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Enhanced cell adhesion to silicone implant material through plasma surface modification

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Abstract Silicone implant material is widely used in the field of plastic surgery. Despite its benefits the lack of biocompatibility this material still represents a major problem. Due to the surface characteristics of silicone, protein adsorption and cell adhesion on this polymeric material is rather low. The aim of this study was to create a stable collagen I surface coating on silicone implants via glow-discharge plasma treatment in order to enhance cell affinity and biocompatibility of the material. Non-plasma treated, collagen coated and conventional silicone samples (non-plasma treated, non-coated) served as controls. After plasma treatment the change of surface free energy was evaluated by drop-shape analysis. The quality of the collagen coating was analysed by electron microscopy and Time-Of-Flight Secondary Ion Mass Spectrometry. For biocompatibility tests mouse fibroblasts 3T3 were cultivated on the different silicone surfaces and stained with calcein-AM and propidium iodine to evaluate cell viability and adherence. Analysis of the different surfaces revealed a significant increase in surface free energy after plasma pre-treatment. As a consequence, collagen coating could only be achieved on the plasma activated silicone samples. The in vitro tests showed that the collagen coating led to a significant increase in cell adhesion and cell viability.

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1 Introduction

For several years now, silicone breast implants play a major role in aesthetic and reconstructive plastic surgery. Although new product development led to significant quality improvements of the implants, lack of biocompatibility still remains a significant problem [1, 2]. The foreign body reaction of the surrounding tissue often leads to the development of an avascular fibrous capsule around the implant [3, 4]. Capsular contracture represents one of the main complications after breast implant operations and causes implant distortion, firmness and severe pain. Hence this complication often requires surgical revisions [5]. Although the exact pathophysiological mechanisms for the development of a capsular contracture still remain unclear, it most likely seems to be triggered by an inflammatory host defence mechanism [6]. It is well known that the chemical and physical characteristics of the implant surface have a decisive influence on the connection between the implant and the adjacent tissue [7]. Therefore a lot of effort has been made to improve the biocompatibility of implants by texturization or polyurethane coating of the outer implant membrane. However, scientific analysis of these modified surfaces could not prove a clear and significant improvement of biocompatibility. Due to these unsatisfying results of surface

modification silicone is still far from being called the ideal implant material [8–10]. For several years now there is ongoing interest in modifying implant surfaces by biomimetic coatings such as matrix protein coatings or growth factor coatings to improve cell adhesion and proliferation [11]. Most materials are coated by conventional dip coating techniques, hot plasma spraying or by using chemical coupling agents. However, there are problems associated with many of the applied techniques. Dip-coating techniques can only be applied on surfaces with adequate roughness and hydrophilicity and due to temperatures of over 2000°C hot plasma spraying techniques can not be applied on heat sensitive materials or for heat sensitive coatings. Another method of achieving good binding properties on the material surface is the use of chemical coupling agents. Most of these agents, however, are rather cytotoxic and consequently their application is limited [12]. Silicone used for breast implants is heat sensitive and extremely hydrophobic. Due to these physical and chemical surface characteristics coating of silicone is one of the greatest challenges in biomedical engineering. A possible solution to many of the problems associated with silicone surface coating could be the use of cold low pressure gas plasma.

Cold plasma is partially ionized low pressure gas comprising ions, electrons and ultraviolet photons as well as reactive neutral species such as radicals and excited atoms and molecules with sufficient energy to break covalent bonds on the material surface [13]. It initiates a variety of chemical reaction pathways which increase surface free energy (SFE). The change of SFE is reflected in an increase in wettability.

This effect is called surface activation. Plasma treatment is a dry, cold (<40°C) and fast process which especially allows the treatment of vulnerable materials. The gas plasma does not affect the bulk mass of the implant but only interferes with the superficial layer of the material. For this reason surface treatment is possible without causing any structural damage to the implant [14, 15]. Another positive aspect of this method is that the materials can be coated under sterile conditions. Recently it has been proven that low pressure gas plasma effectively inactivates spores and germs on medical implant materials [16, 17]. Additionally the increased surface energy of the material surface after low pressure plasma treatment greatly enhances the protein adhesion on the material [18]. Xu et al. [19] demonstrated that surface hydrophilicity and high surface energy strongly influences the protein binding capacity. Proteins adsorbed to the surface from the serum in the culture media, or secreted by the cells, are widely accepted to be involved in the cellular attachment to the implant surface. Therefore low-pressure plasma treatment is a promising technique to create bioactive protein coatings on medical implant surfaces.

