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Technical note

A method to develop mock arteries suitable for cell seeding and in-vitro cell culture experiments

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ABSTRACT

Sylgard® is a biocompatible elastomer which has been widely used in biomedical applications including in simulations of the mechanical response of soft tissues and mechanotransduction investigations. In this study the effect of fabrication parameters including base to curing agent ratio and curing time on the mechanical response of Sylgard® was investigated and a novel fabrication technique for the production of mock arteries with highly uniform thickness, which is essential for mechanotransduction studies, is described. Finally a method for the surface treatment of Sylgard® using sulphuric acid and fibronectin to enhance smooth muscle cell (SMC) adhesion is proposed and examined in vitro. Sylgard® mock coronary arteries fabricated using the proposed technique exhibited a mechanical response close to arterial tissue with cell adhesion enhanced using the surface treatment techniques described.

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1. Introduction

Mock coronary arteries are widely used in many biomedical sectors. The main applications are in validation of numerical simulations of the vascular system, and interaction of medical devices such as stents with the arterial wall (Ramesh et al., 2008).

The increased number of mechanotransduction studies has required the use of mock coronary arteries as a substrate for mechanical stimulation of cells in a 3D environment. A real artery is a complex, multilayered anisotropic soft tissue with chemical, biological and biomechanical factors involved in its development and maintenance. For the majority of mechanotransduction applications, however, the strain cells undergo locally is the most important biomechanical

parameter under consideration. A mock coronary artery may therefore have a simplified structure on condition that it can maintain the cyclic strain that cells undergo in vivo.

Sylgard® Elastomer 184 (poly-dimethyl siloxane (PDMS) — (C₂H₆OSi)_n, Dow Corning, Midland, MI, United States) is a widely used silicon elastomer in many biomedical applications (Brown et al., 2005; Hecker et al., 2005; Moore et al., 1994; Oláh and Vancso, 2005; Rajesh et al., 2007). It is often employed for cell and tissue culture applications due to its biocompatibility and easy-to-mould characteristics (Brown et al., 2005; Hecker et al., 2005; Oláh and Vancso, 2005) and it has been extensively used in experimental flow studies due to its compliance and transparency (Moore et al., 1994; Liepsch et al., 1992). Sylgard® is supplied as a two part kit consisting of a base and a curing agent which are mixed together

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to obtain the required elastic material. Depending on the ratio between the base and curing agent, the level of elasticity can be altered to represent the mechanical characteristics of a real vessel. This makes it a suitable choice to simulate the behaviour of human tissues such as coronary arteries in mechanotransduction applications (Brown et al., 2005; Oláh and Vancso, 2005). Sylgard® cylinders have previously been generated into mock-vessels with varying elasticity and dimensions (Hecker et al., 2005; Rajesh et al., 2007).

Due to interest from other researchers and the absence of such a protocol in the literature, especially for mechanobiology applications, this paper describes a simple and reproducible method of creating Sylgard® sheets and mock arteries. Vessels with an internal diameter of 3 mm, a constant wall thickness of 0.4 mm and a length of 4 cm have been developed as a simplified representation of human coronary arteries, suitable for controlled 3D mechanobiology cell culture studies. The method presented can, however, be applied to represent arterial segments or complete arteries of various diameters, thickness and stiffness and the mechanical properties of various Sylgard® ratios are presented.

2. Methods

2.1. Sylgard® preparation

The base and curing agent were weighed on a laboratory scale and ratios of 10:1, 11:1, 14:1 and 16:1 were prepared. The base and curing agent were mixed for 30 s in a small container. During this process air bubbles formed inside the mixture. To remove all air bubbles from the mixture, the container was placed in a Nalgene vacuum desiccator attached to a vacuum pump. Following removal of the air bubbles the Sylgard® material was moulded into flat sheets and cured in an oven at 120 °C for 1 h. To study the effect of curing time, Sylgard® with a base to curing ratio of 16:1 was retained in the oven for two different time periods, 1 h and 16 h. In addition Sylgard®, with base to curing ratios of 10:1 and 16:1, was moulded into a custom built rig for mock coronary artery preparation and cured for 1 h and 16 h at 120 °C.

