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ORIGINAL PAPER

Analytical methods for detection of small amounts of amino groups on solid surfaces: a survey

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Abstract Various analytical methods have been performed or adapted for the detection of small amounts of amino groups on solid surfaces. As well as the quantification of available amino groups, area-wide coatings should be detectable for applications such as biocompatible coatings of medically relevant materials. In this regard, photometric and fluorometric methods have been used and an enzymatic assay developed, all of which are compared herein.

Keywords Biocompatible coating · TNBS assay · Kaiser test · Enzymatic assay · Sulfo-SDTP assay · OPA assay

Introduction

For medical applications, biocompatible coating of relevant materials is important for the acceptance or rejection of foreign material. The biocompatibility of a long-term implantable medical device refers to the ability of the device to perform its intended function, with the desired degree of incorporation in the host, without eliciting any undesirable local or systemic effects in that host [1].

In the literature, poly(ethylene glycol) (PEG) is one of the best described biocompatible polymers for nonfouling surfaces [2]. It is hydrophilic and is therefore employed extensively in pharmaceutical and biomedical applications. For the attachment of other compounds, it can be chemically modified.

Tests for biocompatibility are performed in vivo in animal experiments, with porcine coronary artery being the most commonly used animal model, or in vitro with the help of cell or organ cultures. In these tests, the chemical, physical, and structural properties of the biomaterial and the tissue responses to it are critical factors. Thus, as well as culture studies (data not shown), area-wide coating with biocompatible bisamino-PEG, NH_2–(CH_2–CH_2–O)$_n$–CH_2–CH_2–NH_2, was evaluated in this study by comparison of different analytical methods for the detection of small amounts of amino groups on solid surfaces.

Results and discussion

Coating procedures

As carriers, stainless steel 316L (12% Ni, 17% Cr, 2.2% Mo, 67% Fe, 1% Cu, 0.25% N) was used as standard for medical implant materials as well as glass slides for fluorescence microscope studies. For photometric methods with TNBS and the Kaiser test, controlled-pore glass powder and Gulsenit, respectively, were used. Gulsenit is an active magnesium silicate mineral having particle size less than 10 μm and density of about 3.2 kg/dm^3.

The first step of coating was conducted by amino-silanization with 3-aminopropyltriethoxysilane (APTS), a popular organosilane for the creation of functional amino groups on inorganic surfaces (Scheme 1). The reaction can be performed by either aqueous or organic solvent deposition. Heating to 110 °C forms the siloxane bond, resulting in a more stable coating.

H. H. Hinterwirth and M. Strobl contributed equally to this work.

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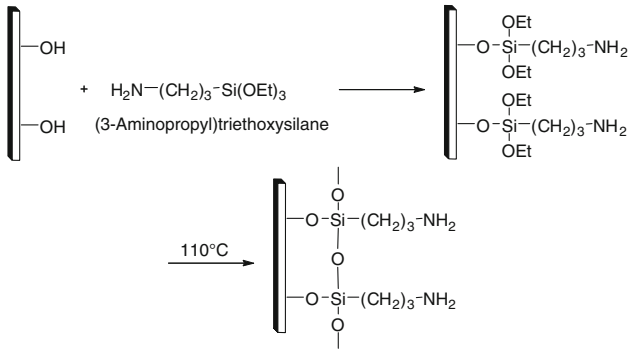
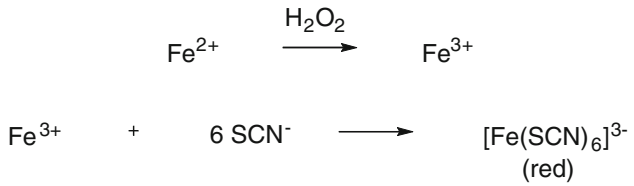


Scheme 1



Scheme 2

For activation of 316L, the stainless steel was etched with 3% HNO_3, 0.5 M HCl, or 6 M HCl for 10 min and soaked for 2 days in double-distilled water (ddH_2O) to obtain hydroxyl groups needed for further amino-silanization, as described above. Effective etching was verified by reaction of Fe^{3+} with NaSCN, forming a red complex (Scheme 2).

For coating with bisamino-PEG, different linkers were used. For linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), the amino-silanized surface was first reacted with succinic anhydride to introduce carboxylate groups (Scheme 3).

