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Biodegradable fiducial markers for X-ray imaging – soft tissue integration and biocompatibility

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This study aims at the development of materials for biodegradable fiducial markers for X-ray based medical imaging and their anchorage in soft tissue. Towards this goal a degradable polymer matrix of poly(L-lactide-co-ε-caprolactone) (P[LAcOCL]) was combined with barium sulfate (BaSO₄) and hydroxyapatite (HAp) as radio-opaque fillers. Low pressure plasma treatment was applied to the composite materials to improve cell adhesion and subsequent tissue integration. In particular, the effects of oxygen and ammonia plasmas were evaluated and compared using X-ray photoelectron spectroscopy, atomic force microscopy and dynamic water contact angle measurements as well as *in vitro* studies using the murine fibroblast cell line L929. To exclude the cytotoxic effects of degradation products of P[LAcOCL] and released BaSO₄ or HAp cytotoxicity assays with the degradation products of the composite materials were conducted. The results obtained by this broad range of analytical techniques suggest the application of composites of P[LAcOCL] with BaSO₄ and HAp as promising material systems for innovative fiducial markers for soft tissue in X-ray based medical imaging.

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1 Introduction

Materials which give a positive or negative contrast during medical imaging are needed for manufacturing various medical devices, *e.g.* for catheters,^{1,2} stents^{3,4} or fiducial markers, that are used to mark the corresponding region of interest in the human body.^{5–7} This attribute facilitates the correct implantation of the device⁴ and gives additional opportunities. These devices can also be used as a point of reference which appears in the medical imaging system, *e.g.* in standard⁸ or real-time tumor tracking⁶ radiotherapy and in surgical navigation systems.⁹

Some of the devices, *e.g.* fiducial markers, are used only for marking during the treatment or therapy. The great majority of commercially available fiducial markers are made of materials that are non-degradable in the human body.^{6,10,11} Therefore, it is required to remove them surgically after therapy. The usage of biodegradable materials supercedes a further surgical intervention. They can degrade *in vivo* and the degradation products can be moved away from the site of implantation.^{12,13}

Synthetic biodegradable polymers, mainly aliphatic polyesters such as polyglycolide (PGA), polylactide (PLA), poly(ε-caprolactone) (PCL), their copolymers and blends, have been widely investigated for their utilization in tissue regeneration as scaffolds that provide the necessary support for cells and tissues.^{14–23} They undergo degradation by random hydrolytic cleavage of ester bonds *in vivo*.^{13,24} They can also be used as contrast devices *e.g.* in X-ray computed tomography imaging, if some radio-opaque component is added.^{25–27} The usage of such materials as fiducial markers also requires appropriate anchorage in the tissue to exhibit sufficient immovability after insertion in the body.^{28–30} This can be obtained by manufacturing the materials in a specific macroscopic shape and also by improving the cell adhesion, *i.e.* changing the characteristics of the material *via* surface modification.

Cell adhesion is preceded by wetting with biological fluids and subsequent protein adsorption on the biomaterial surface.³¹ For that reason it is possible to optimize cell attachment, alignment and spreading by tuning surface characteristics like chemical composition,³² wettability,³³ or roughness.³⁴ In this sense low pressure plasma treatment of polymer materials can be used to modify surface properties and promote the initial interaction with adherent cells and encourage cells to increase their contact area with the surface.^{32,35,36}

In this study a poly(L-lactide-co-ε-caprolactone) (P[LAcOCL]) matrix with barium sulfate (BaSO₄) and hydroxyapatite (HAp) as radio-opaque fillers was tested as a potential material system with improved cell adhesion and biological response for

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biodegradable fiducial markers in X-ray based medical imaging techniques of soft tissue. The copolymer of P[LAcoCL] has good thermal properties which enable good processability,^{37,38} appropriate degradation rate and mechanical properties suitable for soft tissue. Two last statements are important due to the application – proper stability in the human body is required for at least 6 months and low elastic modulus is an advantage because of better cell response.³⁹

