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Analysis of Different Natural and Synthetic Biomaterials to Support Cardiomyocyte Growth

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Abstract

The aim of this study was to scan through several biomaterials to find an optimal biomaterial to support the growth of cardiomyocytes. Neonatal rat cardiomyocytes were cultured on polylactide, chitosan, poly (1,8-octanediol-co-citric acid), copolymer of poly(ethylene oxide terephthalate) and poly (butylene terephthalate), PuraMatrix™ and collagen. The suitability of biomaterials for cardiomyocyte culture was evaluated based on several parameters. The cells were characterized with time-lapse imaging, immunocytochemistry and LIVE/DEAD® staining. Collagen gel was the best biomaterial. It supported well the growth, survival and functionality of the cardiomyocytes. Polylactide and chitosan membranes supported the cell growth and survival, but these biomaterials were too stiff for further cardiac applications. In conclusion, collagen gel is a good biomaterial to obtain a 3D structure to model heart tissue.

Keywords: Neonatal rat cardiomyocytes; Biomaterials; Collagen; Cell-IQ®

Abbreviations: NRCs: Neonatal Rat Cardiomyocytes; POC: Poly(1,8-Octanediol-co-Citric Acid); PEOT: Copolymer of Poly(Ethylene Oxide Terephthalate); PBT: Poly(Butylene Terephthalate); PEO: Poly(Ethylene Oxide); CMI: Culture Medium I; P/S: Penicillin/Streptomycin; CSFM: Complete Serum Free Medium; ITS: Insulin-Transferrin-Sodium Selenite Media Supplement; T_3: 3,3',5-Triiodo-L-Thyronine Sodium Salt; TUT: Tampere University of Technology; BME: Department of Biomedical Engineering at TUT; β-GP: Glycerol-Phosphate Disodium Salt Hydrate; CNT: Carbon Nanotube; NTU: Nanyang Technological University; $NaHCO_3$: Sodium Bicarbonate; $Na2HPO_4$: Disodium Hydrogen Phosphate; α-SMA: Anti-Actin, α-Smooth Muscle; VWf: Anti-Von Willebrand Factor; α-AS: Anti-α-Actinin(Sarcomeric); MAP-2: Anti-Microtubule-Associated Protein 2; PLA10GA90+PLLA: 75 wt% Poly(Glycolide-Co-Lactide) blending with 25 wt% Poly(L-Lactide); PLGA+PEG–PLA: 85 wt% Poly(Lactide-Co-Glycolide) blending with 15 wt% Poly(Ethylene Glycol)-B-Poly(D,L-Lactide)

Introduction

Myocardial damage due to infarction leads to the formation of a non-functional scar in the heart. The use of medication can to some extent improve the function of the heart, but currently no treatments exist to repair the damaged myocardial tissue. Tissue damage can lead to heart failure, and if such damage is severe, the only option is heart transplantation. With the development of stem cell technologies, researchers have suggested that cell transplantation could be a potential new therapy for repairing the damaged tissue and functional impairment [1].

Cell transplant studies have been performed in both animals and humans [2]. In these studies, however, the problem has been either that cells neither remain nor survive at the site of injury, which is a hostile environment for these cells [3]. Biomaterial could offer an advantage to cell transplantation by keeping the cells in the injecting locus, thereby providing structural support and improving their survival [1]. In addition to cell therapy, a 3D heart tissue model would be an optimal platform for studying the pathophysiology of different heart diseases as well as for drug discovery and toxicological testing. Such a heart tissue model would also require a scaffold for structural support.

There are many approaches to regenerating heart tissue, including patches [4], sheets [5], rings [6], sponges [7] and injectable gels [8], among others. The requirements for the biomaterial are high; it must be permeable to enable cell migration, vascularisation and diffusion of substances such as nutrients and oxygen. The mechanical properties of the biomaterial must be elastic, yet strong enough to bear the force of the contracting heart. For the *in vitro* phase, the transparency of the biomaterial would permit monitoring of the cells.

The aim of this study was to screen biomaterials to see whether they would support cardiomyocyte culture and function and prove to be suitable for tissue model purposes as well as for cell transplantation in the future. For these screening tests, neonatal rat cardiomyocytes (NRCs) were used since compared to other cardiomyocytes they can be obtained fairly easily in large numbers making the comparison studies with the biomaterials possible in larger scale [9]. They beat in culture for approximately a week enabling the evaluation of the functionality, but it is not possible to do long term follow-up. Previous studies with NRCs also show that creating a 3D heart tissue-like structure is possible [6].

