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Coating of polyurethane scaffolds with collagen: comparison of coating and cross-linking techniques

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Abstract Collagen has been coated successfully onto numerous hydrophilic polymer scaffolds to improve cell adhesion. Due to the hydrophobic nature of thermoplastic polyurethane (TPU), coating with aqueous collagen solution is problematic for such scaffolds. This study facilitated the coating of TPU with collagen and compared cross-linking and coating techniques. Three different cross-linking methods were compared. Both thermal and glutaraldehyde methods showed proof of cross-linking; however glutaraldehyde seemed to be superior to the other methods. The use of human urine as a wetting agent and the chemical glutaraldehyde had no effect on a cytotoxicity test performed by means of a WST-1 assay with a fibroblastic cell line. Three different coating techniques for porous TPU scaffolds were also investigated: ultrasound, pressurized air and injection. Of these, injection performed best. This method facilitated a coating of 100% of the porous scaffolds examined, which was verified by staining, FTIR and SEM.

1 Introduction

Materials for tissue engineering and implants are desired to encourage attachment, proliferation and differentiation of the desired cells or tissue. A material which has been shown to support such properties is collagen [1]. For example, collagen in fibrillar form has been used to coat titanium surfaces, improving osteoblast spreading, attachment, proliferation and differentiation in vitro [2–5].

Collagens are structural proteins of which 27 types have thus far been identified [6–8]. Collagen has been coated onto numerous hydrophilic polymers (e.g., PLGA) to improve the cell adhesion [9–14]. The hydrophobic nature of thermoplastic polyurethane (TPU) makes collagen coating procedures on scaffold of such material difficult [15–21]. The hydrophobic characteristics of TPU must be reduced before a layer can be deployed. However, the main obstacle is to find an adequate surfactant. Treatment of polyurethane by urine has been demonstrated in to reduce the hydrophobicity of polyurethane, which led to enhanced adhesion of the bacteria *Enterococcus faecalis* and *Escherichia coli* on polyurethane surfaces [22, 23]. The reason for the reduction is likely to be the deposition of polysaccharides and proteins from the urine onto the polyurethane surface, including Tamm–Horsfall protein and alpha-1 microglobulin [24]. To our best knowledge, urea is the only surfactant demonstrated in the scientific literature to lower the hydrophobicity of polyurethane.

Collagen must be cross-linked if it is to be used as a functional replacement in vivo due to its high degradation rate and low biomechanical strength. Several chemical agents have been used to achieve this goal. The toxicity of the cross-linking agent should be taken seriously when developing biomaterials. Glutaraldehyde, the most widely used reagent, has been known to be toxic due to the presence of unreacted functional groups or the release of those groups during enzymatic degradation of the cross-linked biomaterials [25]. CMC (*N*-cyclohexyl-*N*′-2-morpholino-ethyl-carbodiimide-methyl-*p*-toluolsulfonate), however, is known to be less toxic and biocompatible because it

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generates peptide-like bonds and allows direct cross-linkage without incorporation of the reagent [26]. Using CMC or another carbodiimide substance to cross-link collagen can yield biomaterials with good biocompatibility, high cellular differentiation potential and increased resistance against enzymatic degradation [27–29].

The aim of this study was to test the coating potential of collagen on TPU by utilizing urine as a wetting agent and to compare coating and cross-linking techniques on thermoplastic polyether-urethane thin sheets and porous scaffolds. In addition, three methods for the coating of porous scaffolds with collagen were compared, which made use of ultrasound, pressurized air and injection, respectively. Three different cross-linking methods were examined; thermal cross-linking and chemical cross-linking with either CMC or glutaraldehyde.