Even though all components are well known the idea of composition and application has not been studied comprehensively. Moreover improvement of the properties of known materials and development of new materials made from them are very valuable in the biomaterial field. As many people are using similar materials in their research, the idea of easily available composition may solve their problems *e.g.* with monitoring of tissue engineering scaffolds *in vivo*. Other advantages of the composition are the relatively low cost of BaSO₄ and a high atomic number which generates good radiological contrast. There is also a group of scientists who reported about their advanced studies with biodegradable pancreatic stent which consists of BaSO₄.^{40,41} However, some scientists suspected that larger doses of water insoluble BaSO₄ can be hazardous or can accumulate in the body.^{42,43} Therefore, HAP which is biocompatible and soluble in water^{44,45} was used to decrease the amount of BaSO₄ and to obtain the radio-opaque properties comparable to the natural contrast material in the human body – bone. Hydroxyapatite increases not only the radiopacity, but also changes the surface area⁴⁶ and shows good cell adhesiveness due to excellent absorbability of cell-adhesive proteins,⁴⁷ and influences the degradation rate of the matrix material.^{48,49}

Beyond the *in vivo* degradation behaviour of the polymer matrix and the X-ray absorption by the filler material, a proper anchorage of the construct in a soft tissue environment is a crucial prerequisite for a fiducial marker. For that reason, the cell adhesion properties of the material surface were improved by low pressure plasma treatment. Advantage of this technique is the modification of the physico-chemical properties of the uppermost few nanometers of a polymer material without changing its bulk properties.³⁵ By choosing appropriate process parameters (process gas, plasma excitation, pressure, power, *etc.*) the introduction of functional groups dominates over inevitable etching effects. For the purpose of this study microwave excited oxygen (O₂) and ammonia (NH₃) plasmas were evaluated and compared. While O₂ plasma is especially popular and widely available with rather simple devices, NH₃ plasma is known for a better stability of the obtained modification effect, *i.e.*, less pronounced hydrophobic recovery, on similar polymeric materials.³⁶ Because the scope of the study is strongly connected with the surface characteristics, a comprehensive set of surface analytical techniques comprising X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and dynamic water contact angle measurement (DCA) was employed to characterize the impact of different plasma treatments. The net effect on cell adhesion was evaluated by culturing the adherent murine fibroblast cell line L929 as a model for the soft tissue environment of a fiducial marker. Evaluation of the materials cytotoxicity was performed according to ISO 10993-5.

Table 1 Compositions of materials

Material	Composition [wt%]		
	P[LAcoCL]	BaSO ₄	HAp
P[LAcoCL]	100	—	—
P[LAcoCL]1020	70	10	20

2 Materials and methods

Polymeric and composite samples were prepared by the solvent casting technique from P[LAcoCL] with a molar ratio of 70 to 30 (RESOMER[®] LC 703 S, Evonic, Germany) of BaSO₄ (0.5 µm, Acros Organics, Geel, Belgium) and HAp (particle size 33 nm, agglomerate size 5 µm, Ca₅(OH)(PO₄)₃, Merck) by dispersion of the powders in chloroform (CHCl₃, Poch S.A., Gliwice, Poland) and subsequently dissolving the polymer. The compositions of prepared materials are presented in Table 1. The plates prepared by solvent casting were first dried in air at room temperature and then in a vacuum dryer (*T* = 35 °C, *p* = 100 mbar) for three days. The obtained plates had a thickness of 1 mm.

2.1 Microcomputed tomography (microCT)

The extruded 1 mm diameter pins of both materials (Table 1) were placed in a piece of pork neck containing both adipose and muscle tissues. The meat with pins was closed in a polypropylene probe and examined by microCT on a SkyScan 1172 system. The scanner was set at 70 kV and 141 µA. The scan was performed over 180 degrees with a rotation step of 0.4 degree and a frame averaging of 4. A 0.5 mm aluminium filter was used. Additionally, ring artefacts were reduced through selection of a random movement amplitude of 50. The image pixel size was 8.35 µm. Reconstruction of images was done by using the SkyScan NRecon software.

2.2 Model system

To improve the applicability of analytical techniques, model surfaces of the respective materials were prepared. For cell culture experiments transparent, flat and clean samples are required. Flat samples enable for an easy characterization of the modified surfaces, *e.g.* by XPS, DCA measurements and also the analysis of cell cultures by fluorescence imaging methods.

P[LAcoCL] and P[LAcoCL]1020 1% w/v solution/dispersion in CHCl₃ were prepared at 25 °C. Commercially available silicon wafers (10 × 20 mm²) and glass coverslips (*d* = 20 mm) were used as substrates for thin film preparation by spin coating (RC5 by Karl Suss, Saint-Jeoire, France) in a clean room. The parameters of the spin coating process were: velocity 5000 rpm, acceleration 5000 rpm s⁻¹, duration 30 s. Small silicon wafers (5 × 5 mm²) were used to prepare samples for XPS by solvent evaporation of a drop of the same solution on it.