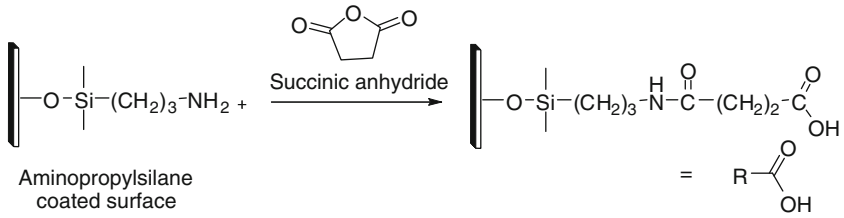
EDC is a zero-length cross-linker and often-used carbodiimide for coupling of carboxylates with amines. Adding additional sulfo-*N*-hydroxysuccinimide (sulfo-NHS) increases the yield of EDC-mediated reactions by several fold (Scheme 4) [3].

For direct cross-linking of two amino groups, *p*-chloranil for PEGylation (Scheme 5) and glutaraldehyde for coupling of alkaline phosphatase (AP) for the enzymatic assay (Scheme 6) were used.

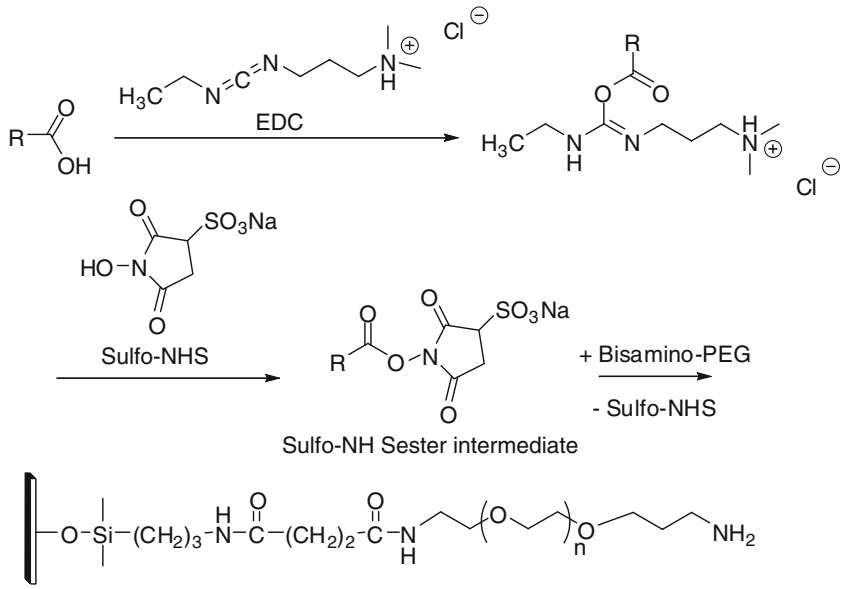
Analytical methods

For detection of the amino groups of the amino-silanized or amino-PEGylated slides, different analytical methods were compared for quantification of the amount of amino groups available on the surface as well as area-wide coating for optimal biocompatibility. Herein, the Kaiser test, an enzymatic assay, assays employing 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) and sulfosuccinimidyl-4-*O*-(4,4′-

