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Cell Response of Flexible PMMA-Derivatives: Supremacy of Surface Chemistry over Substrate Stiffness

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Graphical abstract

Abstract

The present work reports on the development of a range of poly(methyl methacrylate)/poly(ethylene glycol) (PMMAPEG)-based materials, characterized by different elasticity moduli in order to study the influence of the substrate's mechanical properties on the response of human umbilical vein endothelial cells (HUVECs). To render the selected materials cell-interactive, a polydopamine (PDA)/gelatin type B (Gel B) coating was applied. Prior to the *in vitro* assay, the success of the PDA and Gel B immobilization onto the materials was confirmed using X-ray photoelectron spectroscopy (XPS) as reflected by the nitrogen percentages measured for the materials after PDA and Gel B deposition. Tensile tests showed that materials with E-moduli ranging from 37 to 1542 MPa could be obtained by varying the ratio between PMMA and PEG as well as the PEG molecular weight and its functionality (i.e. mono-methacrylate versus di-methacrylate). The results after one day of cell contact suggested a preferred HUVECs cell growth onto more rigid materials. After 1 week, the material with the lowest E-modulus of 37 MPa showed lower cell densities compared to the other materials. No clear correlation could be observed between the number of focal adhesion points and the substrate stiffness. Although minor differences were found, these were not statistically significant. This last conclusion again highlights the universal character of the PDA/Gel B modification. The present work could thus be valuable for the development of a range of cell substrates requiring different mechanical properties in line with the envisaged application while the cell response should ideally remain unaffected.

Keywords: gelatin, PMMAPEG copolymers, mechanical properties, surface modification

1. Introduction

After my PhD covering biopolymer-based hydrogels under the supervision of Prof. Etienne Schacht at Ghent University (UGent), it was an evident choice to apply for a post-doctoral grant from the Research Foundation Flanders in 2008. I decided to stay in the Polymer Chemistry & Biomaterials (PBM) Group, meanwhile headed by Prof. Peter Dubruel, which was known territory, but the increasing number of responsibilities including project writing and PhD supervision rendered my stay very appealing. Given the limited number of professorships available in Belgium, I tried to further consolidate my future in academia, in compatibility with my family life, by accepting a 10% professorship at the University of Antwerp for teaching along with a 10% professorship at Vrije Universiteit Brussel (B-PHOT, Prof. Thienpont) to perform research. These positions enabled me to successfully apply for project funding thereby showcasing my capacity to function as an independant researcher. Crucial aspects associated with the latter included: (1) a perfect match between call topic and project, (2) multiple project submissions, (3) a proper balance between innovation and feasibility.

I always recommend my students to go abroad and shift research groups often when pursuing an academic career. However, my personal experience indicated that the latter is not a necessity when you can establish an extensive international network from your home country, in combination with persistence, ambition, creativity, excellent mentors and the required dose of luck. Indeed, my efforts resulted in a full-time, permanent professorship at Ghent University starting from October 1st 2017.

Over the years, researchers have always tried to adjust the bulk properties of existing materials. In case of polymer materials, two main strategies co-exist: 1) The production of composites: inorganic fillers are used to fine-tune the polymer properties; 2) A copolymerization strategy: the properties of different homopolymers are combined by reacting their respective building blocks.

With respect to PMMA-based composites, a lot of studies have already been conducted in an attempt to improve their physical properties such as their tensile strength, their heat resistance, gas permeability and even their conductivity, all with a minimum loss in transparency [1]. As a result, nanocomposites gained increasing interest over the years. Examples include the introduction of graphite-based compounds [2-4], (multi-walled) carbon nanotubes via in situ bulk polymerization [5-10], sulfides such as ZnS [11, 12] and MoS₂ [13], clays [14-17], alumina [18] and silicon-based compounds [19, 20, 1]. In the medical world, a typical example includes PMMA/hydroxyapatite (HA) composites. More specifically, HA, which is a major inorganic component of bone, stimulates the adhesion of the PMMA cement to bone as well as the subsequent bone growth [21-23]. Furthermore, the introduction of silver nanoparticles has been studied to enhance the antibacterial properties of PMMA [24].