2.2. Mock coronary artery rig design and functionality

A simple rig was designed in Pro/ENGINEER (Parametric Technology Corporation, Needham, MA, USA) to create mock arteries with an inner diameter of 3 mm and outer diameter of 3.8 mm. The rig was designed such that idealised coronary artery geometries could be manufactured consistently and with no artefacts or damage to the Sylgard® surface. The construction rig consisted of two stainless steel end spacers within which a polished stainless steel inner mandrel was placed. The stainless steel mandrel measured 3 mm in diameter. Both end spacers were tapered and slots were cut on the tapered ends to allow for transfer of fluid through these predefined channels at either end of the mandrel. Plastic outer moulds were manufactured from PTFE shrink tubing such that they had an inner diameter of 3.8 mm. To achieve this, 6 mm shrink tubing (Tyco Electronics, Kessel, Belgium) was heated onto a 3.8 mm diameter polished

stainless steel pin using a heat gun at 150 °C. The pin was shaped like a hook at one end to enable the release of the mould. This 3.8 mm PTFE cylindrical tubing was then used to form the outer shell of the Sylgard® mould and was placed over the end spacers as shown in Fig. 1. A non-stick coating was also sprayed onto the stainless steel pin prior to heat shrinking the PTFE tubing. This made it easier to release the heat shrink tubing once it had moulded to the diameter of the pin.

The mock artery mould was constructed by inserting the 3 mm stainless steel mandrel inside the PTFE shell and fixing both ends with stainless steel end spacers such that both cylinders were concentric (Fig. 1). The end spacers were specially designed to fix the mandrel in the centre such that the resulting mock artery had a uniform thickness along its length. The plastic mould was cut such that it was longer than the mandrel and covered the four holes cut into the end spacers. This ensured that the elastomer was able to pass through these holes to fill out the space between the inner mandrel and the outer PTFE tubing. PTFE isolating tape was used to avoid air infiltration between the plastic mould and the holders (Fig. 1).

2.3. Creation of the mock artery mould

The prepared elastomer was drawn into the rig by suction of a vacuum pump connected to one end of the rig with a pneumatic connector. Upon suction, the Sylgard® was able to pass from the container into the mould cavity through four small holes created on the stainless steel end spacers until the cavity was filled. The rig was then placed inside an oven at 120 °C for 16 h to allow the Sylgard® mould to cure. When the entire mould was filled up with Sylgard® an aluminium cover was fastened on top of the base plate to ensure that the concentricity of the inner mandrel and the outer PTFE tubing was maintained (Fig. 1). It was important at this stage not to close off the holders at either end as this prevents the elastomer from expanding longitudinally during curing and could result in non-uniform thickness along the mandrel.

The Sylgard® Product Datasheet (Dow Corning, Midland MI, USA) suggests curing the polymer for one hour at 100 °C; however this temperature was found to be inadequate in this case due to the presence of a plastic tube around the polymer which prevents homogenous heat diffusion. In fact, the level of curing was lower in the centre of the mock artery if the Sylgard® manual instructions were followed. Heat diffusion was found to be higher at the open inlet and outlet in comparison to the surrounding mould.

2.4. Removal of the mock coronary artery from the rig

Once the polymer was cured the rig was taken out of the oven and left to cool down for 30 min at room temperature. To remove the Sylgard® compliant mock artery from the mould, the end spacers were first removed and the PTFE outer shell was gently peeled off. The external PTFE tube was removed by gently unwinding it with a spiral movement taking care not to damage the Sylgard® artery.

The mock coronary artery was subsequently removed from the inner mandrel by injecting acetone between the mock

Fig. 1 – (a) Pro/ENGINEER assembly of the rig designed for the fabrication of mock coronary arteries, (b) Detailed Pro/ENGINEER image of the rig showing the holes through which the uncured Sylgard® is drawn into the gap between the mandrel and the plastic tube. The polymer reaches the mandrel area through four channels grooved into the end spacer. (c) The mandrel linked to the holders and surrounded by the coaxial tube. (d) Assembled rig, ready for the elastomer injection.

artery and the mandrel with a needle. At this stage care was taken not to pierce the elastomer with the syringe needle when trying to inject acetone. The mock artery was then gently slipped off the mandrel to distribute acetone all along the length of the mandrel. The mock artery was then washed and placed in distilled water until use.