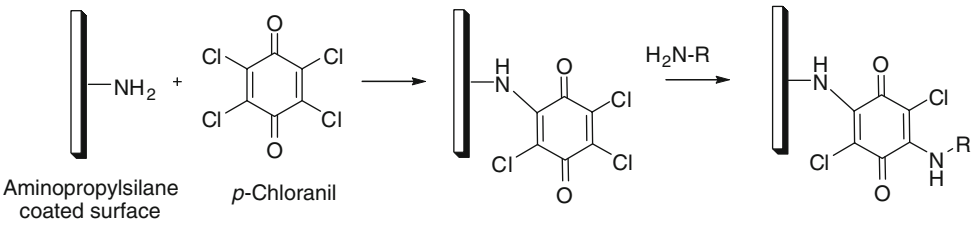
Scheme 3



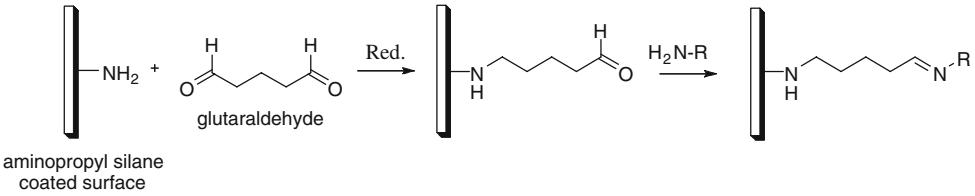
Scheme 4



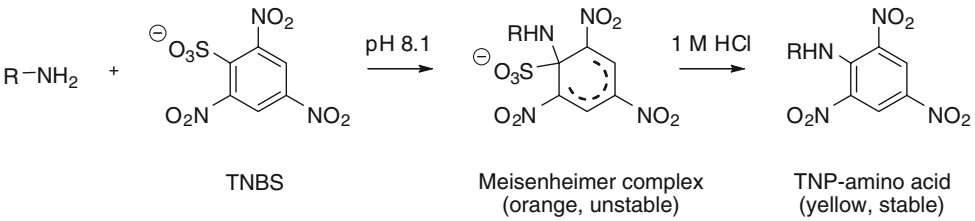
Scheme 5



Scheme 6



Scheme 7



dimethoxytrityl)butyrate (sulfo-SDTP), and fluorescent staining with *o*-phthaldialdehyde (OPA) are discussed.

TNBS assay

TNBS, a reagent introduced by Sateke [4], reacts under relatively mild alkaline conditions with amines, forming an unstable Meisenheimer complex—a highly chromogenic orange-colored derivative with absorbance at 335 nm. Subsequent acidification to pH 1 rapidly converts the unstable orange product to a stable yellow trinitrophenol (TNP) derivative (Scheme 7) [5].

Only slides after amino-silanization by organic solvent deposition show faint yellow staining. However, this method—widely used for amino group tests on controlled-pore glass beads or other substrates with very large surfaces—was not sensitive enough to detect small amounts of amino groups on rather flat solid surfaces and thus was not used further. The much simpler OPA assay, which showed better results for these purposes, was preferred.

Kaiser test

The ninhydrin reaction was developed by Moore and Stein [6] and adapted for detection of amino groups on solid phases by Kaiser et al. [7]. Ninhydrin reacts with primary amines on the surface, forming Ruhemann's blue (Scheme 8). As Ruhemann's blue is slightly soluble it can be found on the surface as well as in solution, and thus it is not useful for quantification.

The Kaiser test was efficient for detection of free amino groups on organic amino-silanized Gulsenit, as this substrate is a porous silicate with large surface. However, this assay is not sensitive enough for detection of amino groups on most carriers or nonporous amino-silanized glass powder (Fig. 1).