Apart from composites, many researchers have applied the principle of copolymerization to alter material properties. A lot of MMA-based materials have already been investigated and examples of co-monomers include methacrylic acid [25], styrene [26] and aldehyde-functionalized poly(ethylene oxide) (PEO) [27]. In the last case, the copolymerization was facilitated by amination of PMMA.

Also in the medical world this strategy has been applied in an attempt to adjust the material properties of PMMA. In the context of contact lenses, MMA has been copolymerized with N-vinylpyrrolidinone to create soft daily contact lenses characterized by a much higher oxygen permeability compared to the original hard ones [28]. For IOL's, sulfonate and carboxylate bearing monomers were used in an attempt to modulate fibroblast proliferation [29, 30]. Furthermore, flexibility and elasticity have already been introduced by the copolymerization of MMA and hydroxyethyl methacrylate (HEMA) [31].

It should be noted that the use of polymer blends can also result in an alteration of the mechanical along with alternative physical properties of a material. In case of PMMA, examples of this strategy include the combination of PMMA with polyaniline [32] and polystyrene [33]. Furthermore, the creation of interpenetrating networks should also be mentioned, such as in combination with isocyanate-functionalized poly(ethylene glycol) [34].

From literature, it is well-known that matrix stiffness influences cell behavior [35, 36]. In this respect, cells not only exert forces on the underlying substrate, but they also experience tension from their environment on which they respond through alterations in their cytoskeleton organization, adhesion, protein expression, post-translational modifications, differentiation properties and cell-cell interactions [37, 38, 36]. It should be noted that these responses are cell-type dependent. In this context, increased proliferation with a rise in substrate rigidity is common for many cell types including neurons, osteoblasts, vascular smooth muscle cells and fibroblasts [36]. Also endothelial cells behave similarly to fibroblasts in the sense that increased substrate rigidity will result in superior cell spreading. Furthermore, for these cell types, it has already been demonstrated that soft materials (E-mod = 0.5 kPa) stimulate the existence of cell-cell contacts, whereas more rigid ones (E-mod = 33 kPa) enhance cell-substrate adhesion [39].

In order to study the influence of the mechanical properties on cell behavior, a range of PMMAPEG-based materials, characterized by different elasticity moduli were selected in the present work and compared for their cell-interactive properties.

2. Materials & Methods

2.1. Production of PMMA- and PEG-based copolymers

Methyl methacrylate (MMA; Sigma Aldrich) was distilled prior to use. Poly(ethylene glycol) monomethylether monomethacrylate (PEGMA) with a molecular weight of 500 g/mol and poly(ethylene glycol) dimethacrylate (PEGDMA) with molecular weights of 550 and 750 g/mol (Sigma Aldrich) were applied as received. In order to produce polymer sheets, an initiator (i.e. Irgacure 2959, BASF) / monomer solution (2 mol% compared to the amount of monomer double bonds) was injected in a pre-shaped silicone spacer (James Walker), which was placed between two teflon foil (Holders technology) covered glass plates. Subsequently, the samples were UV-irradiated at both sides (UV-A, 365 nm, 13 mW/cm², distance UV-lamps: 3 cm). Optimization of the UV irradiation times was performed by the evaluation of polymer samples obtained after different UV irradiation times by ¹H NMR spectroscopy (Bruker 300 MHz spectrometer). Determination of the conversion percentage of PMMA was enabled by comparing the proton integrations of the monomer double bonds (δ = 5.98 ppm and 5.44 ppm) with the sum of the O-CH₃ polymer and monomer protons (δ = 3.53 ppm and 6.69 ppm respectively). In case of PEGMA, similar

calculations were performed, but for PEGDMA, full conversion was assumed when the disappearance of the double bond protons in the soluble fraction was attained.

