width, and 15 μm height was applied to bioactive glasses in this study.

A few mold materials were preliminary discussed and tested with minor success, as we decided to stay with platinum gold. Platinum alloys are commonly used in combination with glass melts and batches, as chemical reactions are minimal up to high temperatures. Platinum gold is further known to be only poorly wetted by oxide glass melts, hence a favorable demolding behavior is expected.

Discontinuous casting does not belong to the major forming methods for mass glass production, but it is typically used to manufacture bulk glass samples for a variety of standard testing methods in glass science, or in the field of glass art.

Microstructured components are for example fabricated in the field of Micro-optics by using hot embossing of borosilicate glass [25, 26]. Surface features of some micrometers are standard requirements for this application. Hot embossing requires heating the glass samples up to a temperature level sufficiently high enough for forming, but due to the high crystallization tendency of bioactive glasses, this process is hardly applicable to these glasses.

Combining the outstanding properties of bioactive glass with those of a triggered surface topography is very promising and holds potential to further improve bone replacement applications. The presented results show that a tailored casting process can be used to realize the demanded glass surfaces.

2 Methods

PtAu5 sheet metal with 8 mm thickness was used as substrate material, micrometer sized grooves were applied by a solid state ultrashort pulsed laser (Timebandwidth Duetto, Zurich, Switzerland). The applied groove depth to be imprinted on the glass was 15 μm, the groove width 30 μm and the ridge width 10 μm.

Two bioactive glasses namely 45S5 [27] and 13–93 [28] were synthesized via melt–quench technique. Batches of the pure chemical components: Fused silica (Aachener Quarz–Glas Technologie Heinrich, Aachen, Germany), CaHPO4 (Merck, Darmstadt, Germany), CaCO3, Na2CO3 (both AppliChem, Darmstadt, Germany) for 45S5, and additionally MgO (VWR Chemicals, Darmstadt, Germany) and K2CO3 (Merck, Darmstadt, Germany) for 13–93, were homogenized and then melted at 1400°C for 2 hours in a platinum crucible. Batch sizes yielding 300 g of glass were prepared and the melts were fritted into water. XRF and XRD analysis were carried out to ensure the correct composition and amorphous nature of the material implying the described melting conditions. The glass frit was sieved to different designated particle size fractions from 63 μm to 1 mm and provided for the following casting procedure respectively.

15 g of glass granules were remelted in a small platinum crucible for 40 minutes at a temperature level Tcast and then cast. The PtAu5 substrate bearing the microstructure was preheated at a temperature Tmold. To ensure the correct mold temperature, the mold was stored in a separate furnace wherein the casting was performed. The temperature of the melt as well as the mold were varied in a series of experiments. Demolding of the solidified glass was accomplished by turning the substrate upside down and carefully pounding from the back. The samples were then annealed for 12 hours at a temperature level 20 K above the glass transition temperature Tg, and cooled down to room temperature within 10 hours.

Polished bioactive glass 45S5 and 13–93 samples to be used as control for cell culture experiments were cast from 1400°C melt into graphite molds which were preheated at 350°C as described elsewhere [29]. The cylinders were then
Table 1: Temperatures (T in °C) crucial for the casting process; subscripts g = glass transition, x = main bulk crystallization peak; m = bulk melting peak, liq = calculated liquidus temperature; stick = sticking temperature, cast = optimal casting temperature, strain = strain point; mold = optimal mold temperature.

