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ORIGINAL PAPER

Cleavage of antibodies using dihydrolipoamide and anchoring of antibody fragments on to biocompatibly coated carriers

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Abstract This study shows the possibility of anchoring antibody fragments on to carrier surfaces with biocompatible coatings. Cleavage of whole antibody molecules into two fragments is performed with immobilized dihydrolipoamide. The proof of successful coating and immobilization of antibody fragments is shown by use of fluorescence microscopy. The biocompatibility of a poly(ethylene glycol) coating, on 316L stainless steel as carrier, was shown in cell culture with Caco-2 cells. Immobilization of antibody fragments can serve another purpose—as a suitable specific binding site for nano or microparticulate drug-delivery systems.

Keywords Lipoic acid · Poly(ethylene glycol) · Medical implant · Drug delivery

Introduction

The concept of this study is the development of carriers containing bio-compatible coatings and binding sites for bio-degradable nano or microparticles that may also serve as reloadable drug-delivery carriers on medical implant materials. Reloadability needs binding systems strong enough to keep the drug-releasing nano or microparticles tightly bound for a certain period of time, but, on the other hand, weak for the degradation products. Thus after degradation the binding system is free again to allow reloading. In this study the chosen specific recognitive site for drug-releasing nano or microparticles is an antibody fragment. Because antibodies (Abs) against the drug carriers (biocompatible and biodegradable polymers) were not yet available for proof of principle, binding conditions were demonstrated with HRP-antibody (Ab). The cleavage of Abs to create Fab′ fragments was carried out using immobilized dihydrolipoamide. The method of reduction of disulfides with dihydrolipoamide immobilized on to a gel material was first used by Gorecki and Patchornick in 1973 [1]. In our approach, lipoic acid (6,8-dithiooctanoic acid) was immobilized on amino-silanized Gulsenit via the carboxylic acid group, by means of the water soluble carbodiimide EDC. The efficiency of the immobilization was monitored by the Kaiser test for free amino groups [2]. Afterwards the cyclic disulfide of the immobilized lipoic acid was reduced to thiols using sodium dithionite ($Na_2S_2O_4$) as reducing agent (Scheme 1).

These thiols are able to cleave disulfide bridges of Abs creating two Fab′ fragments with free sulfhydryl groups. This method is time-saving and economic. The immobilized dihydrolipoamide can easily be removed from the sample solution by centrifugation and the Fab′ fragments are thus free from unwanted by-products. The efficiency of cleavage of Abs is shown by a positive Ellman's assay [3]. Furthermore, after regeneration of the immobilizate with $Na_2S_2O_4$ the reaction can be repeated several times with the same immobilizate.

Medical implant materials should be biocompatible. For this purpose a biocompatible coating or so called "passive coating" for medical implant materials has been developed using poly(ethylene glycol) (PEG) derivatives. PEG is

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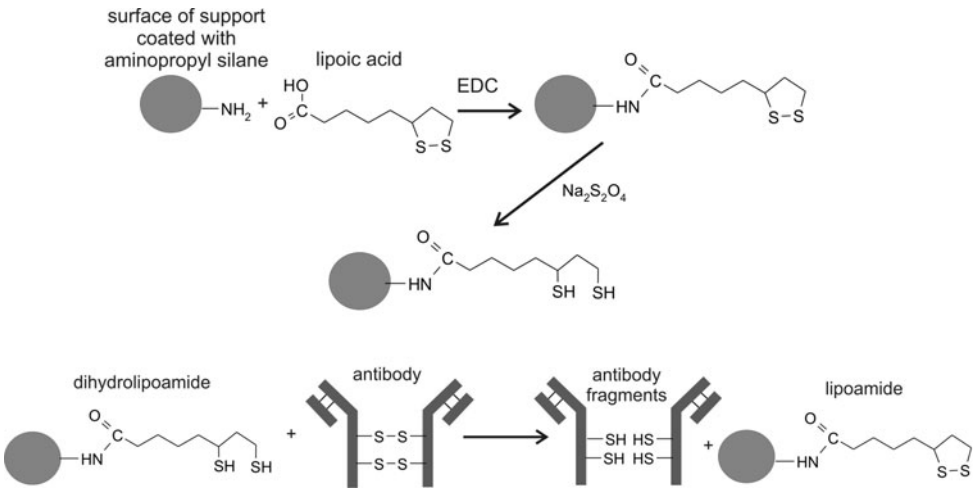
Scheme 1