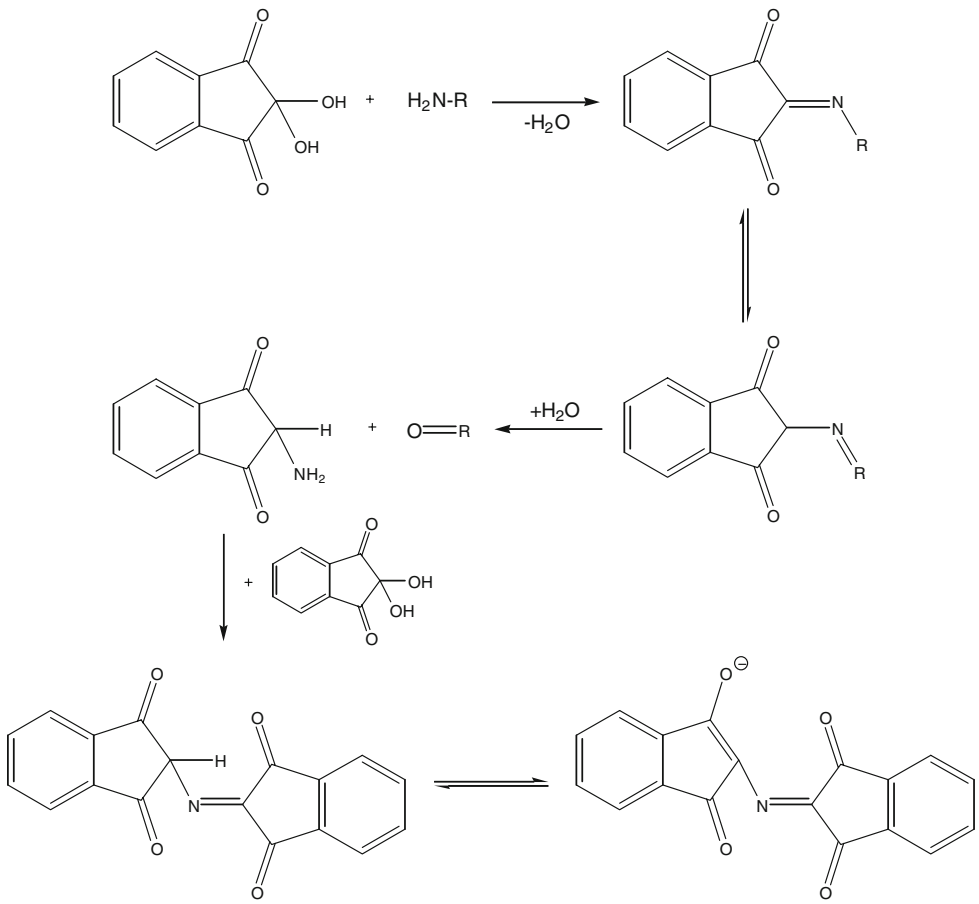
Enzymatic assay

An enzymatic approach was developed to detect very small amounts of amino groups on solid surfaces. The free immobilized amino groups on the carriers are activated with glutaraldehyde followed by coupling of calf intestinal alkaline phosphatase (AP). AP catalyzes the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP), cleaving a phosphomonoester, with a strong yellow color detectable at 405 nm (Scheme 9).

The slope—using a calibration function with different amounts of AP at a constant amount of *p*-NPP—is proportional to the concentration of the coupled AP, which can be correlated to the number of amino groups on carriers. The amount of immobilized enzymes was calculated using a standard calibration curve.

Table 1 shows the results for amino-silanized carriers, showing that etching of 316L stainless steel with 0.5 M HCl and 3% HNO_3 increased the amount of AP that could be immobilized by about fourfold compared with 6 M HCl. For unetched 316L stainless steel no AP activity was measured, which demonstrates the importance of previous etching of stainless steel for subsequent amino-silanization.

Scheme 8



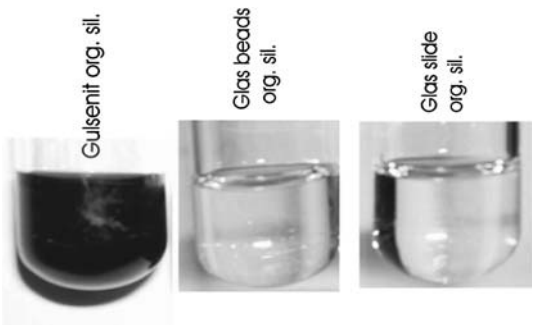


Fig. 1 Detection of free amino groups by Kaiser test. The intensity of the blue color is generated by the reaction of ninhydrin with free amino groups. Amino-silanized Gulsenit provides the most intensive blue color due to the high amount of free amino groups. However, the Kaiser test was of limited value for detection of free amino groups on amino-silanized glass slides or glass beads

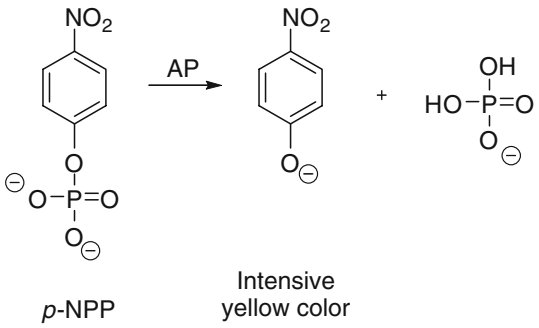
Thus, etching with 0.5 M HCl or 3% HNO_3 was preferred for further immobilizations. The detected amount of amino groups on glass carriers was higher than on 316L stainless steel despite the etching.

Table 2 shows the results for 316L stainless steel after amino-PEGylation using EDC as cross-linker at pH 5 and 10; the different methods for etching the 316L stainless steel and the different pH conditions for cross-linking with EDC show almost the same results. However, the amount of immobilized AP is approximately fourfold less in comparison with the amino-silanized 316L stainless steel; the detected free amino groups on PEGylated glass at pH 5 are five times higher than on 316L stainless steel, and fourfold higher compared with on PEGylated glass at pH 10. This might derive from the fact that the terminal free amino groups of bisamino-PEG (MW 1,500) are protonated and stretched better in acidic buffer than in alkaline buffer. Moreover, to enhance single attachment of the polymer, avoiding the formation of loops, requires a large excess of bisamino-PEG (Table 3).

Glass carriers showed the highest immobilized amount of amino groups; therefore only glass carriers were used for detection of amino groups using sulfo-SDTB.

Sulfo-SDTP assay

Sulfosuccinimidyl-4-*O*-(4,4′-dimethoxytrityl)butyrate (Sulfo-SDTB) is used for colorimetric assay and has high sensitivity to amino groups on solid support [8]. Sulfo-SDTB reacts with amino groups in the presence of perchloric acid to release the 4,4′-dimethoxytrityl cation with a very high



Scheme 9

extinction coefficient (E_{498} = 70,000) (Scheme 10), which makes this method very sensitive [9]. The released cation can be measured at a wavelength of 498 nm.