2 Materials and methods

2.1 Materials

Thermoplastic poly-ether urethane TPU (Texin 986®, Bayer Polymers, Pittsburgh, USA) granulates were dried for 1 h at 105° C in a furnace. The dried granulates were formed into thin sheets (0.08–0.4 mm) by hot pressing (300P, Dr Collin GmbH, Ebersberg, Germany) after 1 min exposure to 190° C and a pressure of two bar. The temperature was gradually lowered to 50° C with sustained pressure. The production method of the porous polymer samples is described in detail by Haugen et al. in previous articles [30, 31] and is, therefore, not included in this section.

2.2 Collagen coating

Thermoplastic polyurethane sheets were placed in urea (human, donated, Munich, Germany) for 24 h prior to coating. TPU sheets not placed in urine were also coated. In the case of porous TPU scaffolds, a needle was pushed into the middle of the scaffold and urine forced through using a syringe to drive air out of the pores. Subsequently the porous TPU scaffolds were likewise placed in urine for 24 h prior to coating. After air-drying, the material was coated with collagen (type I, Bovine Achilles Tendon, C 9879, Sigma-Aldrich, Munich, Germany). The TPU sheets were dip-coated. The thickness of the collagen coating was measured using a calliper. The porous scaffolds were coated using collagen solution with an identical concentration. This solution was forced into the scaffold by three methods: with pulses from an ultrasonic horn (B/0009, Bandelin electronic GmbH und Co.KG, Berlin, Germany); with compressed air and injection with a 10 mL steel needle (VWR, Darmstadt, Germany). In the ultrasonic method, scaffolds were immersed in collagen solution and subjected to ultrasonic pulses to force air out of the pores. In the compressed air method, a droplet of collagen solution was placed on a porous scaffold and forced into pores with a blast of compressed air. In the injection method, a needle was pushed into the middle of the scaffold and collagen solution forced out through the pores using a syringe. Prior to collagen coating, preliminary tests were performed using ink to verify the effectiveness of the three methods in driving liquid into the scaffold pores. All three methods resulted in ink penetration into pores.

2.3 Cross-linking

Three different cross-linking methods were examined; chemical cross-linking by glutaraldehyde, CMC and thermal cross-linking. Cross-linking with glutaraldehyde [32] and CMC [33] has previously been reported, and thus is not explained here. TPU sheets were placed in 20, 40, 70 and 100 mg/ml CMC in CPB (Citric of phosphates Buffer, pH 3.56, Merck, Darmstadt, Germany). Cross-linking was accomplished after 24 h at 4° C. The thermal cross-linking method was closely adapted from a publication by Noshiki and Lee [34]. The TPU sheets were flushed three times with buffer solution and air-dried. Subsequently, the coated TPU sheets were thermally cross-linked at 130 °C for 20 h in a furnace (Narbertherm GmbH, Lilienthal, Germany). Azocarmine staining (Azocarmine G powder, Merck, Darmstadt, Germany) was used to visualize the collagen in the porous TPU scaffolds and prepared according to a protocol [35]. The TPU materials were soaked for 10 min in the staining solution. Excess coloring was removed by means of a paper towel. The TPU were then rinsed with ddH_2O and embedded in a gel, which is liquid at ambient temperature, but is solid at −30° C. After imbedding, the samples were cut with a cryostat (Leica, Bensheim, Germany) to yield slices with a thickness of 80 µm. Afterwards slices were placed on slides, whereby the gel, which became liquid at ambient temperature, served as a fixative. After staining, the slides were examined with a stereolight microscope (Stemi, Zeiss, Germany).

2.4 Contact angle and adhesion of collagen

The static contact angle (OCA 20, DataPhysics, Filderstadt, Germany) was measured by applying 1 µL of ultrapure water (HPLC grade water, Sigma, Dramstadt, Germany). Samples were placed on a smooth base. Four categories of samples were examined: (a) TPU sheets without urine

