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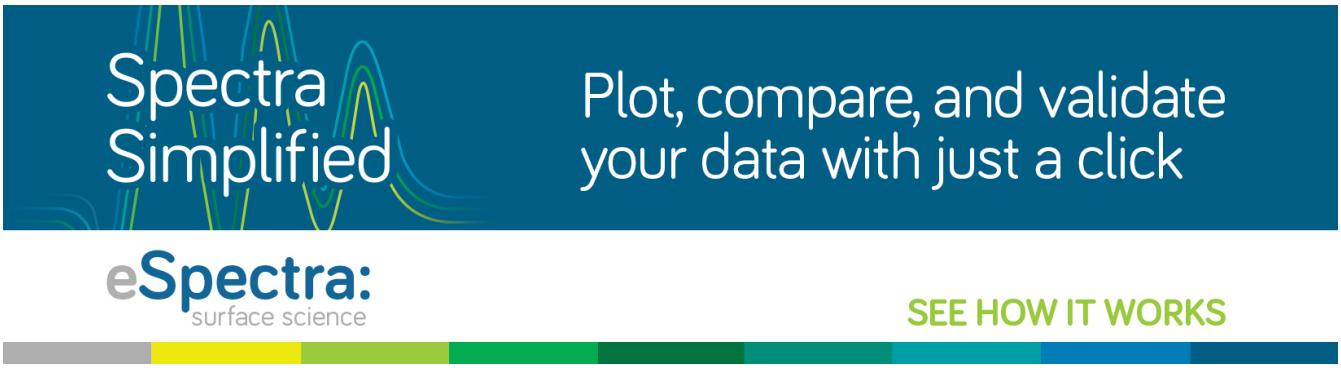

Archaeal tetraether lipid coatings—A strategy for the development of membrane analog spacer systems for the site-specific functionalization of medical surfaces

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The primary goal of our investigation was the development of a versatile immobilization matrix based on archaeal tetraether lipids that meets the most important prerequisites to render an implant surface bioactive by binding specific functional groups or functional polymers with the necessary flexibility and an optimal spatial arrangement to be bioavailable. From this point of view, it appears obvious that numerous efforts made recently to avoid initial bacterial adhesion on catheter surfaces as an important prerequisite of material associated infection episodes have shown only a limited efficiency since the bioactive entities could not be presented in an optimal conformation and a stable density. A significant improvement of this situation can be achieved by highly specific biomimetic modifications of the catheter surfaces. The term "biomimetic" originates from the fact that specific archaeal tetraether lipids were introduced to form a membrane analog monomolecular spacer system, which (1) can be immobilized on nearly all solid surfaces and (2) chemically modified to present a tailor-made functionality in contact with aqueous media either to avoid or inhibit surface fouling or to equip any implant surface with the necessary chemical functionality to enable cell adhesion and tissue integration. Ultrathin films based on tetraether lipids isolated from archaea *Thermoplasma acidophilum* were used as a special biomimetic immobilization matrix on the surface of commercial medical silicon elastomers. A complete performance control of the membrane analog coatings was realized in addition to biofunctionality tests, including the proof of cytotoxicity and hemocompatibility according to DIN EN ISO 10993. In order to make sure that the developed immobilization matrix including the grafted functional groups are biocompatible under *in vivo*-conditions, specific animal tests were carried out to examine the *in vivo*-performance. It can be concluded that the tetraether lipid based coating systems on silicone have shown no signs of cytotoxicity and a good hemocompatibility. Moreover, no mutagenic effects, no irritation effects, and no sensitization effects could be demonstrated. After an implantation period of 28 days, no irregularities were found. https://doi.org/10.1116/1.5008816

I. INTRODUCTION

Numerous, more or less complex investigations published recently were devoted to an important health care related topic by trying to minimize medical device associated infections. Besides severe consequences for the individual patient, infections lead to a prolongation of hospitalization and to increasing costs in the health care system.[1–3] Especially, application of different catheter systems such as central vascular catheter, ureter, or peritoneal dialysis (PD) catheter is often associated with infection and inflammation.[4,5] Accordingly, peritonitis represents the most significant complication of peritoneal dialysis.[6–8] Changes in methodology to continuous ambulatory peritoneal dialysis do not reduce the frequency of peritonitis.[9–11] Despite the development of better connection techniques (double-cuff Tenckhof catheter) exit-site infection dominates with a certain potential to cause subsequent tunnel infections and persistent peritonitis.[8,12]

There is no doubt that the appearance of these infections is associated with bacterial adhesion and biofilm formation. Strategies currently used for biofilm control comprise solutions to (1) prevent initial device contamination, and (2) minimize initial microbial cell attachment or (3) kill biofilm-associated cells, and (4) remove the device.[13] With this in mind, many intervention strategies were developed to control and prevent the formation of an infectious biofilm on surfaces

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of biomaterials used in the production of medical devices. Hence, numerous approaches were introduced which are based on the functionalization of surfaces to render implants that are antiadhesive or antimicrobial. Today, it is common knowledge that such coatings can be subdivided in active and passive coatings depending on whether they interfere with cell metabolism and cell proliferation of the involved microorganisms or if they simply prevent the adherence of pathogenic microorganisms at surfaces.[14]

During the last decades, the development of active antimicrobial medical devices was primarily directed to the modification of implant surfaces with the aim to kill pathogenic bacteria and to combat or eradicate infections.[15] Such new customary antifouling coatings led to an additional long-term stability as well as to an improved bio- and hemocompatibility.[16] However, antibiotic release from biomaterial surfaces as antiinfective approach is often insufficient to hinder biofilm formation and leads apparently to an increased microbial resistance.[17] Especially, for biofilms inside of catheters the resistance to antibiotics is problematic and changes in clinical practice are recommended.[4,18,19] An alternative approach to overcome the problem is based on the passive modification of catheter surfaces to avoid initial bacterial adhesion and subsequent biofilm formation by changing their physicochemical characteristics such as surface energy and surface charge to influence the hydrophilic–hydrophobic balance or to establish a stable surface hydration using specific functional groups.[5,20–22] A lot of publications describe the use of polymer brushes, in particular, polyethylene glycol (PEG) polymers grafted to a surface, where water molecules will be immobilized between the PEG chains, which hinder bacterial adhesion.[23–27]

Another effective approach is the grafting of (poly)zwitterionic polymers to generate a strongly bound hydration layer that leads to an excellent superhydrophilicity and a high resistance against bacterial adhesion.[28] The molecular design of such zwitterionic surfaces is of great importance for antifouling.[29] In the past, the steric repulsion was first shown as a function of the chain lengths of the polymer and the packing density achieved, using the example of the polyethylene glycol.[30–32] In addition, the flexibility of the adherent polymer chains and the chain chemistry also became important to address the development of efficient antifouling layers (Fig. 1).[33–36]

The primary goal of the present study was to establish a new membrane-analog immobilization matrix, which can be used to bind different functional groups and polymers or combinations thereof in order to avoid bacterial adhesion as an important prerequisite of infections. Besides this, it is important that the new biomaterial coatings fulfill the necessary requirements of biocompatibility required by law, respectively, the Medical Device Directive.

As a new approach, surface modification based on covalently coupled tetraether lipids is done to build up a biomimetic immobilization matrix. *Thermoplasma acidophilum* is a thermoacidophilic archaea, which has been isolated by Darland *et al.* from a steaming coal refuse pile.[37] Due to the composition of their membranes, these archaea are extremely resistant against strong acids (pH $\sim$ 2) and high temperatures.[38,39] The general structure of the archaeal lipid is a membrane-spanning tetraether core linked to two glycerol moieties. The inherent monolayer stability compared with known lipid bilayer structures and the absence of double bonds in the hydrocarbon backbone guarantee the resistance toward hydrolytic, oxidative, and other (bio)chemical attacks.[40,41] Bipolar tetraether lipids are provided with two head groups, and therefore, they possess excellent prerequisites to serve as a versatile spacer system.[42] Hence, tetraether lipid molecules can be covalently coupled to substrates and form a well ordered impermeable monolayer by self-assembly. Using the opposite head group of the tetraether lipid, various functional groups/capping ligands can be attached covalently to develop antiadhesive and antimicrobial surfaces.[22,43,44] This study is focused on the surface modification of peritoneal dialysis catheter tubes by a covalently fixed immobilization matrix based on tetraether lipid monolayers. A defined selection of functional groups with varying physicochemistry was coupled to the outermost head group of the lipid spacer system to render commercial medical grade silicone antiadhesive. The major topic in the present work is the performance control, including coating stability against sterilization as well as biocompatibility according to DIN EN 10993.