<table>
<thead>
<tr>
<th>Glass log η</th>
<th>T_g</th>
<th>T_x</th>
<th>T_m</th>
<th>T_liq</th>
<th>T_stick</th>
<th>T_cast</th>
<th>T_strain</th>
<th>T_mold</th>
<th>T_contact</th>
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</thead>
<tbody>
<tr>
<td>45S5</td>
<td>536</td>
<td>691</td>
<td>1217</td>
<td>1198</td>
<td>592</td>
<td>1190</td>
<td>517</td>
<td>500</td>
<td>653</td>
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<td>13–93</td>
<td>589</td>
<td>972</td>
<td>1141</td>
<td>1134</td>
<td>663</td>
<td>1417</td>
<td>580</td>
<td>500</td>
<td>704</td>
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The obtained surface structures of the samples were characterized using laser scanning microscopy. Measurements were performed at 50x magnification using a Keyence VK X100 series microscope (Neu–Isenburg, Germany). A surface area of 200 × 275 μm was analyzed to evaluate the surface quality in three dimensions. In addition, two separate measurements, one of the glass and one of the mold surface, were compared in order to evaluate the shaping accuracy of the casting process: Two individual two-dimensional linescans of 250 μm were superimposed. Laser scanning microscopy was used as a non-destructive analyzing method so that analyzed samples could be used for further testing. Cross sections were additionally studied with a Zeiss Leo 440i scanning electron microscope (Oberkochen, Germany). Microstructured bioactive glass samples were cut and embedded orthogonally to the microgrooves in order to inspect the shape and size of the surface topography.

Thermal analysis of the two bioactive glasses with heating and cooling rates of 10 K/min were performed with a Setaram Instrumentation Setsys DTA 16 apparatus (Caluire, France) using an empty platinum crucible as reference. Data was adapted with a calibration measurement (Caluire, France) using an empty platinum crucible as reference. Data was adapted with a calibration measurement (Caluire, France) using an empty platinum crucible as reference.

Experimental viscosity data from Vedel et al. [30] were reevaluated on the basis of a VFT interpolation using the numerical method of linearized coordinates. For this purpose, a linear regression analysis of the experimental log η data vs. the auxiliary function

\[ f \left( \frac{T_g}{T} \right) = \frac{1 - a_{VFT} \cdot \frac{T_g}{T}}{1 - a_{VFT} \cdot \frac{T_g}{T}} \]

was performed under a numerical variation of the constant \( a_{VFT} \) for minimum square deviation between the experimental data and the regression fit line. The VFT parameters \( A, B, T_0 \) were obtained as follows: \( A \) is the intercept of the fit, \( T_0 = a_{VFT} \cdot T_g, B = (13 - A) \cdot (T_g - T_0) \).

Live/dead stainings were conducted to estimate the initial biological behavior of the microstructured samples in contact with osteoblast-like MG-63 cells. Three structured and polished bioglass samples of each composition as well as borosilicate glass cover discs (Carl Roth GmbH+Co, Karlsruhe, Germany) according to ISO 8255-1 as control were sterilized at 200°C for 2 hours. After the sterilization process, the cells were seeded on the glass samples at a concentration of 20,000 cells/cm² and incubated at 37°C and 5% CO₂ for 24 hours. A mixture of 600 μl Ringer solution (Delta-Pharma, Pfulling, Germany), 10 μl of 5 mg/ml propidium iodide (Sigma-Aldrich, Steinheim, Germany) in Ringer solution and 10 μl of 5 mg/ml fluorescein diacetate (Sigma-Aldrich, Steinheim, Germany) in acetone was prepared. The cells were then stained by giving 20 μl of the mixture onto the samples. They were then analyzed by fluorescence microscopy (AXIO Imager M2m, Zeiss, Wetzlar, Germany) to visualize the number of living as well as dead cells and their orientation.

3 Results

Characteristic temperatures of the two bioactive glasses were obtained from thermal analysis. These are the glass transition temperature, \( T_g \) (as established from the point of inflection of the DTA curves), the bulk crystallization maximum temperatures \( T_x \) during up-scan, and the bulk melting temperature, \( T_m \). The range displaying endothermal melting effects after crystallization comprises three and two consecutive individual peaks for the bioactive glasses 45S5 and 13–93 respectively as indicated in Figure 1. Individual peak minima were averaged to a single value \( T_m \), see Table 1. Note that \( T_m \) must be carefully distinguished from the liquidus temperature, \( T_{liq} \). Calculated values for \( T_{liq} \) using thermochemical modelling are also listed in Table 1.