The aim of this study was to create a stable collagen I surface coating via glow-discharge plasma pre-treatment on silicone implants in order to enhance the cell affinity and biocompatibility of the material. For biocompatibility tests mouse fibroblasts 3T3 were cultivated on the silicone surfaces. Cells were stained with calcein-AM and propidium iodine to evaluate cell viability and adherence.

2 Methods

2.1 Implant material

In this study standard texturized silicone of breast implants (Polytech Silimed, Deissenhofen, Germany) was used. Four different surface properties were compared.

(a) plasma pre-treated and collagen-I coated silcone samples;
(b) collagen-I dip-coated samples without plasma pre-treatment;
(c) plasma treated but not collagen-I coated samples;
(d) regular silicone implant material (non-plasma treated and non-collagen coated) served as control.

2.2 Plasma treatment

A newly developed double inductively coupled plasma reactor (Institute for Plasma Technology, Ruhr-University Bochum, Germany) was used for coating experiments. This reactor with an inner volume of 25 l is equipped with two copper coils. These coils are separately energized which leads to a more stable and homogeneous plasma in the large plasma chamber especially developed to hold large medical implants. The plasma was ignited and heated by a rf-source at 13.65 MHz with a forward power of 1000 W. A gas mixture of argon (100 sccm) and oxygen (5 sccm) with a pressure of 10 Pa was used in this study. Each sample was treated for a duration of 5 min.

2.3 Collagen coating procedure

Collagen type I from rat tail 4 mg/ml in 20 mM acetic acid (BD Biosciences, Bedford, MA, USA) was diluted 1:8 with phosphate buffered saline (PBS) to a final concentration of 0.5 mg/ml. After plasma treatment, 10 implants were incubated with 500 µl per cm^2 for 24 h at 4°C under sterile conditions. After 24 h the supernatant was pipetted off and the probes were incubated for another 48 h at 37°C. After drying the implants were rinsed with PBS buffer and distilled water several times to wash away the non-adsorbed protein.

Another 10 silicone samples were plasma treated but not collagen coated and 10 samples were not plasma treated

but collagen coated. A group of 10 non-coated, non-plasma treated samples served as control.

2.4 Surface characterization and coating analysis

The wettability of the surface, which is a measure of surface energy, is often considered as a factor of biocompatibility of blood-contacting implants. The change of surface free energy and change in hydrophilicity of the silicone surface after plasma treatment was characterized by measuring the contact angles using a drop shape analysis device (Kruess DSA 10, Kruess, Hamburg, Germany). All contact angle measurements were carried out under ambient conditions (50% relative humidity, 21°C). Measurements of the plasma treated silicone implants were performed immediately after surface modification using deionized water and di-iodomethane as test liquids. The surface free energy was calculated based on the Owens–Wendt–Raabe method.

2.5 Scanning electron microscopy (SEM)

For analysis of the structure and adhesion pattern of the collagen layer SEM pictures of the implant materials were taken prior to rinsing. For scanning electron microscopy (LEO Gemini 1530, LEO, Oberkochen, Germany) the collagen coated samples were sputtered with a gold layer.

2.6 Time-of-flight secondary ion mass spectrometry (TOF-SIMS)

In TOF-SIMS analysis (ION-TOF, Muenster, Germany), the silicone sample surface is bombarded with a high energy pulsed primary ion beam. The primary ion energy is transferred to target atoms via atomic collisions and a so-called collision cascade is generated. Part of this energy is carried back to the surface and subsequently atomic and molecular ions are emitted from the outer layers of the coated material. These ions are extracted into a mass spectrometer and their mass is determined by measuring the flight time to the detector. The determination of the precise mass of the secondary ions emitted from the surface allows their distinct chemical identification (e.g. constituent amino acids of the collagen coating). Thus the chemical composition of a surface or a surface coating can be probed with high sensitivity. Additionally the comparison of the peak intensities supplies semi-quantitative data. To analyse the stability and adhesion strength of the collagen coating all samples were rinsed with PBS and distilled water prior to TOF-SIMS analysis. The silicone samples of both groups (plasma pre-treated-collagen coated and non-plasma pre-treated-collagen coated) were analysed by TOF-SIMS as mentioned above.

