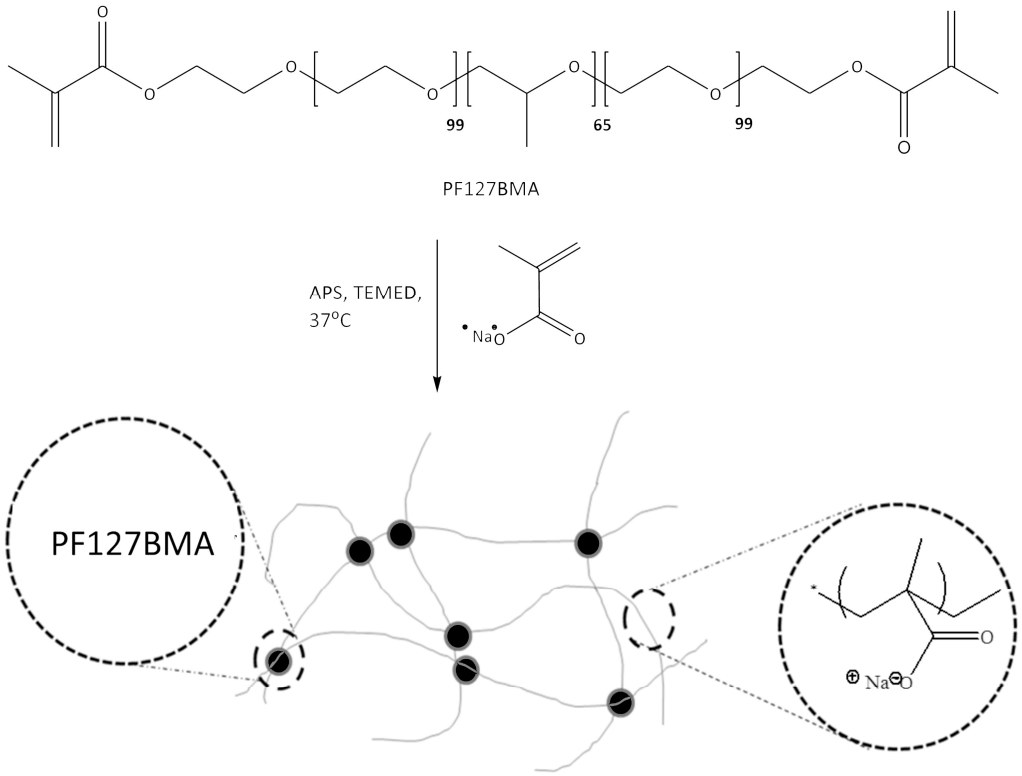

Figure S1 Schematic illustration of PF-127, methacrylic acid sodium salt and polymerized PLMANa hydrogel.

Preliminary biocompatibility tests:

Cell toxicity test

Culture conditions were as follow: Mouse L929 fibroblasts were obtained from European Collection of Cell Cultures (ECACC No. 88102702) and routinely cultured in minimal essential medium supplemented with 10% fetal calf serum, 1% MEAA, 1% penicillin G, 1% streptomycin sulphate and 10 µl/ml L-glutamine. Cultures were maintained in an atmosphere of 5% CO_2 in air at 37 °C, and media was changed every 48 h.

The in vitro cytotoxicity was evaluated by means of the elution test method (extract testing), ISO 10993-5[1], exposing fibroblasts grown to near confluence to fluid extracts from the material under investigation. The cells were cultured for 24 hours until subconfluence in a 96-well microplate; thereafter the medium was exchanged with the extract of the test samples. These extracts were obtained by placing PLMANa hydrogel samples (50x50x5mm) in 25 ml of supplemented culture medium for 24 hours at 37°C. Dilutions (1/1, 1/2, 1/8, 1/10, 1/100, 1/1000) were performed with sterilized culture medium. As negative control, culture medium was used and as positive control, phenol 6,4g/L. For each condition six samples were tested. After 24 h of incubation cell viability was assessed with the quantitative colorimetric MTT

assay (Vybrant MTT Cell Proliferation Assay Kit, Molecular Probes).[2][3] Cell viability was reported as percentage with respect to control.