biocompatible, can act as a barrier against attacks of the immune system on medical implant materials, for example stainless steel or zirconia, and is widely used for this purpose because of its hydrophilic and also hydrophobic character [4]. In the course of this study the previously cleaved antibody fragments are anchored on the PEG coating on the surface of the carrier material used. The anchor of a suitable receptor system (an Ab fragment against the polymer of biodegradable nano or microparticles) to bind drug containing biodegradable nano or microparticles can serve to mediate local pharmacological activity, in the form of a so-called active coating. The nano or microparticles can be injected into patients after implantation. The nano or microparticles circulate in the blood and should bind specifically to their receptor (Ab fragment) on the biocompatibly coated implant surface. The particles can then be degraded either by hydrolysis or by means of enzymes circulating in the bloodstream while releasing the drugs. After complete biodegradation the receptor system is then free again for reloading with new particles containing the respective drugs, making this a completely new means of drug administration on medical implants. Such coatings can be the basis of a number of applications that require controlled, local drug delivery at the interface between an implant and healthy tissue. Because nano or microparticles can be loaded with different drugs, this technology allows easy adjustment to patient-specific medication needs and the administration of compounds easily degradable or difficult to dose. Several studies have already shown the possibility of loading different drugs into nano or microparticles consisting of biodegradable and biocompatible polymers, and their release [5, 6].

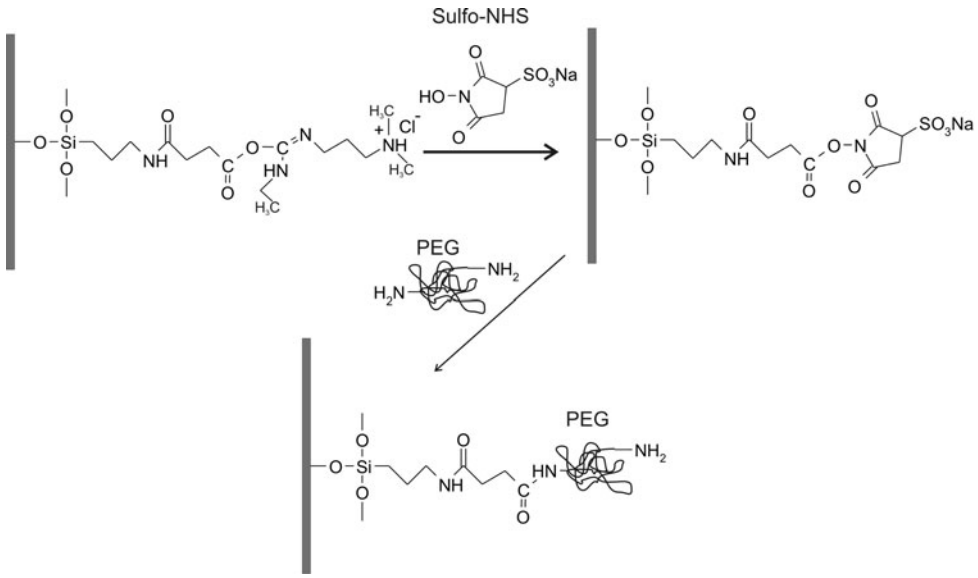
The surface of the carriers used (316L stainless steel, silica glass) needs to be previously activated by chemical etching followed by amino-silanization in order to immobilize the biocompatible NH_2–PEG–NH_2 polymer (amino-PEGylation). Amino-PEGylation was carried out using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) as cross linker. For this purpose succinic anhydride is first coupled to the amino-silanized carrier and then activated with EDC–sulfo-NHS (*N*-hydroxysulfosuccinimide) to immobilize the NH_2–PEG–NH_2 (Scheme 2).