Results of *in vitro*- and *in vivo* toxicity evaluation and implantation tests were proved and evaluated in preparation of the product certification according to Medical Device Directive.

II. EXPERIMENT

A. Materials

All commercial chemicals were used without further purification: sodium hydroxide and acetone (Merck), ammonium hydroxide (25% solution) (J.T. Baker), diisopropylamine (Merck) aminopropyl trimethoxysilane (ATMS, Fluka), pentafluoroaniline (Aldrich), monoamino- and *bis*-amino-polyethylene glycol molecular weight 3400 g/mol (Shearwater), *bis*(3-aminopropyl)amine (Fluka), glycine (Fluka), and propanesultone (Aldrich). Silicone sheets and disks (Raumedic SIK 6504) were kindly provided by Ir. T. Klijn (Humeca B.V.). Borosilicate glass (BOROFLOAT® 33, Schott AG) served as a model surface for several tests.

A bipolar membrane spanning tetraether lipid with a sugar component at one polar end and a glycerol at the other end is the main tetraether lipid of the archaea *T. acidophilum*.[43,44] To isolate caldarchaeol (the core structure of the main tetraether lipid after removal of both head groups) directly from the raw bacterial dry mass, an efficient single-step procedure resulting in high purity was used, as described earlier.[43–45] The caldarchaeol could further be activated by cyanuric chloride at both end groups.[44,46]

B. Methods

1. Lipid preparation and coating procedure

Lipid monolayers were formed by self-assembly. An activation strategy for the modification of the polymeric silicone

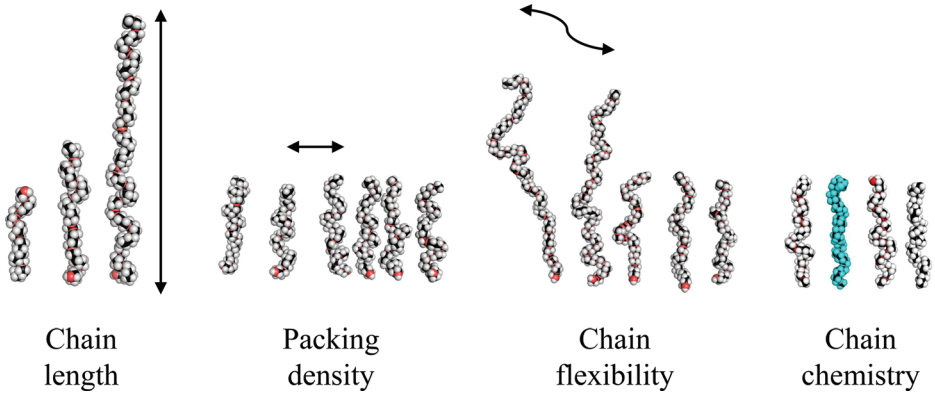

FIG. 1. Illustration of important polymer properties to establish a hydrated chain formation.

implant materials was developed with special emphasis on a process applicable for coating of catheters.[43] Briefly, in a first step, the inert silicone surface has to be activated, which means that the reactive groups are introduced as anchors for the following modification steps (Fig. 2). Hydrolyzed silicone material was coated with aminopropyl trimethoxysilane to introduce amino functional groups. Silanization was confirmed by FTIR measurements. Silicone sheets with superficial amino groups were reacted with cyanuric chloride-activated caldarchaeol in acetone for 2 h at 40 °C in the presence of diisopropylamine (sample name: SIK TL).

Thereby it was possible to attach a dense monomolecular lipid layer on the silicone surface and to provide a sufficient number of chemical binding sites for further modification strategies. The molecules introduced in this step were chemically coupled to the second cyanuric chloride group on the caldarchaeol, and therefore, these molecules preferentially had to contain amino groups. Coating procedures for the various lipid functionalization performed to introduce positive (SIK TL positive) or negative (SIK TL negative) charges, hydrophobic (SIK TL CF) or hydrophilic (SIK TL PEG) entities, and a combination of hydrophilic groups and negative charges (SIK TL Combi) are described elsewhere (Fig. 3).[43,45]

All coatings were characterized by zetapotential and water contact angle measurements.[43] Determination of advancing water contact angles by means of the sessile drop method has been carried out with a computer controlled contact angle measuring system (DCA20, dataphysics, Germany). Zetapotential measurements based on the detection of streaming potentials were carried out in standard KCl-electrolyte (EKA, Anton Paar Germany).

2. Biocompatibility testing

In order to acquire the entire application potential, the new developed tetraether lipid based spacer system was (1) chemically modified to prevent catheter associated infections by surface hydration, and (2) tested in view of its efficiency by means of a static adhesion assay. Therefore, a mixed culture of *Staphylococci* strains was used in order to ensure the desired clinical relevance.

An essential prerequisite for further applications, for example, in the field of biomedicine (e.g., implant coatings to stimulate cellular attachment and tissue integration) and bioanalytics (immobilization matrices for coupling of different ligands with extraordinary sensitivity and selectivity) is (3) an excellent biocompatibility of the membrane analog lipid matrix under *in vitro* and *in vivo* conditions. Initial evaluation of biocompatibility has already been described in an earlier work and underline the general suitability of the membrane-analog coatings.[43] The focus now is directed to a more comprehensive *in vitro* and *in vivo* evaluation as a basis for clinical decisions. Under these circumstances, a comparatively extensive test sequence was designated. Very briefly *in vitro*-biocompatibility testing including cytotoxicity and hemocompatibility testing were carried out with the tetraether lipid modified catheter silicones according to DIN EN ISO 10993. Moreover, genotoxicity, sensitization, irritation, and local effects after implantation were analyzed by means of animal tests. According to the requirements of DIN EN ISO 10993-2 to minimize animal tests, further investigation of risk potentials was carried out on selected silicone modifications, SIK, SIK TL PEG, and SIK TL negative, since these modifications showed the most promising combination of coating stability, cytocompatibility, and antiadhesive performance. It

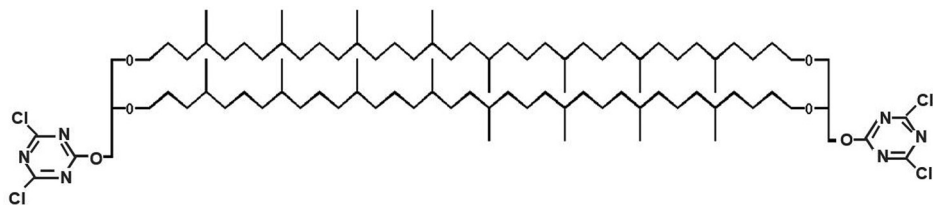

FIG. 2. Structure of caldarchaeol activated on both head groups by cyanuric chloride (CyCl).