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The biomaterials tested included biomaterials of natural origin (chitosan and collagen) and synthetic polymers, such as polylactide and poly(1,8-octanediol-co-citric acid) POC as well as commercially available polymers PuraMatrix™, and copolymer of poly(ethylene oxide terephthalate) (PEOT) and poly(butylene terephthalate) (PBT) (PEOT/PBT, Polyactive®). The current study examined the compatibility of a range of both natural and synthetic biomaterials to support cardiomyocyte culture in both 2D and 3D.

Materials and Methods

Cell sources

Neonatal rat cardiomyocytes were harvested from two to six-day-old Sprague Dawley rat hearts as described in Uusimaa et al. [10]. First, the hearts were removed and dissected with a collagenase solution and then isolated and plated onto different biomaterials in Culture Medium I [CMI, Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12, Sigma-Aldrich, USA), 10% Foetal bovine serum (FBS, Invitrogen, USA), 100 IU/ml Penicillin/0.1 mg/ml Streptomycin (P/S, Lonza, Belgium), 2.56 mM L-glutamine (Sigma-Aldrich)]. At day one and thereafter every day or every second day, Complete Serum Free Medium [CSFM, DMEM/F-12, 10% Bovine serum albumin (BSA, Sigma-Aldrich), 2.8 mM Sodium Pyruvate (Lonza), 2.56 mM L-glutamine, Insulin-transferrin-sodium selenite media supplement (ITS, Lonza; 1 µM insulin, 5.64 µg/ml transferring, 32 nM selenium), 100 IU/ml P/0.1 mg/ml S, 0.1 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3, Sigma-Aldrich)] was changed. Uncoated commercial well plate (Nunc, Thermo Fisher Scientific, USA), on which the cells grew well, served as a control in each experiment. NRCs usually beat for one week under standard culture conditions.

Biomaterials

Polylactide: The polylactide membranes were manufactured at the Tampere University of Technology, Department of Biomedical Engineering (TUT, BME, Finland), where medical-grade Polylactide P(L/DL)LA (70/30) (Resomer® LR 708, Boehringer Ingelheim, Ingelheim am Rhein, Germany) was extruded using a single-screw extruder with a slit die (50 mm x 1.0 mm). The band was cut to pieces and compression moulded to uniform membranes. The membranes were then cut into samples (ø = 15 mm), which were sterilized prior to use with 70% ethanol (EtOH) and then washed with Dulbecco's Phosphate Buffered Saline (DPBS, Lonza) and CSFM.

Chitosan: The chitosan membranes were done by dissolving chitosan powder (Protasan UP B 90/500, medical grade, deacetylation ratio: 91%, BioPolymer, Norway or Novamic™, deacetylation ratio: 73% Novasso Oy, Finland) into either lactic acid (Oriola, Finland) or acetic acid (J.T. Baker, The Netherlands), and casting the mixture in teflon moulds. After the membranes dried, they were detached and neutralized with 1 M sodiumhydroxide (NaOH, Sigma-Aldrich). Then the membranes were washed with deionised water and DPBS, and sterilized with 70% EtOH. Prior to cell culture, the membranes were cut into samples (ø = 15 mm) and washed with CSFM to remove EtOH residues.

The chitosan gel was done by first dissolving chitosan powder (Novamic™) into 0.1M hydrochloric acid (Sigma-Aldrich) and then neutralizing the mixture with 56 v-% glycerol-phosphate disodium salt hydrate (β-GP, Sigma-Aldrich) as described by Chenite et al. [11]. The gelling occurred due to a change in pH and temperature.

Poly(1,8-octanediol-co-citric acid): Poly(1,8-octanediol-co-citric acid) [3] was used in these experiments as such and as a coating on carbon nanotubes (CNT). Also CNT coated on POC was used. All the biomaterials were manufactured at Nanyang Technological University (NTU, Singapore). POC was synthesized by condensing citric acid and octanediol as described previously in a study by Lee et al., with minor modifications [12]. Briefly, first, the molar ratio (1:1) of citric acid to diol was mixed and purged with high-purity nitrogen gas and then heated to 140°C. Once the chemicals were fully dissolved and mixed, the temperature was decreased to 120°C until the solution became viscous, and then it was post-cured at 120°C. Then the cured sample was rinsed with distilled water for a few days to remove any unreacted monomers. All the chemicals were purchased from Sigma-Aldrich.

The CNT was prepared and a cover slip (ø = 13 mm) was coated as previously described [13]. For the POC-coated CNT, the POC solution was spun onto the CNT-coated cover slip and left it to dry. The thickness of the POC coating was about 300-400 nm. For the CNT-coated POC, a similar method was adapted of coating the cover slip whereby, instead of using the cover slip as the substrate for the deposition, a film of POC was used.