For the direct testing, cells seeded with a concentration of about 5 x 10^4/ml were cultured 24 hours in a 6-well plate. PLMANa samples (10x10x2mm), being submersed in culture medium for 24 hours at 37°C, were placed in each well and cultured for another 24 hours. To assess viability with the Countess Automated Cell Counter (Invitrogen, Carlsbad, CA, US), the sample was removed and the cells were detached by trypsinization. To assess cell morphology the well plates were fixated with formalin for 5 minutes and stained with crystal violet. Morphology was evaluated microscopically and changes in general morphology, vacuolization, detachment, and cell lysis were evaluated. As a negative control glass discs of comparable size were used. For the positive control we used phenol dissolved in culture medium (6.4g/L).

The direct cytotoxity testing focuses on the toxic features of the entire hydrogel, including the materials surface properties which are particularly important in the interaction of a biological environment and artificial materials.[4] Extract experiments can give an indication on cytotoxic or haemolytic components leaching out the polymer. As residual unreacted monomers, oligomers and initiators might leach out after polymerization.[5] Figure S2 shows fibroblast viability after 24 hours of incubation in an extract of the hydrogel. Viability after contact with an extract of PLMANa is 100% in relation to the negative control. Viability of the positive control, phenol, is low till a dilution of 1 over 8. This clearly indicates that no toxic compounds are released from the gel.

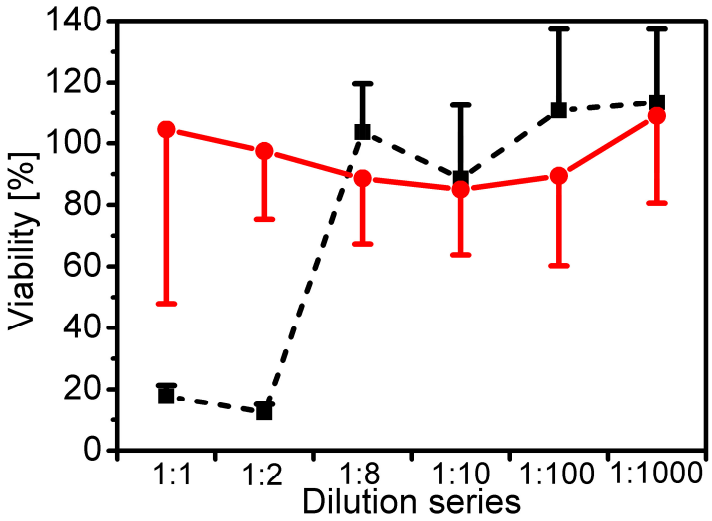

Figure S2 Viability, with standard deviation, of cell culture after contact with dilution series of extract of PLMANa (red-solid line), versus the positive control (black-dashed line).

As illustrated in Figure S3, assessment of viability after direct contact of the hydrogel showed a viability of 64% (SD: ± 27), which is comparable to the negative control (glass) - 89% ± 5.6. Viability is higher with respect to the positive control (phenol dissolved in culture medium) - 13.3% ± 3.5. This again, gives direct evidence that PLMANa gel itself is non-toxic.

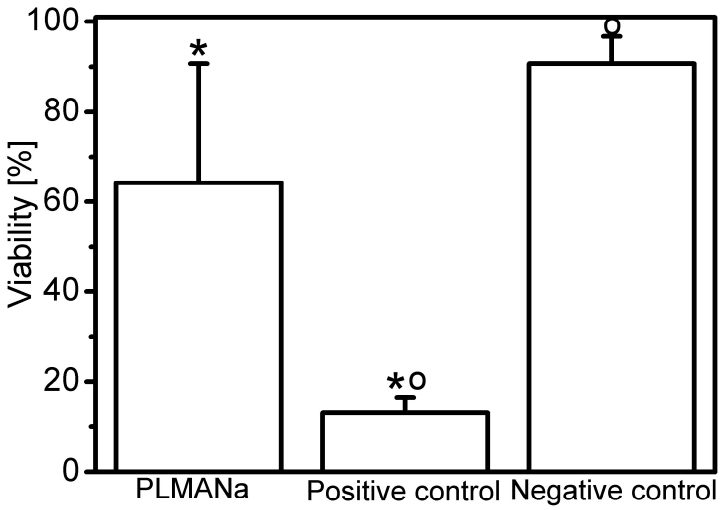

Figure S3 Mean viability, with standard deviation, after direct contact of the sample with cultured cells (*, °: p-value < 0,05).