The detected amounts of 4,4′-dimethoxytrityl cations after reaction of sulfo-SDTB with amino-silanized glass slides and amino-PEGylated glass slides—using the *p*-chloranil coupling procedure at pH 10—were similar. However, a higher amount of amino groups was detected by amino-PEGylation at pH 5 for both reagents, EDC and *p*-chloranil. A similar amount of amino groups was detected after amino-PEGylation at pH 10 using EDC.

Table 1 Quantification of AP immobilized with glutaraldehyde onto amino-silanized carriers and the etching effect of stainless steel 316L (surface of glass slides and 316L: 2.88 cm^2)

Sample description	Slope	Amount of coupled AP (ng/carrier)	Calculated amount of NH_2 (pmol/cm^2)
Unetched 316L	8E−5	0.0	0
316L etched with 0.5 M HCl	6E−4	2.5	5
316L etched with 6 M HCl	2E−4	0.5	1
316L 3% HNO_3	5E−4	2.0	4
Amino-silanized glass	7E−4	3.0	6

The amount of immobilized AP enzyme was calculated using a standard calibration curve ($y = 0.0002x + 0.0001$) as described in "Experimental"

Table 2 Quantification of amino groups via glutaraldehyde-immobilized AP onto etched amino-silanized and amino-PEGylated carriers

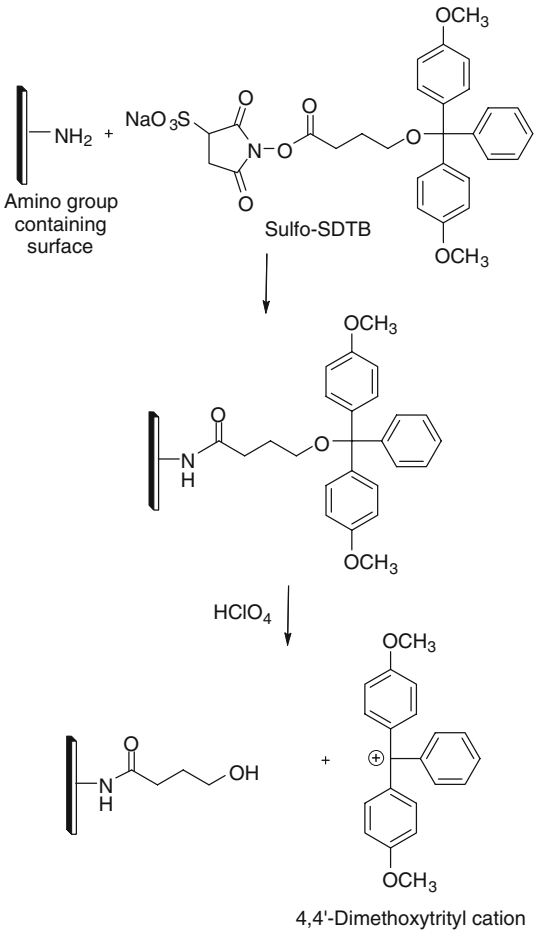
Sample description	Slope	Amount of coupled AP (ng/carrier)	Calculated amount of NH_2 (pmol/cm^2)
316L etched with 0.5 M HCl, PEGylated at pH 5	2E−4	0.5	1
316L etched with 3% HNO_3, PEGylated at pH 5	2E−4	0.5	1
316L etched with 0.5 M HCl, PEGylated at pH 10	2E−4	0.5	1
316L etched with 3% HNO_3, PEGylated at pH 10	2E−4	0.5	1
Amino-silanized glass, PEGylated at pH 5	7E−4	3.0	6
Amino-silanized glass, PEGylated at pH 10	3E−4	1.0	2

See legend of Table 1 and "Experimental" describing the calculation of the detected amino groups

Table 3 Average amounts of amino groups on the solid surface for different coupling methods (all coating experiments done in triplicate)

	Silanized slides	Slides amino-PEGylated by means of			
		p-chloranil pH 5	*p*-chloranil pH 10	EDC/sulfo-NHS, pH 5	EDC/sulfo-NHS, pH 10
Absorption (498 nm)	0.651	1.019	0.72	1.045	0.903
Concentration of sulfo-SDTB (μg/slide)	0.0211	0.0373	0.0232	0.0232	0.0341
Calculated amount of NH_2 (nmol/cm^2)	0.0155	0.0272	0.0169	0.0281	0.0249

The concentration of sulfo-SDTB per slide was calculated using the calibration curve of sulfo-SDTB giving the equation $y = 22.814x + 0.1685$. Slide surface area was 2.26 cm^2