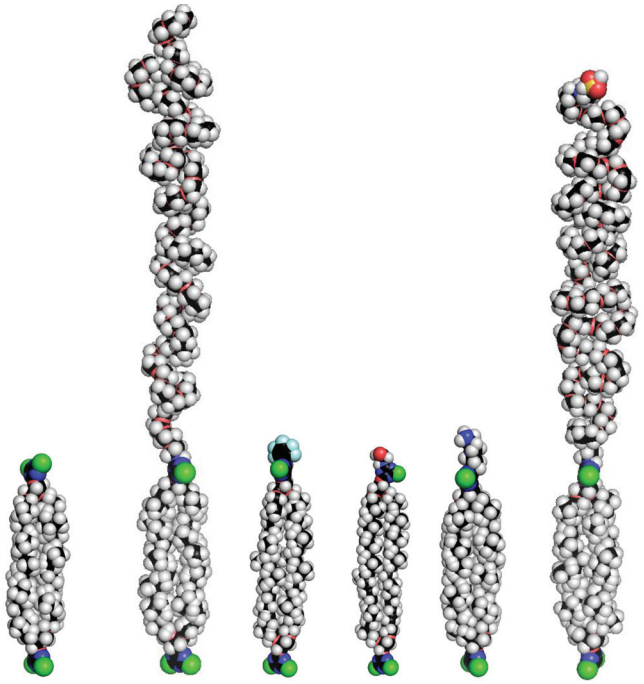

FIG. 3. Models of caldarchaeol molecules functionalized with different head groups: from left to right: bis-cyanurchloride, PEG3400-OMe, pentafluoraniline, glycine, *bis*(aminopropyl)amine, and PEG3400-NH-SOOH.

is worth to point out that here only a short description of the different tests will be given. Note that all biocompatibility tests, except the performance control, were carried out in a certified laboratory (BMP Competence GmbH, Alsdorf). The details of the performed tests are part of the comments in DIN EN ISO 10993 and correspond to a common practice in medical device evaluation and approval.

a. Cytotoxicity testing. According to DIN EN ISO 10993-5: 2009, L929 cells were incubated for 24 h directly on the surfaces of the samples to be tested for determining the influence of the test samples on the membrane integrity. By testing the membrane integrity, the vitality of the cells after contact with the test sample was investigated. The membrane integrity is determined by double staining with fluorescein diacetate and ethidium bromide. Fluorescein diacetate easily permeates the membranes of living cells. Thereby it is converted into fluorescein and can thus no longer leave vital cells. As a consequence, vital cells appear green. Within short periods of time, ethidium bromide only penetrates cells with damaged membrane integrity, intercalates with the DNA of the cell, and labels the cell nucleus red.

For the indirect investigations, the extraction of the samples in the cell culture medium was carried out for 72 h at 37 °C using a surface/volume ratio of 3 cm^2/ml according to DIN EN ISO 10993-12:2012. L929 cells were incubated with the extraction medium for 24 h to determine the influence of the extracts on mitochondrial activity [tetrazolium salt splitting test (XTT test)]. This test is suitable for determining the metabolic activity of the cell culture. The mitochondrial activity of the cells is determined by colorimetric quantification of tetrazolium salt (XTT) converted by the mitochondrial dehydrogenases.

b. Hemocompatibility testing. The application of lipid-coated silicone tubes for peritoneal dialyses requires an excellent hemocompatibility. In addition to the study of coagulation and platelet activation, these tests must also include the assessment of inflammation and thrombosis, represented by the number of leukocytes at the implant surface after the blood was brought in contact with the biomaterial. In order to investigate the hemocompatibility according to DIN EN ISO 10993-4:2009, the test samples were incubated with human whole blood or blood components under static conditions in a blood chamber at a constant temperature (37 °C) for 30 min. Hemocompatibility indices were restricted to a quantitative evaluation of the blood cell number, the coagulation activation [thrombin–antithrombin (TAT) complex formation], and hemolysis (free plasma hemoglobin). Blood samples taken before incubation were used as baseline values. Test samples were proved in separate test series.

c. Genotoxicity testing. Bacterial reverse mutation assays serve to detect point mutations, which involve substitution, addition, or deletion of one or a few DNA base pairs. In order to investigate the potential of the test sample for its capability to induce gene mutations, the preincubation test was performed with the *Salmonella typhimurium* strains. The test samples were extracted in 0.9% NaCl for 72 h at 37 °C at a surface/volume ratio of 3 cm^2/ml according to DIN EN ISO 10993-12:2013. Subsequently, the sample extracts were tested at several concentrations (10%, 20%, 40%, 60%, 80%, and 100%) according to DIN EN ISO 10993-3:2009. The assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate.

d. Sensitization testing. Sensitization tests were performed in the guinea pig according to DIN EN ISO 10993-10:2010. The assessment of the test sample to produce skin sensitization is achieved by multiple expositions under standardized conditions. The exposure can result in allergic or sensitization reactions. The extraction of the test sample was performed taking into account a total ratio of 60 cm^2 of the test sample to 20 ml at 37 °C for 72 h, according to DIN EN ISO 10993-12:2012. During the induction phase, the test animals were intradermally injected and topically treated with a polar extract of the test samples. After a latency of 2 weeks to allow a potential reaction of the immune system, the animals were challenged with the extract of the test samples on the flank. The grade of skin reactions was compared to control animals (negative control), which were treated with the extraction medium during the induction phase and the challenge phase with the same extract than that used for the test sample.

e. Irritation testing. According to DIN EN ISO 10993-10:2010, in each of these irritation tests, rabbits were injected with 0.2 ml of the polar solvent containing the

extract at five sites on one side of each animal and similarly were injected with 0.2 ml of the polar solvent (reagent control) at five posterior sites on the same side of each animal. The procedure was repeated for the nonpolar solvent containing the extract and the nonpolar reagent control on the other side of each animal. Observations were recorded and compared to the reagent control injection sites immediately after injection and 24, 48, and 72 h after injection.

f. Testing of local effects after implantation (tissue compatibility). According to DIN EN ISO 10993-6:2009, the tests for local effects after implantation were conducted in three rabbits for each test sample and an implantation period of 7, respectively, 28 days. Three pieces of the test sample (approximately $10 \times 1 \times 1$ mm) were implanted with a hypodermic needle into the paravertebral muscle of one side of the spine of each rabbit, in order to yield a total of at least eight samples of the test sample. Three strips of negative control were implanted with a hypodermic needle in the opposite muscle of each animal in order to yield eight control samples for the evaluation of the biological response. After the implantation period, the biological response of the test sample was evaluated by macroscopic and histopathological assessment in comparison to the control material.

3. Performance control

a. Sterilization stability. Sterilization stability was tested to guarantee the mechanical stability of the tetraether lipid immobilization matrix during, respectively, after sterilization. The analysis was based on lipid detection before and after the sterilization procedure by confocal laser scanning microscopy (CLSM). Two sterilization methods were considered: steam sterilization (Varioklav Thermo Scientific, 121 °C, 20 min) and gamma sterilization (BGS Wiehe Germany, 40 kGray). One slide of an uncoated reference glass and a tetraether lipid coated glass (TL) was divided into three parts: untreated, steam sterilized (typical laboratory procedure), and gamma sterilized (typical industrial or clinical method).

b. Bioadhesion testing. Bioadhesion experiments were performed under static conditions. Biofilm analysis was adapted to the requirements of peritoneal dialysis as far as possible. A mixed culture of *Staphylococci* strains was used to make sure, that the most relevant pathogens found at infection sites are involved. Both strains, *Staphylococcus aureus* and *Staphylococcus epidermidis*, are responsible for 60%–80% of implant related infections in orthopedic surgery.[11,21] Different studies have been shown that *S. aureus* is the most frequent infection germ for peritoneal dialysis-related peritonitis.[5,7,47] On the other hand, the microorganism of *S. epidermidis* was the most commonly found species of a recent study in patients treated with peritoneal dialysis,[48] and as a natural skin colonizer, this strain possesses a high prevalence for medical device related infections.[19]

The idea behind the screening tests on bacterial adhesion is to evaluate the ability of microorganisms to attach and grow on different surfaces under static conditions. The testing procedure is subdivided into four sequential steps: (1) inoculation of the test samples with microorganisms, (2) removal of nonattached microorganisms from the sample surface, (3) static incubation to allow a material specific proliferation, and (4) determination of the final number of adherent microorganisms after defined proliferation periods. The growth behavior of the mixed culture (50:50) of *S. aureus* (DSMZ 1104) and *S. epidermidis* (DSMZ 9269) was observed during an incubation period of 24 h at 37 °C using a synthetic dialysis effluent designated peritoneal dialysis fluid according to Holmes.[49] The tests were performed in 24 multiwell plates using a bacterial concentration of 10^7 cells/ml and a medium volume of 1 ml per test position. The test samples were placed at the bottom of the wells. Each modification was tested in relation to the uncoated substrate reference in triple precision with $n = 5$ parallels. Cell counting was carried out in combination with the evaluation of the cell viability by means of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits to be able to differentiate between vital and dead cells.

III. RESULTS AND DISCUSSION

A. Lipid coating characterization

Tetraether lipids from archaea *T. acidophilum* were used to establish new types of surface coatings onto silicone rubber. For covalent immobilization of the lipid spacer molecules to polymer surfaces, the end groups have to be activated. In the present study, activation with cyanuric chloride followed by coupling to amino surface groups was chosen to enable a covalent immobilization.