Morphological evaluation, as seen in Figure S4a, showed low reactivity for the direct contact test. Around the sample there was no detectable zone of limited cell growth or malformed cells. Under the specimen there was a reduction in cell growth with an increase of round and loosely attached cells. These changes were not visible in the glass control. There was no cell growth on top of the gel sample. Morphological evaluation of the glass control depicted normal morphology, even underneath the glass disc. In the toxic (positive) control there was a nearly complete destruction of the cell culture with detachment of the fibroblasts and cell lysis (Figure S4b).

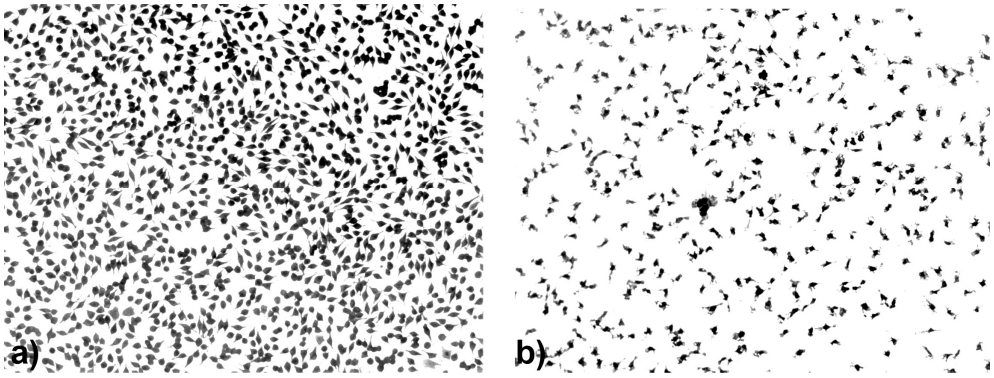

Figure S4 Fibroblast culture after direct contact with hydrogel (a), or with positive control (b) (Crystal violet staining, magnification 10x).

Haemolytic test

In order to determine free haemoglobin concentration the Drabkins reagent method is used in accordance with the international standard (ASTM F756-08).[6] The Drabkins reagent converts haemoglobin to the corresponding cyanoderivative.[7] Fresh human blood, from three blood donors, was anticoagulated with acid citrate dextrose. The free plasma haemoglobin concentration was lower than 2 mg/ml (0.575 mg/ml). The blood was diluted with an appropriate amount of calcium- and magnesium-free PBS to adjust the total haemoglobin content to 10 ± 1 mg/ml. For the indirect test and extract test a haemolytic index was calculated using the following equation:

$$\%\text{Haemolysis} = \frac{(\text{Hb test sample - Hb negative control})}{(\text{Hb positive control - Hb negative control})} \times 100\%$$

To test the extract, a 0.1 ml of diluted blood was added to 0.7 ml of the extract. The solution was then placed in a test tube rack for 3 hours at 37°C. Each tube was gently inverted three times every 30 minutes to maintain a good contact of the blood and the extract. At the end of the incubation time the samples were centrifuged for 15 minutes at 750 G. The supernatant was carefully removed and 0.250 ml was added to 0.250 ml of Drabkins solution. This mixture was allowed to stand for 15 minutes at 37°C. Afterwards 0.1 ml was placed in a 96 well plate and the absorbance of the solution was recorded with a spectrophotometer at a wavelength of 540 nm. The haemoglobin concentration of the supernatant was calculated using a standard curve. Six test samples, 6 positive controls (water), and 6 negative controls, extract of high-density polyethylene (HDPE), were evaluated.

In the direct testing a PLMANa samples of 10x10x2 mm were immersed in a mixture of 0.7 ml PBS and 0.1 ml of diluted blood for 3 hours at 37°C. Each tube was gently inverted three times every 30 minutes. The rest of the methodology is similar to indirect haemolytic testing. Six test samples, 6 positive controls (water), and 6 negative controls (HDPE) were evaluated.

Indirect and direct testing of the hydrogel showed no haemolysis. As shown in Table 3, the amount of free haemoglobin after contact with an extract of the gel or the gels surface was comparable with the negative control for this test - HDPE.