treatment; (b) urine-treated sheets; (c) urine-treated sheets subsequently coated with collagen; (d) urine-treated sheets subsequently coated with collagen and cross-linked using glutaraldehyde. Adhesion of the collagen on TPU sheets were validated by the adhesive tape test described in EN ISO 2409 [ASTM-D- 1000]. The tape, which had a strength of 10 ± 1 N and a width of 25 mm, was applied to the collagen-coated TPU sheets. The number of the chipped-off squares was classified into four groups (0–4). For example, in class 0 the edge of the cuts are completely smooth and none of the squares of the lattice are detached, whereas in class 4 the collagen layer is removed along the edges of the cuts and/or it is removed on different parts of the squares. In addition, coated-collagen TPU sheets were placed in Ringer's solution (VWR, Darmstadt, Germany) for 14 days, then withdrawn and rinsed with water. The sheets were examined as described above.

2.5 Chemical analysis

The Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR FT-IR) (Spectrum One, Perkin Elmer Instruments, Rodgau-Juegesheim, Germany) with an ZnSe crystal was utilized to verify the cross-linking and chemical alteration within the collagen [36]. A mean spectrum of ten different spectra from each sample was made. An ANOVA one tail student *t* test with a confidence interval of 0.05 was carried out at each wavenumber.

2.6 Cytotoxicity tests, WST-1

The in vitro WST-1 cytotoxicity test based on the European norm ISO 10993-5: 1999 was performed on the material. The in vitro cell studies were performed with fibroblasts from a permanent cell line, (Detroit 551, CCL-110, ATCC, Manassas, USA), taken from the skin of a female Caucasian, and was cultivated in MEM-Earle-Medium (Biochrom AG, Berlin, Germany) with supplementary ingredients [37]. The samples (30 mg) from the porous samples (coated and uncoated) were cut into fine pieces, each 1 ± 0.01 mm. These pieces were washed in sterile water and later incubated in culture medium (eludate). This eludate was added to a well plate containing a monolayer of fibroblasts in four different dilutions: undiluted and 1:2. Fibroblasts were cultivated for 3 days at 37° C in a 5% CO_2 atmosphere. 10 vol.% of cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) was added directly to the medium. The medium was changed every third day. The behavior of cell monolayers was analyzed after 3 h. The positive control was the culture medium and 10% WST-1 held under the same conditions as mentioned above. The photometric measurement of the control took place in a 96-well-plate with 450 nm (reference wavelength 620 nm) on an ELISA reader (Sunrise, Tecan GmbH, Crailsheim, Germany). The absorption of the control was subtracted from the measured samples. The photometric measurements of the samples were performed three times and with the same parameters as the control. An average optical density (OD) with standard deviation was calculated. The difference in OD percentage from the samples was compared to the control well, which was taken to be 100% (negative control). Uncoated TPU was taken as a positive control. Significant levels between the groups were analyzed with an ANOVA one tail student *t* test with a confidence interval of 0.05.

3 Results and discussion

3.1 Contact angle and FTIR

The contact angle of TPU sank from an average value of 99.50° ± 4.44° to 21.00° ± 9.51° after urine treatment (Table 1). The collagen-coated, non-cross-linked had a contact angle of 110.75°°± 8.98° and collagen-coated TPU cross-linked with glutaraldehyde 80.48° ± 13.80°. The group of Gorman also managed to reduce the contact angle of a polyurethane, however not as low as our values [22]. ATR FT-IR could distinguish between the TPU and the collagen (Fig. 1). The following peaks were detected and quantified for collagen: the peak at 3,320 cm^{-1} (*s*NH) higher and at 1,600 cm^{-1} (*s*C = C). TPU had six distinctive peaks: 1,733 cm^{-1} (*s*C = O, free), 1,703 cm^{-1} (*s*C = O, bonded), 1,530 cm^{-1} (*b*NH + *s*CN), 1,250 cm^{-1} (*s*COC + *w*CH_2) and 1,080 cm^{-1} (*s*COC from the hard segment). *s* denotes stretching, symmetrical, *w* indicates wagging and *b* stands for bending of the chemical bonds. The beam from the ATR FT-IR penetrated between 0.1 and 1 μm into the measured samples [38]. The thickness of the collagen layer was measured and found to range between 20 and 40 μm ($n = 20$). Thus the equipment would only measure the collagen layer, if present on the surface.