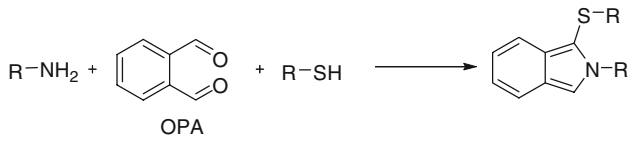


Scheme 10

OPA assay

o-Phthaldialdehyde (OPA) reacts with amino groups in the presence of thiol-containing molecules such as 2-mercaptoethanol, generating a fluorescence product with extinction wavelength of $\lambda_{exc} = 360$ nm and emission wavelength of $\lambda_{em} = 455$ nm (Scheme 11). Detection limits for proteins in liquid are in the µg/cm^3 range.

A very important advantage of OPA is the formation of a fluorescent reaction product on the surface of the tested materials. Thus, the OPA method is also applicable for visualization of the distribution of amino groups on surfaces. The efficiency of aqueous and organic silanization appears to be similar on staining with OPA whereas—according to the TNBS assay—the organic solvent deposition method for amino-silanization showed higher amounts of amino groups. Thus, for all further coatings and experiments, the organic solvent deposition method was preferred due to its simpler and faster procedure (Fig. 2).



Scheme 11

The introduction of carboxylate groups onto the surface of amino-silanized surfaces was tested under different pH conditions (pH 6 and 8.4). Several amino groups did not react with succinic anhydride at pH 6 and hence they can be detected by reaction with OPA, in contrast to pH 8.4, where almost all available amino groups reacted (Fig. 3).

The immobilization of bisamino-PEG or carboxyl-PEG (H_2N-PEG-COOH, as negative control) was carried out with the cross-linker EDC and with EDC plus sulfo-NHS at pH 10. A positive signal can be seen with the bisamino-PEG-coated carrier after reaction with OPA. Thereby, sulfo-NHS is important to increase the reaction by several fold. However, no signal is observable with the carboxyl-PEGylated carrier (Fig. 4).

Conclusion

Etching of stainless steel 316L is necessary for further coating. Thereby, etching with 3% HNO_3 or 0.5 M HCl showed the best results with the enzymatic assay. Amino-silanization by organic solvent deposition was preferred to aqueous deposition because of the simpler and faster procedure. However, both showed similar results on staining with OPA. The additional of sulfo-NHS to an EDC-mediated reaction is important for successful area-wide coating.

The different methods confirmed the effectiveness of biocompatible coating despite their different sensitivities. The TNBS assay and the Kaiser test were not sensitive enough for detecting very small amounts of amino groups. However, the enzymatic assay, the fluorometric assay with sulfo-SDTB, and fluorescent staining with OPA enabled the quantification as well as the visualization of free amino groups on the solid surfaces. The sulfo-SDTB reaction procedure is more sensitive for quantitative detection of amino groups immobilized on carriers compared with the enzymatic assay.

Experimental

All used reagents were of analytical grade. The stainless steel was received from Goodfellow, Gulsenit from MAGINDAG, and the glass powder was a controlled-pore glass from Sigma.

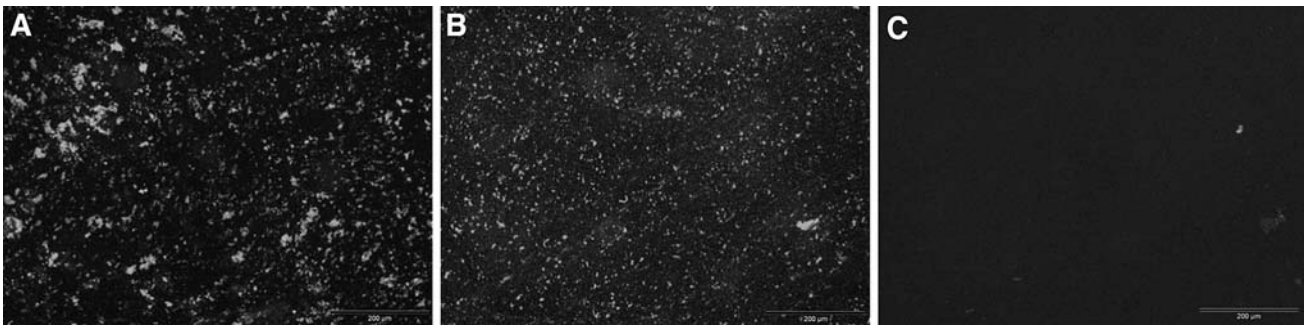


Fig. 2 Visualizations of the fluorescent molecule OPA after reaction with amino groups after amino-silanization by: **a** aqueous deposition, **b** organic solvent deposition, and **c** unsilanized slides (negative control)