2.5. Cleaning of the rig

Once the mock coronary artery was removed it was possible to reuse the rig. It was however necessary to remove the residual cured Sylgard® from the end spacers. This was carried out using alcohol and a needle to clean the nozzles. It is important at this stage not to scratch the outlet channels on the holders as these micro-abrasions could alter the fluid dynamics of the system and lead to the formation of bubbles during the flow of Sylgard®.

2.6. Mechanical tests

Following removal from the oven all of the Sylgard® samples were left at room temperature to rest for three days. Subsequently, uniaxial tensile tests were carried out on dogbone-shaped samples of the Sylgard® with gauge dimensions of 4×20 mm. The samples were obtained from the Sylgard® sheets (0.5 mm thickness) and the mock coronary arteries (0.4 mm thickness) and tested using a displacement controlled tensile testing machine (Zwick Material Testing, Germany) and utilising a 20 N load cell. This enabled the mechanical properties of Sylgard® sheets and tubes to be compared to the properties of porcine coronary arteries. In this way the influence of fabrication parameters, such as base to curing agent ratio and curing time, could be investigated to

generate Sylgard® which best matched coronary artery properties. The samples were marked and a precise video extensometer (MESSPHYSIK Materials Testing, Austria) was utilised to measure the strain. A preload of 0.001 N was chosen as the criterion for the testing machine to initiate recording the strain. Samples were preconditioned by five cycles of extension to 10% strain and then loaded to failure. Strain rates of 0.5, 1 and 1.5 mm/s were used for each batch in order to analyse the viscoelastic properties of the material. The thickness of the mock coronary arteries was measured before every test ($n = 3$) under an optical microscope to confirm its uniformity.

2.7. Inflation and compliance tests

Mock coronary arteries of 10:1 and 16:1 base to curing agent ratio were mounted onto the fittings of a custom built inflation rig where the arteries were tethered longitudinally without any axial pre-stretch applied. The mock arteries were then inflated using a hydrostatic luminal pressure ramping up from 0 to 200 mmHg incrementally and the external diameter of the mock arteries was measured at each pressure level using a videoextensometer. Using the same rig and a pump system, the artery was inflated using a mean luminal pressure between 50 and 80 mmHg having a heartbeat pattern. The cyclic strain was recorded with a videoextensometer.

2.8. Treatment of the Sylgard mock arteries for cell attachment

Sylgard® is a naturally hydrophobic material, not suited for medium wetting and cell attachment. Cell adherence was tested by seeding the same number of cells on Sylgard® surfaces with different surface treatments and measuring the

number of adherent cells after 48 h in culture inside an incubator.

The silicon elastomer was dipped into a 70% sulphuric acid bath inside a fume hood for 5 min similar to that previously described by Moore et al. (1994). Care was taken to completely wet the artery surface without any bubble formation. The artery was then washed in distilled water. The ends of the mock arteries were attached to Polyvinylidene Fluoride (PVDF) plastic connectors (Value Plastics, Fort Collins, CO, USA) and the system was sterilised in an autoclave.

The mock coronary artery was dipped into PBS (phosphate buffer solution) containing 8 µg/ml of bovine plasma fibronectin (BioSciences, Ireland) for 30 min to improve cell adherence. The mock coronary artery was then inserted into a 15 ml tube and was rotated overnight in a spinning device (STR4/4, Stuart) to obtain a uniform distribution of fibronectin. Thereafter, PBS was removed and bovine aortic smooth muscle cells (SMC) were injected into the 15 ml tube for cell seeding. The tube was returned to the spinning device for SMC seeding for additional 8 h.