Copolymer of poly(ethylene oxide terephthalate) and poly(butylene terephthalate): In this study, six different segmented block copolymers of poly(ethylene oxide terephthalate) and poly(butylene terephthalate) were used. The initial lengths of the poly(ethylene oxide) (PEO) segments were either 300 or 1000 g/mol, and the molar ratios of the copolymers were either 60/40, 70/30 or 80/20.

The copolymer membranes were compression moulded from copolymer granulates at 110-170°C and 10 MPa, depending on the copolymer, according to the procedure described by Kellomäki et al. [14]. Compression moulding was carried out with a small laboratory-scale hydraulic press. After compression moulding, the membranes were rapidly cooled and cut into samples with a diameter of 20 mm and sterilized using Gamma irradiation (29.6 kGy). The final samples were 90-130 µm thick.

PuraMatrix™: BD™ PuraMatrix™ Peptide Hydrogel (BD Biosciences) is a synthetic matrix containing amino acids and water. Gelation was achieved by increasing the pH with additions of CMI to the PuraMatrix™ and raising the temperature to 37°C according to the manufacturer's instructions. Briefly, the PuraMatrix™ stock solution was first vortexed and then diluted with CMI at room temperature. Gelation occurred with CMI at 37°C.

Collagen gel: In addition to self-isolated collagen, commercial rat tail collagen, type 1, (BD Biosciences) was used. The commercial collagen was gelled by increasing the pH with 1M NaOH and raising the temperature to 37°C according to the manufacturer's instructions.

The self-isolated collagen was isolated as described previously [15] with some modifications. Briefly, the tails were harvested from adult Sprague Dawley rats and stored at -20°C. First the tails were thawed and then cut into pieces. Then the skin was removed, and the collagen fibres pulled out and placed in 0.1% acetic acid. The fibres were dissolved in acetic acid, and the collagen was precipitated with the addition of 25 v-% sodium chloride. The solution was then centrifuged, and the collagen pellet dissolved again in 0.1% acetic acid. To balance the salinity, the solution was dialyzed at 4°C. Lastly, the concentration of collagen (1.043 mg/ml) was determined with a Pierce® BCA Protein Assay kit (Thermo Scientific) using the BSA as the standard.

Gelation of the self-isolated collagen was carried out as described previously [16] with minor modifications. First, the collagen solution

was placed on ice and two different neutralizing buffers [12 mg/ml sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich) in 0.1 N NaOH (Merck, Germany) and 1.3 M sodium chloride (NaCl, J. T. Baker) in 0.2 M disodium hydrogen phosphate ($Na2HPO_4$, J. T. Baker)] were added. The solution was then incubated at 37°C for 30 minutes to allow the gel to form.

Cardiomyocyte characterization and evaluation of the biomaterials

In each experiment, NRCs were always plated in equal amounts to biomaterials and uncoated control wells to enable the comparison of samples. Cells were microscoped daily and several qualitative parameters were evaluated and scored to assess the suitability of biomaterial for cardiomyocyte culture. Parameters were always evaluated compared to cardiomyocyte control wells, which also had to fulfil certain criteria for an experiment to be accepted and continued.

Evaluation criteria for the suitability included: 1. Cell attachment, 2. Cell spreading (after 24-48 hours), 3. Beating (beating rate and strength, synchronicity, regularity), 4. Growth/detachment (how the culture looks like from day 4 onwards, do the cells start to detach), 5. Final evaluation on day 7 compared to control. Additionally LIVE/DEAD assay for vitality was done when possible and staining for cardiomyocyte markers to help to asses the alignment and spreading of cardiomyocytes.

Time-lapse imaging

The Cell-IQ® (Chip-Man Technologies Ltd., Finland) is a cell-culturing system which includes phase-contrast microscopy, environmental control and machine vision [17]. Cell-IQ® was used to follow cell attachment, behaviour and viability on selected biomaterials, usually for one week. Also the proliferation and growth were observed.