The various NMR peak annotations of the starting monomers are given below (data not shown):

MMA: ^1H NMR (CDCl_3 ; 300 MHz): $\delta = 6.02$ ppm (1H, s, $\text{H}_2\text{C}=\text{C}-$), $\delta = 5.47$ ppm (1H, s, $\text{H}_2\text{C}=\text{C}-$), $\delta = 3.69$ ppm (3H, s, $\text{CH}_3-\text{O}-$), $\delta = 1.83$ ppm (3H, s, $\text{CH}_3-\text{C}=\text{CH}_2$);

PEGMA: ^1H NMR (CDCl_3 ; 300 MHz): $\delta = 6.02$ ppm (1H, m, $\text{H}_2\text{C}=\text{C}-$), $\delta = 5.47$ ppm (1H, m, $\text{H}_2\text{C}=\text{C}-$), $\delta = 4.19$ ppm (2H, t, $-\text{C}=\text{OO}-\text{CH}_2-\text{CH}_2-$), $\delta = 3.64$ ppm (2H, t, $-\text{C}=\text{OO}-\text{CH}_2-\text{CH}_2-$), $\delta = 3.54$ ppm (2H, m, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), $\delta = 3.44$ ppm (2H, m, $\text{CH}_2-\text{O}-\text{CH}_3$), $\delta = 3.27$ ppm (3H, s, $-\text{O}-\text{CH}_3$), $\delta = 1.84$ ppm (3H, s, $\text{CH}_3-\text{C}=\text{CH}_2$).

PEGDMA: ^1H NMR (CDCl_3 ; 300 MHz): $\delta = 6.02$ ppm (1H, s, $\text{H}_2\text{C}=\text{C}-$), $\delta = 5.47$ ppm (1H, m, $\text{H}_2\text{C}=\text{C}-$), $\delta = 4.19$ ppm (4H, t, $-\text{C}=\text{OO}-\text{CH}_2-\text{CH}_2-$), $\delta = 3.64$ ppm (4H, t, $-\text{C}=\text{OO}-\text{CH}_2-\text{CH}_2-$), $\delta = 3.54$ ppm (13H, m, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), $\delta = 1.84$ ppm (6H, s, $\text{CH}_3-\text{C}=\text{CH}_2$).

2.2. Tensile tests

A universal tester 10-KM (Hounsfield Test Equipment Ltd), equipped with a load cell of 100 and 1000 N was used for mechanical analysis. All measured specimens were dog bone shaped (25 mm in gauge length and 4 mm in width, according to ISO 37-2). The sample thickness was assessed using a caliper and was averaged at 1 mm. The unidirectional tension speed was 25 mm/min. For each composition, 10 measurements were performed and averaged. The elastic modulus (MPa), the elongation (%) and force at break (N) were recorded using the QMAT software.

2.3. Swelling experiments

To determine the swelling percentage of the materials, three disc-shaped samples ($d = 1$ mm) per condition were freeze-dried and weighed (initial mass, m_0). Subsequently, the samples were incubated for 24 hours in deionized water at 37 °C. After swelling, the samples were again weighed (m_t) and swelling degrees were calculated using the following equation:

$$\text{Swelling}(\%) = \frac{m_t - m_0}{m_0} * 100\%$$

2.4. Surface functionalization with Gel B layer.

The surface functionalization of PMMAPEG-based materials with gelatin B has been optimized and described earlier.[40] More specifically, the deposition of polydopamine (PDA) was established by applying the protocol described by Lee et al [41]. Using this protocol, the samples were incubated for 24 hours in a 2 mg/ml dopamine.HCl (DA) (Sigma Aldrich) solution in Tris buffer (10^{-3}M , pH 8.5; prepared from Trizma base, Sigma Aldrich). The deposition of Gel B (Rousselot) onto PMMAPEG was established afterwards. More specifically, the samples were incubated overnight at 40°C in a gelatin B solution, by dissolving the appropriate amount of Gel B (0.5, 1 or 2 w/v%) in Tris buffer (10^{-3}M , pH 8.0). After incubation, the samples were rinsed with milliQ water and gently dried with N_2 gas.