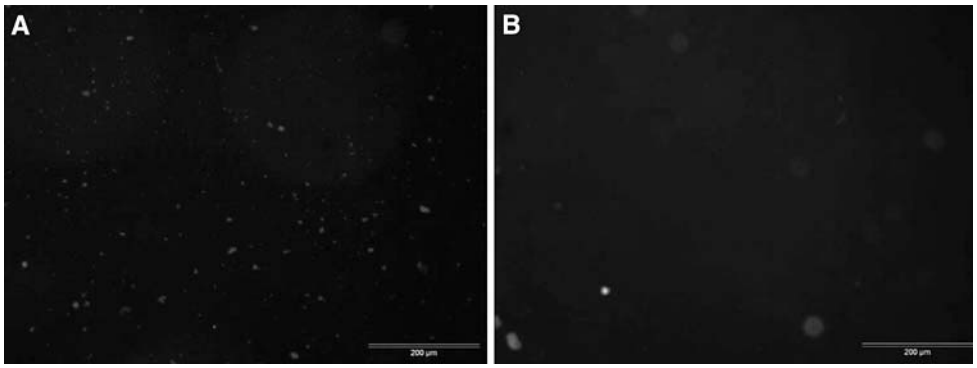


Fig. 3 Effect of pH on coupling of succinic anhydride to amino groups at: **a** pH 6, and **b** pH 8.4, which is preferred because of better reactivity

Fig. 4 Reaction of the fluorescent molecule OPA with **a** carboxyl-PEGylated carrier (negative control), **b** amino-PEGylated carrier linked only with EDC, and **c** amino-PEGylated carrier linked with EDC plus sulfo-NHS

Coating

Etching of stainless steel 316L

To clean the surface of fatty material the carriers were washed with isopropanol and rinsed several times with ddH_2O. Etching occurs under mild conditions with 3% HNO_3. After repeated washing with ddH_2O, the steel was soaked for 2 days in water. For successful etching of stainless steel 316L a spot test for Fe^{3+} was used. A Whatman® filter paper was moistened with 5% NaSCN and then dried at room temperature (rt). To 1 cm^3 of the supernatant of the 3% HNO_3 solution over steel, 50 mm^3 1% H_2O_2 was added. A few mm^3 of this solution were dotted onto the Whatman® filter paper. The color changes to red in the presence of Fe^{3+} ions.

Amino-silanization

For amino-silanization by organic solvent or aqueous deposition the glass slides were cleaned of fatty material by isopropanol and washed with ddH_2O several times.

Amino-silanization by organic solvent deposition

The support materials (glass slides or stainless steel 316L) were shaken in 5% (v/v) APTS (Fluka) solution in 95%

EtOH at rt for 1 h. After 3× 5 min washing with 95% EtOH, they were cured overnight at 110 °C.

Amino-silanization by aqueous deposition

The support materials were shaken in 10% (v/v) APTS solution in ddH_2O at 75 °C for 2 h. After 3× 5 min washing with ddH_2O, they were cured overnight at 110 °C.

Coupling of succinic anhydride as a linker

Succinic anhydride (1 g, Merck) was suspended in 25 cm^3 phosphate-buffered saline (PBS, pH 6; avoid buffers containing primary amino groups such as Tris) and the pH adjusted with 1 M NaOH to either pH 6 or 8.4. Even in buffered solutions, the pH should be monitored to prevent severe acidification of the reaction solution. This solution was added to the silanized carriers and reacted at rt overnight to ensure complete blocking of all amino groups. After washing 3× 5 min with buffer and 3× with ddH_2O the carrier can be activated with carbodiimides.

EDC combined with sulfo-NHS

EDC (400 mg, Sigma, 0.1 M) and sulfo-NHS (Pierce, final concentration 5 mM) were dissolved in 50 cm^3 ddH_2O, and the pH was adjusted to 10 with 1 M NaOH. The carboxylated carriers prepared with succinic anhydride were incubated in this solution for about 1–2 h (maximum) at rt under gentle shaking and thereafter washed several times with ddH_2O.

p-Chloranil as a linker

The amino-silanized carriers were washed 3× 5 min with toluene under shaking and thereafter incubated in 1% (w/v) solution of *p*-chloranil (Fluka) in toluene for 1 h under gentle shaking. After washing 2× with toluene, acetone, and ddH_2O the carrier is ready for amino-PEGylation.