Immunocytochemistry

The cells were first fixed with 4% paraformaldehyde (Fluka, Italy) and then stained with the following primary antibodies: anti-actin, α-smooth muscle (α-SMA, 1:1000) (Sigma-Aldrich), anti-vimentin (Vimentin, 1:1) (Abcam, UK), anti-von Willebrand factor (VWf1, 1:1000, 1:1500) (Abcam,), anti-von Willebrand factor (VWf2, 1:200) (Sigma-Aldrich), anti-α-actinin (Sarcomeric) (α-AS, 1:1500) (Sigma-Aldrich), anti-microtubule-associated protein 2 (MAP-2, 1:400) (Millipore, USA) and anti-Pax-6 (1:100, DSHB, USA). The secondary antibodies used included Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:800), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:400, 1:800), Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:400) (Invitrogen) and Rhodamine Red-conjugated donkey anti-mouse IgG (1:800) (Jackson Immuno Research Laboratories, Inc., USA). Lastly, the cells were mounted and the nuclei stained with Vectashield (DAPI, 4',6-diamidino-2-phenylindole) (Vector Laboratories, UK).

LIVE/DEAD® staining

A LIVE/DEAD® Viability/Cytotoxity kit for mammalian cells (Invitrogen) enables one to separate live and dead cells. The staining kit contains Calcein AM, which stains live cells green and is visible at a wavelength of 488 nm, and Ethidium homodimer-1, which stains dead cells red and is visible at a wavelength of 568 nm. Staining was carried out according to the instructions of Invitrogen with some modifications. Briefly, the stains were added to the culture medium and incubated for 30 min at room temperature, after which the staining medium was changed to DPBS.

Results

Table 1 summarises all the biomaterials, testing conditions, main conclusions and problems encountered in this study.

Polylactide and chitosan membranes

An equal number of NRCs was plated on each well (275 000-300 000 cells/cm^2). The behaviour of the cells was monitored with the Cell-IQ® for 2-12 days. NRCs attached and grew well on the chitosan (Figure 1 A-D) and polylactide (Figure 1 E-H) membranes. Occasionally the cells started to detach from the membranes at later stages of culture (Figure 1H) which was not detected on the uncoated control wells (Figure 1 I-J). Based on the Cell-IQ® videos some proliferation was also evident. Considering other criteria, synchronous beating of the cells on membranes was comparable to controls. Furthermore, whether the chitosan was made with acetic acid (n = 13, Figure 1 A-D) or with lactic acid (n = 13, data not shown) made no difference in cell behaviour. There were also no major differences between the polylactide (n=11,

material	the form of material	sample size	cell dencity (cells/cm^2)	n	days in culture	technical problems	results / conclusions	cell survival
polylactide	membrane	ø 15 mm	275000-300000	11	2-12	-	too stiff material	++
chitosan	membrane	ø 15 mm	275000-350000	13	2-12	immunocytochemistry: background	the cells survived but did not attach as well as on controls	++
chitosan	gel	nd	nd	nd	nd	gelation	the cells did not spread	-
POC	membrane	ø 13 mm	300000-400000	2 (POC, POC on CNT), 3 (CNT on POC)	6-7	LIVE/DEAD: background	the cells survived but there were not as much cells as on controls	-
PEOT/PBT	membrane	ø 20 mm, thickness 90-130 µm	335000-414000	3 (1000 g/mol), 4 (300 g/mol)	6	-	the cells survived but there were not as much cells as on controls	-
PuraMatrix™	gel	250 µl in 24 well plate	300000-400000	10 (*)	4-9	uneven gelling, the gels detached	the cells did not migrate inside the gel	+
collagen	gel from commercial	250-500 µl in 24 well plate	331500-350000	10 (**)	nd	the gels detached	the cells were not evenly spread	+
collagen	gel from self isolated	250 µl in 24 well plate	400000 (on) / 1400000 (in)	14 (***)	4-10	-	the cells were evenly spread and migrated inside the gel	+++

POC = Poly(1,8-octanediol-co-citric acid), CNT = carbon nano tube, PEOT = poly(ethylene oxide terephtalate), PBT = poly(butylene terephtalate)
(*) gel concentration = 0,15-0,4 %; (**) gel concentration = 0,784-1,960 mg/ml; (***) gel concentration = 0,8384 mg/ml

Table 1: The biomaterials used in the study. Summarizing table of detailed information about the experiments with different biomaterials.

Figure 1 E-H) and chitosan membranes (Figure 1 A-D).

Immunocytochemical stainings were challenging on the chitosan membrane due to the high background staining, even though the stainings were repeated five times. The cells on the polylactide membrane (Figure 2 A) stained against α-actinin similarly to those on the uncoated control wells (Figure 2 B). The NRCs on the chitosan and polylactide membranes were also characterized with LIVE/DEAD® staining, which stains live cells green and dead cells red, and shows the overall biocompatibility of the biomaterials. The number of live (ca. 80%) and dead (ca. 20%) cells was almost the same on both biomaterials as in the uncoated control well (data not shown).