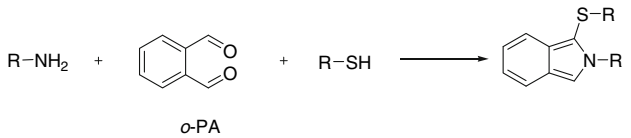
Fab′ fragments of polyclonal anti-peroxidase-Ab were immobilized on to amino-PEGylated carriers. The immobilization of Fab′ fragments on to amino-silanized and amino-PEGylated carriers is shown using *o*-PA (*o*-phthaldialdehyde) as reagent. *o*-PA reacts with amino groups in the presence of thiol-containing molecules such as *β*-ME (*β*-mercaptoethanol) generating a fluorescent product with an excitation wavelength of 360 nm and an emission wavelength of 455 nm (Scheme 3).

In this approach *o*-PA was used as a crosslinker between amino groups on the carrier and sulfhydryl groups on the Fab′ fragments. For this purpose Fab′ fragments were used instead of *β*-ME, which is used normally for detection of amino groups by means of *o*-PA (Scheme 3). This approach has the advantage that it enables, at the same time, detection of positive Ab cleavage and its immobilization on to amino-silanized or amino-PEGylated slides.

The chemical setup for biocompatible coating was shown with 316L stainless steel and zirconia ceramics as widely used medical implant materials. Analytical proof of principle on these materials with regard to Ab fragment immobilization is difficult, because the necessary optical methods (*o*-PA assay) available could not be applied, because of the quenching effects of steel and the very uneven surface structure of the zirconia ceramics used. Therefore specific binding of the antibody fragments to the carrier surface was tested by means of fluorescence microscopy on silica glass precoated by use of the same chemical procedures as steel and zirconia. However, the biocompatibility of amino-PEGylated 316L stainless steel was tested in vitro with Caco-2 cells.

Scheme 2





Scheme 3

Results and discussion

Detection of amino-PEGylation and immobilization of Fab fragments by means of o-PA assay

Cleavage of HRP-Abs was carried out by means of dihydrolipoamide immobilized on amino-silanized Gulsenit. The efficiency of lipoic acid immobilization on to organic silanized Gulsenit was shown by means of the Kaiser test for amino group detection. An intense blue color is generated by reaction of ninhydrin with free amino groups. The efficiency of cleavage of Abs by means of immobilized dihydrolipoamide is observed by a positive Ellman's assay. A positive signal of Ellman's assay is observed in the presence of sulfhydryl groups of Fab′ fragments and no signal with amino-silanized Gulsenit including lipoamide, because of the absence of free thiol groups. *o*-PA is used to crosslink the sulfhydryl groups of cleaved Abs to amino groups of NH_2–PEG–NH_2 on the surface of slides. This approach is very efficient as it serves at the same time for the detection of successful Ab cleavage and also its immobilization on to amino-PEGylated slides. Anti peroxidase-IgG was cleaved with immobilized dihydrolipoamide to create two Fab′ fragments containing free sulfhydryl groups in the hinge region. *o*-PA crosslinks these Fab′ fragments with amino groups on amino-PEGylated slides. The immobilization of cleaved Abs on to amino-PEGylated carriers is shown in Fig. 1.

Biocompatibility test of amino-PEGylated 316L stainless steel using Caco-2 cells

The biocompatibility of the plain and amino-PEG-coated stainless steel was evaluated by cell proliferation studies, 2 and 3 days postseeding. A proliferation study revealed that both amino-PEG-coated surfaces had a tremendous impact on the proliferative activity of Caco-2 cells, as shown in Fig. 2.

A glass slide was used as reference substrate for cell proliferative activity and furnished a signal of 0.1881 AU (absorbance units) on day 2 and 0.3722 AU on day 3 post seeding. Plain high-grade steel presented itself as possible growth substrate, with no toxicity. However, generally it resulted in lower cell proliferation of 0.1516 AU on day 2 and 0.2179 AU on day 3 post seeding, which indicated a need for some improvements. These improvements were made in form of amino-PEGylation. Both amino-PEG-coated substrates seemed to be suitable growth supports for Caco-2 cells with twofold proliferation relative to glass as reference on day 2 post seeding. On day 3, these results are slightly different—whereas amino-PEG coating at pH 5 resulted in further constant cell growth, and 1.5-fold higher proliferation than on the glass reference, the amino-PEG coating at pH 10 resulted in lower proliferation than on day 2, being as much as a factor of two lower than on the glass reference on the same day.