2.9. Comparison of Sylgard® mock arteries with different surface treatments

Bovine aortic smooth muscle cell adherence on the Sylgard® was tested for different surface treatments. Treatment of a polymer surface with sulphuric acid increases the number of charged surface groups favouring cell attachment and proliferation by hydrophilising the Sylgard® (Moore et al., 1994). BASMC were cultured to 80/90% confluence in a T-75 flask and seeded on autoclaved flat Sylgard® layers treated with/without sulphuric acid and with/without fibronectin. After 24 h of culture the cells were fixed in 3% formaldehyde and their membranes made permeable with 0.2% Triton X-100 to enable the nuclei to be stained with DAPI (4',6-diamidino-2-phenylindole). Pictures were taken under a fluorescence microscope (Olympus DP-50) and the images were analysed. The number of cells in constant regions of 300 × 300 pixels was analysed. Cells were blind counted in three random areas of the Sylgard® surface ($n = 3$) for the different surface treatments and results were compared.

In a separate test, the resistance of cell attachment to 10 dyne/cm² shear stress was also measured to establish the suitability of the mock coronary artery for cell seeding under culture medium or blood flow conditions. BASMC were cultured on Sylgard® sheets which were previously treated with sulphuric acid and fibronectin. Some sheets were left in static conditions while other sheets were placed on a rotator at 10 dyne/cm².

Images were taken using a fluorescence microscope after DAPI staining of SMCs and cells were counted ($n = 3$) in a region of 300 × 300 pixels with magnification 40×.

3. Results

3.1. Mechanical tests

The mechanical tests revealed that both the base to curing agent ratio and the curing time have a significant influence

Fig. 2 – Stress–strain response of dogbone-shaped samples of Sylgard® sheets cured at different base to curing agent ratios.

Fig. 3 – Stress–strain response of dogbone-shaped samples of Sylgard® sheets at different strain rates cured for 16 h and fabricated with different base to curing agent ratios of 16:1 and 10:1.

on the mechanical properties of the Sylgard®. While a higher base to curing agent ratio softens the material (Figs. 2 and 3), longer curing duration considerably stiffens the Sylgard® for both the 10:1 and 16:1 ratios, see Figs. 4 and 5 respectively. The variation in the mechanical properties of Sylgard® due to curing time is more significant for a base to curing agent ratio of 16:1 than for 10:1, see Figs. 4 and 5. In addition, the role of strain rate within a physiologically relevant range was investigated and proved insignificant, see Figs. 4 and 5.

Given that the curing level depends on the heat transfer from the preparation rig throughout the Sylgard®, the rig design was also found to have an important effect on the final mechanical properties. In Fig. 6, the flat specimens show considerably higher stiffness compared to the tubular mock coronary arteries fabricated with the same ratio and curing time for both ratios of 10:1 and 16:1. The use of the 16:1 ratio and a curing time of 16 h at 120 °C produced more compliant mock coronary arteries. The mechanical properties of these tubes is in the range of the circumferential stress–strain response of porcine coronary arteries, most notably in the low physiological stress range (10–13 kPa or 80–120 mmHg), see

Fig. 4 – Stress–strain response of dogbone-shaped samples of Sylgard® sheets at different strain rates cured for different durations and fabricated with a base to curing agent ratio of 10:1.

Fig. 5 – Stress–strain response of dogbone-shaped samples of Sylgard® sheets at different strain rates cured for different durations and fabricated with a base to curing agent ratio of 16:1.

Fig. 7. It is clear, however, that specimens can be fabricated to represent a range of arterial properties.

The thickness of all mock coronary arteries was measured using an optical microscope and found to be 0.4 ± 0.04 mm.

3.2. Inflation and compliance tests

Further investigation of the mechanical properties of the mock coronary arteries using inflation tests showed results consistent with the tensile test results. Mock coronary arteries prepared with a base to curing agent ratio of 16:1 were found to be more compliant than arteries prepared with a base to curing agent ratio of 10:1, see Fig. 8.

In addition, a physiologically realistic strain pattern, with 5% cyclic strain amplitude, was recorded using a videoextensometer in the 16:1 mock coronary artery in response to a heartbeat pressure waveform, see Fig. 9.