Since chitosan seemed quite a promising biomaterial for the cardiomyocyte culture, the chitosan was gelatinized. This, however, proved very challenging. Several conditions were tested (different pH as well as chitosan and β-GP concentrations), but none seemed optimal, neither for the gel formation nor for our cells.

Poly(1,8-octanediol-co-citric acid)

Previous studies [12] show that L929 cells and human mesenchymal stem cells can grow on POC, so the suitability of the biomaterial for cardiomyocytes was examined. 300 000-400 000 NRCs/cm^2 were plated on POC biomaterials and on uncoated control wells and the cells were grown for one week. The cells attached to POC (n = 2, day four, Figure 2 G) and to CNT-coated POC (n = 3, day two, Figure 2 H), but not as evenly as on uncoated control wells (day two, Figure 2 I). In addition, the cells did not spread and grow during culture as they did on the controls, and to some extent the cells even detached from the biomaterials. The beating of the cardiomyocytes on POC was not as strong as on control wells, due to a smaller amount of cells attached to POC. The behaviour of the cells was similar whether CNT was coated on POC or whether POC was coated on CNT. LIVE/DEAD® staining proved impossible on POC because of the high background (data not shown). To conclude, POC did not support cardiomyocyte attachment and survival and thus it is not an optimal biomaterial for cardiomyocyte culture.

Copolymer of poly(ethylene oxide terephthalate) and poly(butylene terephthalate)

PEOT/PBT copolymer has served previously mainly as scaffolds for cartilage and bone applications [18, 19]. In aqueous conditions some of the compositions form hydrogel, so its suitability for cardiomyocyte culture was tested. 335 000-414 000 NRCs/cm^2 were plated on PEOT/PBT copolymer with different initial lengths of PEO segments and molar ratios. Same amount of cells were also plated on uncoated control wells. Initially, the cells attached to PEOT/PBT copolymer but did not survive well, especially on the copolymers with short PEO segments [segments of 300 g/mol (n = 4, data not shown)]. LIVE/DEAD® stainings (Figure 2 C) performed after six days of culture revealed even fewer cells attached to copolymers with PEO segments of 1000 g/mol biomaterials (n = 3) than to uncoated controls (Figure 2 D). The molar ratio did not affect cell adhesion (data not shown). In conclusion, these biomaterials were unsuitable for cardiomyocyte culture.

PuraMatrix™

PuraMatrix™ is a commercially available biomaterial that is used for cell cultures both as a coating and in gel form, so it seemed a promising biomaterial for cardiomyocyte culture. However, although the gelling was performed according to the manufacturer's instructions and tested four times with different concentrations (0.15-0.4%), no homogenous gels were obtained. The cells (600 000-800 000 NRCs/250µl) remained either on the surface of the gel or migrated through the gel to the bottom of the well plate. The gel also easily detached from the cell culture well during the four to nine days of culture. As the Cell-IQ® pictures show on day four (Figure 2 E), the gel was three dimensional, but the cells remained only on the surface and were less evenly spread than on the uncoated controls (Figure 2F). Thus, the homogenous cell culture could not be obtained on PuraMatrix™ with the gelling protocol. Cardiomyocytes either grew on top of the material or migrated through the material, but did not stay in the gel. However, the cells which remained on the surface of PuraMatrix™ grew nicely and continued to beat for the whole follow-up period.

Collagen gel

Collagen is a natural, gelable biomaterial widely used in many applications, including cardiac studies. Self-isolated collagen gelled well and nicely supported cardiomyocyte growth (Figure 3). If the cells were plated in the gel made of commercially available collagen (n = 10, data not shown), however, they were less evenly spread than in the self-isolated collagen gels. Thus, the experiments described below were carried out using our self-isolated collagen.

Initially, NRCs (800 000 NRCs/250µl) attached well on collagen and during culture, formed a uniform network of cells on the surface of the collagen gel (Figure 3A day six). Beating was comparable to controls. When the cells were plated on the surface of the gel, migration inside the gel was observed. When the cells were mixed with the gel (2 800 000 NRCs/250µl) even distribution of the beating cells was observed inside the gel (Figure 3 B) forming a uniform tissue-like structure. LIVE/DEAD® staining, performed at day seven, revealed that most of the cells were alive (Figure 3 C, D). In addition to the cellular population, also tube-like structures were observed in the collagen (Figure 3, arrows). After four to ten days of culture, the characteristics of the tubes were analysed with immunocytochemistry: Vimentin for