2.3 Low pressure plasma treatment

For plasma treatments a computer controlled MicroSys apparatus (Roth & Rau, Wüstenbrand, Germany) was used. The cylindrical vacuum chamber, made of stainless steel, has a diameter of 350 mm and a height of 350 mm. The base pressure obtained

Table 2 Parameters applied for low pressure plasma treatment

Process gas	O ₂	NH ₃
Power [W]	250	800
Duty cycle [%]	100	25
Gas flow [standard cm ³ min ⁻¹]	15	15
Pressure [mbar]	7 × 10 ⁻³	7 × 10 ⁻³
Time of treatment [s]	7, 15, 30	30, 60, 120, 240

with a turbomolecular pump is less than 10⁻⁷ mbar. On the top of the chamber a 2.46 GHz electron cyclotron resonance (ECR) plasma source RR160 (Roth & Rau) with a diameter of 160 mm and a maximum power of 800 W is mounted. The plasma source can be operated in a pulsed mode with different duty cycles. The process gas is introduced into the active volume of the plasma source *via* a gas flow control system. When the plasma source is on, the pressure is measured by a capacitive vacuum gauge. The samples are introduced by a load lock system and placed on a grounded holder near the centre of the chamber. The distance between the sample and the excitation volume of the plasma source is about 200 mm. Low pressure O₂ and NH₃ plasma treatments were carried out. The used parameters are presented in Table 2.

2.4 X-ray photoelectron spectroscopy (XPS)

XPS was carried out using an Amicus spectrometer (Kratos Analytical, Manchester, UK) equipped with a non-monochromatic Mg K_α X-ray source operated at 240 W and 8 kV. The kinetic energy of the photoelectrons was determined using an analyser with a pass energy of 75 eV. The take-off angle between the sample's surface normal and the electron-optical axis of the spectrometer was 0°. Under these conditions the information depth is approximately 8 nm. Spectra were referenced to the C_{1s} peak of aliphatic carbon at a binding energy of 285 eV.⁵⁰ A satellite subtraction procedure was applied. Quantitative elemental compositions were determined from peak areas using experimentally determined sensitivity factors and the spectrometer transmission function (typical accuracy ± 0.5%). C_{1s} spectra were deconvoluted by means of the software CasaXPS (Casa Software Ltd, Devon, UK). The fit procedure was allowed to vary component energies, component intensities and a common value for the full width at half maximum.

2.5 Atomic force microscopy (AFM)

The topography of the samples was examined using an atomic force microscope MFP 3D Bio (Asylum Research, Oxford Instruments, Santa Barbara, USA) operating in tapping mode. All images were obtained in air at room temperature. Surface roughness was calculated as an arithmetic average of the absolute values of the profile height deviations from the mean line, *R_a*, of 5 × 5 μm² scanning area. O₂ and NH₃ plasma modified P[LAcOCL] samples, as well as untreated samples, were measured in five different areas.

2.6 Dynamic contact angle measurements (DCA)

Dynamic water contact angle measurements were performed to analyse changes in wettability of the model surface upon

plasma treatment. The sessile drop method was carried out using the video-based optical contact angle meter (OCA 30, Dataphysics, Germany). Subsequently, the volume of a water drop of 10 μl was changed with a rate of 0.1 μl s⁻¹ to determine advancing (Θ_A) and receding (Θ_R) contact angles.

2.7 Cell culture and analyses

2.7.1 Culture and subculture of cells. The murine fibroblast cell line L929 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in RPMI medium 1640 (1×) with GlutaMAXTM supplement (RPMI, ThermoFisher Scientific, Darmstadt, Germany) plus 10% v/v fetal bovine serum (FBS, Merck Millipore, Berlin, Germany) and 1% v/v penicillin-streptomycin (PS, Sigma-Aldrich, Steinheim, Germany). When the cells reached subconfluence they were subcultured by incubating them for 3 min at 37 °C with 0.05% v/v trypsin and 0.02% v/v ethylenediamine-tetraacetic acid (Sigma-Aldrich). Cells were collected in growth medium, centrifuged at 1000 rpm for 5 min in the Labofuge 400 R (Heraeus, ThermoFisher Scientific) and seeded at a density of 2 × 10⁵ cells per growth area of 75 cm² in culture flasks with a filtered cap (TPP, Trasadingen, Switzerland). L929 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Growth medium was changed three times per week.