2.5. XPS measurements

To determine the elemental compositions, an ESCA S-probe VG monochromatised spectrometer equipped with an Al Ka X-ray source (1486 eV) was used.

Survey scans were recorded on three spots of each sample and the elemental composition of the top surface could be determined by calculating the peak areas in the obtained spectra. A minimum of two samples per test condition were measured.

2.6. In vitro biological evaluation

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth medium 2 (EGM-2, Clonics®, Lonza), at 37°C. This medium was composed of endothelial basal medium (EBM-2) and SingleQuots™

(Clonics®, Lonza) containing several supplements and growth factors. The concentration of growth factors and supplements in 500 ml basal medium was: 0.5 ml human epidermal growth factor (hEGF), 2 ml human recombinant fibroblast growth factor (hFGF-β), 0.5 ml vascular endothelial growth factor (VEGF), 0.5 ml insulin-like growth factor (R3-IGF-1), 0.2 ml hydrocortisone, 0.5 ml ascorbic acid, 0.5 ml heparin, 10 ml FBS and 0.5 ml gentamicine/amfotericine (GA-1000).

Prior to cell seeding, all samples were sterilized by ethylene oxide treatment (cold cycle, AZ Sint-Jan, Brugge, Belgium). Gel B was subsequently immobilized under sterile conditions.

HUVECs of passage 7 were seeded on top of the samples with a cell density of 20000 cells/cm². Cell adhesion at day 1 and 7 was evaluated by a live/dead assay. To this end, after rinsing the films, the supernatant was replaced by 1 ml PBS solution supplemented with 2 μl (1 mg/ml) Calcein AM (Anaspec, USA) and 2 μl (1 mg/ml) propidium iodide (Sigma). Cultures were incubated for 10 minutes at room temperature, washed and evaluated by fluorescence microscopy (Type U-RFL-T, Olympus, XCellence Pro software, Aartselaar, Belgium). Evaluations were done 1 day and/or 7 days post cell seeding.

Cytoskeletal organization was evaluated after 2 days of incubation by immunolabeling and confocal microscopy. Actin filaments and focal adhesion points were (immuno-)labeled. All steps were performed at room temperature and a washing step consisted of 3 times 5 minutes washing in PBS buffer, unless stated otherwise. After removal of the medium, the cells were washed briefly with PBS and fixed in 4 % buffered paraformaldehyde solution for 20 minutes at room temperature. After washing the samples, the cells were permeabilized with a 0.5 % Triton X-100 in PBS solution for 5 minutes. Subsequently, cells were blocked with a 50 % FBS solution in PBS for 1 hour, washed, incubated with the primary antibody, mouse anti-paxillin (Millipore, Overijse, Belgium; 1:1000 diluted in 50 % FBS in PBS) for 1 hour and washed again. Afterwards, the cells were incubated with the secondary antibody AlexaFluor 488 goat anti-mouse IgG (A11001, Life Technologies, Merelbeke, Belgium) for 30 minutes and washed. Next, the cells were stained with a phalloidin-rhodamine (Life Technologies) solution (1:100 diluted in PBS) and a 1 μg/ml DAPI solution (Life Technologies, Merelbeke, Belgium) for 10 minutes and washed again. The samples were mounted on a cover slide prior to microscopy measurements. All immunolabeling experiments were performed in duplicate.

The samples were investigated by means of a Nikon A1r confocal microscope mounted on a Nikon Ti Body (Nikon Instruments, France) and magnified with a 40x Plan Fluor oil objective (numerical aperture 1.3). In order to obtain a representative image of each test condition, three separate regions were randomly acquired per sample.