(Amino-)PEGylation

O,*O*′-bis(3-aminopropyl)polyethylene glycol (1 g, bisamino-PEG, MW ≈ 1,500, Fluka) was dissolved in 50 cm^3 carbonate-bicarbonate buffer (pH 10) or in 50 cm^3 citric acid-sodium citrate buffer (pH 5). The slides prepared with EDC plus sulfo-NHS or with *p*-chloranil, respectively, were shaken in this solution overnight and afterwards washed several times with ddH_2O.

TNBS assay

A 0.01% (w/v) TNBS solution was prepared freshly by dilution of 20 mm^3 5% (w/v) stock solution (Sigma) with 0.1 M sodium bicarbonate buffer (pH 8.4). The amino-group-containing slides were shaken for 2 h at 37 °C in this solution, and afterwards 1 cm^3 10% SDS and 0.5 cm^3 1 M HCl were added to each sample.

Kaiser assay

Solution A: 8 g phenol was dissolved in 2 cm^3 EtOH. Solution B: KCN solution (10 mM) in ddH_2O; 200 mm^3 was diluted to a volume of 10 cm^3 with pyridine. Solution C: 50 mg ninhydrin (Merck) was diluted to 1 g with EtOH and dissolved. Then 500 mm^3 of each solution was added to a test tube containing the sample and boiled for 5 min in a water bath.

Enzymatic assay

For immobilization of AP the amino-silanized carriers were shaken gently in 3 cm^3 2.5% glutaraldehyde (Fluka) solution in 0.05 M PBS buffer (pH 7) for 1 h and washed several times with ice-water. AP solution (40 mm^3) was dissolved in 300 mm^3 0.05 M PBS (pH 7), and 100 mm^3 of this solution was added to each carrier and incubated overnight in a humid chamber at rt. After washing the carriers with buffer (3× 5 min), AP activity can be measured as follows. The carrier was placed in a disposable cuvette and filled to 4 cm^3 with 0.1 M Tris-buffered saline containing 0.15 M NaCl and 5 mM $MgCl_2$ (pH 9.5, TBS). The reaction was started by addition of 10 mm^3 *p*-NPP solution (Koch Light Ltd., 50 mg/cm^3 ddH_2O) and the absorption measured against a blank at 405 nm every 30 s for seven times. The cuvette content was mixed with a Pasteur pipette before every measurement.

For generation of a calibration curve, a stock solution of AP with a concentration of 4.5 μg/cm^3 in 0.1 M TBS was prepared. Different aliquots (1.12, 2.24, and 4.49 mm^3) were added in a disposable cuvette and filled to 4 cm^3 with TBS, and the reaction was started and measured as described before.

The calibration curve was calculated by measuring the extinction of several concentrations of AP over time separately. The definite concentration of each AP and its slopes are used to give the equation: $y = 0.0002x + 0.0001$. The slope is proportional to the concentration of the enzyme, which should be approximately equal to the amount of amino groups on the surface of the carrier. Using the equation and the slope of each sample the amount of immobilized amino groups can be calculated.

Sulfo-SDTP assay

A 2.33 mg/cm^3 sulfo-SDTB (Pierce) stock solution (42 mg SDTB dissolved in 2 cm^3 DMF and 16 cm^3 50 mM sodium bicarbonate buffer) was prepared freshly. For calibration the stock solution was diluted several times with 35% (v/v) perchloric acid to concentrations from 7 to 46.7 μg/cm^3 and the absorbance measured after 10 min at 498 nm against a blank of 35% perchloric acid.

For the detection of amino groups on solid surfaces, the carriers were soaked in 1 cm^3 stock solution for 10 min, then rinsed first with 1 cm^3 ddH_2O, and then soaked in

10 cm^3 ddH_2O to remove excess sulfo-SDTB. Perchloric acid (1 cm^3, 35%) was added to each carrier and reacted for 10 min. The observed orange color indicated the release of 4,4′-dimethoxytrityl cations. The 1 cm^3 solution was transferred to a cuvette and the absorbance measured as described above.

OPA test

A solution of 1 cm^3 50 mM borate buffer (pH 9.2), 250 mm^3 OPA (Fluka, 20 mg/cm^3), and 250 mm^3 2-mercaptoethanol (Fluka) were added to the amino-group-containing carrier and reacted for 1 h at rt. After washing several times with ethanol, the fluorescent marking of amines by OPA was observed under an Olympus BX41 fluorescence microscope using extinction wavelength of 360 nm and emission wavelength of 436 nm. Images were captured by ColorView Soft Imaging System (Olympus Soft Imaging Solutions, Münster, Germany) with magnification of 10× (N.A. 0.25) and edited with Cell^D life-science documentation software.

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