2.7.2 Cell adhesion assay. The model system described above was used for cell adhesion analysis. The samples prepared by spin coating on glass coverslips and modified by plasma treatment were sterilized by UV light treatment for 20 min and put into sterile 12-well plates (Corning Costar/ Sigma-Aldrich). L929 cells grown to subconfluence were subcultured by trypsinization and seeded onto the samples at an initial cell density of 1 × 10⁴ cells per cm². The samples with the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cell adhesion to the model surface was evaluated by (a) assessing the spreading of cells (ratio of the number of elongated and flat cells to the total number of all cells) by transmission light microscopy (Olympus IX73, Hamburg, Germany) and (b) determining the number of nuclei of the cells attached to the model surface after flushing with Dulbecco's PBS (Merck Millipore, Berlin, Germany). Staining was performed with the Hoechst 33342 dye (ThermoFisher Scientific). Hoechst stock solution (10 mg ml⁻¹ in deionized water) was used to prepare 2 μl ml⁻¹ Hoechst staining solution in Dulbecco's PBS. Cells were washed in Dulbecco's PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min, washed with Dulbecco's PBS and then incubated in staining solution for 10 min. Observation was carried out using a fluorescence microscope (Observer.Z1, Zeiss, Jena, Germany). Both commercially available adhesive 12-well plates (TPP) (evaluation (a)) and glass coverslips spin coated with polystyrene treated in O₂ plasma (PSO2)⁵¹ (evaluation (b)) served as reference surfaces for assessing the percental content of adhered cells. Three samples of both materials P[LAcOCL] and P[LAcOCL]1020 after plasma modification were analysed. Statistical analyses were performed using ANOVA single factor analysis of variance (alpha = 0.05), in which the values of

experimental groups were compared with the values of controls and within groups.

2.7.3 Cytotoxicity assay. Cytotoxicity experiments of P[LAcoCL] and P[LAcoCL]1020 plates were carried out using the murine fibroblast cell line L929 as advised by ISO 10993-5. The first part of the assay was to obtain extracts from UV-sterilized (20 min per each side) material pieces ($15 \times 5 \times 1 \text{ mm}^3$). For this, incubation in a closed container for two and eight weeks in culture medium (RPMI w/10% v/v FBS and w/1% v/v PS) at 37°C was performed. The ratio of the mass of the test sample to the volume of culture medium was 50 mg:1 ml. In order to avoid accumulation of degradation products which could accelerate hydrolysis^{44,45} and to eliminate the negative effect of not fresh culture medium, the medium was changed every two weeks during this part of experiment. There were three samples per each time point. Additionally, pure culture medium was stored in a closed container in the incubator for the same duration and then used to obtain four logarithmic dilutions of the extracts ($c = 1, 1/10, 1/100, 1/1000$). Medium with extract, named treatment medium, was used for treating the cells in the cytotoxicity tests.

L929 cells were seeded at a concentration of 1×10^4 cells per well in a 96-well plate. The cells were cultured for 24 hours with the culture medium. Afterwards, the culture medium was aspirated and changed against the treatment medium containing the (diluted) material extracts. Each sample was triplicated. Cells that were treated with fresh culture medium without extracts served as control samples. Cell growth was observed by transmission light microscopy (Olympus IX73) after 24 hours. Then the treatment medium was removed and a viability assay was carried out using the WST-1 reagent according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany). The WST-1 assay provides measurement of cell proliferation and consequently the viability of cells. For the assay 100 μl of a 10% v/v WST-1 reagent in RPMI per each well were used. The samples were incubated for 55 min at 37°C . The measurement of absorbance at 450 nm was performed using a plate reader (GENios, TECAN, Crailsheim, Germany). The cell viability was calculated according to eqn (1).

$$\text{Viability}[\%] = \frac{\text{OD}_{450}}{\text{OD}_{450c}} \times 100\% \quad (1)$$

OD_{450} – mean value of the measured optical density of treated samples, OD_{450c} – mean value of the measured optical density of control samples.

Toxic potential was confirmed when the viability of cells was reduced to <70% of control.

The cytotoxicity test was performed for the pure fillers (BaSO_4 and HAp), too. Extracts were obtained by incubating the autoclaved powders in the culture media at a concentration of 25 mg ml^{-1} for three days at 37°C . Three samples of each type of powder were tested. Additionally, each sample was triplicated for WST-1 assay which was performed as described above.