A new process for coating of silicone surfaces with caldarchaeol monolayers has been developed with a special emphasis on a process applicable for coating of tubular catheters and other medical devices in an industrial production line. The process consists of two initial steps: (1) activation of the inert silicone polymer surface by hydrolysis with sodium hydroxide and (2) introduction of amino groups by aminosilanization of the hydrolyzed silicone surface. FTIR analysis of silanized silicone (see Fig. 4) shows clearly that amino groups are superficially introduced as reported by Kim *et al*. and Vandenberg *et al*.[50,51]

The tetraether lipid was then covalently attached to the amino-functionalized silicone by reaction with the cyanuric chloride (SIK TL). Further functionalization of the outermost cyanuric chloride group of the tetraether lipid bilayer was used to introduce various surface properties: hydrophilic surface through PEG-derivatization (SIK TL PEG), hydrophobic modification by means of a fluorinated surface through derivatization with pentafluoroaniline (SIK TL CF), positively charged surface through introduction of bis(3-aminopropyl) amine (SIK TL positive), and negatively charged surface through introduction of glycine (SIK TL negative). Finally, a combination of hydrophilic and negatively charged surfaces by derivatization of the lipid coating with PEG and propane sultone was prepared (SIK TL Combi).

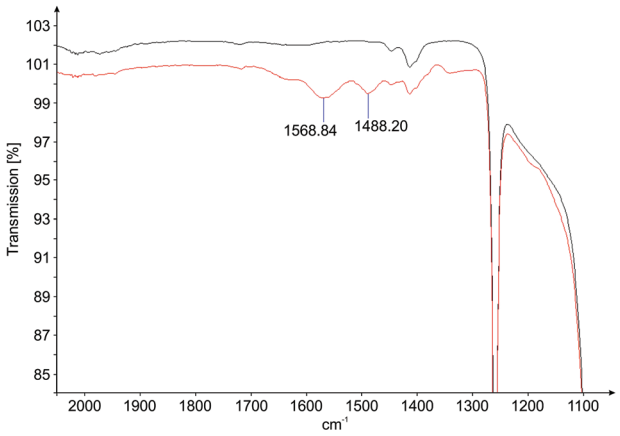

FIG. 4. FTIR-spectra of the uncoated silicone surface (black line) and the ATMS coated silicone surface (red line).

Table I summarizes water contact angles and zetapotentials of modified silicone surfaces including standard deviations and data resulting from a significance test [analysis of variance (ANOVA)—analysis related to the uncoated silicone, significance interval $p = 0.05$] to document statistically significant differences due to lipid coating. Corresponding significant changes become apparent for both, the wetting behavior measured by water contact angles and the zeta potential measurements. Referring to the zeta potential data, a statistically significant reduction was observed between the uncoated and the lipid coated samples. According to previous data shown in Ref. 26, zetapotential/*p*H-functions of lipid modified silicone displayed a clear reduction of the surface potential coupled with a shift of the isoelectric point (IEP) to higher, more alkaline values (data of IEP not shown). As expected, the resolution and macroscopic character of the applied streaming potential method does not allow a clear discrimination between the various tetraether lipid (TEL) capping ligands except the TL positive sample which shows a slightly positive zeta potential of 0.2 mV. A similar result can be observed for the investigated wetting behavior. Compared to the superhydrophobic character of the uncoated silicon surface, all lipid coated samples have shown a statistical significant reduction of the water contact angle. As expected, the introduction of the fluorinated surface group (SIK TL CF) does not follow this trend and a comparable superhydrophobicity as for the uncoated reference could be observed. Again we must state that the determined standard deviation does not allow a differentiation between the various TEL capping ligands except for the fluorinated sample which shows a water contact angle of 118°. Despite the statistical significance it is obvious that some of the differences found are rather moderate. Nevertheless both sets of data allow the conclusion, that the covalent coupling of the TEL based capping ligands was successful.

B. Biocompatibility testing

1. Cytotoxicity testing

SIK TL PEG, SIK TL positive, SIK TL negative, SIK TL Combi, and SIK TL CF showed no relevant cytotoxic effect according to DIN EN ISO 10993-5:2009 after direct cell contact. Parts of the surface of the test samples SIK and SIK TL showed a mild cytotoxic effect. This might be due to an inhomogeneous surface texture. SIK, SIK TL, SIK TL PEG, and SIK TL positive showed a marginal antiadhesive effect and mostly rounded cells on the surface of the test samples. For use in catheters, this antiadhesive effect can be assessed as positive. No significant effect on cell metabolism was seen after exposure of the cells to the extracts to all test samples. All modifications have passed the indirect contact test as shown in Fig. 5.

2. Hemocompatibility testing

For the direct static human blood contact investigation, all test samples were incubated for 30 min with human whole blood or plasma. Under the chosen experimental conditions, no test sample did significantly influence the leukocyte, erythrocyte, and platelet counts compared to the baseline and the control (see Figs. 6–8). Equally, no increased activation of the coagulation system measured as TAT complex generation could be observed. Compared with the negative control, the test samples SIK TL PEG and SIK TL positive showed a tendency to increased hemolysis rate. The other test samples were in the range of the control and did not show evidence for a significantly increased hemolysis. To summarize the results of the *in vitro* experiments, no significant evidence for a blood incompatibility of the test samples used according to DIN EN ISO 10993-4:2009 was observed. The negative and/or positive controls showed the expected reactions.

TABLE I. Physicochemical data of the prepared coatings (Bonferroni-correction, ANOVA-test, significance level $p = 0.05$, + significant differences to SIK, and − no significant differences to SIK).

	Water contact angle (deg)	Standard deviation (deg)	Significance vs SIK [±]	Zetapotential (mV)	Standard deviation (mV)	Significance vs SIK (±)
SIK	120.8	1.5	—	−25.0	4.0	—
SIK TL	103.5	3.7	+	−8.5	1.3	+
SIK TL PEG	107.0	6.5	+	−11.1	2.9	+
SIK TL CF	118.5	0.7	−	−12.1	3.1	+
SIK TL negative	107.9	4.4	+	−10.3	2.4	+
SIK TL positive	101.5	3.1	+	0.2	1.2	+
SIK TL combi	103.6	7.0	+	−5.7	2.2	+

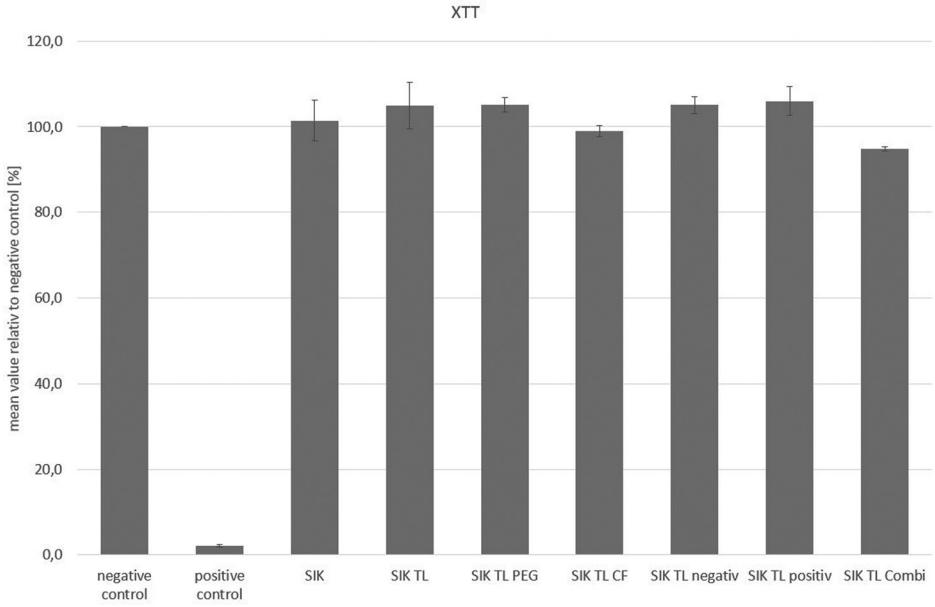

FIG. 5. Mitochondrial activity of L929 cells relative to the negative control, negative control extraction medium without material contact, and positive control: dilution of the negative control with 20% ethanol v/v.