The BrdU proliferation study provided insight into the biocompatibility of amino-PEGylated high-grade steel. Glass slides are commonly used as cell growth support [7].

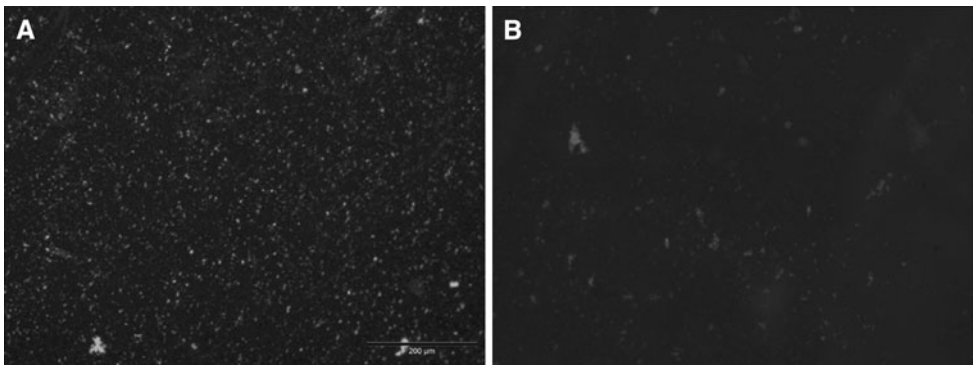


Fig. 1 HRP-IgG Fab fragments immobilized by means of *o*-PA on to silica glass slides. **a** Activated via EDC–sulfo-NHS and amino-PEGylated at pH 5; **b** activated via EDC–sulfo-NHS (negative control)

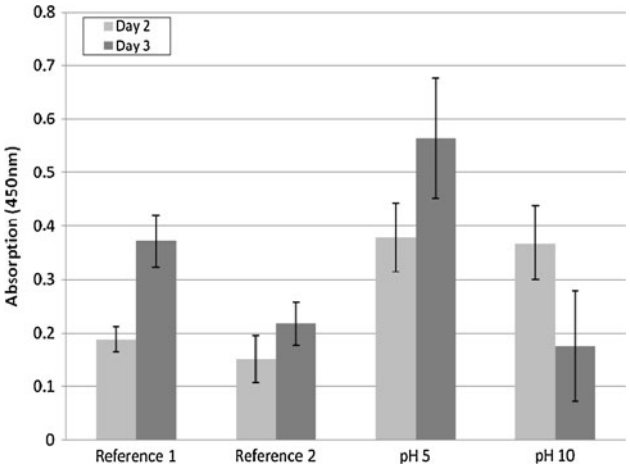


Fig. 2 Proliferative activity of Caco-2 cells on days 2 and 3 post seeding for amino-PEGylated stainless steel coated at pH 5 and pH 10. *Reference 1* refers to proliferation on uncoated glass slides and *Reference 2* is proliferation on plain high-grade steel as growth support ($n = 6$, mean ± SD)

On day 2 the initial adhesion and proliferation activity on glass and on plain high-grade steel is comparable, whereas further cell growth on day 3 shows only a small increase for plain high-grade steel. Coating with PEG is commonly known to reduce protein adsorption and cell adhesion [8]. In contrast, amino-PEGylated stainless steel resulted in improved cell growth. The initial cell adhesion is higher, and the cells showed stable attachment and kept proliferating at the same rate as on glass slides. Results revealed amino-PEGylation at pH 5 as the best combination.

This study confirmed amino-PEGylated high-grade steel as a biocompatible substrate that induces stable cell attachment and results in further cell growth.