Statistical analyses were performed using ANOVA single factor analysis of variance ($\alpha = 0.05$), in which the values of experimental groups were compared with the values of controls and within groups.

Fig. 1 MicroCT reconstruction of P[LAcoCL] and P[LAcoCL]1020 pins in the surrounding tissues and air.

3 Results and discussion

3.1 X-ray based imaging

The results of microCT (Fig. 1) confirmed the theory that pure polymers need the addition of radio-opaque components to be visible in soft tissue.^{25–27} The presented image showed very low contrast between muscle tissues and P[LAcoCL]. Whereas composite material P[LAcoCL]1020 was easily noticeable in the surrounding tissue.

3.2 Preparation of the model system

Since the interaction between cells and materials depends on the properties of the material surface, it is necessary to evaluate the surface characteristics. In this context, the preparation of thin films of the materials as model systems allowed for the detailed analysis of the surface characteristics. By spin coating the dissolved/dispersed materials were obtained as thin films on either silicon wafers or transparent glass coverslips. Following this route, transparent and flat surfaces which are needed for live cell observation by transmission light microscopy were obtained. Moreover, optimizing the parameters of the spin coating process of the composite materials enabled obtaining model surfaces with the same chemical composition and wetting properties in comparison to plates prepared by solvent casting techniques (Table 3). Presumably, BaSO_4 and HAp particles were covered by the P[LAcoCL] matrix and for that reason Ba and Ca atoms

Table 3 Comparison of preparation of P[LAcoCL]1020 samples by spin coating and solvent casting

	XPS				DCA	
	Atomic composition [at%]				Contact angle [$^\circ$]	
	C	O	Ba	Ca	θ_A	θ_R
Spin coating	66.6	33.3	—	0.1	81.7 ± 1.9	46.6 ± 0.7
Solvent casting	67.6	32.4	—	—	81.5 ± 3.9	50.5 ± 1.3

were out of range for XPS measurements (sampling depth is approx. 8 nm).

3.3 Surface modification and characterisation

O₂ and NH₃ plasma treatments were carried out to improve cell adhesion and proliferation on the composite surface of fiducial markers. As a first step, both treatments were applied to well-defined model surfaces of the matrix material P[LAcOCL] only. This ensured a better applicability of the analytical techniques and thereby more reliable results. Subsequently, the impact of the filler dispersed in P[LAcOCL] was evaluated to conclude on the behaviour of the marker's composite surface.

The untreated P[LAcOCL] model surface closely resembled the atomic composition expected from the structural formula of the copolymer (Fig. 2a). This also applied to the binding energies and relative intensities of the XPS C_{1s} components (Fig. 2b, Table 4, superscripts in the structural formula indicate the assignment of chemical environments to the three components of C_{1s} peak deconvolution). The untreated material was found to be rather hydrophobic with an advancing and receding water contact angle of $\theta_A = 75.1^\circ \pm 0.6^\circ$ and $\theta_R = 59.3^\circ \pm 0.5^\circ$, respectively. The small contact angle

hysteresis $\theta_A - \theta_R = 15.8^\circ \pm 0.8^\circ$ of the untreated material was consistent with the very low roughness of less than 0.4 nm (R_a).

Table 4 and Fig. 3 illustrate the surface analytical results of O₂ plasma treatment. Here, the most prominent effect was a change in wetting behaviour that was fully established after a treatment time of 7 s and remained constant for longer treatments. The large increase of contact angle hysteresis from $15.8^\circ \pm 0.8^\circ$ to $54.3^\circ \pm 1.0^\circ$ is hard to explain only in terms of roughness which increased slightly but did not exceed 1 nm. However, a supposed change in surface chemistry (which is also known to contribute to an increased hysteresis due to added heterogeneity) is not evident in XPS data. This can occur when the untreated polymer structure comprises a certain amount of oxygen. In this situation the equilibrium between functionalization and degradation, which is established during oxygen plasma treatment, can leave behind the same O:C ratio as before along with a similar C_{1s} peak shape. Therefore, the only indirect hint to a modified surface chemistry is the hydrophobic recovery⁵² that was clearly observed after 30 days.