3. Genotoxicity testing

In the genotoxicity test, no toxic effects of test sample extracts were observed in any of the five tested strains. Therefore, the highest extract concentration evaluated with and without metabolic activation in the experiment was used. The reference mutagens induced a distinct increase of reverting colonies indicating the validity of the experiment. In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, no test sample extracts caused gene mutations by base pair changes or frameshifts in the genome of the test strains used. Therefore, the extracts of all investigated samples are considered to be nonmutagenic in this bacterial reverse mutation assay.

4. Sensitization testing

The sensitization rate after application of all extracts of the test samples was 0%. Under the applied test conditions, no test samples showed signs of allergenic potency. Considering the data of these tests for delayed-type sensitivity, it can be evaluated that the test sample extracts caused no reactions identified as sensitization. Therefore, all investigated test samples are considered to have no sensitizing properties.

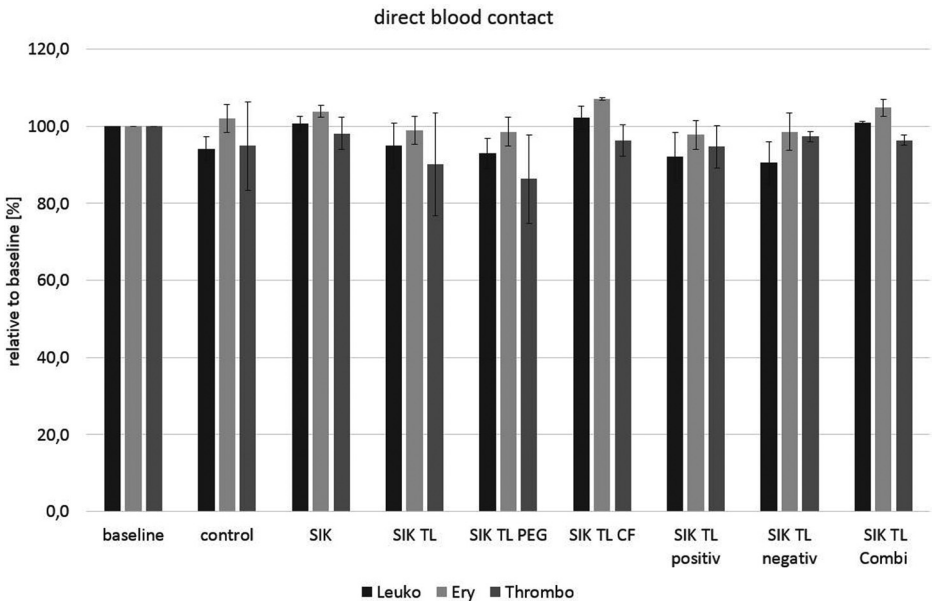

FIG. 6. Amount of blood cells after direct human blood contact relative to baseline, control: commercially available polytetrafluoroethylene (PTFE) foil.

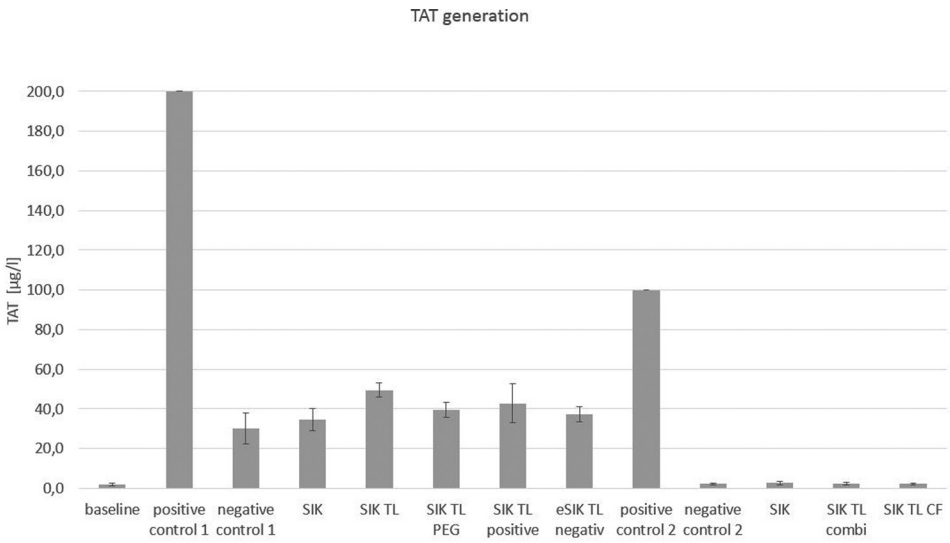

FIG. 7. TAT complex formation, data arising from two test protocols performed with different concentrations, negative control: commercially available PTFE foil, and positive control available glass.

5. Irritation testing

The injection of two extracts of all test samples at doses of 0.2 ml led in total to no signs of irritation compared to injection sites of the reagent control. The average score was zero. Therefore, all test samples met the requirements of the tests and will be classified as nonirritant. Considering the reported data of this irritation studies, it can be stated that the polar and nonpolar extracts of all investigated test samples showed no irritation/corrosive effects.

6. Testing of local effects after implantation (tissue compatibility)

With respect to the conducted implantation tests, the overall response to test and to control samples was in general rather moderate. Tissue response in all groups and all investigated samples was limited to moderate fibrosis and fibrous capsule response. The inflammatory infiltrate was mild, and basically, all interfaces showed a moderate infiltration of macrophages and in most instances of lymphocytes and plasma cells. Infiltrates of polymorphonuclear neutrophile granulocytes could be detected in some of the test or control samples. Moreover, some samples indicated small calcifications at the interface. Under no circumstances infection, necrosis and/or degeneration of the skeletal muscle could be observed.

In the present experimental setting no microscopically significant pathological alterations could be observed after the implantation period. In regard to the short implantation period, test and control samples indicated a normal chronic inflammatory reaction with a moderate fibrous capsule

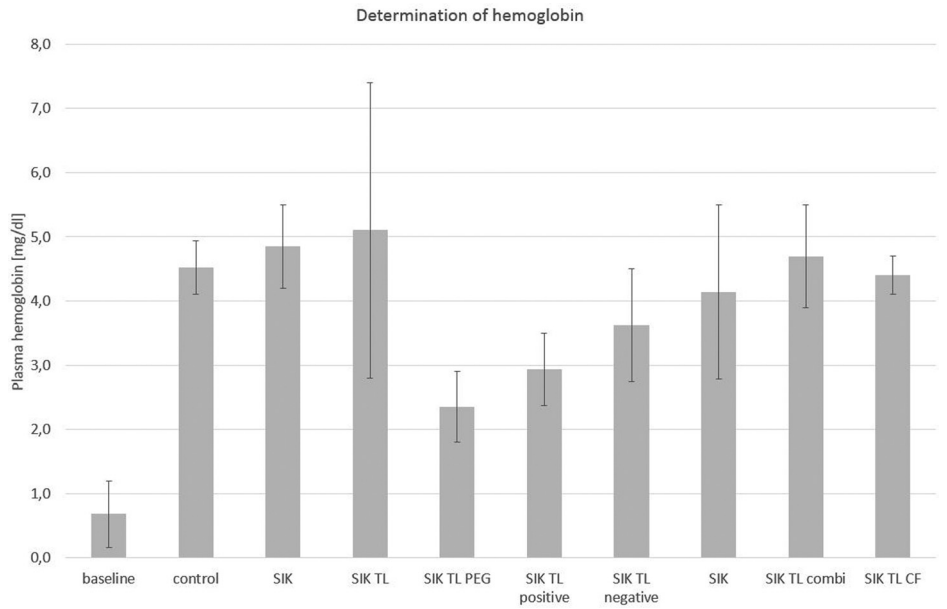

FIG. 8. Data representing the hemoglobin detection, control: commercially available PTFE foil.

formation. After the implantation period of 7 and 28 days no significant differences of the tissue reaction could be observed between the several investigated material modification test groups (Figs. 9 and 10). In conclusion, it can be stated that the investigated lipid based modifications reveal good tissue compatibility in the present *in vivo* implantation study.