The casting temperature was set to a level \( T_{cast} \) corresponding to \( \log \eta = 1.3 \). This viscosity level was found
Figure 1: DTA up-scan analysis of bioactive glasses 45S5, sample mass 39.109 mg (left) and 13–93, sample mass 32.490 mg (right), performed with Setsys TG-DTA 16 apparatus, heating rate 10 K/min. $T_g$ = glass transition temperature from point of inflection, $T_x$ = maximum crystallization temperature, $T_m$ = average melting temperature resulting from individual peak minima.

Figure 2: 2D-linescans of glass surfaces from samples cast from varying viscosity levels. 1420°C: $\log(\eta) = 1.29$; 1400°C: $\log(\eta) = 1.35$; 1380°C: $\log(\eta) = 1.42$; $\eta$ in dPas.

Figure 3: Viscosity-temperature relation from VFT interpolation for bioactive glasses 45S5 and 13–93; data taken from Vedel et al. [30]; temperatures $T_{strain}$, $T_{stick}$, and $T_{cast}$ correspond to viscosity levels of $\log \eta = 14.5$, 9.8, 1.3, respectively; $\eta$ in dPas.

... was avoided by taking into account the results by Rieser et al. [32]. They identified so-called contact sticking conditions, suggesting that mold sticking during demolding is safely avoided at temperatures below a viscosity level $T_{stick}$ corresponding to $\log \eta = 9.8$ ($\eta$ in dPas). The temperature level $T_{stick}$ addresses the contact temperature $T_{contact}$ at the interface between glass melt and mold material. $T_{contact}$ was calculated according to Rieser [33]. Thus, sticking was indeed safely avoided by setting the mold temperature well below $T_{stick}$ at 500°C for both glasses. The significant effect of a variation in the mold temperature from 500°C to 580°C can be seen in Figure 3. The higher mold temperature leads to sticking of the glass samples and complica-
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Figure 4: Surfaces of two cast 13–93 glass samples with varying mold temperature. $T_{\text{mold}} = 580^\circ$C (left) led to sticking whereas $T_{\text{mold}} = 500^\circ$C (right) ensured proper demolding and therefore a high quality glass surface.

Figure 5: Surfaces of glass 13–93 after remelting 125–180 µm particle size precursor material (left) yielding defects from remaining bubbles and 355–1000 µm particle size precursor material (right) achieving a high quality glass surface.

Further process parameters, such as $T_{\text{cast}}$, $T_{\text{stick}}$ and $T_{\text{strain}}$ related to viscosity levels are marked in Figure 4 and a complete set of relevant temperatures yielding from thermal analysis and the viscosity temperature curve is compiled in Table 1.

Further attention was given to the effect of the quality of the glass to be remelted from powders with constant particle size fractions. In accordance with the intended rheological parameter and melting time, glass granules from 355–1000 µm were found to yield a bubble free melt. This is essential for the quality of the surface obtained. Figure 5 compares the quality of the glass surface when using 125–180 µm and 355–1000 µm granules of the 13–93 glass composition as precursor material. Holes on the surface resulting from bubbles in the glass melt appeared for the smaller particle size fraction.

2D laser scanning microscopy linescans of glass surfaces and mold and 3D images confirm the accuracy of the developed casting process, see Figure 6. The acute peaks in the 2D linescans, especially at the edges of the grooves, are no real surface features but artifacts of the laser technique itself. The periodical and defect-free microtopography was also confirmed by cross sectional SEM analysis (Figure 7).

The comparison of the laser scanning results from a series of cast glass samples verifies the stability of the process. Figure 8 shows how the microstructure could be reproduced on the surface of ten different 13–93 glass samples manufactured under identical conditions.

Live/dead stainings (Figure 9) show predominately green fluorescent cells, indicating a high cell viability on the structured as well as on the polished bioactive glasses (here: 45S5). In addition, when compared to cells on polished bioactive glass samples, the cells spread and formed long directional extensions. Notably, the cellular morphology appeared polarized and oriented in the direction of the grooves, revealing that microstructured bioactive glass surfaces influence cellular guidance. The cell culture experiments using 13–93 showed similar results.