Image processing was performed in Fiji (<http://fiji.sc>), a packaged version of ImageJ freeware (W.S. Rasband, U.S.A. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2014). The focal adhesion points were quantified by means of a custom-designed image processing pipeline, which is essentially based on a high-content analysis workflow described earlier [42] and available upon request. In brief, the analysis consists of a few image preprocessing steps, followed by hierarchical segmentation of nuclei, cells and focal adhesion points (FA) to allow region-specific analysis of objects. First, image hyperstacks are projected according to the maximum axial pixel intensity and corrected for lateral illumination heterogeneity and background signals by means of a pseudo-flat field correction, which consists of dividing the image by a duplicate image smoothed with a Gaussian blur of large radius (sigma = 50). A subsequent local contrast enhancement balances for more subtle intensity fluctuations. Next, nuclear regions of interest (ROIs) are segmented in the DAPI channel after filtering with a Gaussian kernel of small radius (sigma = 3), automatic thresholding according to Otsu's algorithm and watershed-based separation. Cellular boundaries are then delineated by combining a segmentation of the median filtered Phalloidin channel, using the Triangle auto-thresholding algorithm, with a Voronoi tessellation on the nuclear ROIs (Boolean AND operation). Finally, FA are specifically enhanced in the FA channel by means of a Laplacian operator and binarized using the Triangle auto-thresholding algorithm. Segmentation of FA can be further refined by cell-dependent peak selection, which is based on preserving only those objects (presumed FA) for which the average signal surpasses a threshold (typically defined as the average signal + 1 x std of the FA channel in the cell ROI). This feature avoids detection of noise pixels and allows for accurate segmentation of FA in cells with varying background fluorescence. The resulting ROIs were used for analyzing shape (area, aspect ratio circularity) and intensity metrics of objects larger than a predefined size (> 5 pixels) on the original image.

2.7. Statistical analysis

Except for the cell tests, the average and standard deviations were calculated for each condition tested and statistical differences were determined via a one way ANOVA-test in the 95% confidence interval, followed by a Sidak post hoc test, for multiple comparisons. IBM SPSS Statistics Version 20 (IBM Corporation, New York, United States) software package was used as statistical software and two values were considered significantly different when $p < 0.05$. Error bars represent the standard deviations in all graphs.

In case of cell-data, the average values of various parameters such as number of focal adhesions and circularity were statistically compared by means of a non-parametric Kruskal-Wallis test. IBM SPSS Statistics Version 20 (IBM Corporation, New York, United States) software package was used as statistical software. The boxplots were generated with R statistical freeware (The R Development Core Team) [43]. Two values were considered significantly different when $p < 0.05$. Error bars represent standard deviations.

3. Results

3.1 Material development and physico-chemical characterization

In a first part, material optimization occurred to ensure full conversion of the material constituents PMMA, PEGMA and PEGDMA. For PEGMA, irradiation times of 2, 6, 10 and 20 minutes were evaluated and after 20 minutes, all double bond protons disappeared (figure 1, left). Compared to PMMA which shows 99% conversion after 90 minutes,[40] the PEG-based oligomer is characterized by superior conversion kinetics (figure 1, right), due to the lower viscosity of the reaction mixture. For PEGDMA, a similar optimization was performed (data not shown). Based on the material composition (i.e. ratio between PMMA, PEGMA and PEGDMA, see table 1), a UV irradiation time of 55 minutes was selected for the development of the copolymers.

Figure 1: Overlay of the ^1H NMR spectra of unreacted (a) and reacted PEGMA(500) after 6 (b), 10 (c) and 20 minutes (d) of UV irradiation (left). PEGMA conversion percentage as a function of UV irradiation time (right).

The material composition together with the corresponding E-moduli, swelling behavior and the surface composition of the selected materials are presented in table 1. Different mechanical properties were obtained by varying the PEG chain length (550 vs 750 g/mol) or by varying the molar composition of linear PEG (PEGMA) vs PEG cross-linker (PEGDMA). As reference materials, materials 1 and 3 (see table 1 for their exact composition) were included, because of their similar E-moduli. Material 5, on the other hand, was included to study the effect of a substantially lower E-modulus. To render the selected materials cell-interactive, the PDA/Gel B coating was applied. Regarding the experimental setup, two assumptions could be made. First, if the PDA/Gel B coating is indeed characterized by a universal character, it is expected that regardless of the different mechanical properties, a similar cell response would be provoked. Secondly, if a difference in cell response would be observed between materials 1 to 4, it can be anticipated that the latter is due to differences in the mechanical properties of the substrate materials, as similar compositions were selected (90 mol% MMA and 10 mol% PEG).