C. Performance control

1. Sterilization stability

In order to test the suitability of the tetraether lipid spacer system and their modifications as coating of medical products, the application-specific testing of the stability during sterilization was carried out. Fluorescence microscopy analysis could not demonstrate detectable changes of the investigated lipid coatings after both sterilization procedures compared to the untreated coating (Fig. 11). As expected, the layers have shown a high stability during steam and gamma sterilization due to their covalent bonding to the silicon surface. Defects in the original coating can be enlarged by the influence of steam sterilization. After gamma sterilization the layer is still homogeneous and mechanically stable. Lipid coatings are stable under clinical relevant sterilization procedures.

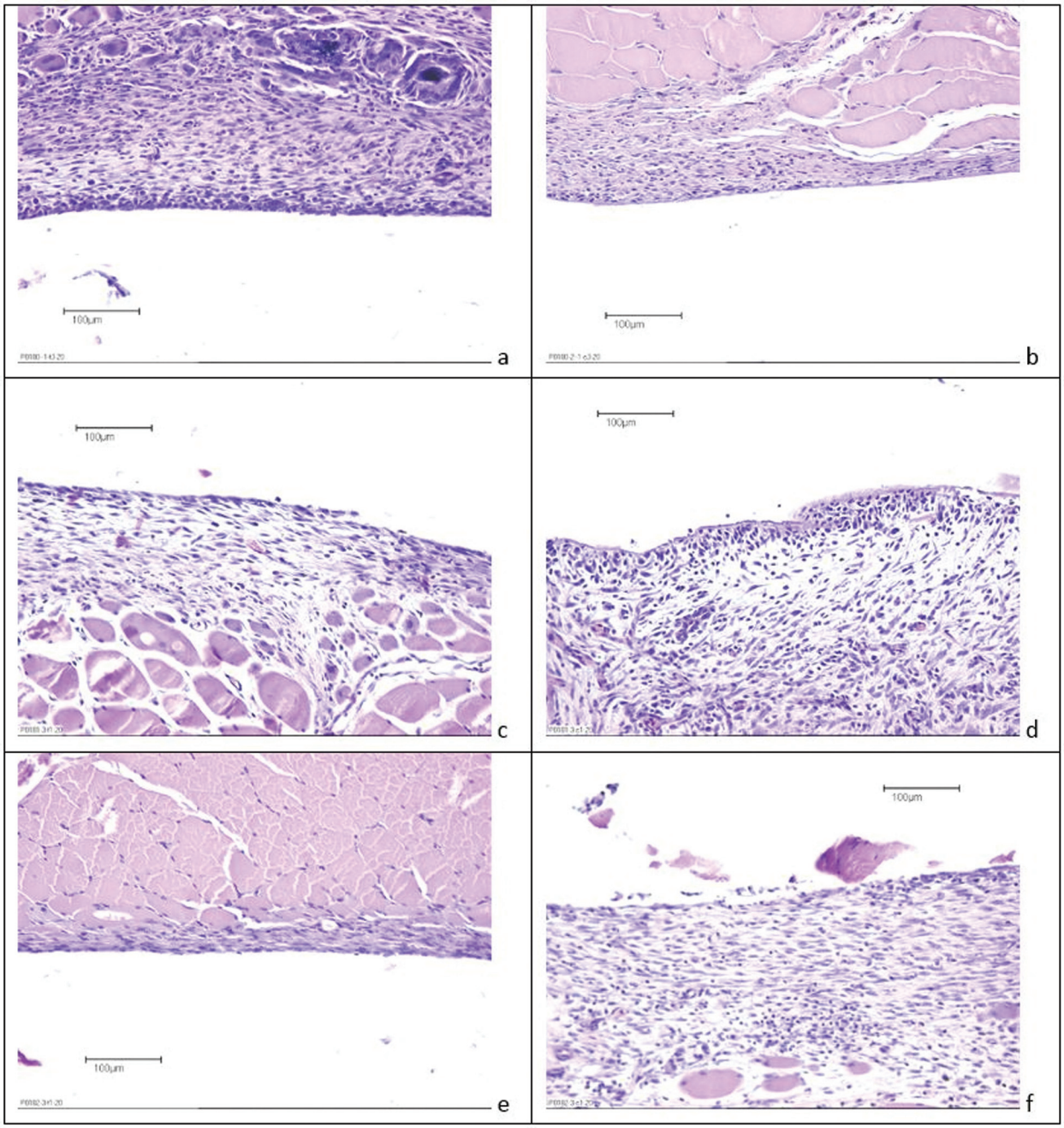

FIG. 9. Histology images after 7 days implantation period (magnification ×200, hematoxylin and eosin staining), [(a), (b)] SIK TL and control, [(c), (d)] SIK TL PEG and control, and [(e), (f)] SIK TL negative and control.

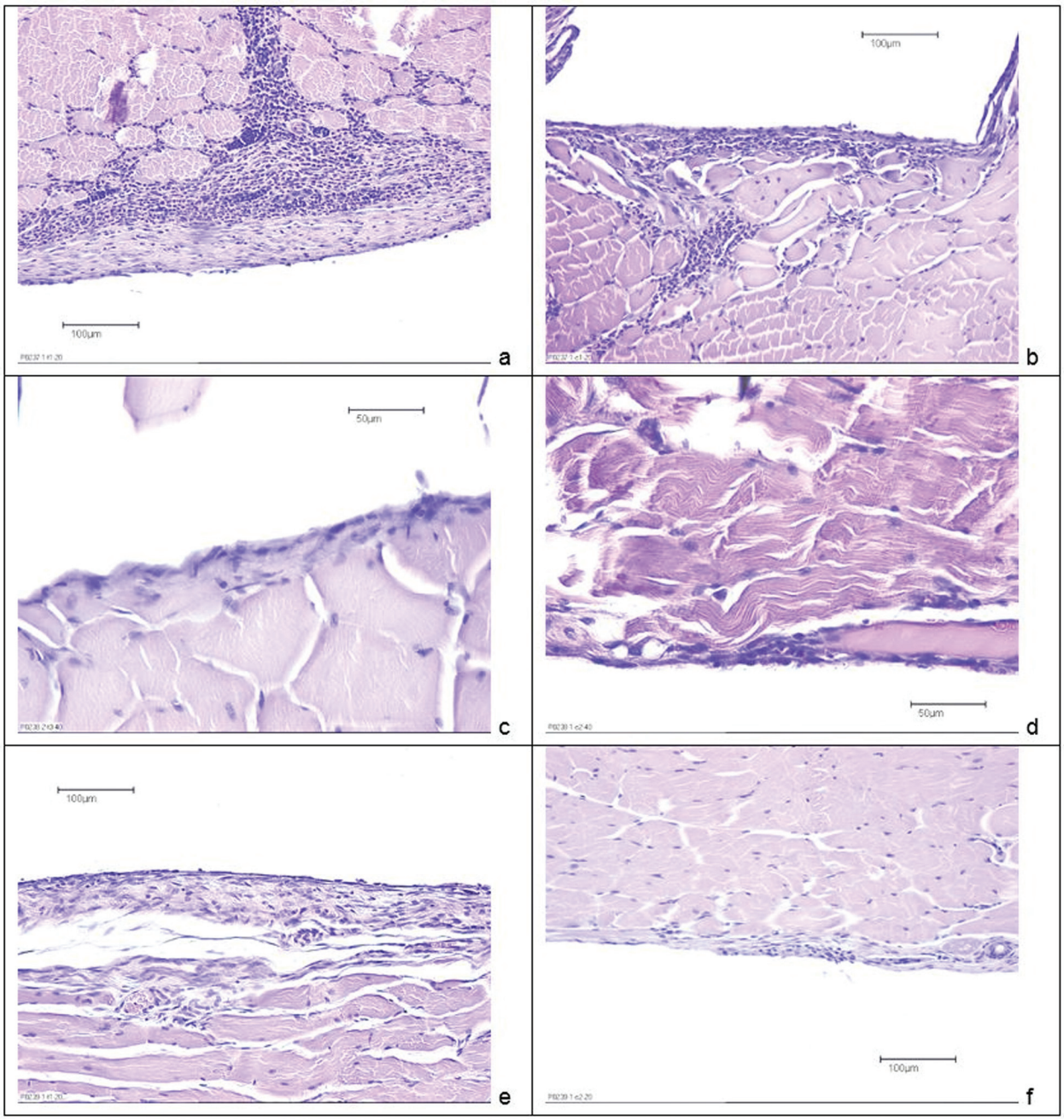

FIG. 10. Histology images after 28 days implantation period (magnification ×200, hematoxylin and eosin staining), [(a), (b)] SIK TL and control, [(c), (d)] SIK TL PEG and control, and [(e), (f)] SIK TL negative and control.