Table 1 Contact angle of TPU after different surface treatments ($n = 40$)

Sample	Contact angle (°) ± SD (°)
Untreated TPU	99.50° ± 4.44°
Urine treated	21.00° ± 9.51°*
Collagen coated, without cross-linking	110.75° ± 8.98°*
Collagen coated, cross-linked with glutaraldehyde	80.48° ± 13.80°*

* $p < 0.05$ compared to untreated TPU

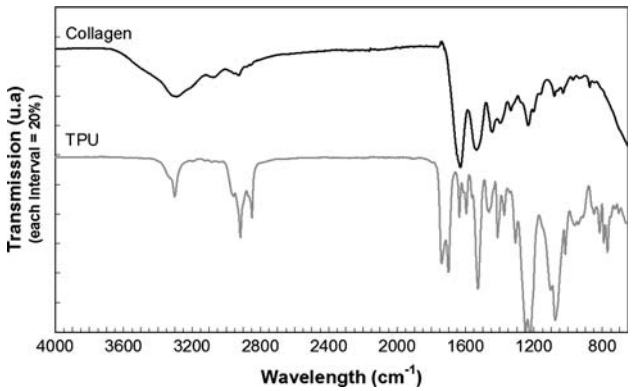

Fig. 1 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy of pure TPU and collagen. The two materials can be easily distinguished from each other. The following peaks were detected and quantified for collagen: the peak at 3,320 cm^{-1} (*s*NH) higher and at 600 cm^{-1} (*s*C = C). TPU had six distinctive peaks: 1,733 cm^{-1} (*s*C = O, free), 1,703 cm^{-1} (*s*C = O, bonded), 1,530 cm^{-1} (*b*NH + *s*CN), 1,250 cm^{-1} (*s*COC and *w*CH_2) and 1,080 cm^{-1} (*s*COC from the hard segment). *s* stretching, *w* wagging, *b* bending

3.2 Cross-linking methods

Different cross-linking methods were analyzed by ATR FT-IR (Fig. 2). The IR spectra for thermal cross-linking showed a distinctive peak in the area of 3,450–3,350 cm^{-1}, which are from the *s*NH bonds. The other methods, glutaraldehyde and CMC cross-linking, showed weaker peak amplitude in this region. In the so-called fingerprint region the *s*C = C, *b*NH and *s*CN bonds were present for both glutaraldehyde- and thermally cross-linked collagen.

Usually, two major peaks in the 1,700 cm^{-1} to 1,500 cm^{-1} region appear in the type I collagen spectra, one at 1,660–1,630 cm^{-1} (Amide I peak from C = O stretching vibrations) and the other at 1,553 cm^{-1} (Amide II peak for amide N–H bending vibrations coupled with C–N stretching). Peaks were detected at 1,250 and 1,080 cm^{-1}. These could be from any of the three chemical bonds: CC 1,150–1,250: CN 1,030–1,230: CO 1,020–1,275. A CN signal would indicate peptide bonds. Both thermal and glutaraldehyde cross-linking showed adsorption for 1,250 and 1,080 cm^{-1} (*s*C = C, *b*NH and *s*CN bonds). However, these were less pronounced after CMC treatment. Thermal cross-linking may have induced a denaturing of the collagen since some peaks are missing for the amide I and II. In addition three "extra" peaks (a, b and c, Fig. 2) were also detected. Extracted type I collagen from bovine Achilles tendon mainly contains bundles of fibers made of packed collagen helices. The fibers will partially unfold in acid aqueous solution (preferably below pH 3.5), or upon heating, but at high temperatures (130° C) the collagen helices will be strongly degraded [39]. This could be the reason for the denaturing symptoms. The FT-IR data showed stronger peaks for the chemical bonds of *s*N–H, *s*C = C, *b*N–H and *s*C–N for glutaraldehyde- and thermally cross-linked collagen. These bonds can be taken as proof of cross-linking since they show a chemical peptide cross-link between the amino acids. Glutaraldehyde induces cross-linking for the amino acid where lysine is present [40]. The C = C bond appears due to the formation of benzol rings. It seems that both glutaraldehyde and thermal cross-linking were successful, whereas the CMC cross-linking failed due to the fact that *b*N–H + *s*C–N are missing. In addition the glutaraldehyde cross-linking was shown to be more effective since the presence of *b*N–H + *s*C–N bonds was more pronounced. This can be confirmed by Fig. 2 since the IR peak is lower for glutaraldehyde compared to thermal cross-linking. Studies von Meade et al. came to the same conclusion [41]. Since the CMC cross-linking was shown to be less successful, a range of different concentrations were tested (Fig. 3). The unwanted peaks (a, b and c, Fig. 2) were found for the three CMC concentrations, 20, 40 and 70 mg CMC/mL buffer (labeled b and c, Fig. 3). The