2.7 Cell culture

Mouse fibroblasts (3T3) (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH—DSMZ Braunschweig, Germany) were maintained in 75 cm^2 tissue culture flasks (Falcon, BD Biosciences, Le Pont de Claix, France) at 37°C in a humidified 5% CO_2 atmosphere, using RPMI 1640 culture medium (Invitrogen, Karlsruhe, Germany) with 10% heat inactivated fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany), sodium bicarbonate (concentration 2 g/l), 4 mM L-glutamine and 20 mM HEPES, (*N*-(2-hydroxyethyl)-piperazidine-*N*′-(2-ethansulfonacid), (Sigma, Deisenhofen, Germany). The cells were passaged every 2–3 days depending on cell proliferation rates. After twofold washing with phosphate buffered saline solution (PBS) adherent cells were detached from the culture flasks by addition of 0.2 ml/cm^2 0.25% trypsin/0.1% ethylenediamine tetraacetic acid (EDTA) for 2 min at 37° C. Cells were collected and washed twice with growth medium.

2.8 Cell seeding experiments

The different silicone samples were placed in 6-well culture plates (BD Biosciences). Subsequently 10000 3T3 cells (counted in a Neubauer cell chamber) in 1 ml culture medium were added to the silicone samples. The 3T3 fibroblasts were allowed to grow for 24 h. Afterwards the medium was exchanged and the probes were incubated for another 72 h under cell culture conditions.

2.9 Fluorescence microscopy

For fluorescence analysis the silicone implants were removed from the culture plates and were washed 3 times with RPMI 1640. Subsequently, adherent cells were stained with 14 μg ml^{-1} Calcein-AM (Calbiochem-Novabiochem, Bad Soden, Germany) and propidium iodine (50 μg ml^{-1}, Molecular Probes Inc., Eugene, OR, USA). After a twofold washing with RPMI 1640 the stained cells were microscopically photographed using a fluorescence microscope (Photomicroscope 3, Zeiss, Oberkochen, Germany) with a digital camera (Camedia C3030, Olympus, Hamburg, Germany). Images were processed using AnalySIS 3.2 software (Soft Imaging System, Muenster Germany) and Photoshop 5.0 software (Adobe, Unterschleissheim, Germany). Statistical analysis was performed by Student's *t*-test for independent samples.

2.10 Analysis of cell adhesion pattern by scanning electron microscopy (SEM)

The cell covered samples were fixed with 3.7% glutaraldehyde (Sigma-Aldrich, Deisenhofen, Germany) in PBS

for 15 min. After a twofold washing with PBS, the fixed cells were dehydrated with an ascending sequence of ethanol (40, 60, 80, 96–98%). Subsequently, after aspiration of ethanol, the samples were left at room temperature for 24 h. Finally the samples were sputtered with a gold layer.

3 Results

3.1 Drop shape analysis

Contact angle measurement is a convenient method for evaluating the hydrophobicity or hydrophilicity of a material surface. Figure 1 shows the change of surface free energy (contact angle to water) between the untreated ($\approx$16.5 mN/m) and plasma-treated ($\approx$55.6 mN/m) silicone samples. This decrease of contact angel verifies that argon/oxygen plasma treatment leads to a significant increase in hydrophilicity and surface free energy of the silicone probes.

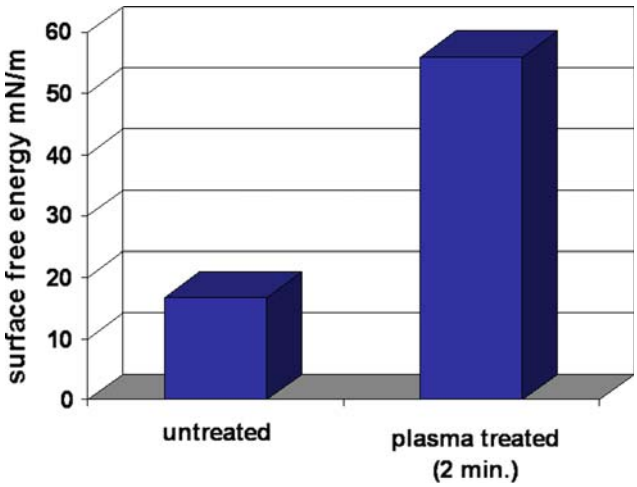

Fig. 1 Change of surface free energy (contact angle to water) after 2 min of plasma treatment. *Left bar* untreated silicone samples (SFE $\approx$ 16.5 mN/m). *Right bar* plasma-treated silicone samples (SFE $\approx$ 55.6 mN/m)