Fig. 6 – Stress–strain response of dogbone-shaped samples of Sylgard® sheets compared to dogbone-shaped samples of Sylgard® tubes, both prepared with a base to curing agent ratio of 16:1 and 10:1 for 16 h curing duration, $n = 3$.

3.3. Treatment of the Sylgard arteries for cell attachment

In order to optimise cell attachment to the mock coronary artery, the 16:1 Sylgard® surface was treated with 70% sulphuric acid solution and/or fibronectin. BASMC were seeded on the Sylgard® surfaces and after 12 h they were fixed in formaldehyde and stained with DAPI. Cells were counted in a standard region of 300×300 pixels under a fluorescent microscope with magnification $40\times$. Where the Sylgard® surface was untreated with sulphuric acid solution, 18.66 ± 0.57 cells were counted. When Sylgard® was treated with the sulphuric acid solution, 21.33 ± 1.52 cells were found in the selected region. The use of fibronectin increased the number of BASMC to 27.66 ± 1.15 whilst treatment with sulphuric acid followed by fibronectin resulted in maximum cell adhesion whereby 36 ± 2 BASMC attached to the Sylgard® surface. Cell counts were statistically significant (ANOVA, $p < 0.01$) showing that treatment of mock coronary arteries with sulphuric acid improved cell adherence and was further enhanced when bovine plasma fibronectin was used as a binding protein (Fig. 10).

The influence of shear stress on cell adhesion to the Sylgard® surface was assessed by exposing the cells to a physiologically relevant shear stress of 10 dyne/cm^2 . Cell numbers showed no statistically significant difference in samples exposed to shear stress (92.66 ± 2.51 cells per 300×300 pixels) and samples which were not exposed to shear (90.66 ± 2.08 cells in 300×300 pixels), see Fig. 11.

4. Discussion

The increasing number of mechanotransduction studies has led to a need for 3D cell straining platforms. Sylgard® mock coronary arteries are a suitable option where a cell monolayer is cyclically strained. In spite of the fact that Sylgard® mock arteries are uniform and homogeneous, they can mimic the cyclic strain levels that cells experience *in vivo* (Wedding et al., 2002) during the physiological intraluminal pressure range, see Fig. 9.

Fig. 7 – Strain/stress response of dogbone-shaped samples of Sylgard® sheets at base to curing agent ratios of 10:1, 11:1, 14:1 and 16:1, and cured for 16 h at 120 °C in comparison to porcine coronary arterial tissue. The strain/stress response of a dogbone-shaped sample obtained from a 16:1 ratio mock coronary artery (MCA) is also shown.

Fig. 8 – Pressure–strain response of Sylgard® mock coronary arteries cured for 16 h and fabricated with different base to curing agent ratios of 16:1 and 10:1. (Error bars represent standard deviation among 3 tested tubes).

Fig. 9 – Strain pattern of a Sylgard® mock coronary artery exposed to a heartbeat pressure waveform recorded with a videoextensometer.

This paper provides a detailed description of a simple and repeatable method to prepare mock coronary arteries using a common polymer, Sylgard® 184. To the authors' knowledge a simple way to construct such a mock coronary artery has never been thoroughly described. In fact, several requests have come to the authors for a full process description, the goal of this work. The in-house system described enables a specific base to curing agent ratio and curing time to be selected to represent the different mechanical properties that may be required. The process can be replicated and adopted for segments or complete arteries and veins of different sizes and with different mechanical properties.

Mock arteries are widely employed in research and in the biomedical industry, especially for the mechanical evaluation of endovascular stents (Hecker et al., 2005; Rajesh et al., 2007) or for the evaluation of imaging techniques such as ultrasound, CT, and angiography (Douville et al., 1983; McDicken, 1986). Most of these studies, however, do not give a clear explanation of the production process or focus more

Fig. 10 – Influence of surface treatment with sulphuric acid and fibronectin coating on SMC adhesion to Sylgard®. Every blue dot represents the nucleus of a cell stained with DAPI under the fluorescence microscope (magnification 40×).