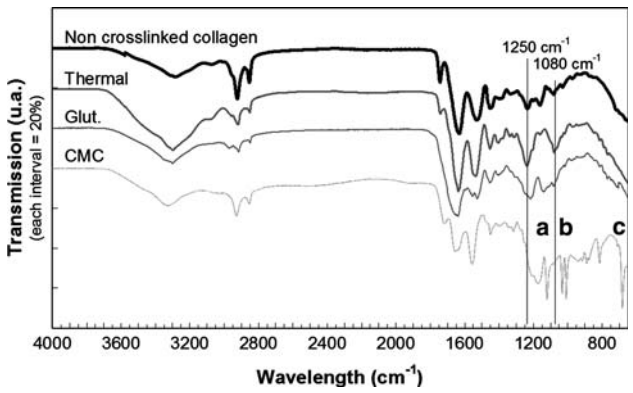

Fig. 2 Comparison of different cross-linking methods by ATR FT-IR, where glutaraldehyde and thermal cross-linking were shown to be effective. CMC appears not to cross-link collagen well. All spectra are the average of five separate measurements

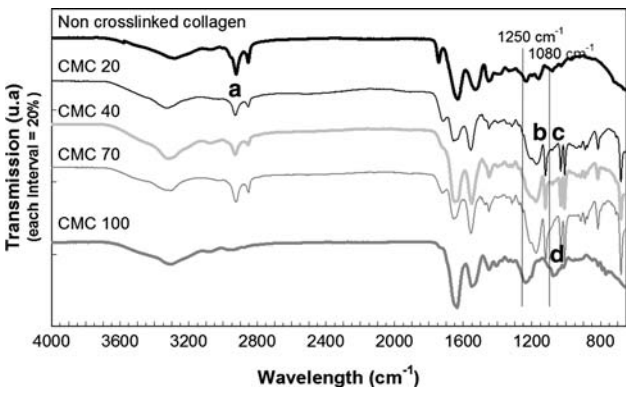

Fig. 3 Attenuated Total Reflectance Fourier Transform Infrared spectra of cross-linked collagen with different concentration of CMC (mg CMC/ml citric acid phosphate buffer)

*s*C = C, *b*NH and *s*CN bonds were visible, but not as marked as in Fig. 2. No peaks were detected for the wavelengths 1,250 and 1,080 cm^{-1}. The peaks at 2,930 and 2,850 cm^{-1} (a, Fig. 3) disappeared when a concentration of 100 mg CMC/mL buffer was applied. Additionally, a peak at 1,070 cm^{-1} emerged in the spectrum for the same treatment. From a visual evaluation of the cross-linked collagen-coated TPU sheets, the CMC method seems to be the best, due to the stickiness of the collagen layer to the TPU sheet. The collagen seemed more fragile with CMC treatment at a concentration of 100 mg/mL. After cross-linking with glutaraldehyde as well as by the thermal method, the collagen did not adhere as well to the surface as on uncrosslinked samples. The tape-test showed that the collagen layer detached from the TPU sheets after all cross-linking methods (Table 2). The Ringer solution test proved that the collagen layer was fully intact after immersion in Ringer solution for 14 days.