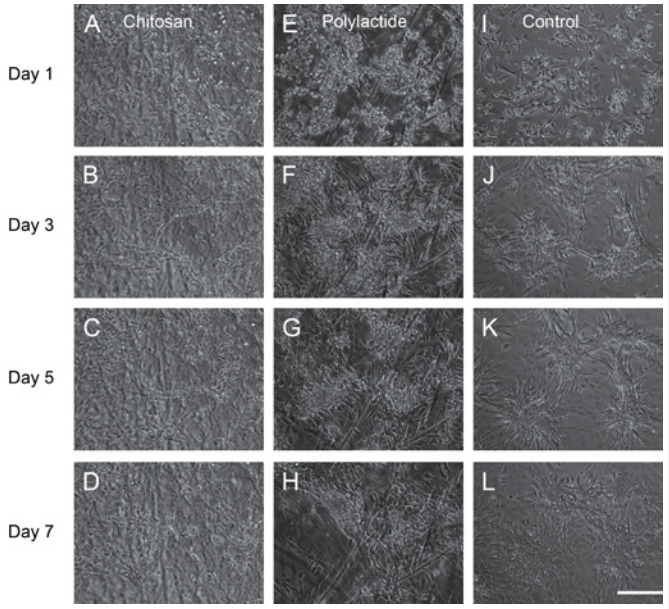

Figure 1: The growth and behaviour of NRCs were monitored with the Cell-IQ® platform. Growth was analyzed every other day (days 1, 3, 5 and 7). The cells were on (A-D) chitosan, (E-H) polylactide and (I-L) uncoated control wells. The scale bar is 200µm.

fibroblasts, endothelial and smooth muscle of mesenchymal origin, α-AS for myotubes in skeletal and cardiac muscles, α-SMA for smooth muscle, VWf1 for endothelial, VWf2 for endothelial, Pax-6 for neural and MAP-2 for neural, but they did not stain positively against any antibodies used (data not shown), thus suggesting that they are neither muscle-derived nor vascular nor neural origin. The nature of those tubes remains unknown. In conclusion, from the studied biomaterials, collagen was clearly the best in supporting cardiomyocyte culture within the biomaterial and also enabled forming a 3D tissue-like structure.

Discussion

A three-dimensional cardiac tissue model would be highly desirable for drug discovery, studying cardiac diseases and in the future for cell transplantation treatments. Finding a suitable supportive biomaterial, however, has been challenging. In this study, the survival and growth of neonatal rat cardiomyocytes on six different biomaterials were analysed. The biomaterials used included natural biomaterials (collagen and chitosan) and synthetic biomaterials [polylactide, poly(1,8-octanediol-co-citric acid), copolymer of poly(ethylene oxide terephthalate) and poly(butylene terephthalate), and PuraMatrix™]. The best biomaterial for cardiomyocyte culture was the collagen gel made of self-isolated rat tail collagen type 1. Polylactide and chitosan membranes also supported cardiomyocyte growth, but due to their stiffness would not be suitable for 3D cardiac applications.

The collagen gel was the best to support the growth and survival of cardiomyocytes. During culture cells were able to migrate and to form a uniform 3D network, which indicates that collagen could be utilised in making tissue-like cardiac structures. The gelling technique was easy to perform, and the cells also survived nicely inside the gel. Interestingly, tubes formed inside the collagen gel. Other studies have previously shown that collagen promotes angiogenesis [4]. In our study, however, tubes did not stain positively for endothelial markers, neither for mesenchymal, myotube, smooth muscle nor neural markers. What exactly these tubes are remains unclear.

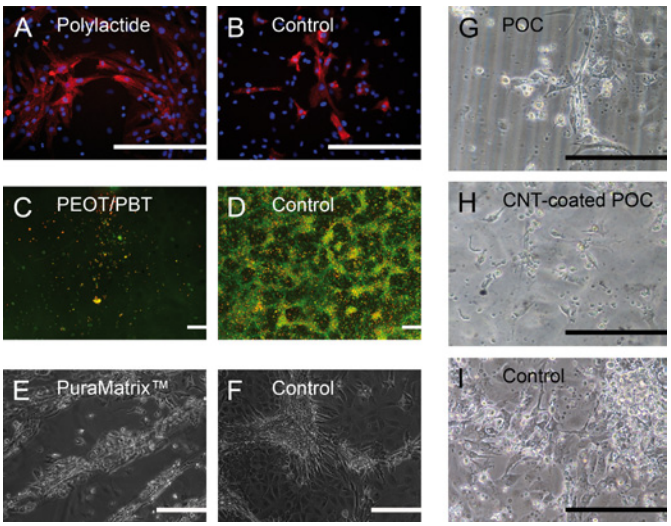

Figure 2: The growth of NRCs on biomaterials. (A) NRCs stained positively against α-actinin equally well, whether the cells were on polylactide or on (B) uncoated control wells. NRCs failed to survive on (C) PEOT/PBT as compared to (D) uncoated control wells according to LIVE/DEAD® staining. NRCs did not spread as evenly on (E) PuraMatrix™ as on (F) uncoated control wells. NRCs failed to attach and grow properly on (G) POC and failed to spread evenly on (H) CNT-coated POC compared to (I) uncoated control wells. The scale bars are 200 μm.