The effect of NH₃ plasma treatment is shown in Table 4 and Fig. 4. Contrary to O₂ plasma, the atomic composition changed remarkably. A decrease of the O:C ratio went along with an introduction of up to 2 at% nitrogen. A pronounced impact on

Fig. 2 (a) Structural formula of P[LAcOCL]. Superscripts indicate different chemical environments of carbon. (b) High resolution XPS C_{1s} and N_{1s} spectra of untreated P[LAcOCL].

Table 4 XPS results of surfaces before and after O₂ and NH₃ low pressure plasma treatment

Sample	Plasma gas	Time of treatment [s]	Atomic composition [at%]					C _{1s} components [%]		
			C	O	Ba	Ca	N	A	B	C
Theoretical composition calculated from the structural formula of P[LAcoCL] 70:30	—	—	66.5	33.5	—	—	—	48.7	25.6	25.6
P[LAcoCL]	—	0	68.1	31.9	—	—	—	44.6	27.2	28.2
P[LAcoCL]-O ₂ -7s	O ₂	7	67.4	32.6	—	—	—	45.9	26.5	27.6
P[LAcoCL]-O ₂ -15s	O ₂	15	67.2	32.9	—	—	—	45.3	26.6	28.1
P[LAcoCL]-O ₂ -30s	O ₂	30	67.6	32.4	—	—	—	46.4	26.1	27.5
P[LAcoCL]-NH ₃ -30s	NH ₃	30	71.3	28.1	—	—	0.5	58.2	20.7	21.1
P[LAcoCL]-NH ₃ -60s	NH ₃	60	71.7	27.6	—	—	0.8	60.2	20.0	19.9
P[LAcoCL]-NH ₃ -120s	NH ₃	120	71.4	27.6	—	—	1.3	60.6	20.2	19.2
P[LAcoCL]-NH ₃ -240s	NH ₃	240	71.7	26.0	—	—	2.2	63.1	19.9	17.0
P[LAcoCL]1020	—	0	66.6	33.3	0	0.1	—	44.5	27.2	28.4
P[LAcoCL]1020-NH ₃ -30s	NH ₃	30	68.8	30.8	0	0.1	0.3	49.9	25.0	25.1
P[LAcoCL]1020-NH ₃ -60s	NH ₃	60	69.6	29.9	0	0.1	0.4	52.6	23.4	24.0
P[LAcoCL]1020-NH ₃ -120s	NH ₃	120	71.1	28.0	0	0.1	0.8	59.8	17.7	22.5
P[LAcoCL]1020-NH ₃ -240s	NH ₃	240	71.3	26.9	0	0.1	1.8	63.9	14.7	21.5

Fig. 3 (a) High resolution XPS C_{1s} spectra of 30 s O₂ plasma treated P[LAcoCL]. (b) Advancing and receding water contact angles of P[LAcoCL] directly after O₂ plasma exposure with different treatment times and after 30 days of storage (hydrophobic recovery). (c) Roughness of the P[LAcoCL] model surface after O₂ plasma exposure with different treatment times.

the C_{1s} peak shape was observed (Fig. 4a vs. Fig. 2b). The decrease of the high energetic components B and C as well as the overall loss of oxygen suggest a degradation of the respective copolymer structures within the sampling depth of XPS (the expected signal for C–N is not considered as a separate component due to the low amplitude and the close proximity to component A). The change in wetting behaviour directly after plasma treatment is comparable to the case of O_2 plasma treatment. The receding contact angle decreased almost immediately and remained constant for longer treatment times. Again, a remarkable increase

in contact angle hysteresis indicates surface heterogeneities beyond roughness which amounts to 0.9 nm after plasma treatment. No hydrophobic recovery was observed within 30 days which is a known advantage of NH_3 plasma treatment of hydrocarbon polymer surfaces.³⁶

In summary, P[LAcOCL] surfaces modified in O_2 plasma showed only very slight changes in chemical composition. The roughness of polymer surfaces modified in O_2 and NH_3 plasmas was comparable (Fig. 5). Moreover, ammonia plasma treatment allowed introducing a considerable density of functional

Fig. 4 (a) High resolution XPS C_{1s} and N_{1s} spectra of 240 s NH_3 plasma treated P[LAcOCL]. (b) Advancing and receding water contact angles of P[LAcOCL] directly after NH_3 plasma exposure with different treatment times and after 30 days of storage. (c) Roughness of the P[LAcOCL] model surface after NH_3 plasma exposure with different treatment times. (d) Introduction of nitrogen by NH_3 plasma exposure with different treatment times.