Use of human urine for surface modification of biomaterials raises concerns regarding the biocompatibility and immunogenicity due to likely polysaccharide and protein deposition. However, the cytotoxicity test showed no significant different between the controls and the glutaraldehyde cross-linked collagen coated TPU scaffold (Table 3).

3.3 Coating methods for porous scaffolds

The aim of this study was to compare coating and cross-linking techniques for porous TPU scaffolds. Thus, after successful coating of the smooth TPU foils was achieved, three different coating techniques using injection, ultrasonic pulses and pressurized air were evaluated. As predicted, non-urine treated TPU sheets did not give a successful coating (data not shown). No significant difference ($p > 0.05$) was found in the IR spectra from the different coating techniques when compared to pure collagen (Fig. 4). A comparative first derivate analysis on all spectra showed a difference of less than 0.8% (data not shown) compared to pure collagen.

Table 2 Adhesive test of collagen on the TPU sheets after different cross-linking methods

Sample	Tape test	Ringer solution test
Glutaraldehyde	4	1
Thermal	4	1
CMC 40	4	1
CMC 100	4	1

Numbers denote classification of the intactness of the collagen coating as described in "assessment of collagen adhesion to TPU" in the Experimental section

Table 3 Optical density measurements with the WST-1 assay ($n = 5$, $p < 0.05$) on no scaffold (negative control), uncoated TPU scaffold (positive control) and glutaraldehyde fixated collagen on TPU scaffold

Sample	Optical density ± SD
Negative control	100 ± 8.7
Positive control	103 ± 9.6
Glutaraldehyde	96 ± 11.2

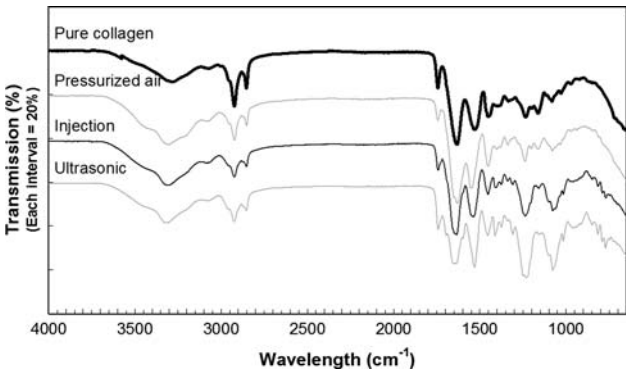

Fig. 4 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy spectra of the porous scaffold after coating with collagen with different techniques

The collagen coating was successfully and evenly distributed in the porous TPU structure (Fig. 5). The staining confirmed that this coating was uniform (Fig. 5, a and b) with the exception of an area in image B, where a region at the bottom right corner shows uncoated TPU. Interestingly, more collagen was found at the rougher surface edges as the staining color is more profound for these regions. A SEM image shows the coating inside the pores after coating with the injection method (Fig. 5, c), which also demonstrates a uniform coating. Arrows show interconnective pores which were closed by the coating.

However, the coverage of the collagen differed depending on the technique used, and the success of the coating cannot be judged merely from sliced images. Thus a large number ($n = 25$) of TPU scaffolds were coated using different techniques and the percentage of scaffolds with collagen-coated pores was determined by staining. The result is displayed in Table 4. The best coating technique was found to be injection of the collagen solution into the porous scaffold. With this method coated pores were found in 100% of the scaffolds tested. The pressurized air method was significantly slightly less successful with coated pores achieved in only 12.5% of cases. The ultrasonic technique was unsuccessful for all samples.