2. Bioadhesion tests

With respect to the results described above (coating stability, cytocompatibility, and implantation performance), the most promising candidates to establish a suitable coating technology were selected: SIK TL, SIK TL PEG, and SIK TL negative. Accordingly, these final lipid-based modifications on medical silicone were investigated by means of a static bioadhesion test.

A significant difference in bacterial adhesion between the uncoated sample and the lipid surfaces can be observed. Unsurprisingly, the modification (SIK TL PEG) shows the strongest antiadhesive effect in comparison with the other coatings (Fig. 12). The vitality of adhered bacteria seems not to be influenced. This indicates a specific antiadhesive surface effect without an antibacterial influence. Subsequently scanning electron microscopic images illustrate the reduction of microbial adhesion on the investigated modifications (Fig. 13).

D. Discussion

The technique of PD is the preferred dialysis method compared to hemodialysis due to its accepted economic

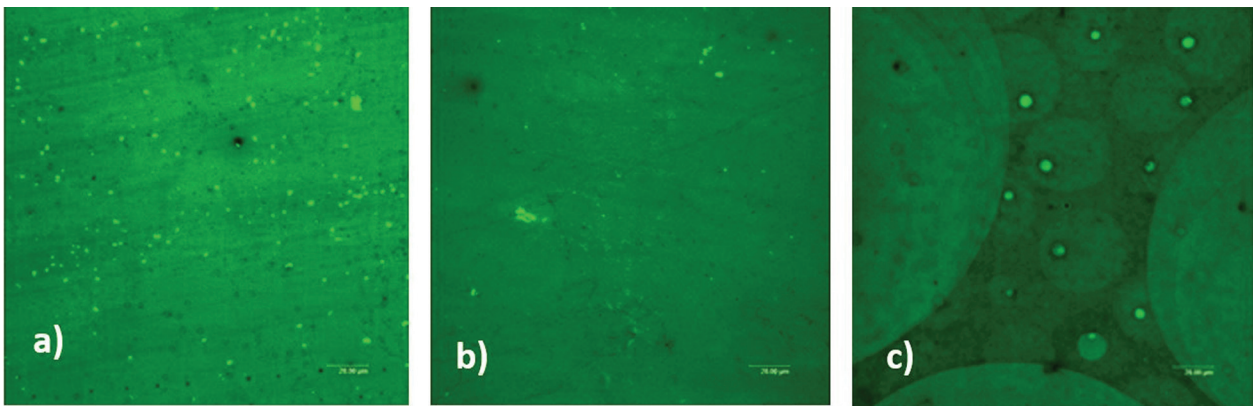

FIG. 11. Fluorescence mages of tetraether lipid coatings (200 × 200 μm, stained with $DiOC_{18}$), (a) reference image without sterilizaiton, (b) after gamma sterilization, and (c) after steam sterilization.

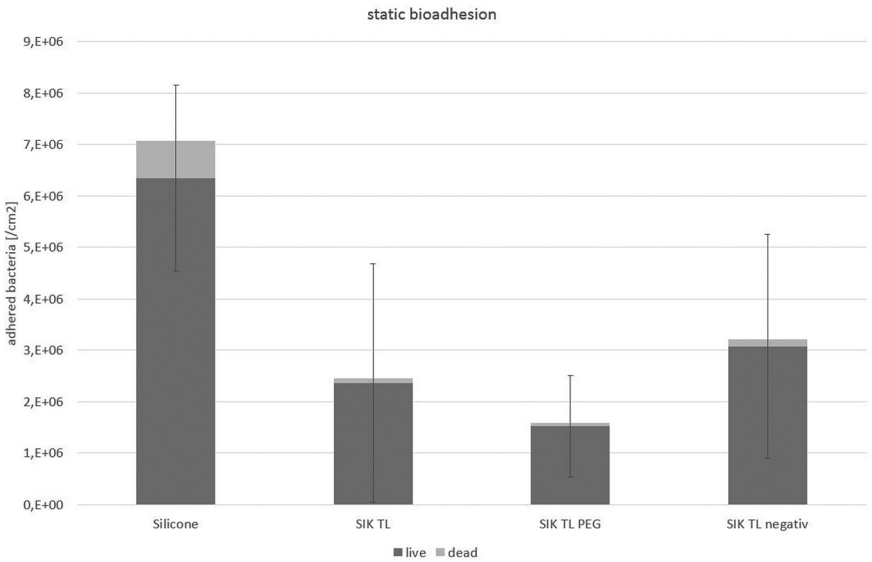

FIG. 12. Number of adhered cells per cm^2 after 24 h under static conditions.

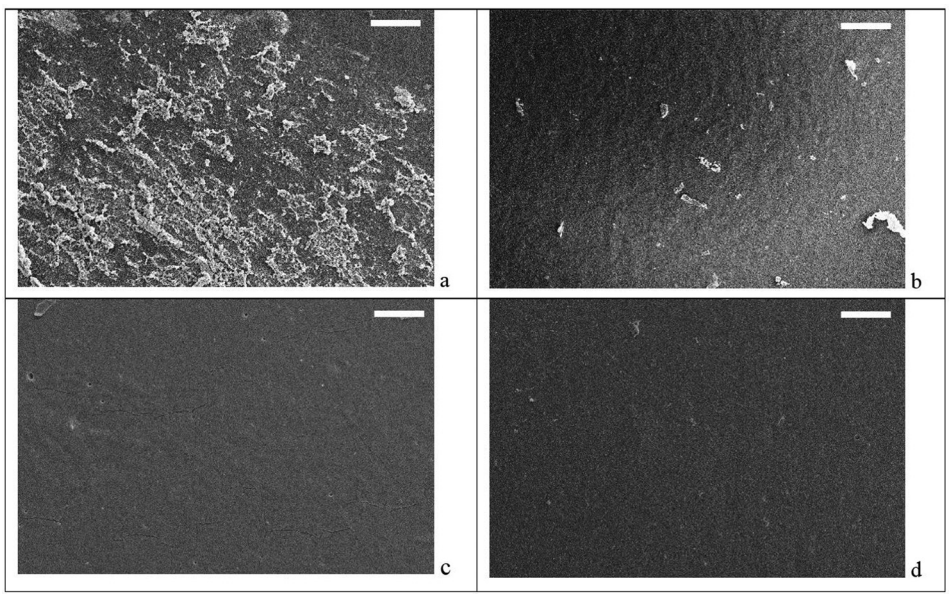

FIG. 13. Representative SEM-images of the sample surfaces colonized with microorganisms (scale-bar 300 μm), (a) SIK, (b) SIK TL, (c) SIK TL PEG, and (d) SIK TL negative.

benefits and offers more autonomy for the afflicted patients. However, the main disadvantage of PD can be described as follows:

(1) PD is as always related to a high risk of infection. A recent study in peritoneal dialysis in Japan, for example, points to a mean incidence of peritonitis of 0.22 per patient per year.[52] Essentially the peritoneum, the subcutaneous tunnel and in numerous cases the catheter exit site are concerned. (2) Frequently infection episodes lead to mechanical and metabolic complications inherent in the technique and a higher rate of technique failure associated with a transfer to hemodialysis.[10,12] (3) Unfortunately, the current clinical treatment options try to reduce the burden of planktonic cells and do not take into account the biofilm as preferred mode of growth of pathogens.

For this reason, peritonitis, the most challenging cause of technique failure, remains the major problem in clinical use. A lot of approaches for surface modification of polymer surfaces are published, most of them demonstrate good anti-infective properties in laboratory experiments, and other concepts were evaluated in specific clinical studies. Only a few medical devices reached necessary accreditation and marketability.[53–55]

One reason is the lack of a complete performance and biocompatibility control of the optimized antimicrobial coatings according to DIN EN ISO 10993 taking into consideration the design of the final device and animal tests to proof the *in vivo* performance of the medical device.