3.2 Implant surfaces analysis via SEM

The SEM picture analysis of the material surface showed that only the plasma treated samples were covered with a compact and consolidated collagen layer. The collagen on the non-plasma treated samples was not adhered and washed away by rinsing with buffer. Compared to the plasma treated silicone samples it was obvious that the coating on the non-plasma treated samples was neither homogeneous nor adherent showing obvious cracks and delamination signs (Fig. 2a–d).

3.3 TOF-SIMS surface analysis

Only on the plasma-treated collagen-I coated silicone samples high intensity peaks of collagen specific amino acids were detectable on the material surface. Even after intensive rinsing of the samples the collagen components

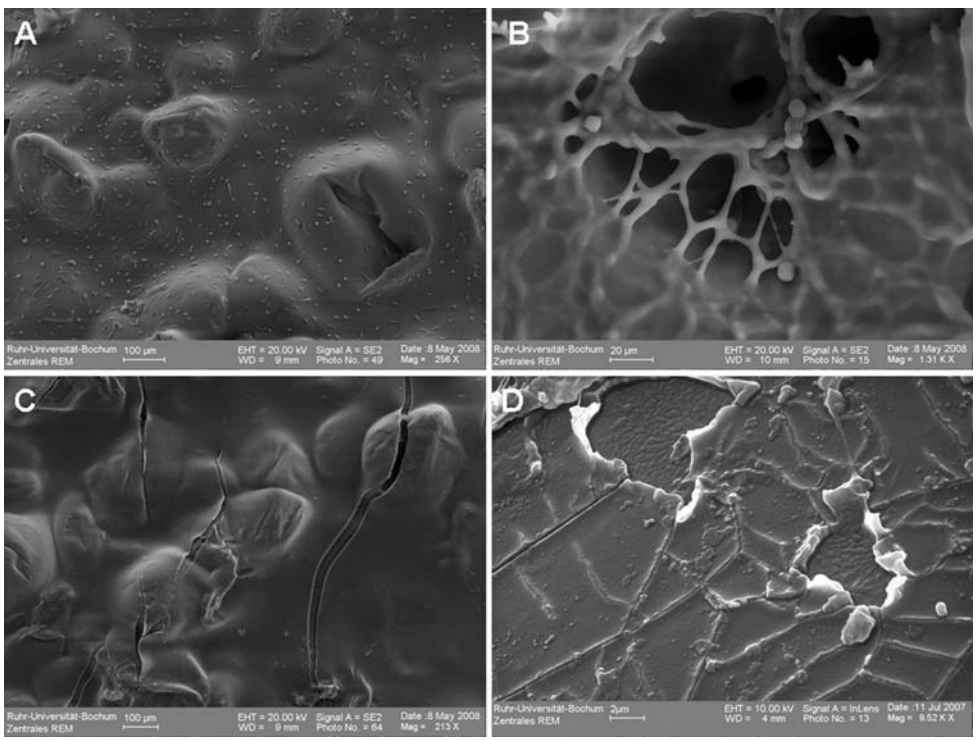

Fig. 2 SEM analysis of the collagen-I-coating (before rinsing procedure was performed). **a**, **b** (higher magnification) showing samples after plasma-treatment; **c**, **d** showing non-plasma treated silicone samples. Note the obvious cracks and delamination signs of the collagen-I coating on the non-plasma treated samples. **b** Shows collagen fibres spanning a pore in the surface of the silicone material