Table S1 Averages and standard deviation of the haemoglobin concentration (mg/mL) of the test hydrogel sample, positive (phenol), and negative (HDPE) control after indirect and direct haemolysis testing, and percentage of haemolysis of the hydrogel in respect to the positive control.

	[Hb] PLMANa	*[Hb] Pos. control*	*[Hb] Neg. control*	*Haemolysis (%)*
Indirect	0.30 ± 0.006	1.35 ± 0.015	0.30 ± 0.007	0 %
Direct	0.30 ± 0.016	1.40 ± 0.017	0.30 ± 0.006	0 %

Protein adhesion test

To test, protein adhesion, PLMANa samples were placed into 12-multiwell plates and incubated in 2 ml of a 0.05M bovine serum albumin (BSA) solution in PBS at 37°C for 1 h.[8] After an incubation period the sample was carefully rinsed twice with 2ml of PBS, and then placed in a new 12-multiwell plate. Two millilitres of a SDS 1% (w/v) aqueous solution was added to each well and kept under stirring for 1 hour at 37°C to remove the absorbed proteins from the surface. The amount of proteins was determined by the Qubit method (Invitrogen, Carlsbad, CA, US). The experimental data were reported as the protein concentration per unit sample's surface (μg/ml/mm^2). Six hydrogel samples were tested (10x10x2 mm), 6 HDPE control samples (10x10x1 mm), and 6 circular glass samples (10x0.1 mm).

Evaluation of albumin adhesion to the PLMANa surface shows, as depicted in Figure S5, that the amount of protein adhesion is low (3.8 μg/ml/mm^2 ± 0.70).

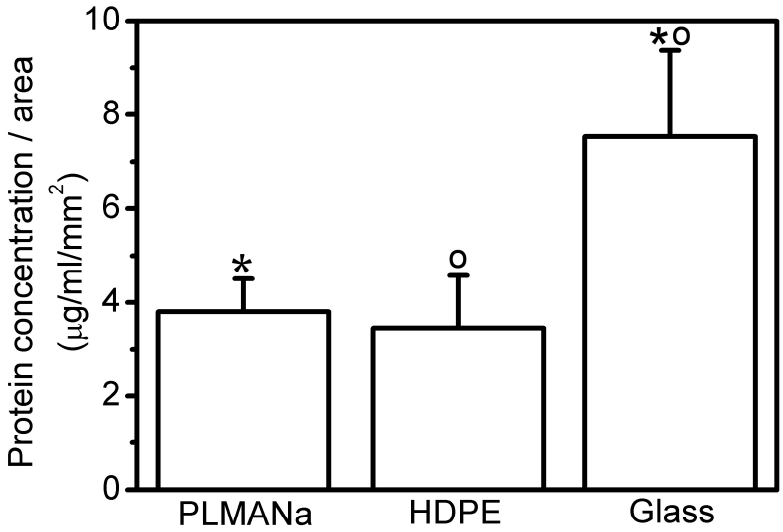

Figure S5 Amount of adherent proteins corrected for the area of the sample for the polymer, HDPE, and glass (*, °: p-value < 0,05).

The amount of the proteins adherent to PLMANa hydrogel is comparable to the adhesion to a HDPE surface (3.4 µg/ml/mm^2 ± 1.21), and signifcantly lower than the amount of adherent proteins to a glass surface (7.5 µg/ml/mm^2 ± 1.81).

We showed above that direct cytotoxicity testing of PLMANa hydrogels indicated no reduction in cell viability. Morphological evaluation showed no reactivity around the PLMANa samples. Although a reduction in cell growth and increase in loosely attached cells directly underneath the hydrogel samples was seen. This might be due to a reduced availability of nutrients for fibroblasts under the sample. Direct and extract tests showed no haemolytic properties of PLMANa; results were comparable to the negative control, HDPE. PLMANa hydrogel resists adhesion of albumin, the most abundant protein in blood, very well. This result is of a high importance in terms of the gel durability as the adhesion and infiltration of biomaterial surfaces with proteins is believed one of the first events after implantation that can change the characteristics of a hydrogel.[9]

We can therefore conclude that PLMANa gel shows no cytotoxic nor haemolytic effects and resists protein adhesion.

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