First, the influence of PEGMA on the mechanical properties of the developed materials was assessed. Table 1 clearly shows that an increased amount of PEGMA resulted in a decrease of the E-modulus (material 3 versus 4 respectively) along with an increase in the swelling percentage going from 8% to 16% ($p < 0.05$). Furthermore, the molecular weight of the PEGDMA incorporated also affected the E-modulus of the final materials. More specifically, increasing the molecular weight of PEGDMA from 550 g/mol to 750 g/mol resulting in a decrease of the E-modulus from 1533 MPa to 324 MPa (see table 1, material 1 versus 2). A similar effect was observed for the swelling potential of the latter PMMAPEG-based materials with an increase in swelling going from 7% to 19% respectively. Finally, the incorporation of higher amounts of bifunctional building blocks (i.e. PEGDMA) resulted in higher E-moduli along with lower swelling percentages (i.e. material 4 versus 1 respectively) ($p < 0.05$).

Table 1: Overview of the chemical composition, the E-moduli and the swelling percentage of the selected PEG containing materials. The atomic nitrogen percentages, as determined by XPS, after PDA deposition and Gel B immobilization are also shown. Herein, the standard deviations, shown between brackets, are a measure of the homogeneity of the coatings applied on the sample surfaces.

Material	MMA (mol%)	PEGDMA (550) (mol%)	PEGDMA (750) (mol%)	PEGMA (500) (mol%)	E-mod (MPa)	Swelling (%)	N _{PDA} (at%) (stdev)	N _{GelB} (at%) (stdev)
1	90	10	0	0	1533	7 ± 1	5 (0.2)	10 (0.4)
2	90	0	10	0	324	19 ± 2	4 (0.2)	9 (0.4)
3	90	8	0	2	1542	8 ± 1	5 (0.6)	10 (0.7)
4	90	2	0	8	678	16 ± 1	5 (0.4)	9 (0.2)
5	50	40	0	10	37	24 ± 0	5 (0.1)	7 (0.4)

In a subsequent part of the present work, the effect of the material composition on the respective forces at break and elongation percentages was also determined (figure 2). The above observed correlation between the material composition and the corresponding E-moduli was also confirmed for the alternative characteristic mechanical properties. Indeed, a higher molecular weight of the PEGDMA (750 versus 550 g/mol) resulted in significantly lower forces at break and higher elongation percentages. Moreover, higher PEGMA amounts gave rise to lower forces at break and higher elongation percentages, albeit not significant. When incorporating 2% PEGDMA (cfr. material 4) versus 10% PEGDMA (cfr. material 1), the force at break reduces accordingly while the elongation increases. However, given the large standard deviation on the elongation percentage of material 4, the results do not differ significantly.

Figure 2: Force at break (grey bars) and elongation percentage (black line) of the PMMAPEG-based materials overviewed in table 1.

Prior to the *in vitro* assay, the success of the PDA and Gel B immobilization onto the materials was confirmed using XPS as reflected by the nitrogen percentages measured for the material series after PDA and Gel B deposition (see table 1). It can be concluded from table 1 that PDA deposition resulted in a similar nitrogen content for the different material surfaces (i.e. $\pm 5\%$). In case of Gel B immobilization, the different materials also resulted in a similar nitrogen content (i.e. $\pm 10\%$ N) which resembled the values as described in earlier work reporting on this surface modification strategy.[40] An exception was observed for material 5, since only 7% of nitrogen was detected after Gel B immobilization.

3.2. *In vitro* biological evaluation

Evaluation of the live-dead images, presented in figure 3, showed that after 1 day of cell contact, all materials, except material 5, resulted in high cell densities. However, for materials 2 and 4, areas of lower cell density were also observed (data not shown). After 7 days, confluent cell layers were observed on all materials, although material 5 again showed lower cell densities compared to the other materials. Furthermore, more dead cells were observed for this material. If material 1 and 3 are compared, more dead cells were present on the latter.