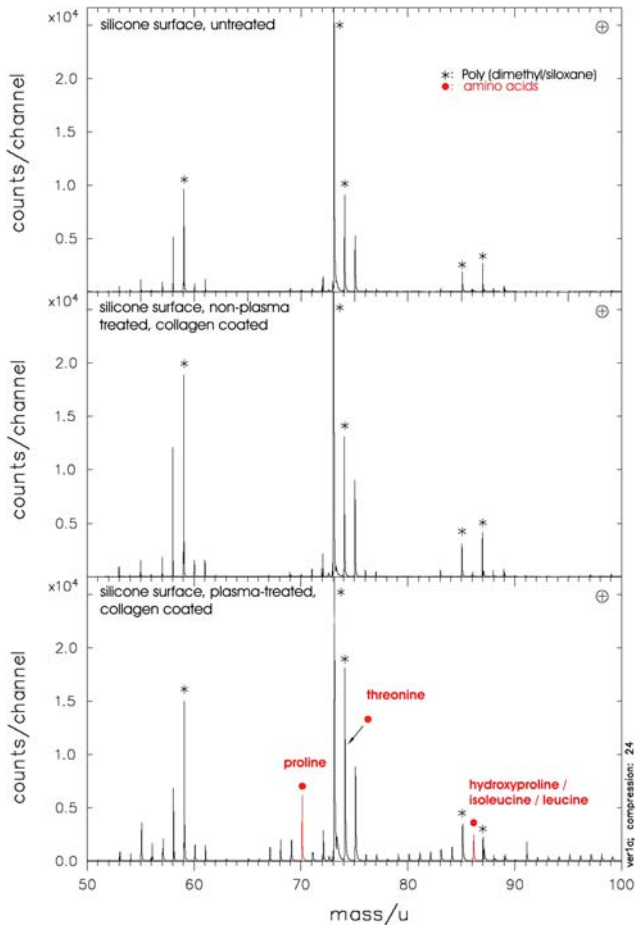

Fig. 3 TOF-SIMS spectrum of positive secondary ions. After rinsing procedure amino acids were solely detectable on the plasma-treated silicone samples

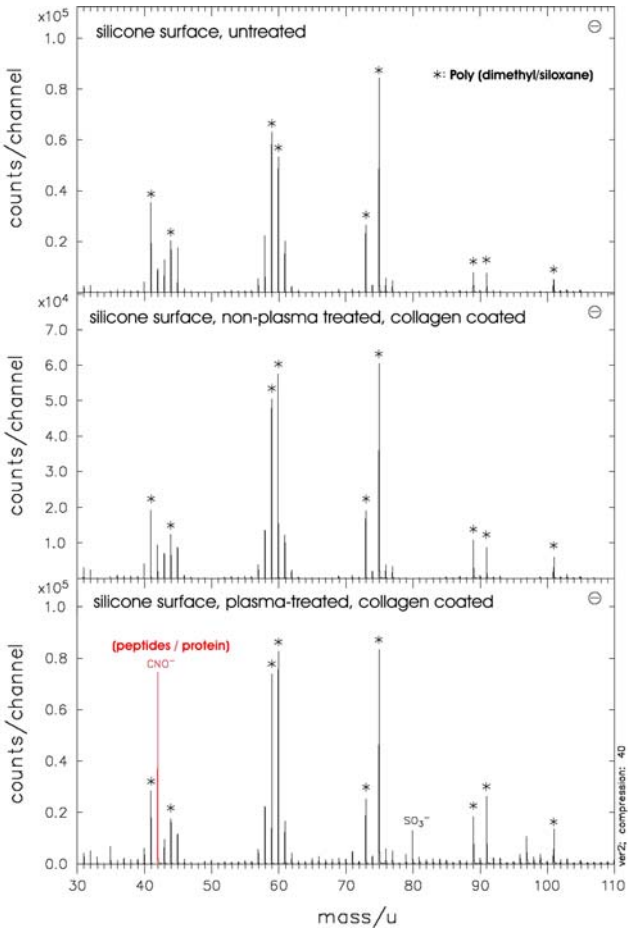

Fig. 4 TOF-SIMS spectrum of negative secondary ions. Note that only plasma-treated samples showed surface bound peptides

(proline, hydroxyproline, isoleucine, leucine and threonine) were clearly detectable by TOF-SIMS analysis. In contrast after rinsing of the non-plasma treated collagen-I coated silicone samples the TOF-SIMS ion mapping showed no surface bound amino acids (Figs. 3 and 4).