Fig. 5 AFM images of P[LAcoCL] model surfaces: untreated (a) and treated with O₂ (b) or NH₃ (c) plasma; all AFM maps have the same scale in the Z-direction.

groups what was proved by the appearance of N atoms. Therefore ammonia plasma modification was chosen for detailed studies of composite materials for fiducial markers.

For the untreated composite material P[LAcoCL]1020 containing the fillers BaSO₄ and HAp a big contact angle hysteresis $\Theta_A - \Theta_R = 37.7^\circ \pm 0.5^\circ$ occurred. It was the result of the higher roughness of about 100 nm (R_a) caused by the particles of the fillers protruding from the surface and covered by a thin layer of polymer. It was also reported that the addition of hydroxyapatite radically changes the surface morphology which reflects *e.g.* in the mat surface roughness.⁴⁶ As the consequence the roughness of the composite material did not depend on plasma treatment time because the introduced nano-roughness in the polymeric matrix had a very low influence on this result. However, NH₃ plasma modification caused a strong decrease of Θ_R to $9.4^\circ \pm 0.3^\circ$ (30 s of treatment) (Fig. 6).

Fig. 6 Advancing and receding water contact angles of P[LAcoCL]1020 after NH₃ plasma exposure with different treatment times.

3.4 Cell adhesion

Spreading as part of the cell adhesion process was used as a parameter for comparing the different materials and their surface modifications with respect to their ability for stable integration into soft tissue (Fig. 7a and b). The obtained results were compared to the adhesion of L929 on 12-well plate growth area surfaces (Fig. 7c). After one hour approx. 60% of the cells seeded on the control samples were spread; after four hours and 24 hours almost 90% showed spread morphology (Fig. 7c).

In the case of untreated P[LAcoCL] samples approximately 60% of all attached cells were spread after 24 hours, while on treated P[LAcoCL]-NH₃-240s samples almost 80% and 90% of all cells were spread after four and 24 hours, respectively. L929 cells spread significantly faster on P[LAcoCL] samples after NH₃ plasma modification. The comparison of the results after one hour obtained for P[LAcoCL] and P[LAcoCL]-NH₃-240s showed a four times stronger promotion of cell spreading on the modified surface.

On the untreated composite material P[LAcoCL]1020 the percentage of spread cells was significantly lower than for the control surface. In contrast, an enhanced cellular spreading behaviour after four hours on composite surfaces modified for 60 s and 240 s with NH₃ plasma was observed. Increased treatment times appeared to cause more favourable properties for cell spreading. After one hour the cell spreading on P[LAcoCL]1020-NH₃-240s was two times lower than on the control sample, whereas after 24 h it was almost the same.

In the case of untreated P[LAcoCL]1020 the percentage of spread cells after one hour was slightly higher than for untreated P[LAcoCL], but after four and 24 hours the results were comparable. Different results were obtained by the determination of the number of attached cells *via* cell nucleus staining (Fig. 7d). After four and 24 hours the number of adhered cells to P[LAcoCL]1020 was approx. three times higher than to P[LAcoCL]. The percentage of spread cells was almost independent of the type of material. Only the treatment time was the parameter which caused a change in the number of spread cells. However the results presented in Fig. 7d showed a higher number of attached cells on modified P[LAcoCL]1020 than on modified P[LAcoCL]. Furthermore, the results presented in Fig. 7d confirmed the

Fig. 7 Cells on untreated P[LAcoCL] (a) and P[LAcoCL]-NH₃-240s (b) surface after 4 hours; black arrows highlight the examples of spread cells. (c) Percentage of spread cells after 1, 4 and 24 hours, (d) the number of adhered cells determined by staining of the cells nuclei; results are shown as % of control mean $\pm$ SD; * when ns indicate $p > 0.05$.

findings from the transmission light microscopy analyses shown in Fig. 6c regarding the strong increase in the number of adherent cells on modified surfaces. In the case of P[LAcoCL]-NH₃-60s the number of adhered cells was approx. four times higher than for untreated samples. For P[LAcoCL]1020 the difference between unmodified samples and samples after 60 s of NH₃ plasma modification was not so big but still easily noticeable. It was approx. 3200 and 5300 cells per cm² respectively. The reason for these observations could be the high surface roughness of P[LAcoCL]1020 (R_a approx. 100 nm) in comparison to P[LAcoCL], while the surface chemical composition is almost the same. Moreover, despite the similar surface roughness and wettability measured for a modified series of P[LAcoCL] (Fig. 4b and c) and P[LAcoCL]1020 (Fig. 6), cells attached and spread faster with increasing treatment time and introduction of N atoms. The maximal amount of N atoms in performed modifications was 2.2% (Fig. 4d, Table 4). This seems to be the optimum concentration of N atoms which is essential for protein adsorption and cellular adhesion.⁵³