Figure 3: Live dead assay with propidium iodide (red color, dead cells) and calcein AM stained (green color, living cells) HUVEC cells.

In order to obtain more information about cell adhesion, the focal adhesion (FA) points were stained and quantified. Figure 4 shows a representative confocal image for the different substrate materials, as well as the boxplots, which represent the distribution of the number of FA counted for different cells on a substrate material. Visual observation of the FA shape showed that for all material compositions, stable, elongated focal adhesions were obtained. Quantification of the number of focal adhesion points showed that materials 4 (median FA = 236), 2 (median FA = 207) and 3 (median FA = 198) resulted in the highest median number of focal adhesion points, as compared to

material 1 (median FA = 126) and 5 (median FA = 124). Nevertheless, based on statistical analysis, it could be concluded that the focal adhesion distribution is the same across the different material categories ($p > 0.05$).

Figure 4: Immunostaining assay with HUVECs. Stainings are performed to visualize the actin filaments (red), the nuclei (blue) and the focal adhesions (green). The boxplots representing the FA distributions are also shown.

Discussion

In the present work, different crosslinked PMMAPEG-based materials have been developed and characterized resulting in a broad range of flexible, hydrophilic materials based on PMMA, PEGMA and PEGDMA with different molecular weights. The effect of several parameters including the PEG molecular weight, the ratio between monofunctional and bifunctional methacrylates and the overall PEG(D)MA content (with respect to PMMA) on the physico-chemical properties (i.e. mechanical and swelling properties) of the final materials was screened.

The results showed that increasing PEGMA amounts, resulted in lower E-moduli and more swelling which was anticipated, because of the presence of higher amounts of long chains in the materials developed. The longer side chains increase the free volume between the polymer backbones as well as their freedom to move. As a result, a higher flexibility of the material is obtained. Moreover, a less densely crosslinked network corresponds with more free volume enabling greater swelling. Similar observations were already made for complete hydrogel networks based on PEG. For example, McGann et al have reported earlier on photo-crosslinkable networks consisting of thiolated multi-arm PEG and resilin-like polypeptides functionalized with norbornene.[44] They have shown that more densely crosslinked networks resulted in higher storage moduli, as observed via rheology. In addition, Hwang et al have shown that higher PEGDMA quantities incorporated in hydrogel networks result in higher elasticity moduli along with lower swelling capacities.[45] The same holds for natural hydrogels. Indeed, Van Vlierberghe et al have shown that the swelling and mechanical properties of gelatin hydrogels crosslinked via oxidation of the incorporated thiols into disulfides can be tuned by varying the degree of substitution (DS) of the thiols.[46] More specifically, DS's of 15 % and 60 % result in storage moduli of 10 kPa and 22 kPa respectively, along with swelling degrees of 14,000 % and 1,000 % respectively. Furthermore, the effect of the ratio between PMMA and PEG-based constituents was anticipated to affect the swelling capacity of materials developed. More specifically, higher PMMA/PEG ratios result in lower swelling degrees given the hydrophobicity and the hydrophilicity of PMMA and PEG respectively. The latter observation was in agreement with previous reports on hydrogel blends containing compounds with varying water affinity. Indeed, Van Vlierberghe et al have already shown earlier that the incorporation of 1 w/v% chondroitin sulphate, being a highly hydrophilic compound, in 10 w/v% crosslinkable gelatin hydrogels resulted in higher swelling degrees going from 1,000 % to 1,300 %.[47]

The XPS results showed that similar nitrogen contents were obtained after PDA and GelB immobilization, irrespective of the sample composition, with the exception of the nitrogen content after GelB immobilization on the sample coating the highest PMMA/PEG ratio (i.e. material 5). It might be anticipated that the somewhat higher swelling degree (i.e. 24% cfr a maximum swelling of 16%) could be responsible for this fact, although further investigation is needed to elucidate this effect in greater detail. The effect of swelling degree on the modification efficiency of hydrogels has only been reported scarcely to date. However, Primo et al have also shown that the swelling potential of a hydrogel can affect the subsequent surface functionalization, albeit through single pulse laser irradiation.[48]

In a subsequent part, the *in vitro* biocompatibility of the materials with varying E-moduli was assessed after applying a cell-interactive gelatin B coating via an intermediate polydopamine layer along with assessing the effect of the substrate stiffness on the cell response.