3.4 Cell adherence and cell viability

Calcein AM staining of 3T3 fibroblasts revealed that there was a significant ($P < 0.05$) higher rate of adherent, viable cells on the plasma treated, collagen coated samples compared to controls (Fig. 5a–d). On the non-plasma treated collagen coated silicone implants no significant increase of cell adherence could be noticed compared to the non-plasma treated non-collagen coated materials.

The statistical analysis of the plasma treated but non-collagen coated material showed that there was a slight increase of adhered cells on the surface compared to the regular implants although this effect was not significant (Fig. 6).

In correlation to that the computer assisted analysis of the propidium-iodine staining showed that the percentage of non-viable apoptotic cells was significantly higher on the non-plasma treated, non-coated silicone probes compared to the plasma pre-treated and collagen coated samples ($P < 0.05$) (data not shown).

Furthermore the fibroblast cell morphology on plasma-treated, collagen-coated samples was clearly different compared to the non-treated silicone probes. Whereas the fibroblasts on the non-coated implants exhibited the typical rounded morphology of apoptotic cells, the cells on the plasma-collagen coated implants were mainly spread out and adherent to the collagen fibers. This feature is typical for viable non-apoptotic cells. These results were confirmed by SEM. As shown in Fig. 7a, b, the rounded fibroblast morphology dominated the cells on non-plasma treated–non-coated silicone surfaces. In distinct contrast, on plasma-collagen coated implants, the cells were viable and spread out.

In summary the adherence and viability of 3T3 fibroblasts was significantly enhanced on the plasma treated,

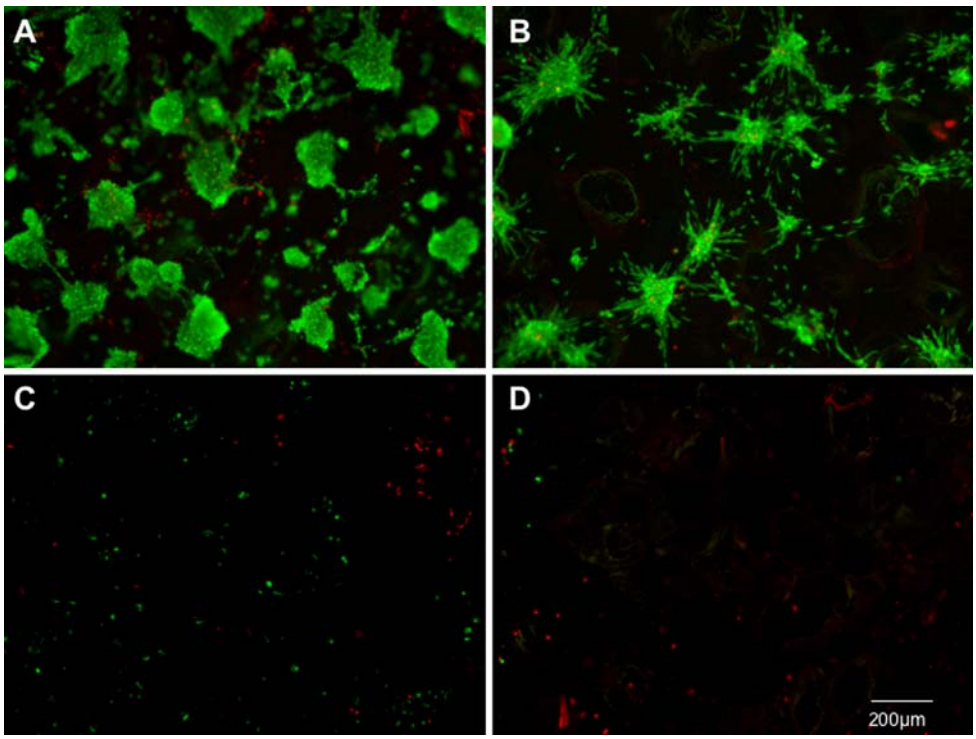

Fig. 5 Representative micrographs of calcein-AM stained 3T3 fibroblasts on plasma-treated, collagen-I coated (**a**, **b**) and non-plasma treated, collagen-I coated (**c**, **d**) silicone probes. The different growth patterns of the cells in **a** and **b** are due to different focal planes of the 3-dimensional surface structure of the textured silicone implant material. Under microscope magnification the material exhibits pronounced irregular grooves. The two pictures exhibit two different focal planes. In **b** the focal plane shows more of the cells growing along the grooves resulting in a star like pattern