Cell behaviour on P[LAcoCL] has recently been reported^{54–57} and it was not as good as for tissue culture polystyrene. The presented analysis of cell adhesion has shown that NH₃ plasma modification increased the number of spread cells on the surfaces for both materials P[LAcoCL] and the composite P[LAcoCL]1020. Furthermore, P[LAcoCL]1020-NH₃-240s after four hours led to an increased cell adhesion behaviour comparable to control samples and therefore would be favoured for future applications. Increasing the interaction between cells and the surface of the contrast material is needed not only in the case of fiducial markers but also in the case of different applications, like tissue engineering scaffolds (e.g. during *in vivo* monitoring^{58,59}).

3.5 Cytotoxicity assay

Fig. 8a and b show the influence of the degradation products of P[LAcoCL] and the composite material P[LAcoCL]1020 after the first two and eight weeks of degradation on cells. After incubation with the undiluted (C1) treatment medium from two-weeks of degradation of P[LAcoCL] the viability of the cells was approx. 100%. In the case of P[LAcoCL]1020, it was approx. 80%. Interestingly, the viability of cells decreases with a decrease of extract concentration. It is more clearly visible during the first 2 weeks of degradation. It can be explained by the cumulative effect of degradation products of P[LAcoCL] and the release of the residual monomers from the polymeric matrix,⁴⁵ which can influence the metabolic function of cells,^{60,61} e.g. being an energy substrate.⁶⁰ The viability of cells after treatment with degradation products from eight-week degradation was similar and high for both materials.

Higher cell growth inhibition and concentration of extract dependence occurred after the first period of extraction from P[LAcoCL]1020. It can be suspected that differences in the viability of cells after incubation in undiluted P[LAcoCL] and P[LAcoCL]1020 extracts were induced by the presence of fillers in the surface area of the composite materials. Despite the fact that the extract of BaSO₄ in the culture medium seems to be

Fig. 8 Cytotoxicity results of four logarithmic dilutions of extracts from: (a) P[LAcOCL], control = $100 \pm 8\%$; (b) P[LAcOCL]1020, control = $100 \pm 11\%$; (c) BaSO₄, control = $100 \pm 8\%$; (d) HAp, control = $100 \pm 7\%$; the results are shown as % of control mean $\pm$ SD; * – when P -value was $p < 0.05$.

non-toxic (Fig. 8c), the undiluted extract of HAp resulted in a cytotoxic effect (Fig. 8d). The absence of serious hazards in the presence of BaSO₄ was also observed in other works^{42,62–64} and the negative effect of the extracts of calcium phosphate ceramics was reported.^{65,66} This could be caused by the high concentration of calcium ions, which has been reported to be hazardous^{67–69} or by the uptake of HAp nanoparticles^{70,71} by cells. However, dilutions of extracts were not cytotoxic. Additionally, embedding the filler in the polymeric matrix led to a slow release of BaSO₄ and HAp which should be phagocytosed and removed from the implantation site.^{70,72} Nevertheless, the results obtained for the composite material P(LAcOCL)1020 were satisfactory.

Degradation products of the tested composite material may cause a slight negative effect on cells. However a continuous circulation of body fluids in living organisms is suspected to remove the by-products (L-lactic acid, ϵ -hydroxy caproic acid, BaSO₄, HAp and their solubility products) from the implantation site.^{12,73–76}

4 Conclusions

Polymer matrices of poly(L-lactide-co- ϵ -caprolactone) with barium sulfate and hydroxyapatite as radio-opaque fillers were identified as promising material systems for biodegradable fiducial markers in X-ray based medical imaging. No cytotoxic effects were found in systematic assays according to ISO 10993-5. The crucial problem of proper marker anchorage in soft tissue applications was addressed by low pressure plasma surface modification of the marker corpus. Beyond a solely shape-based fixation, this strategy facilitates an improved cell-surface interaction. Multi-technique surface

analytical studies of plasma-treated P[LAcOCL] revealed ammonia plasma as the superior option compared to the more common oxygen plasma.

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