The obtained results after one day of cell contact suggest a preferred HUVECs cell growth onto more rigid materials, an observation which was also made in literature, although, in the latter case rigid materials were characterized by an E-modulus of 30 kPa [36], while the current work covers E-moduli ranging from 37 to 1542 MPa. After 1 week, the material with the lowest E-modulus of 37 MPa showed lower cell densities compared to the other materials, which resulted in comparable cell densities despite their different E-moduli ranging from 324 MPa to 1542 MPa. Upon comparing the materials containing PMMA and PEGDMA versus PMMA, PEGDMA and PEGMA, more dead cells were present on the PEGMA-containing samples. Since both materials are characterized by similar elasticity moduli (i.e. 1533 MPa versus 1542 MPa respectively), it cannot be excluded that the introduction of PEGMA into the material's composition is responsible for this phenomenon. Since material 5 contains the highest amount of PEGMA(500), it can also be questioned whether the difference in cell behavior is caused by a difference in mechanical properties or by the presence of a higher amount of PEGMA. Additionally, for material 5, a third cause could be the less efficient Gel B immobilization (cfr. 7% N versus 10% N for the other materials). In literature, a similar observation was already made for acrylamide-based gels. In this study, more rigid substrates (E-mod of 30-100 kPa) resulted in mature elongated focal adhesions, whereas on more flexible substrates (~1kPa), the FA appeared as more diffuse dot-like structures [49]. When comparing these E-moduli to the values in our study, it is clear that all materials reveal an E-mod > 100 kPa (i.e. all materials in this study) and thus they can be considered as rigid substrates, which are characterized by an organized F-actin stress fiber structure [37]. A comparison with literature results describing similar cell types and materials characterized by an E-mod > 40 MPa would have been more relevant, but these are lacking in the current state-of-the-art. Furthermore, it should be noted that the mechanical analysis procedures generally described in literature are in great part different from the techniques applied herein (e.g. the use of rheology [50] or indentation tests [51] versus tensile testing in the current study).

Apart from substrate stiffness, the presence of Gel B will also influence cell adhesion. In this respect, a previous study has shown that a decreased density of collagen type I onto polyacrylamide gels resulted in decreased cell-matrix adhesions but in increased cell-cell connections [52]. Since Gel B immobilization resulted in a similar surface composition (cfr. XPS results) for the different materials, except for material 5, this variable could be considered as a constant. No clear correlation could be observed between the number of focal adhesion points and the substrate stiffness. Although minor differences were found, these were not statistically significant. This last conclusion again highlights the universal character of the PDA/Gel B modification.

Conclusions & future perspectives

In the present work, a universal modification strategy was applied to introduce cell-adhesive properties onto a range of PMMAPEG-based materials featuring different stiffness. More specifically, the combination of PDA and gelatin B was applied onto PMMAPEG-based materials with E-moduli ranging from 37 MPa to 1542 MPa. Application of the bi-layer system revealed similar cell-interactivities irrespective of the underlying substrate material's (physico-)chemistry. Indeed, upon applying PDA/Gel B onto different PMMAPEG-based materials with varying elasticity moduli, similar numbers of cell focal adhesions were obtained, which again stresses the universal character of the coating. In order to influence the cell response, PMMAPEG-based materials could be developed with lower E-moduli by decreasing the PMMA/PEG ratio and/or by increasing the PEGMA/PEGDMA

ratio. In addition, it can be anticipated that stem cell differentiation is likely to become affected to a greater extent upon varying the substrate's stiffness compared to HUVEC's.

The present work could thus be valuable for the development of a range of cell substrates requiring different mechanical properties in line with the envisaged application while the cell response should ideally remain unaffected.

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