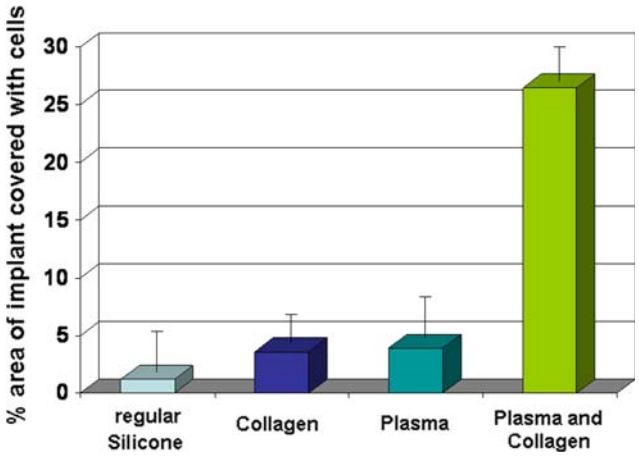

Fig. 6 Computer assisted statistical analysis of cell adherence and cell viability. Note the significant ($P < 0.05$) increase of viable and adherent cells on the plasma-treated, collagen-I coated implants compared to controls

collagen coated silicone implant materials compared to controls.

4 Discussion

In recent years there is ongoing interest in modifying implant surfaces by applying bioactive coatings to improve cellular adhesion and proliferation. Extracellular matrix molecules such as collagen, fibronectin or laminin are known to mediate cell adhesion and proliferation. Especially collagen is known to be one of the best matrices for cell migration and growth [20–22]. The positive cellular response induced by collagen is mainly mediated through the amino acid sequence arginine-glycine-asparagines which is recognized by the integrin receptors located in the cell membrane [23]. These facts well correlate with the results of enhanced cell adherence on plasma modified collagen coated silicone described in this study. Hence collagen has been tried to immobilize on surfaces of many implant materials such as titanium alloys or polyethylene to improve their biocompatibility [24]. Due to its chemical and physical surface properties coating of silicone implant material is a challenging task. The high hydrophobicity, inadequate wetting behaviour and surface condition of silicone causes extreme adhesion problems [25]. Ksander et al., who examined the incidence of capsule contracture of collagen coated silicone implants in animals, describe a significantly reduced capsule formation in the group of collagen coated implants. The collagen coating described in the study of Ksander et al. was cross-linked with formaldehyde and glutaraldehyde [26]. In addition to the fact that these chemical additives are problematic in terms of cytotoxicity and their carcinogenic properties, the authors were faced with the fact that the coatings were imperfect. The authors described the coatings as rather thick, inflexible and showed defects due to adhesion problems [27]. In their study they hypothesize that the development of better, more persistent coatings will permit