The injection method may have been superior by displacing air bubbles from the scaffold pores more successfully than the ultrasonic and pressurized air methods.

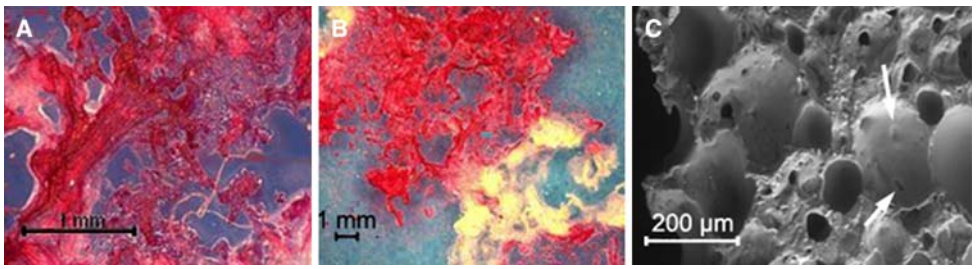

Fig. 5 Sliced and stained TPU scaffolds coated with collagen by injection (**a** and **b**). SEM image of a cross-section of the collagen-coated TPU (**c**). The collagen is evenly distributed with the exception of the bottom right corner of image B. In this region, the pores were not interconnected, and the injected collagen solution was unable to coat the closed pores. After slicing, these pores became accessible. Image **c** also shows that the collagen solution closed pores (marked with *arrows*)

Table 4 Comparison of different coating techniques ($n = 8$)

Coating type	Percentage coated
Ultrasonic	0
Pressurized air	12.5
Injection	100.0

4 Conclusion

This study verified that urea was an effective wetting agent for TPU surfaces, and the coating of TPU with an aqueous collagen solution was feasible. Surface treatment with urine for 24 h lowered the contact angle on the TPU from 102.57° to 20.24°. This reduction facilitated the coating of TPU with a collagen with a thickness between 20 and 40 µm. The coating was verified by spectroscopy (ATR FT-IR).

The collagen needed to be cross-linked prior to in vivo use. Three methods were compared, glutaraldehyde, thermal and CMC cross-linking on TPU sheets. The adhesion of the collagen layer to the surfaces of the TPU foils failed for all cross-linking methods through the Tape-test (EN ISO 2409) and in all cases was completely torn away. A dissolving test in Ringer's solution showed that the collagen layer was intact after 14 days of immersion. The FT-IR spectra from the different cross-linking methods were compared with non-crosslinked collagen. The chemical alteration of the collagen was observed for the CMC treatment and was found to be dependent of the CMC concentration. On the other hand it was not clear on the basis of the FT-IR curves whether cross-linking took place by means of CMC, and hence this method is not to be recommended. Nor was any connection found between increased concentration of CMC and cross-linking within the collagen. Cross-linking by the thermal and glutaraldehyde methods, however, was proved using FT-IR. However, irregular IR peaks were found for the thermal cross-linking. Cross-linking of the collagen layer with glutaraldehyde seemed to be superior to the other methods. The use of humane urine as a wetting agent and the chemical glutaraldehyde had no effect upon a cytotoxicity test performed by a WST-1 assay with a fibroblastic cell line.

Three different coating techniques for porous TPU scaffolds were investigated. Aqueous collagen solution was forced into the scaffold by three means, needle injection, use of pressurized air and ultrasonic pulses. FT-IR investigation confirmed that the collagen remained chemically unchanged after all three coating methods. Staining with Azocarmine G followed by light-microscope investigations demonstrated the presence of collagen in the open pores. SEM imaging also showed the collagen coating. Some of the interconnected pores were closed due to the coating.

Injection seemed to be the best coating method and resulted in coating of the open porous region within the scaffolds in 100% of cases ($n = 8$). The pressurized air method achieved coverage in 12.5 % of cases ($n = 8$), whereas the ultrasonic method failed in all cases ($n = 9$).

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