4 Discussion

A successful casting process depends on the choice of crucial temperature levels in a most sensitive way. As typical in glass technology, these temperature levels are communicated in terms of temperatures at which specific viscosity values are reached, making these settings useful for glasses of different chemical compositions and viscosity temperature curves. A casting temperature $T_{\text{cast}}$ corresponding to a viscosity level of $\log \eta = 1.3$ ($\eta$ in dPas) was found to be sufficiently high to ensure mold filling. The mold sticking level of $\log \eta = 9.8$, originally obtained for smooth mold surfaces [31], was also applicable to mold structures at the 10 µm scale. The remaining temperatures related to viscosity, i.e., $T_g$ and $T_{\text{strain}}$ are commonplace levels of glass technology and processing in general [34, 35]. $T_{\text{mold}}$ at 500°C was a compromise found for both glass compositions regarding chill ripples, sticking and cracking due to thermal stress.

Taking into consideration that the platinum gold alloy is known to be only poorly wetted by glass melts and the temperature dependence of the surface tension of the melt is not significant, we conclude that viscosity is the decisive parameter for form filling on the low micrometer scale. Figure 2 confirms the high sensitivity of the process of form filling on viscosity.

It comes to notice, that the temperature corresponding to the casting viscosity $T_{\text{cast}}$ for bioactive glass 45S5 is very close to the liquidus temperature of the crystallized material and below the experimentally derived parameter $T_m$. Comparable rheological conditions for both glasses were
Figure 6: 2D laser scanning profile of the surface microstructure on bioactive glass 45S5 (top) and 13–93 (bottom) superimposed with 2D scan of the mold surface; as well as 3D laser scanning profile of the glass surfaces (right).

Figure 7: SEM micrograph of the cross section of cast bioactive glass 45S5 showing the surface microstructure; BSE mode, 15 kV accelerating voltage.

Figure 8: Ten superimposed 2D-linescans of different 13–93 glass samples manufactured with identical conditions, showing the high reproducibility.

requested, however the corresponding viscosity levels of glass 13–93 are at significantly higher temperatures than those of glass 45S5. Though setting the temperature at $T_{\text{cast}}$ for glass 45S5 and preventing crystallization was possible by first heating above the bulk melting temperature $T_m$ and cooling down to $T_{\text{cast}}$ after that.

The results show that with sufficient considerations regarding the material preparation, as well as time temperature conduction and mold melt interaction direct casting can be used to produce bioactive glass samples with an oriented microtexture on the surface. Results from thermal analysis and the viscosity temperature curve are very convenient tools to set process parameters. Continuance in the provided glass material for remelting is also decisive to prevent remaining bubbles. A particle size fraction of 355–1000 µm was found to be suitable.

Our cell culture results on 45S5, which is a well-known cytocompatible material and the most widely used bioactive glass composition [36], are in accordance with earlier observations in literature. All cells appeared green, indicating they were all alive, showing an excellent cy-
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5 Conclusion

We elaborated process parameters allowing to generate periodically and defect-free structured bioactive glass surfaces at the 10 µm scale by direct casting. Casting temperature and mold temperature were defined under consideration of viscosity levels and crystallization temperatures. As a result, the negative effects of chill ripple formation, crystallization, mold sticking, residual bubbles, and destruction of the sample by thermal stresses could be controlled and mostly eliminated. The initial response of the structured bioactive glass surfaces on osteoblast-like cells was also estimated. The cellular morphology appeared oriented in the direction of the grooves known as contact guidance. Though our investigations are still fundamental and restricted to planar samples, the derived results indicate that the microstructuring of bioactive glass surfaces might offer a promising method to enhance bone healing processes.

Acknowledgement: Thanks to Univ.-Prof. Dr. med. dent. Hendrik Meyer-Lückel and PD Dr. Marcella Esteves-Oliveira, Department of Operative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen University Hospital for their help with the experiments on the laser scanning microscope. We also thank Andreas Dohrn, Fraunhofer ILT Aachen for his help concerning structuring of the molds with laser.

We are grateful to the Deutsche Forschungsgemeinschaft (DFG) for financial support (grant # FI 975/21-1; CO 249/14-1, BU 1072/36-1).

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