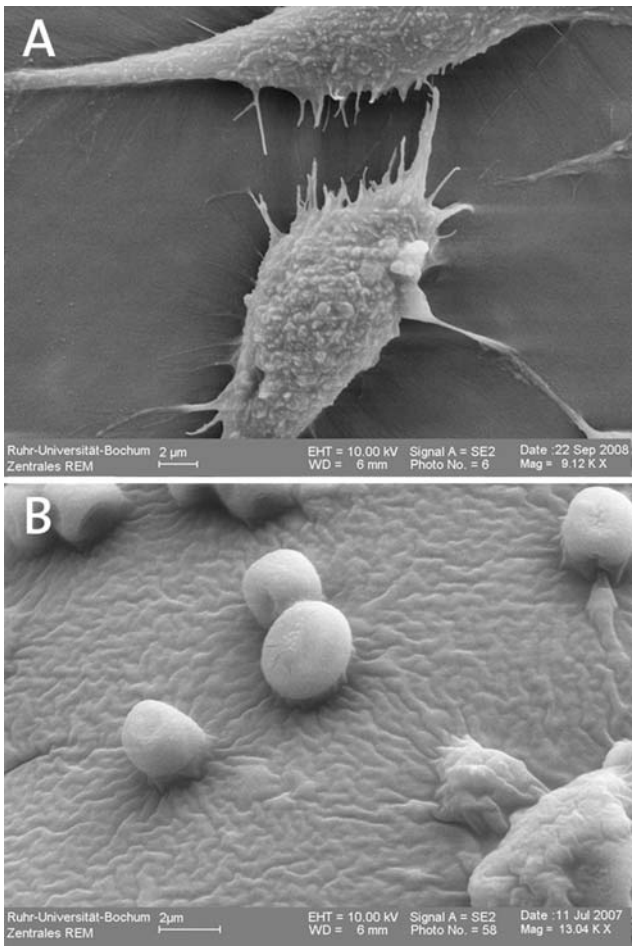

Fig. 7 Morphological cell appearance on plasma-coated (**a**) and non-coated (**b**) silicone implant material. The fibroblasts on the non-coated implants exhibit the typical rounded morphology of apoptotic cells, the cells on the plasma-collagen coated implants were mainly spread out and adherent to the collagen coated surface

prolonged inhibition of capsule formation. Habal who examined the biocompatibility of medical silicone implants likewise expressed in his manuscript that changes of its surface properties, such as an improvement of the wetting behaviour could lead to a new dimension in clinical application of silicone [8]. These statements well correlate with the results presented in this study. The TOF-SIMS surface analysis shows clearly that the plasma induced activation of the silicone implant material leads to a drastic optimization of the collagen binding capacity on its surface. TOF-SIMS is a very sensitive technique which provides semi-quantitative data on the surface chemistry of a material. As mentioned above the chemical mapping of the silicone surface via TOF-SIMS analysis revealed that even after extensive irrigation with PBS and water the constituent amino acids of the collagen coating (e.g. proline, leucine, threonine), are clearly detectable on the plasma treated silicone samples whereas on the non-plasma treated samples bound residues of the collagen coating were not detectable.

The results of increased collagen adsorption and improved collagen adhesion to plasma treated silicone surfaces is in accordance with the study of Gölander et al. [28] who examined the influence of surface free energy on the protein binding capacity of polymers. They established that the extent of surface free energy is a decisive factor in terms of the protein adsorption characteristics of an implant surface. The precise mechanisms responsible for the enhanced protein adhesion are still discussed in the literature. It is believed that plasma treatment of surfaces influences the adhesion properties by changing several chemical and physical adhesion parameters. A change of the surface polarity and the introduction of functional binding groups are discussed as possible mechanisms of action for the enhanced protein adsorption on plasma activated surfaces [29, 30]. The plasma induced change of surface polarity has also been demonstrated by Wilson et al. Their study on plasma treated polyetherurethane confirmed that the plasma process increases the polar component of the surface. They also found that Argon and Oxygen plasma treatment results in the incorporation of oxygen containing groups [31]. Baier et al. [32] describe, that high surface free energy levels drastically enhance the adsorption of hydrophilic proteins such as collagen and fibronectin. It is a well known fact that these hydrophilic proteins (in contrast to lipophilic proteins) promote cell adhesion and cell growth on surfaces. Coating with poly-lysine or collagen causes adsorption and subsequent cell-protein interactions as well as cell attachment [33, 34]. The cell experiments on silicone materials conducted in our study support these findings. The statistical analysis revealed that fibroblast adhesion was significantly ($P < 0.05$) enhanced on the plasma treated and collagen coated silicone implants compared to controls. In addition it appears that cell morphology on the materials differed significantly. SEM micrographs revealed that cells attached to the non-coated/non-plasma treated samples were rounded up in shape whereas cells on plasma-collagen surfaces appeared to be wide spread which is a morphology indicative of viable and healthy cells.

5 Conclusion

In summary the results presented in this study demonstrate that a resistant collagen coating of silicone implant material can be achieved by cold plasma surface modification. In addition we were able to demonstrate that collagen coating significantly enhances the cell adhesion on the modified silicone surface.

Given the fact that the adhesion of different cell types to the implant surface is a mandatory prerequisite for tissue integration, we firmly believe that this study serves as a good indicator that plasma mediated collagen-I coating increases the biocompatibility of silicone implants. Although it is obvious that the in vitro data do not directly correspond to an in vivo situation the reported results still hold promise for reduced implant associated complications in clinical use.

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