There is a general agreement (1) that microbial biofilms have emerged as a novel pathogenic principle and (2) that the recently observed clinical focus on relapsing peritonitis have brought a new interest to especially investigate the role of biofilms in PD. This led inevitably to the introduction of concepts, suitable to avoid or hinder initial bacterial adhesion and biofilm formation on catheter materials. The fact that passive hydrophilic surfaces in association with a stable surface hydration possess a great potential to reduce bacterial adhesion and to regulate cellular attachment has been published by numerous authors.[22,23,56–58]

The most established approach is to create hydrophilic or polyhydrophilic surfaces by means of poly(ethylene glycol) (PEG) or oligo(ethylene glycol) (OEG).[58–60] However, there is a certain risk that PEG/OEG can degrade in dependence of time by (auto-)oxidation. An interesting alternative to overcome this drawback is the application of poly(2-oxazoline)s (POx), respectively, the water soluble poly(2-methyl-2-oxazoline)s (PMeOx) and poly(2-ethyl-2-oxazoline)s as new antiadhesive agents.[27]

A second passive approach is based on the experimental evidence that surface charges influence biofilm formation and cell adhesion. In most of these publications, the surface potential is modified by attaching charged functional surface groups[61,62] or by surface charges fabricated by plasma polymerization.[63] Finally, some approaches are focused to use self-assembled monolayers or polymer brushes as a strategy for new coating platforms.[26,64–66]

Moreover, it was demonstrated that zwitterionic or polyzwitterionic materials, which contain both positively and negatively charged groups show excellent antiadhesive properties.[28,67] This bio-inspired approach relies on the observation that cellular membranes contain amphiphilic lipids with polar zwitterionic head groups that resist nonspecific adsorption.

Interpretation of adhesion data of *Staphylococcus* strains on catheter polymers underlines the hypothesis that a promising approach needs to be a combination of antiadhesive and antimicrobial strategies.[68] Zare *et al*. give a similar recommendation, using the example of urine catheters.[69] They describe the combination of antibacterial silver with a hydrophilic polymer as the most promising antifouling strategy.

After some decades of intensive scientific debate, it can be assumed that the antiadhesive efficiency of polyhydrophilic as well as polyzwitterionic polymers is correlated with the formation of a more or less stable surface hydration zone. In the case of using polyhydrophilic polymers, the interaction with water is mediated by hydrogen bonding. In contrast, polyzwitterionic polymers undergo ionic solvation by a homogenous distribution of zwitterionic charge groups with a strong affinity to water molecules.[70,71]

In principle, it can be assumed that the way a surface interplays with water molecules will influence the biological response to the material surface. Regardless whether the observed surface hydration is formed by hydrogen bonding or ionic solvation, the intensity and the stability of surface hydration correlates with the physicochemical properties of the coating material and the achieved packing density. Consequently, it can be hypothesized on the basis of a thermodynamic context that the strength of the surface hydration seems to be a function of enthalpic components (chain dehydration) and entropic components (chain compression) and therefore depends on parameters like chain thickness, chain length (molar mass), chain conformation, chain flexibility, grafting density, and surface chemistry to name the most important properties to achieve an excellent fouling resistance.[72]

It seems to be clear on this background that a careful molecular design of the material surface is necessary to address all the above-mentioned properties of functional polymers and to tailor-made a specific biointerface. In this study, a membrane-analog immobilization matrix consisting of tetraether lipids is proposed that can be modified very easily by an extensive toolbox of functional polymers (capping ligands). Thereby exists the option to tailor-made the self-assembling process of the tetraether lipids itself such that specific functional molecules can be integrated by self-assembling in order to get mixed self-assembled monolayers to influence the grafting density on the one hand or to provide additional functionalities on the other hand.[73]

This strategy has become particularly favorably since it was necessary to establish such a polymer coating covalently on silicone. Medical grade polymers like silicone are in principle chemically inert and hydrophobic, thus creating an adhesive bond between the polymer surface and coating becomes difficult. A marked damage of the adhesion is often

observed after hydration in a physiological environment.[74] Nevertheless, a couple of papers cover promising coating concepts on silicone substrates.[74–77] For example, the preparation of a mannose-functionalized silicone to promote the adherence of benign *Escherichia coli* 83972 with a certain potential to reduce the adherence of the uropathogenic *Enterococcus faecalis* was reported by Lopez *et al.*,[77] while Jang *et al.* developed a chitosan and vancomycin coated catheter tube.[76]

To overcome the challenge of chemically inert silicone, an activation step to introduce hydroxyl groups as basis for further chemical modifications was used. A self-assembly method to introduce the tetraether lipid based spacer system was chosen because it is easy to produce, forms relatively dense layers, and is suitable for up-scaling. By further functionalization of the outermost cyanurchloride group of the tetraether lipid bilayer, a wide spectrum of surface modifications could be prepared as described earlier by Frant *et al.*[43] The coatings were characterized by FTIR, water contact angle measurement and surface charge calculation.

Based on the lipid core structure (caldarchaeol) a dense and homogeneous layer was obtained by self-assembling. In addition, the TEL system will be referred to as a flexible layer due to its nanoscale dimension (length of the molecule: ~5 nm) and the resulting potential to form a smooth and defect free coating, in particular, on rough surfaces such as silicone.

Significant changes in the water contact angles and mainly in the zeta potentials confirm a successful modification at the outermost cyanurchloride group of the tetraether lipid matrix. A good coating stability due to the covalent immobilization of the lipid monolayer plays an important role for later application as a medical device. Appropriate data indicating an excellent long term stability against chemical, mechanical, and thermal stresses were published by Tauhardt *et al.*[27] The resistance against salt water exposure was investigated over 12 weeks in the North Sea. The following analysis by means of CLSM confirmed an excellent stability of the TEL coatings.

Comparable small differences in water contact angle were published by Chen *et al.* who used a directly coupling of polyethylene oxide onto silicon films,[78] as well as by Cao *et al.* who studied silanized silicone sheets.[75] Emoto *et al.* hypothesized an increased antifouling effect of core-polymerized block copolymer micelles having aldehyde-ended PEG shells on amino-propylene in comparison to linear PEG coatings due to the higher PEG density.[79] Sateesh *et al.*[44] have published similar results of polymer coating on polyurethane. To solve the problem of establishing autoclavable layers on the inert silicone, Li *et al.* proposed silver heparin films.[80] In the present study, the immobilization strategies are based on wet-chemistry and self-assembly methods for surface modifications of silicone PD-catheters leading to sterilizable, biomimetic tetraether lipid based layers.

Tetraether lipids are biomimetic, nontoxic, and immunologically inert.[38,41,46] Biocompatibility tests of this study confirm the excellent compatibility in the fields of cytotoxicity, genotoxicity, irritation, sensitization, local effects after implantation, and biofunctionality of the lipid and its outermost modifications. As expected, the investigated coatings showed no cytotoxic effects in direct cell contact. In the hemocompatibility tests, no test samples showed significant influence on leukocyte, erythrocyte, and platelet adhesion. Correspondingly, no increased activation of the coagulation system was observed. In conclusion, there was no finding which indicated a blood incompatibility of the tested lipid-based coatings. These excellent outcomes of blood contact are in accordance with former published results of phospholipid coatings.[34]

IV. CONCLUSION

Finally, it could be concluded that lipid based coatings consisting of a membrane-analog immobilization matrix and various functional polymers that form a stable surface hydration possess a high potential to reduce the amount of adhering bacteria on catheter surfaces as illustrated, for example, by Tauhardt *et al.*[27] The vitality of bacteria is not substantially influenced. This indicates a specific antiadhesion mechanism without significant antibacterial activity as expected due to the near-surface zone of "hydration water", whose dynamic nature is still subject of intensive scientific discussion.[81]

The developed strategy using biomimetic membrane-analog spacer systems as matrix for further chemical modification was successfully tested on silicone polymers and should be extended to a general procedure for the controlled coating of medical devices with tailor-made functionalities.

In general, the authors concluded that further studies are necessary to investigate this potential *in vivo* and to reach necessary accreditation and commercialization.

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