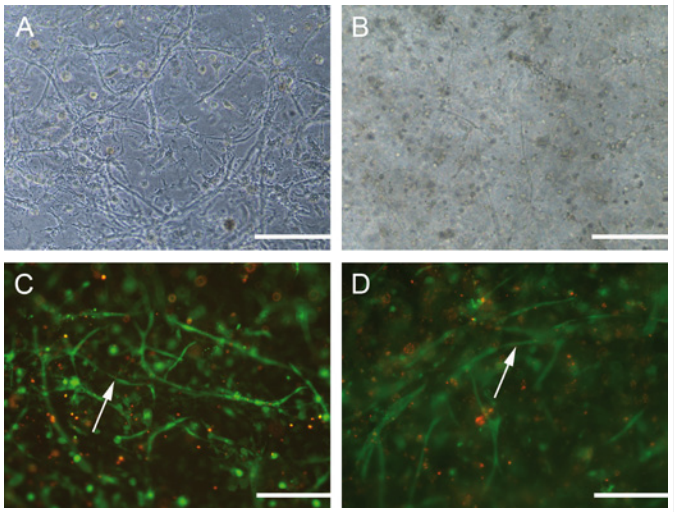

Figure 3: NRCs grown with collagen gel. (A, C) NRCs plated on the surface of collagen gel and (B,D) mixed with collagen gel. (C, D) The cells were stained with a LIVE/DEAD® kit. Tube-like structures (arrows) were observed on the surface and inside the collagen gel. The scale bars are 200μm.

Collagen is a natural, biodegradable biomaterial with low antigenicity and good cell-binding properties [20, 21] that promotes angiogenesis *in vivo* [4]. Given these properties, collagen is a potential biomaterial for developing a heart tissue model which was also supported by our findings. Also previous studies have shown that collagen gel supports cardiomyocyte growth in 3D models [15] and has served as a scaffold material in studies aiming to create cardiac muscle constructs [22]. However, collagen gel is typically combined with Matrigel™, a protein mixture secreted by mouse sarcoma cells, and not used alone, as in our study. Although collagen seems to be a promising biomaterial, it has limited mechanical properties, and being a natural polymer, batch variation can occur [23]. Largely due to these downsides, other biomaterials besides collagen were tested, but our results show that of these biomaterials, collagen works best with NRCs.

In our study, the cells grew quite well on the polylactide membranes. Also Zong et al. have shown that electrospun poly(L-lactide) (PLLA) membrane supports the growth of NRCs despite its hydrophobic nature [24]. The PLLA membrane was compared to 75 wt-% poly(glycolide-co-lactide) blending with 25 wt-% poly(L-lactide) (PLA10GA90+PLLA) and 85 wt-% poly(lactide-co-glycolide) blending with 15 wt-% poly(ethylene glycol)-b-poly(D,L-lactide) (PLGA+PEG–PLA); the PLLA proved to be the best of them [24]. However, these results are not fully comparable to our results due to the different physical appearance of the membranes and the different raw materials. In general, polylactides are well tolerated because their degradation product (l-lactide) is also involved in the metabolism of all animals [25]. Polylactides are widely used and studied biodegradable polymers that have already successfully served in medical applications and are considered safe, non-toxic and biocompatible materials [26]. In one recent study, a scaffold consisting of 50%PLLA and 50%PLGA combined with human cardiomyocytes, endothelial cells and fibroblasts, was transplanted into a rat heart. The scaffold was shown to vascularise and functionally integrate to form a stable cardiac graft, thus indicating that PLLA could be suitable for cardiac applications

[27]. Although in our study NRCs grew quite well on polylactide, polylactide as such was too stiff a material for cardiac applications. However, if lactide copolymers could be modified with monomers of another, softer material, polylactide could be an option for cardiac applications.