Experimental

Organic amino-silanization of inorganic carriers

Inorganic carriers silanized in this study were stainless steel 316L (Goodfellow, Germany), silica glass slides (Ø 12 mm, Assistant, Austria), zirconia ceramics (ZrO_2-TZP-A BiO-HIP, Z-Systems AG) and Gulsenit (Magindag, Austria). Gulsenit is an active magnesium silicate mineral with a particle size less than 10 µm and a density of about 3.2 kg/dm^3. Stainless steel 316L and silica glass need to be chemically etched before amino-silanization to introduce hydroxyl groups necessary for amino-silanization. Gulsenit and zirconia were silanized directly. Stainless steel 316L (12% Ni, 17% Cr, 2.2% Mo, 67% Fe, 1% Cu, and 0.25% N) was etched with 3% HNO_3 for 10 min. Silica glass slides may be etched with 3% HNO_3 for 10 min or 1 M NaOH for 30 min. The carriers were washed several times with double-distilled water (ddH_2O) and then soaked for at least 2 days in ddH_2O, with gentle shaking. To enhance hydroxyl group formation, replacement of ddH_2O several times is highly recommended. The surface of the carriers is then modified by amino-silanization with (3-aminopropyl)triethoxysilane [9] in order to immobilize NH_2–PEG–NH_2. The carriers used were covered with freshly prepared 5% (v/v) (3-aminopropyl)triethoxysilane solution (APTS, Sigma, Austria) in 95% EtOH and reacted for 1 h, with gentle shaking, at room temperature. The carrier was washed with 95% (v/v) EtOH three times for 5 min with gentle shaking and cured at 110 °C overnight.

Binding of succinic anhydride to the amino-silanized surface

Succinic anhydride is used to introduce carboxylate groups on to the surface of amino-silanized material. Succinic anhydride (Merck, Austria; 1 g) was suspended in 25 cm^3 PBS buffer pH 8.4. The pH was monitored and adjusted to 8.4 with 1 M NaOH to prevent severe acidification of the reaction solution, which might also damage the molecule to be coupled in the next step. This solution was added to the amino-silanized carrier and reacted at room temperature overnight to ensure complete blocking of all amino groups (the pH was controlled over the first few hours of the reaction). The carrier was washed three times for 5 min with buffer followed by three times with ddH_2O.

EDC combined with Sulfo-NHS

EDC (0.1 mM, Sigma, Austria) and Sulfo-NHS (Pierce, Switzerland) at a final concentration of 5 mM were dissolved in 50 cm^3 ddH_2O and the pH was adjusted to 10 with 1 M NaOH. The carboxylated carrier prepared with succinic anhydride was incubated in this solution for about 1–2 h (maximum) at room temperature with gentle shaking. The activated carrier was washed several times with ddH_2O.

Amino-PEGylation

O,*O*′-Bis(3-aminopropyl)poly(ethylene glycol) (1 g, NH_2–PEG–NH_2, MW 1500, Fluka, Austria) was dissolved in 50 cm^3 of 0.1 M citric acid–sodium citrate buffer pH 5 or 50 cm^3 0.1 M carbonate–bicarbonate buffer pH 10. The carrier, activated with EDC–Sulfo-NHS, was incubated in the above prepared solutions separately at least overnight. Each amino-PEGylated carrier was washed three times for 5 min with the buffer, with shaking, to remove unreacted PEG and then washed several times with ddH_2O.

Cleavage of disulfide bonds in the hinge region of antibodies with the help of immobilized dihydrolipoamide