Fig. 11 – Resistance to physiological shear stress of adherent SMCs on the Sylgard® surface treated with sulphuric acid and fibronectin (magnification 40×).

on the fluid dynamics inside the lumen than on the control of the cyclic strain of the arterial wall. The focus of this work was to obtain mock coronary arteries with cyclic strains in the circumferential direction close to the physiological values by tuning the mechanical properties of Sylgard®.

Sylgard® was found to have minimal strain rate dependence but its mechanical properties are strongly dependent on the curing time. As the Sylgard® curing level depends on the temperature obtained, the preparation rig can also play an important role in the final mechanical properties. Given that in the mock coronary artery preparation rig Sylgard® is encapsulated within PTFE tubing, the heat transfer to the Sylgard® is significantly lower than in the rig used for the

preparation of flat sheets of Sylgard® where the elastomer is moulded between two thin stainless steel plates. As a result, the mock coronary artery is much more compliant than the flat sheets of Sylgard® produced with the same base to curing agent ratio and curing time. This may be explained by looking at the influence of curing time on the properties of the elastomer. For the same set-up the longer the curing time the stiffer the elastomer obtained, indicating that higher temperatures leads to stiffening of the material. Using an insulating polymer as opposed to steel for the mould in the mock artery rig insulates the curing elastomer from the surrounding heat of the oven and therefore reduces the temperature reached by the Sylgard®. The different thermal diffusion rates however

do not affect the final composition and uniformity of the elastomer provided that the material is completely cured. This is as evidenced by the repeatability of the mechanical test data.

The structure of a real artery is very complex compared to a mock coronary artery. The inflation test results presented for the Sylgard® tubes are different from those given for porcine and human coronary arteries under various static pressures (Van Andel et al., 2003). These differences are due to the use of a simple homogeneous and isotropic elastomer to represent the anisotropic structure of coronary arteries. However, selecting the right parameters, such as the base to curing agent ratio and the curing temperature and time, it was possible to create mock coronary arteries with cyclic strains close to physiological values for specific pressure values. As shown in Fig. 9, when exposed to a physiological pressure waveform the mock coronary artery exhibited a periodical strain waveform close to that of a real coronary artery (Wedding et al., 2002).

The system described thus allows the creation of mock coronary arteries of constant wall thickness suitable for cell culture bioreactors where the artery deformation is caused by the pulsatile pressure of flowing culture media. The constant wall thickness is of extreme importance for mechanotransduction studies to obtain uniform cell elongation. Moore et al. (1994) produced mock coronary arteries by pouring Sylgard® on a 6 mm diameter rotating cylinder maintained at 100 °C in the oven. Although Moore et al. outlined that the application of liquid Sylgard® was carefully controlled to maintain the mock coronary artery wall thickness constant the system cannot always guarantee a uniform distribution and we encountered many difficulties in attempts to replicate this protocol. Furthermore, for small diameters, as in the case of coronary arteries, the Sylgard® distribution is even more critical. An attempt was made to use both a stainless steel internal mandrel and outer casing for the mould but this was unsuccessful because of the difficulty in removing the artery from the inner and outer steel casings. The protocol presented in this work overcomes all of these problems by precisely encasing the mock artery between an internal mandrel and an external plastic wall which can be easily removed.

Mock coronary arteries were treated with sulphuric acid and coated with fibronectin as suggested by Moore et al. (1994). Sulphuric acid alone, however, had only a marginal effect on SMC attachment but it increased cell adhesion when used in association with fibronectin. Cell number was significantly increased in all tests where the Sylgard® was coated with fibronectin. Tests have also demonstrated that cell adherence on Sylgard® using this protocol is resistant to physiological levels of shear stress (10 dyne/cm²).

In conclusion, the presented method enables fabrication of biocompatible and transparent mock coronary arteries with mechanical properties which can be easily tuned to mimic the mechanical response of any specific vessel for mechanotransduction studies. In addition, with some minor adaptation of the rig it would be possible to generate composite vessels with specific properties to represent the multilayered nature of arteries.

The Sylgard® can also be used for 2D or 3D cell studies to investigate the influence of strain on cell activity, using sheets and tubes respectively, but care must clearly be taken

to consider the influence of the mould material on the final stress-strain properties of the material as demonstrated in this study.

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