Chitosan is a natural, bioactive material, and its suitability for biomedical applications is well studied. Chitosan stimulates cell proliferation and is naturally involved in the regeneration of soft or hard tissue; it is also hemocompatible, and cells attach well to its surface [28]. In our studies, NRCs grew rather well on chitosan membranes, but like polylactide membranes, were too stiff. Chitosan was also gelled, and although LIVE/DEAD® staining in preliminary tests indicated the cells were alive, they remained round-shaped and did not spread evenly on the gel (data not shown). Our results are opposite to those of Karp et al., who demonstrated that NRCs grow well in photocrosslinkable chitosan gel [29]. One should note, however, that in their study, the gel formed with photocrosslinking, whereas in ours, it formed chemically; this difference could explain the difficulties observed. In the future, mixing chitosan with other materials, such as synthetic polymers, could improve its application. Studies have shown that with chitosan, it is possible to modify the cell affinity and biocompatibility of poly(ε-caprolactone) to improve the adhesion and proliferation of fibroblasts [30].

Studies have shown that L929 mouse fibroblasts [12] and HL-1 cardiomyocytes [31] grow rather well on POC, a soft, biodegradable and biocompatible material which could be used in soft tissue applications. One should note, however, that fibroblasts and cell lines are more robust and easier to culture than primary cardiomyocytes. Research has also shown that human mesenchymal stem cells grow well on CNT film [13]. In our study, however, when POC was combined with CNTs, NRC grew no better than on POC alone. The CNTs could prove useful as a material for cardiac applications because they are conductive, which could benefit heart applications. POC in general could have been a promising biomaterial because it is also suitable for mechanical stimulation (e.g. stretching), which some researchers consider useful for culturing and differentiating cardiomyocytes [31]. In our study, however, the cells seemed to grow poorly on POC materials, and CNT coating failed to improve the survival and growth of the cells.

Two commercially available biomaterials that were tested were PuraMatrix™ and Polyactive® (PEOT/PBT copolymer). NCRs grew well on PuraMatrix™, but the gelation of the biomaterial was problematic. Gels were very uneven, and most of the cells were found at the bottom of the well plate or on the surface of the gels, but not inside the gels, as was intended. However, one study showed that PuraMatrix™ gel improved the long-term survival of human microvascular endothelial cells and inhibited endothelial cell apoptosis more than did collagen type 1 [32], and could thus serve as a potential tissue matrix if technical problems could be overcome. In addition, another study by Narmoneva et al., showed that mouse endothelial cells improved the survival of neonatal mouse cardiomyocytes in PuraMatrix™ gel [33]. Another recent study showed that PuraMatrix™, in combination with cardiac progenitor cells, improved cardiac function when injected into the border area of a myocardial infarction. In addition, the study showed PuraMatrix™ to be angiogenic *in vivo* also, thus improving the potential of tissue graft to engraft [34].

PEOT/PBT copolymers have been used mainly in cartilage and bone applications [18,19], but some of the compositions form a hydrogel-like structure in aqueous conditions, so their suitability for cardiomyocyte culture was tested. However, the NRCs grew poorly on these biomaterials, with their studied compositions and structures; in fact, of all the biomaterials tested, PEOT/PBT copolymers seemed to be the least suitable for cardiomyocytes. Even though some of the materials may form a hydrogel-like structure, they cannot gel with cells inside them, and are therefore unsuitable for a 3D heart tissue model.

None of the biomaterials tested in this study proved fully optimal for cardiomyocyte growth and survival. It may be necessary in the future to concentrate on modifying or combining different biomaterials to identify an optimal combination for each cell type or application. For example, it has been shown that adding chitosan can improve the properties of a collagen gel, especially its stability. Combined biomaterials also stimulated more angiogenesis than did collagen alone [35]. In addition, hydrogels, combined with whole extracellular matrix proteins such as collagen or laminin, proved beneficial for cardiomyocyte maturation and, thus, cardiac tissue engineering [36]. One important aspect when developing 3D cardiac model would be to study the combination of cardiomyocytes with other cell types, such as endothelial cells and fibroblasts that could further support the formation of a tissue-like cardiac structure. In the future, especially when cells are cultured in 3D, in optimal biomaterial it is also important to confirm that the cells are functional. The functionality can be verified with electrophysiological measurements.

Conclusions

During this study collagen was the most suitable biomaterial for cardiomyocyte culture. However, unsuitability of commercial collagen to support the growth of cardiomyocytes points out the importance of the starting material. During culture cardiomyocytes were able to migrate and form a uniform 3D network of cells inside the gel, which indicates that collagen could be utilised in making tissue-like cardiac structure. The results of this study encourage continuing research with collagen or collagen-like materials in the future.

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