To immobilize lipoic acid on to Gulsenit, 40 mg lipoic acid (Sigma) was dissolved in 20 cm^3 EtOH and 50 mg EDC was dissolved in 10 cm^3 EtOH. Dissolved lipoic acid and EDC were added to 5 g carrier (amino-silanized Gulsenit) and incubated overnight at room temperature with stirring. The carrier was rinsed three times with ddH_2O then three times with EtOH, using a suction funnel (pore size 4) to remove solvent from carrier after every rinse. We made sure the immobilized carrier showed no signal with the Ellman's test for free –SH groups. The efficiency of immobilization was determined with the Kaiser test, which detects fewer amino groups in the immobilizate than with amino-silanized Gulsenit. The lipoamide bound to the carrier was reduced to dihydrolipoamide as follows. $Na_2S_2O_4$ solution (0.16 g/cm^3 ddH_2O, 20 cm^3) was added to 5 g of the carrier containing immobilized lipoic acid and reacted for 30 min at room temperature, with shaking. The carrier was rinsed twice with ddH_2O followed by twice with EtOH using a suction funnel (pore size 4) to remove washing solutions from the carrier after every rinse. The carrier was dried at room temperature. Successful reaction was detected by a positive Ellman's test for free –SH groups. The carrier was stored in a sealed vessel at room temperature. For cleavage of Abs with the help of immobilized dihydrolipoamide 1 cm^3 0.1 M PBS, pH 8 was added to 0.5 g dihydrolipoamide immobilizate followed by 2 mm^3 Ab (e.g. pc anti-peroxidase Ab, 41 mg/cm^3). The splitting reaction of the Ab was performed for 30 min, with shaking at room temperature. The supernatant containing split Abs was isolated from traces of carrier by brief centrifugation. Ellman's reagent (50 mm^3) was added to 100 mm^3 of the centrifuged supernatant. If the test was positive, the rest of the supernatant was used for further experiments. Ellman's reagent (50 mm^3) was added to about 60 mg carrier. The test should give no color change. For regeneration of the carrier it can be reduced with $Na_2S_2O_4$ (Sigma) as described above.

Ellman's test

Proof of immobilization of lipoic acid on to the carrier was given by Ellman's test. Ellman's reagent (50 mm^3) (5,5′-dithiobis(2-nitrobenzoic acid), Pierce) at a concentration of 4 mg/cm^3 in 1 cm^3 0.1 M PBS, pH 8, was added to about 60 mg carrier containing either immobilized lipoic acid or dihydrolipoamide (after reduction). Ellman's reagent and carrier were mixed gently using a yellow pipette-tip avoiding air bubbles. It is recommended to carry out the test in white microtiterplates to facilitate visualization of color difference between samples and Ellman's reagent.

Immobilization of cleaved antibody on to silanized or amino-PEGylated carrier via o-PA

o-PA (250 mm^3, 20 mg/cm^3, Fluka) was added to 1 cm^3 50 mM borate buffer pH 9.2 and 500 mm^3 cleaved antibodies (via immobilized dihydrolipoamide). This mixture was added to the carrier containing free amino groups, reacted for 1 h at room temperature, then washed with EtOH. The fluorescent product on the carrier was visualized under a Olympus BX41 fluorescence microscope using an excitation wavelength of 360 nm and an emission wavelength of 436 nm. The pictures were taken with Color View (Soft Imaging System, Olympus Soft Imaging Solutions, Münster, Germany) at 10× magnification and edited with Cell^D life science documentation software.

Biocompatibility test of amino-PEGylated 316L stainless steel using Caco-2 cells

Cell culture

Caco-2 cells (human epithelial colorectal adeno carcinoma) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). The cells (passage number 37) were grown in RPMI-1640 cell culture medium containing 4 mmol L-glutamine, 10% fetal calf serum, and 150 μg/cm^3 gentamycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were

grown to 80–90% confluence and subcultured with TrypLE® select.

Proliferation studies

The proliferation rate was determined by use of the BrdU cell proliferation ELISA test kit (Roche diagnostics GmbH, Vienna, Austria) according to the manufacturer's instructions. The incorporation of 5-bromo-2-desoxyuridine (BrdU) into DNA of proliferating cells was quantified at 450 nm by using a microplate reader (Spectrafluor reader, Tecan, Groedig, Austria). Cells were seeded on high-grade steel supports coated with amino-PEG at pH 5 or 10. Cell proliferation on glass slides and on plain high grade steel served as a reference.

Prior to cell seeding, Flexiperm®, plain stainless steel, and glass slides were disinfected in 70% ethanol for 30 min, and amino-PEG coated high grade steel for 1 min, and left to dry. After forming wells by attachment of Flexiperm® the cells were seeded (17,000 cells/well) and the BrdU proliferation test was performed 2 and 3 days postseeding.

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